A RECOMBINANT APPROACH TO THE ISOLATION AND CHARACTERIZATION OF A PRIMARY DEGRADER OF TRICHLOROETHYLENE

By

YOUNG-JUN KIM

Bachelor of Science
Yonsei University
Seoul, Korea
1983

Master of Science
Yonsei University
Seoul, Korea
1985

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Thesis Approved

Thesis Adviser

Dean of the Graduate College

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TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Large quantities of various chemicals are used routinely by industries and consumers. Production levels of man-made organic chemicals have increased tremendously in recent years (31). One of the problems inherent in the extensive use of chemicals is the release of large quantities of various toxic chemicals into the environment, causing serious pollution problems (16).

Microorganisms play a major role in the breakdown and mineralization of many pollutants. Even though microbial degradation of the toxic chemicals is considered to be a very desirable process, this process is found to be too slow to satisfy many public health concerns. Many harmful organic compounds which are slowly degraded have been identified in laboratory experiments and field tests (16, 107). These chemicals include halogenated aromatics, halogenated aliphatics, and many pesticides. Many factors can have considerable effect on the biodegradation rates of these compounds. These factors might include unfavorable physicochemical conditions (such as temperature, pH, oxygen concentration, salinity, etc.) or the availability of other nutrients (41, 103). Limited degradation rates may likewise be due to the inability of microorganisms to metabolize synthetic chemicals whose structures are uncommon in nature. Many have been designated to be recalcitrant (107).

Microorganisms are, however, well known for their adaptability to extreme or novel environments. Microbial communities exposed to so-called xenobiotics, which are alien to existing enzyme systems, can also adapt to such compounds. The process of microbial adaptation to these compounds, although known to occur, is poorly understood at the molecular levels. Better knowledge about how metabolic pathways involved in biodegradation of these recalcitrant compounds evolve in nature and how they are regulated may enable us to produce the genetically engineered microorganisms which are capable of degrading novel or more complex compounds.

The pathways involved in aromatic degradation have been extensively studied and form a good model for the genetics of xenobiotics degradation (1, 81, 90). The strain *Alcaligenes eutrophus* JMP134 has been reported to degrade various aromatic compounds (such as phenol, benzoate, p-cresols, m-cresols) and many mono- and di-chlorinated aromatics (such as 3-chlorocatechol, 2,4-dichlorophenoxy acetic acid, 3,5-dichlorocatechol) (84, 85). This strain possesses a plasmid-encoded pathway for the degradation of the herbicide, 2,4-dichlorophenoxyacetate (TFD). A number of studies have been performed to elucidate the genetic and molecular basis of the TFD pathway on this plasmid (26, 27, 44, 56, 82, 83, 101). However, little is known about the chromosomally encoded aromatic ring cleavage pathways. Considerable studies have been performed to elucidate the regulatory mechanisms of phenol degrading *meta*-cleavage pathways at the protein level by using various mutant strains in other *Alcaligenes eutrophus* strains (48,

49) and *Pseudomonas putida* U (7, 8, 116). This may partly due to the absence of suitable genetic techniques.

In this study, *Alcaligenes eutrophus* JMP 134 was found to cometabolize trichloroethylene (TCE), a highly toxic pollutant, by two separate and distinct pathways under inducing conditions. One is a chromosomal phenol-dependent pathway and the other is a plasmid TFD-dependent pathway. Two enzymes, phenol hydroxylase and 2,4-dichlorophenol hydroxylase from the chromosome and plasmid respectively, are likely candidates because of similarity to other known TCE degrading enzymes. In this thesis, as a part of the global concern about bioremediation of xenobiotics, chromosomally encoded phenol catabolic pathway has been investigated in *A. eutrophus* JMP134. The gene responsible for TCE degradation was identified, cloned, and its regulatory mechanism characterized. A transposon induced mutant AEK301 which contains the cloned phenol hydroxylase gene was found to efficiently degrade TCE in the absence of the aromatic inducer when the gene is uncoupled from its regulatory gene.

The Purpose of this Research

The overall goal of this research is to create through recombinant DNA techniques a bacterium which can effectively degrade TCE in the absence of any inducer. This study requires the isolation, characterization and modification, if necessary, of the structural gene(s) and its regulatory system which are responsible for TCE degradation in *Alcaligenes eutrophus* JMP134. The understanding of regulatory mechanisms and isolation of the

structural gene may contribute to the construction, through the genetic engineering techniques, of "superbugs" which are capable of degrading more recalcitrant compounds with broader substrate specificity.

Specific Objectives and Strategies of this Research

Specific objectives and strategies for the accomplishment of this research are as follows;

1. Screening of the strains for the ability to degrade TCE.

Two different pathways in *Alcaligens eutrophus* JMP134, the chromosomally encoded phenol pathway and, the plasmid-borne TFD pathway, are screened for TCE removal under the appropriate inducing conditions for each pathway.

2. The identification and cloning of gene(s) involved in TCE degradation in plasmid pathway.

The genetic organization of TFD pathway is well documented. *TfdB* gene, which is the likely candidate, is cloned, expressed in *Pseudomonas aeruginosa,* and checked for TCE removal.

3. The identification and cloning of phenol hydroxylase gene and its regulatory gene(s).

* *A. eutrophus* AEO106 is mutated to isolate mutants defective in phenol metabolism through transposon mutagenesis.

* Each isolated mutant is characterized and screened for TCE removal.

* Colony hybridization and replica triparental mating methods are used for the identification of the positive cosmid clones from the gene bank. For this experiment, a cosmid gene library is

prepared and transposon flanking DNA is cloned and used as a probe for colony hybridization.

* The cosmid clones isolated are characterized through the complementation test. These clones are mapped and subcloned to localize the structural and the regulatory genes.

4. Characterization of regulatory mechanisms of phenol metabolism.

Gene expression is characterized under both heterogenetic and homogenetic background using subcloned structural and regulatory genes.

5. The identification of the enzyme(s) responsible for TCE degradation

Each cloned gene is screened for TCE degradation.

6. Construction of a primary degrader of TCE.

This goal is accomplished by uncoupling the structural gene from its regulatory system.

CHAPTER II

LITERATURE REVIEW

Overview of the Degradation of Phenol

Phenol

Phenols are present in the effluents of oil refineries, petrochemical plants, and other industrial processes (31). This chemical and structurally related compounds, such as cresols, alkylphenols, xylenols, and catechol are listed by the U.S. Environmental Protection Agency as high priority pollutants (52).

Phenol Metabolism in Microorganisms

A number of microorganisms have been found to degrade phenol (3, 6, 42, 48, 59, 76, 84, 94, 102). These are shown in Table 1. Phenol is first metabolized into catechol by various phenol hydroxylases. Hydroxylases are monooxygenases. These enzymes have the property of incorporating one atom of molecular oxygen into their aromatic substrate while the second oxygen atom is reduced to H_2O by an appropriate hydrogen donor such as $NADPH_2$ (74). Catechol, which is formed by phenol hydroxylase, is a key intermediate in many aromatic degradation pathways, and is further metabolized by two distinct sets of enzymes. The *ortho*-cleavage pathway (*beta*-ketoadipate pathway) and the *meta*-cleavage

pathway (*alpa*-ketoacid pathway) convert catechol to TCA cycle intermediates. Typical phenol degradation pathway is depicted in Figure 1. Most phenol-degrading bacteria are known to catabolize catechol through *meta*-cleavage pathway. Involvement of *ortho*-cleavage pathway for phenol degradation was recently reported in *Pseudomonas sp.* EST1001 (29).

Phenol hydroxylase

Phenol hydroxylase was originally purified from the soil yeasts *Trichosporon cutaneum* (30), and *Candida tropicalis* (32). The enzyme of *Trichosporon cutaneum* has been extensively characterized. This enzyme is a bright-yellow single flavoprotein with a molecular weight of 148,000 daltons. NADPH is needed as a hydrogen donor for this enzyme.

Even though phenol metabolism in bacteria has been studied since the 1930s, it was not until recent years that the characterization of bacterial phenol hydroxylases have been published owing to the cloning of phenol hydroxylase genes. Partial purification of bacterial phenol hydroxylases has been reported in two pseudomonas strains. Olsen et al. (59) has isolated the phenol hydroxylase gene and characterized a novel polypeptide with a molecular mass of 80,000 daltons from *Pseudomonas pickettii* PKO1. Phenol hydroxylase of this strain was found to share the similarity with that of *T. cutaneum* and many microbial hydroxylases, which are simple flavoprotein monooxygenases. The oxygenation mechanism of these enzymes has been known to be basically the same, attacking the enzyme-bound flavin-hydroperoxide by the

substrates which are activated by the electron-donating hydroxyl group (22). A multicomponent phenol hydroxylase has been reported by Singler et al. in *Pseudomonas* CF 600 (76, 86). This enzyme was demonstrated to be composed of six polypeptides encoded by six distinct genes on a 5.5 kb DNA fragment. One of these polypeptides was purified and found to be a flavin adenine dinucleotide containing iron-sulfur protein. Phenol hydroxylase from this organism shares a number of similarities to other multicomponent oxygenase systems which consist of one or more redox components. Phenol is also shown to be metabolized by different enzyme systems. Gibson et al. (98) suggested that phenol is degraded by toluene dioxygenase, a multicomponent enzyme, through an alternative monohydroxylation which is different from conventional monohydroxylation in *Pseudomonas putida* F1. A toluene-degrading bacterium, *Pseudomonas cepacia* G4 was known to metabolize phenol by the same enzyme which is responsible for the the degradation of toluene, p-cresol, and m-cresol (94). Phenol hydroxylase of *P. pickettii* PKO1 was also reported to degrade toluene, benzene, and cresols in addition to phenol (59).

Regulation of Phenol Metabolism

Although phenol metabolism has been extensively studied in *Alcaligenes eutrophus* and *Pseudomonas putida* U by using various mutant strains (7, 8, 48, 49, 116), little is known about the regulatory mechanisms of chromosomally encoded phenol pathway at the molecular level. Recently Olsen et al. (59, 60) have revealed the genetic organization of phenol pathway and characterized its

regulatory system from the chromosome of *Pseudomonas pickettii* PKO1. All these strains are known to degrade phenol through catechol *meta*-cleavage pathway. Bayly et al. (48) have proposed that the genes encoding phenol hydroxylase is in a separate operon from the genes encoding catechol *meta*-cleavage pathway in *Alcaligenes eutrophus* 335, with the former under positive control and the latter under negative control. They also proposed that at least one regulatory gene exerts a controlling effect over the expression of all phenol pathway enzymes. Wigmore et al. (116) have reported similar observations from *P. putida* U, suggesting the presence of two separate operons for phenol catabolic pathway. Molecular analysis of *meta*-cleavage pathway in *P. pickettii* PKO1 has revealed that two different regulatory genes, *tbuR* and *tbuS*, act on the expression of phenol hydroxylase gene and of catechol *meta*-cleavage pathway enzymes, respectively. These regulatory genes are localized within a 13 kb DNA fragment which contains all structural genes for phenol catabolic enzymes. It was suggested that the regulatory protein TbuR acts positively for the expression of *tbuD*, the gene responsible for hydroxylation of phenol, whereas TbuS acts as both an activator and a repressor for the *tbuEFGKIHJ*, the genes encoding enzymes for *meta*-cleavage of catechol (60).

Genetic analysis of phenol catabolic pathway in *Pseudomonas sp* CF600 has shown that the genes of the pathway may be encoded in a single operon. The *dmpKLMNOP* genes, which encode a multicomponant phenol hydroxylase in this strain, has been sequenced and the promoter region was analyzed (76). An inverted repeat was found upstream of the promoter region, which is similar

to the symmetrical recognition sequence proposed to be involved in the binding of *E. coli* repressors and activators.

Although chromosomal encoded *meta*-cleavage pathway has not been well characterized, extensive studies of plasmid encoded pathways have been published (15, 35). Many regulatory genes in the plasmids appear to share similarities in sizes, DNA sequences and their modes of regulation. Most of the regulatory proteins encode transcriptional activator proteins (6, 43).

Overview of the Degradation of Trichloroethylene (TCE)

Trichloroethylene

TCE is a low-molecular-weight, volatile chlorinated aliphatic hydrocabon which is one of the most commonly detected halogenated organic contaminants in groundwater along with tetrachloroethylene (PCE),1,1,1-trichloroethane, carbon tetrachloride, and chloroform (23, 40, 88). These compounds are commonly used in the manufacture of plastics, as solvents in aerosols, and as degreasers. The Environmental Protection Agency has classified TCE as a priority pollutant due to its suspected carcinogenicity (105). TCE is degraded to vinyl chloride under anaerobic conditions through its incomplete transformation. Vinyl chloride is known to be tumorogenic and is as much a problem as TCE (50).

TCE degradation in Microorganisms

Biodegradation of TCE occurs in two different environments by

very different processes (Fig. 2). Aerobic or oxidative degradation occurs in surface waters or soils at contaminated sites prior to migration or transport of TCE to the groundwater. Anaerobic or reductive degradation occurs in anoxic environments within the underground water.

Anaerobic Degradation of TCE

TCE is degraded under the highly reducing environment by methanogens (58, 108, 114). Reductive dechlorinations are thought to be involved in this reaction. All of the polychlorinated ethenes are transformed to vinyl chloride which is a greater health threat than the original contaminants.

Aerobic Degradation of TCE

Oxidative transformation of TCE does occur among various bacteria which oxidize toluene, methane, ammonia, and propane (4, 20, 29, 55, 61, 70, 94, 104, 110, 117). These microorganisms are shown in Table 2. All these microorganisms share a common feature that they posses enzymes called oxygenases. The oxidation by the enzymes is initiated by incorporating oxygen from the atmosphere into their substrates. There are two kinds of oxygenases, monooxygenases and dioxygenases. Both classes of oxygenases are implicated in bacterial TCE degradation. Aerobic degradation of TCE was first reported by a consortium of methylotrophs in the presence of methane (33, 115). Methane monooxygenase was found to degrade TCE. The enzyme performs both methane hydroxylation and

propylene epoxidation activities. The first isolation of single bacterium to aerobically degrade TCE was reported in an aromatic compound degrader, *Pseudomonas cepacia* G4 (71, 72). Toluene monooxygenase was found to be involved in TCE degradation in this bacterium. Toluene dioxygenase of *Pseudomonas putida* F1, which is also known to oxidize TCE degradation, is reported to have the ability to hydroxylate phenol (98). This fact suggests that the alternative mechanism of this mono-hydroxylation may be operational on TCE. The activity of monooxygenase-type enzymes toward TCE is thought to result in the formation of TCE epoxide (Fig. 3). TCE epoxide is extremely unstable and reactive. It spontaneously decomposes with a half life of 12 seconds at pH 7.7 in phosphate buffer (67). Under basic conditions, the decomposition products of TCE epoxide hydrolysis are predominantly one carbon compounds such as formate and carbon monoxide whereas under acidic conditions, dichloroacetic acid and glyoxylate become major decomposition products. The fate of TCE degradation was monitored using ^{14}C-labelled TCE. Under the mixed culture conditions of methane utilizers, 34% of the label in biomass, 23% as CO_2, and the remainder in non-volatile non-halogenated compounds were detected (33). The deleterious effect of TCE oxidation on the growth of cells was also reported (34). Inhibitory effect could be caused by the interaction between the natural substrates and the fortuitous substrate, TCE, through the competitive binding to the responsible enzymes. More basic and harmful effect reported was that cellular materials were covalently modified by the possible reactive intermediates of TCE oxidation such as glyoxylyl chloride and formyl chloride (87, 111).

These compounds are proposed to be the precursors of glyoxylic acid and formic acid in studies on the mammalian metabolism of TCE (67).

Degradation of TFD by the pJP4 Encoded Pathway

TFD pathway on pJP4

Alcaligenes eutrophus JMP134 possesses a plasmid pJP4. Plasmid pJP4 is an 80-kilobase, broad-host range, P1 incompatibility group plasmid (44). This plasmid carries the genes for the catabolism of 2,4-dichlorophenoxyacetic acid (TFD). TFD is a chlorinated aromatic hydrocarbon, used as an herbicide to kill broad leaf plants (63). Plasmid pJP4 carries genes essential for the degradation of 3-chlorobenzoate and expression of mercury resistance, in addition to the degradation of TFD. Extensive studies have been performed to elucidate the genetic organization and regulatory mechanisms of TFD pathway. These have been performed largely by transposon mutagenesis (26, 44, 82, 101,). The *tfdA* and *tfdB* gene products are TFD monooxygenase and 2,4-dichlorophenol (DCP) hydroxylase, respectively. TFD pathway is depicted in Figure 4. TFD is first converted to DCP by TfdA, which is subsequently hydroxylated to chlorocatechol by the enzyme TfdB. Chlorocatechol is then further metabolized into an intermediate of the tricarboxylic acid cycle owing to a modified *ortho*-cleavage pathway on plasmid and chromosomally originated enzymes. Recently Hausinger et al. (37) reported that *tfdA*, which is generally known as monooxygenase, encoded α-ketoglutarate dependent dioxygenase,

converting TFD to 2,4-DCP and glyoxylate. They did not observe any reductant-dependent activity from this enzyme, suggesting that the enzyme is not a typical monooxygenase.

2,4-Dicholrophenol Hydroxylase

Although chromosomally encoded phenol hydroxylase has not been purified yet, DCP hydroxylase has been purified and well characterized from both *A. eutrophus* JMP134 (62) and *Acinetobacter* species (10). The enzyme isolated from *A. eutrophus* JMP134 appeared to be a simple flavoprotein with molecular mass of 224,000 daltons. NADH or NADPH is needed for the hydroxylation of various substituted phenol. However, the enzyme shows no activity toward the unsubstituted phenol. Similar enzyme activity was also observed from *Acinetobacter* species. The gene *tfdB* was found to exhibit significant sequence similarity to the gene *pheA* which encodes the phenol monooxygenase from *pseudomoans sp.* EST1001 (78).

Regulation of 2,4-dichlorophenol hydroxylase

The gene *tfdB* was known to be under different regulatory control from *tfdA* and *tfdCDEF* operon. Studies on the regulatory mechanism for *tfdB* have come from Olsen et al. (56) who cloned and characterized the gene *tfdS*, the regulatory gene for *tfdB*. They proposed that *tfdS* gene product is a repressor-activator protein. This gene lies between *tfdA* and *tfdR* which is known to negatively regulate *tfdA* and *tfdCDEF*. In the presence of an effector molecule,

which is presumed to be one of the downstream-metabolites of chlorocatechol, the *tfdS* gene product activates the expression of *tfdB*, but in the absence of an effector, the regulatory protein completely represses *tfdB* gene activity. Recently it was found that the *tfd*S is an identical copy of *tfdR* by sequencing analysis (65). The physical map of pJP4 and the genes for TFD pathway are shown in Figure 5.

CHAPTER III

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Bacterial strains, plasmids and cloning vectors used throughout all the experiments in this study are described in detail in Table 3. All recombinant cosmids and plasmids generated in this study are described in Table 4.

Biotransformation Assays

Media and Growth Conditions.

Cells were routinely grown on tryptone-yeast extract-glucose medium (TNA) (80). All strains were grown on minimal salts medium (MMO) (99) supplemented with the appropriate carbon sources (0.05% 2,4-D, 0.025% 2,4-DCP, 0.025% to 0.05% phenol or benzoate, 0.1% ethanol, or 0.3% casamino acids). Antibiotics were used at the following concentrations (in micrograms per milliliter): tetracycline, 25 (*A. eutrophus*) and 50 (*P. aeruginosa*); carbenicillin, 50 (*A. eutrophus*) and 500 (*P. aeruginosa*); kanamycin, 100 (*A. eutrophus*). Typically, 40 ml cultures in 250 ml Erlenmeyer flasks were grown at 30°C and 37°C with shaking at 180 rpm for *A. eutrophus* and *P. aeruginosa*, respectively. Alternatively cells were

directly used for TCE degradation assay.

Chemicals and Reagents.

TCE, 2,4-D, 2,4-DCP, phenol, benzoate, and pentane were purchased from Aldrich Chemical Company. Casamino acids and all antibiotics were purchased from Difco and Sigma, respectively.

TCE Degradation Assay.

Cells grown in an appropriate inducing medium were removed from cultures in mid-log phase, harvested by centrifugation at 10,000 xg for 10 min, and suspended in MMO. These cells were used to inoculate into an appropriate fresh medium to an optical density (as determined at 425 nm) of approximately 1.0. For overnight analysis, cells were grown on TNA agar plates. Overnight-grown cells were suspended in MMO and diluted to an OD_{425} of 0.1. This suspension was used for the assay. 20 ml amount of the respective cell suspension was dispensed into 100-ml serum bottle and sealed with a Teflon-lined stopper. Various concentrations of TCE from stock solution in pentane was added by injection through the septa with a gas-tight syringe (Hamilton, Reno). The culture was incubated at 30°C and 37°C with shaking at 180 rpm for *A. eutrophus* and *P. aeruginosa* respectively. For interval analysis to measure the rate of TCE removal, 0.5 ml samples were removed every two hours and injected into sealed 2-ml serum vial containing an equal amount of pentane. The mixture was extracted by centrifugation at 10,000 xg for 10 min. 1 µl of pentane phase was removed and analyzed on the

GC. For overnight analysis, 1ml of sample after overnight incubation was extracted with the same volume of pentane and 1 µl of pentane phase was removed for GC analysis.

Gas Chromatograhy.

GC analysis was accomplished using a Hewlett-Packard 5890 gas chromatograph equipped with a 25 m cross-linked methyl silicone gum capillary column (Hewlett-Packard) and electron capture or flame ionization detection systems. 1µl of each sample was injected with 10-ul syringe, and peak integrations were obtained with a Hewlett-Packard 3390A integrator. Operating conditions were as follows: injector temperature, 150°C; electron capture detector temperature, 300°C; oven temperature, 35° to 100°C at 15° / min interval; and nitrogen carrier gas flow, 25 ml/min. Under these conditions TCE had a retention time of 2.2 min.

Mutant Isolation by Transposon Mutagenesis

Transposon Mutagenesis.

The recipient strain, *A. eutrophus* AEK101, was grown overnight in TNA broth (TNB) supplemented with rifampicin (150 µg/ml) at 30°C. The donor strains, *E. coli* S17 (pSUP2021) and *E.coli* HB101 (pUW964), were grown to exponential phase in Luria broth (LB) supplemented with kanamycin (50 µg/ml) at 37°C. 1 ml of each donor strain was mixed with 0.5 ml of recipient strain in an 1.5-ml eppendorf tube. The mixture was centrifuged for 1 min in a Beckman microfuge and suspended in 100 µl of TNB medium. The

cell mixture was spread onto nitrocellulose filters (Milllipore Co.), which were placed on TNA plates, and incubated for 4 to 6 hours at 30°C. After incubation each filter was washed with 2 ml of MMO medium. Samples of 0.1 ml of the suspension were spread on TNA plates with rifampicin (150 µg/ml) and kanamycin (100 µg/ml). The plates were incubated at 30°C. Km^r Rif^r colonies were visible after 48 hrs. Alternatively, the donor and the recipient cells were streaked on LB plates and TNA plates with the appropriate antibiotics, respectively. A loopful of overnight grown donor and recipient cells were mixed in 0.5 ml TNB in an 1.5-ml eppendorf tube. An 100 µl aliquot was spread on TNA agar medium and incubated for 4 to 6 hours at 30°C. After incubation, the lawn of bacterial growth on the plates was transferred by replica plating method using sterile velvet clothes onto the selective media.

Screening of Phenol Hydroxylase Deficient Mutants.

Transconjugants grown on selective media (TNA agar plate with rifampicin (150 µg/ml) and kanamycin (100 µg/ml)) were transferred as patch streaks to the same media so that each plate had one hundred colonies. The resulting colonies were transferred by replica plating method to MMO agar plate containing Km (100 µg/ml) and Rif (150 µg/ml) with one of three different combinations of carbon sources, phenol (0.05%), benzoate (0.05%), and phenol (0.05%) plus casamino acids (0.3%). Casamino acids supported the growth of the cells on phenol-containing medium and the phenol utilizing cells turned a dark brown color on this medium due to the formation and

auto-oxidation of catechol. This was an effective preliminary screen for phenol non-utilizing mutants. Limited growth of the transconjugants on phenol as sole carbon source made clear isolation of mutants difficult. After two to three days incubation at 30°C, mutants showing no growth on phenol but growth on benzoate were selected for further study.

DNA Manipulation

Plasmid DNA Isolation.

Rapid isolation of plasmid DNA was done using the modified method of Birnboim and Doly (12) as follows. A loopful of freshly grown cells on plates was suspended in 100 µl to 200 µl of solution A (2 mg/ml lysozyme, 50 mM glucose, 10 mM EDTA, and 25 mM Tris-HCl, pH 8.0) in an eppendorf tube. After 5 min incubation at room temperature, two volumes of solution B (0.2 N NaOH, 1% SDS) was added, mixed well and held on ice for 10 min. 1.5 volumes of solution C (5 M potassium acetate) was added and incubated for further 5 min. The mixture was centrifuged for 2 min at room temperature. The supernatant was extracted with 2 volumes of ethanol, put on ice for 5 min and centrifuged for 2 min. The pallet was vacuum dried and suspended in 80 µl of TE buffer (0.05 M Tris, 0.002 M EDTA, pH 7.5). Further purification of plasmid DNA from *Alcaligenes* and *Pseudomonas* strains was done by extraction with 1 volume of the phenol-chloroform-isoamyalcohol solution before ethanol extraction. The DNA solution was used for plasmid identification, restriction mapping and in some cases, for cloning

experiments.

Large scale isolation was basically the same as a mini-lysis protocol except for the scale-up preparation and the use of CsCl. Cells gathered from 5 to 10 plates were suspended in 5 to 10 ml of solution A in a 40-ml polypropylene tube, followed by adding of solution B and C. The cell mixture was centrifuged at 18,000 xg for 20 min in a Sorvall SS34 rotor at 4°C. The supernatant was extracted by two volumes of 95% ethanol and incubated for 1 hour on ice. The mixture was centrifuged at 18,000 xg for 10 min at 4°C and the pallet was suspended in TE buffer. 1.01 g of CsCl and 0.5 mg of ethidium bromide per 1 ml of TE solution were added and the DNA was separated by centrifugation at 60,000 rpm in a VTi80 rotor for 16 hours or at 100,000rpm in a table top ultra-centrifuge (Optima TL, Beckman) for 4 hours at 15°C . The plasmid DNA was extracted several times with 20 x SSC (3.0 M NaCl and 0.3 M NaCitrate) saturated isopropanol, added with three volumes of distilled water and precipitated with two volumes of 100% ethnol.

Chromosomal DNA Isolation.

Chromosomal DNA for the construction of a gene library and cloning experiments was prepared essentially as described by Frederick et al. (5). Cells grown on TNB broth (100 ml in a 500-ml flask) were harvested by centrifugation at 8,000 xg for 10 min in a Sorvall SS34 rotor at 4°C in a 50-ml polypropylene tube. The pellet was suspended in 9.5 ml TE buffer(10 mM Tris.Cl, 1 mM EDTA, pH 8.0) with 0.5 ml of 10% SDS and 50 µl of 20 mg/ml proteinase K (Sigma). After mixing and incubating for one hour at 37°C, 1.8 ml of

5 M NaCl and 1.5 ml of CTAB/NaCl solution (10% hexadecyltrimethyl ammonium bromide in 0.7 M NaCl) were added, mixed thoroughly and incubated for 20 min at 65°C. The mixture was extracted with an equal volume of chloroform/isoamyl alcohol and centrifuged at 8,000 xg for 10 min at room temperature to separate phases. The aqueous phase was transferred to a fresh tube and DNA was precipitated with 0.6 volume of isopropanol. DNA pellet was transferred into an 1.5-ml eppendorf tube, washed with 1 ml of 70% ethanol, centrifuged in a microfuge for 2 min, and resuspended in 4 ml of TE buffer. 4.3 g of CsCl and 200 µl of 10 mg/ml ethidium bromide were added in 4 ml DNA solution and the chromosomal DNA was banded in a 4-ml sealable centrifuge tube at 55,000 rpm for overnight at 15°C in a Beckman VTi80 rotor.

Chromosomal DNA band was removed using a 16-G needle and a 3-ml plastic syringe, extracted with 20xSSC saturated isopropanol and dialyzed overnight against 2 liters of TE buffer. The dialyzed DNA solution was precipitated by adding 1/10 vol of 3 M sodium acetate and 0.6 vol of isopropanol, and resuspend in STE buffer (10 mM Tris.Cl, 10 mM NaCl, 1 mM EDTA, pH 7.5).

Miniprep of genomic DNA for Southern blot experiment was done according to Frederick et al. (5). Cells grown on plates were suspended in 567 µl of TE buffer with 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K in an 1.5-ml eppendorf tube. After mixing and incubating for one hour at 37°C, 100 µl of 5 M NaCl and 80 µl of CTAB/NaCl solution were added, mixed thoroughly and incubated for 10 min at 65°C. The mixture was extracted with an equal volume of chloroform/isoamyl alcohol and centrifuged for 5 min in a microfuge

at room temperature. After the aqueous phase was transferred to a fresh tube, DNA was precipitated with 0.6 volume of isopropanol, washed with 1 ml of 70% ethanol, centrifuged for 2 min, and resuspended in 4 ml of TE buffer.

Construction of Genomic Library

Partial Digestion and Size Fractionation

Partial digestion and size fractionation of genomic DNA from *A. eutrophus* AEO106 were performed according to Maniatis et al. (64). 500 µl (0.1 mg/ml) of genomic DNA suspended in STE buffer was partially digested with 0.1 U of *Hind*III for 20 min at room temperature. This condition gave the best result to obtain the fragments in the range of 20 to 30 kb. Partially digested DNA solution was loaded on two 12-ml SW-41 tubes in which linear 10% to 40% sucrose gradient was prepared, and centrifuged at 25,000 rpm for 20 hours at 20°C in an SW-41 rotor (Beckman). The gradient was fractionated by carefully removing every 500 µl of aliquots from top to bottom of the tubes. The size of the collected DNA fractions was analyzed by electrophoresis of 40 µl samples of the gradient aliquots on a 0.7% agarose gel. The fractions containing correctly sized DNA were combined and divided into eppendorf tubes, each containing 180 µl of DNA solution. Each tube was added with 320 µl of water and 1 ml ethanol, and then incubated at -80°C for more than two hours. DNA was precipitated in a microfuge for 10 min and resuspended in 100 µl of TE buffer.

Preparation of Vector DNA.

100 µg of cosmid vector pVK102 was completely digested with 5 U of *Hind*III for 2 hours at 37°C. After the reaction, the enzyme was inactivated by heating 15 min to 75°C. 5 U of calf intestinal alkaline phosphatase (CIP) (Promega) was added, and the mixture was incubated for 1 hour at 37°C. CIP was also inactivated by heating 15 min to 75°C. Vector DNA was extracted with an equal volume of phenol/chloroform solution, centrifuged for 5 min, precipitated the upper phase with 2 vol of 100% ethanol, washed with 1 ml of 70% ethanol, and resuspended in 50 µl of TE buffer.

5 µg to 15 µg of chromosomal DNA were ligated with 5 µg of vector DNA using T4 DNA ligase and 5x buffer (30 mM Tris.HCl ,pH 7.8, 10 mM $MgCl_2$, 10 mM DDT and 1 mM ATP) at 15°C for 24 hours in a total volume of 50 µl in an eppendorf tube.

Packaging and Transduction

Ligated DNA was packaged with lambda DNA packaging extracts (Promega) according to manufacturer's recommendation. 25 µl of Lambda packaging extracts was mixed with 10 µl of ligated DNA (~1µg) in a microcentrifuge tube and incubated for 2 hours at room temperature. After incubation, 0.5 ml of phage buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MgSO_4$) and 25 µl of chloroform were added, then it was mixed gently by inversion and allowed the chloroform to settle to the bottom of the tube. The supernatants were diluted in phage buffer.

Bacterial cells for phage adsorption were prepared as follows.

0.5 ml of *E. coli* LE392 grown overnight on LB broth was inoculated into 50 ml of LB medium supplemented with 0.5 ml of 20% maltose (Difco) and 1 M $MgSO_4$ in a 250-ml flask. This was shaken at 37°C until the OD_{600} has reached 0.6 to 0.7, then stored at 4°C until needed. 100 µl of prepared cells was mixed with 100 µl of appropriately diluted packaging reactions, and incubated at 37°C for 30 min for phage adsorption. The mixture was shaken at 37°C for 1 hour after adding 800 µl of NB medium for the expression of antibiotic resistance. The aliquots were spread on the LB agar plates supplimented with Tc^{20} and incubated at 37°C until cosmid clones appeared.

Gene Amplification

The cosmid clones were amplified as follows. The bacteria containing cosmids were spread on 85 mm nitrocellulose filters (5×10^3 to 1.0×10^4 per each filter) (Millipore Co.) on LB plates with Tc^{20}. 15 independent plates were incubated for 12 to 14 hours until colonies (0.2-0.3 mm diameter) appeared. The colonies were scraped from each plate and suspended in 50ml of LB medium with 15% glycerol. Each aliquot (1ml) was dispensed into an 1.5-ml eppendorf tube and stored at - 80°C.

Alternatively cosmid libraries were amplified in liquid culture. *E. coli* LE392 infected with approximately 2×10^4 of the packaged cosmids were incubated in 20 ml of LB broth containing Tc^{20} at 37°c until the culture has reached mid-log phase. The culture was then added with 15% glycerol and 1 ml of each aliquot was dispensed into an eppendorf tube, and then stored at - 80°C.

Mutant Complementation Test

Triparental Replica Plating Method

Triparental replica plating method was used to screen the gene bank for complementation of *A. eutrophus* mutants as described by Andersen et al. (2). An appropriate titer of gene bank (~1,000 to 3000 colonies per plate) was spread on LB plate containing Tc^{20}. After overnight incubation, the resulting colonies (0.5 to 1.0 mm) were transferred by replica plating onto TNA plates containing a newly spread lawn of *E. coli* HB101(pRK2013) plus *A. eutrophus* mutant to be complemented. The cells used for preparing the lawn were from overnight grown cells on TNA plate containing Km^{100} plus Rif^{150} for *A. eutrophus* mutant and on LB plate containing Km^{100} for *E. coli*. A loopful of each strain was mixed in 200 µl of TNB medium and 100 µl of the mixture was used to prepare for the lawn. The mating plates were incubated 4 to 6 hours at 30°C. Cells from the mating plates were then transferred to MMO plus 0.03% phenol plates supplimented with Km^{100}, Rif^{150} and Tc^{20} by replica plating. *A. eutrophus* colonies resulting from complementation appeared after incubation for 2 to 3 days at 30°C. The suspected area of *E. coli* clones, if too crowded, were scraped from the master plates, and then spread again on LB plates to obtain 50 to 100 colonies per plate. These plates were used for the same triparental mating experiment as described above. The complementing *E. coli* gene bank clones were then identified and purified for further study.

Triparental Conjugation

Triparetal mating method (24) was performed to transfer the recombinant pVK102 plasmids or pMMB67 plasmids from *E. coli* to *A. eutrophus* in the presence of another donor strain *E. coli* HB101 (pRK2013). Loopfuls of parental cells which were grown overnight on the appropriate plates were mixed in TNB medium in the ratio of 1:1:1. An aliquot was spread on TNA, then incubated for 3 to 5 hours at 30°C. Transconjugants were selected by streaking or plating dilutions of the mating mixture onto the appropriate selective media.

Genetic Manipulation (Mapping, Cloning, and Transformantion)

Cloning of *Tn*5 Flanking Sequences

*Tn*5-flanking DNA was cloned from *A. eutrophus* mutants by completely digesting total DNA isolated with *Eco*RI. The vector plasmid, pGEM (Promega Co.) was completely digested with *Eco*RI, dephosphorylated with alkaline phosphatase and purified as described previously. 4 μg of chromosomal DNA was mixed with 1 μg of pGEM in the presence of T4 DNA ligase overnight at 15°C. 40 μg of the ligated DNA was used to transform 90 μl of *E. coli* JM109 competent cells prepared as described below. Transformants were selected on LB medium containing kanamycin (50 μg/ml) and ampicillin (50 μg/ml). The plasmid from transformants was isolated and the presence of *Tn*5 was confirmed by the analysis of restriction mapping and southern hybridization.

Subcloning Procedure

Cosmids and plasmids for cloning experiments were purified as described above. The purified recombinant cosmids were partially digested with the appropriate restriction enzymes, and the fragments to be cloned were isolated in 0.7% low melting agarose gel (SeaPlaque, FMC). Vector DNA was single- or double-digested with restriction enzymes. Single-digested vector DNA was treated with alkaline phosphatase as previously decribed. The digested vector DNA was also isolated in 0.7% low melting agarose gel. After running the gels with different time interval at 4°C, the gels were stained with ethidium bromide, visualized under UV light, and then the fragments of DNA to be cloned were excised from the gels. The gel slices containing the desired fragments were melted at 68°C for 10 min in microcentrifuge tubes, and frozen quickly by putting the tubes at -80°C for 10 min . These were microcentrifuged for 5 min, and the supernatants were directly used for ligation or further purified by phenol/chloroform extraction and ethanol precipitation as described previously.

DNA fragments cloned into pTZ18R or pGEM were transformed into *E. coli* HB101 or JM109 and transformants were selected on LB plates containing ampicillin (50 μg/ml), 0.5 mM IPTG and X-Gal (40 μg/ml). White colonies were picked after 12 to 18 hours incubation at 37°C. The presence of the recombinant plasmid DNA was confirmed by rapid isolation of plasmid and restriction endonuclease digestion.

DNA fragments cloned into pMMB67 vector plasmid were

transformed into both *P. aeruginosa* PAO1c and *E. coli* HB101 or JM109. *P. aeruginosa* transformants were selected on TNA plate containing carbenicillin (500 μg/ml) and *E. coli* transformants were selected on LB plates containing ampicillin (100 μg/ml).

DNA fragments cloned into pRO1727, pRO2321 and pRO1769 were transformed into *P. aeruginosa*. The transformants were selected on TNA medium containing carbenicillin (500 μg/ml) or tetracycline (50 μg/ml), trimetoprim (600 μg/ml), and gentamycine (20 μg/ml) for pRO1727, pRO2321 and pRO1769, respectively. Transformants were initially picked up by insertional inactivation, and then confirmed by restriction enzyme analysis with isolated plasmids.

Agarose Gel Electrophoreses and
Mapping of Recombinant DNA

Agarose gel electrophoresis was performed as described by Maniatis et al. (64). Various DNA samples, which were generated by partial digestion and/or double digestion with different restriction enzymes, were separated on 0.5 to 1.5% horizontal agarose gels (depending on the size of the fragments to be analyzed) using TAE buffer (pH 7.4, 40 mM Tris-acetate, 2 mM EDTA) at room temperature. After running, the gels were stained in deionized water containing ethidium bromide (1 μg/ml) and visualized under UV light. Fragment sizes were estimated by comparison with λ DNA cleaved with *Hind*III or *Hind*III and *Eco*RI, which was used as a size standard.

Preparation of Competent Cells and Transformation

competent cells for *E. coli* HB101 and JM109 were prepared basically as described by Maniatis et al. (64). A single colony of cells was inoculated into 40 ml of LB medium, and grown at 37°C overnight with shaking. 2 ml of this culture was transferred to 400 ml of fresh LB medium and grown at 37°C with shaking. When the culture reached an OD_{590} of 0.3 to 0.4, 40 ml of aliquots was dispensed into prechilled centrifuged tubes and left on ice for 10 minutes. The tubes were centrifuged for 5 minutes at 2,000 xg at 4°C, and each tube was suspended in 10 ml of ice-cold, sterile 0.1 M $CaCl_2$ solution. The cells were centrifuged at 1,600 xg at 4°C, resuspended in the same solution, and the tubes were left on ice for 30 minutes. These were centrifuged again at 1,600 xg at 4°C, and each tube was finally resuspended in 2 ml of 0.1 M $CaCl_2$ solution containing 15% of glycerol. 400 µl of aliquots of competent cells were dispensed into prechilled, sterile tubes and stored at -80°C until needed. For transformation, competent cell aliquots were rapidly thawed and then 90 µl of cells were mixed with 5 µl of ligated DNA. After 30 min incubation on ice, the mixture was heat-shocked at 42°C for 2 min, placed on ice for 2 min, added 1 ml of LB medium, and then shaken at 37°C for 1 or 2 hours. A portion of the cells was plated on the appropriate selective media.

Competent cells for P*seudomonas aeruginosa* were prepared basically as described by Mercer et al. (66). 1 ml of overnight grown cells was inoculated into 80 ml of TNB medium and incubated at 30°C by vigorous shaking. When the cells reached an OD_{425} of 0.4, these

were shifted into 42°C water bath and incubated without shaking until an OD_{425} reached at 0.8 to 0.9 to make the cells restrictionless. The cells were then dispensed into 40 ml centrifuge tubes and placed on ice for 10 min. These were centrifuged for 10 min at 2,000 xg at 4°C, and each tube was suspended in 10 ml of ice-cold, sterile 0.15 M $MgCl_2$ solution. The cells were again centrifuged for 10 min at 2,000 xg at 4°C, resuspended in the same solution, and the tubes were left on ice for 30 minutes. These were centrifuged again at 2,000 xg at 4°C, and each tube was resuspended in 2 ml of 0.15 M $MgCl_2$ solution containing 15% of glycerol. 200 µl of aliquots of competent cells were dispensed into prechilled, sterile tubes and stored at -80°C until needed. For transformation, competent cells were rapidly thawed and then 60 µl of cells were mixed with 5 µl of ligated DNA. After 50 min incubation on ice, the mixture was heat-shocked at 41°C for 2 min, placed on ice for 5 min, added 500 ml of TNB medium, and then incubated at 37°C for 2 hours without shaking. A portion of the cells was plated on the appropriate selective media.

Southern Hybridization

DNA-DNA hybridization experment was performed according to the method by Southern. (97).

Preparation of Membrane-bound Denatured DNA

Chromosomal DNA from *A. eutrophus* mutants was isolated as described before. DNA was completely digested with *Eco*RI or *Bam*HI and run on 0.7% agarose gel at 4°C overnignt. After electrophoresis, DNA was depurinated by soaking the gel in 0.25 M HCl twice for 15

minutes each. It was then denatured by soaking the gel twice for 15 minutes each in a solution containing 0.5 M NaOH and 1.5 M NaCl. It was neutralized in 0.5 M Tris and 1.5 M NaCl (pH 7.0) twice again for 15 minutes each. The DNA was transferred to a Nylon membrane (Zeta probe GT, BioRad) with vacuum blotter (MilliBlot-V, Millipore) using transfer solution 20x SSC (3.0 M NaCl and 0.3 M sodium citrate). After transfer, the DNA-binding membrane was briefly rinsed in 2x SSC and air dried. It was then dried in a vacuum-oven at 80°C for 2 hours.

Prehybridization and Hybridization

Prehybridization was performed by sealing the membrane into a heat sealable plastic bag containing 10 ml of hybridization solution (0.25 M Na_2HPO_4 [pH 7.2] and 7% SDS), and then the bag was incubated at 65°C for more than 4 hours. One corner of the bag was cut and the solution was removed and replaced with 2 ml of fresh hybridization solution. Heat denatured DNA probe was then added into the bag and air bubbles were removed as much as possible before resealing the bag. Hybridization was conducted by incubating the bag at 65°C for 16 hours with agitation. The membrane was removed from the bag and washed in 20 mM Na_2HPO_4 (pH 7.2) and 5% SDS solution at 65°C twice for 30 minutes each. It was washed again twice in 20 mM Na_2HPO_4 (pH 7.2) and 1% SDS solution at 65°C for 30 minutes each. After washing, the wet membrane was wrapped in a plastic wrap and exposed to X-ray film (Kodak XR) overnight at - 70°C with an intensifying screen. The film was developed according to manufacturer's instructions.

Preparation of Probe DNA

Plasmid pSUP2021 was digested with *Hind*III and Cosmid pYK301 was cut with *Eco*RI. These were separately run on a 0.7% low melting agarose gel. Gel slices containing 3.7 kb *Hind*III fragment or 5.5 kb *Eco*RI fragment was cut off each gel and the DNA fragments were purified by phenol/chloroform extraction and ethanol precipitation as described previously. DNA pellet washed with 70% ethanol was dissolved in TE buffer and used for nick translation.

Nick Translation

Nick translation was performed with a kit purchased from Promega. A typical reaction mixture contained 0.5 to 1.0 µg of the DNA, 10 µl of nucleotide mix (5 µM each of cold dCTP, dGTP, dTTP), 5 µl of nick translation buffer (50 mM Tris-HCl, pH 7.2 and 10 mM $MgSO_4$), 7 µl of [α-^{32}P] dATP (400 Ci/mM at 10 mCi/ml), 5 µl of enzyme mix (DNA polymerase I at 1 U/µl and DNase I at 0.2 ng/µl) and sterile water to attain a final volume of 50 µl. The reaction mixture was incubated at 15°C for 1 hour and then stopped by adding 5 µl of 0.2 M EDTA (pH 8.0). The labeled probe DNA was purified by ethanol precipitation after addition of 1/10 volume of 3M ammonium acetate. The DNA pellet was washed with 70% ethanol and dissolved in TE buffer. The radiolabeled probe was immediately used for hybridization after denaturation by heating at 95 to 100°C for 5 min or stored at -80°C until needed.

Colony Hybridization Experiment

Colony hybridization to localize the cosmid clones which complement *A.eutrophus* mutant strains was performed as described by Frederick et al. (5).

Preparation of Probe DNA

DNA probe for colony hybridization was prepared as follows. *Tn*5 containing recombinant plasmid cloned from *A.eutrophus* mutant chromosomal DNA was isolated as described before. The 14.5 kb pAEK201 was completely digested with *Eco*RI and *Hind*III, and then run on 0.7% low melting agarose gel. After running the gel, a gel slice containing 5.0 kb *Eco*RI-*Hind*III DNA fragment was excised and purified by phenol/chloroform extraction and ethanol precipitation, then used for nick translation as described previously. Alternatively a 14.5 kb *Hind*III insert DNA was used as a probe.

Preparation of Membrane-bound Colonies

82 mm nitrocellulose filters (Millipore, Triton-free, HATF) were used to prepare the cosmid clones. The appropriate dilution of cosmid clones (2,000 to 5,000 colonies per filter) from gene bank was grown on autoclaved nitrocellulose filters laid on LB plates plus ampicillin (100 μg/ml). When the sizes of the colonies were about 0.1 to 0.2 mm, the filters were used as master filters to make replica filters. Both master and replica filters were laid on fresh agar plates and these were incubated at 37°C until 1 to 2 mm of colonies appeared. The master plates were sealed with parafilm and stored

at 4°C until needed. The replica filters were peeled off the plates and stored at 4°C for 20 min to cool down the colonies. The filters were then placed on 3 MM paper (Whatman) saturated with 10% SDS for 3 min, transferred onto the same paper saturated with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 min, and finally transferred onto the third sheet of 3 MM paper saturated with neutralizing solution (1.5 M NaCl, 0.5 M Tris. Cl [pH 8.0]) for 5 min. After drying DNA binding filters at room temperature, these were baked for 2 hours at 80°C in a vacuum oven.

Prehydridization and Hybridization

Hybridization was performed as follows. Five DNA-binding filters were prehybridized with 10ml of prehybridization solution (1% SDS, 1 M sodium chloride, and 10% dextran sulfate) in a sealed plastic bag at 65°C overnignt with constant agitation. The prehybridization solution was discarded and a bag was refilled with 5 ml of the same solution containing denatured salmon sperm DNA (100 μg/ml) and denatured radioactive probe (100 ng/ml) prepared by nick translation. A resealed plastic bag was incubated overnignt with constant agitation at 65°C. After hybridization, the filters were washed with 100 ml of 2x SSC (0.3 M NaCl and 0.03 M sodium citrate) twice for 5 min each at room temperature with agitation, with 200 ml of 2x SSC containing 1% SDS twice for 30 min each at 65°C with agitation, and finally washed with 100 ml of 0.1x SSC (15 mM NaCl and 1.5 mM sodium citrate) twice for 30 min each at 65°C with agitation. After washing, the filters were dried at room temperature, wrapped in a plastic wrap and exposed to X-ray film

(Kodak XR) overnight at -70°C with an intensifying screen. After development the film according to manufacturer's instructions, the position of positive hybridization signals was located from the master plates. When the growth was too congested, the suspected area was cut and resuspended in LB medium, and then an appropriate dilution (~100 colonies per plate) was made on LB agar plate containing ampicillin (50 μg/ml). After the growth (2 to 3 mm of colonies), the plate was stored at 4°C for 20 min, and then a nitrocellulose filter was contacted with the colonies grown on plates for 3 min. The filter on which colonies attached was treated for hybridization as described above. Each positive colony was picked and isolated. The cosmid DNA from each colony was isolated and characterized by mapping and complement test.

Analysis of Enzyme Activities

A. eutrophus and *P. aeruginosa* for enzyme assays were grown on MMO supplimented with various carbon sources at 30°C and 37°C, respectively, with vigorous shaking. The cells were harvested in late-log phase and washed twice with MMO. Phenol hydroxylase activity was assayed with the resting cells using a Clark oxygen electrode as described by Sala-Trepat et al. (91). The cells were suspended in MMO to an optical density (OD_{425}) of 1.0. After shaking for 1 to 2 hours to remove any remaining carbon sources, cell suspension was added to the electrode chamber (Gilson Medical Electronics), and the concentration of dissolved oxygen measured with a Clark oxygen electrode (Yellow Springs Instrument Co.). Base-line consumption of oxygen in MMO alone was established followed

by injection of 0.3 mM of phenol. Enzyme activity was expressed as the increase above basal levels of oxygen consumption upon addition of phenol in nmol per hour at a culture density of OD_{425} = 1.0. Catechol 2,3-dioxygenase was assayed by the method of Nozaki (77) and catechol 1,2-dioxygenase was measured by the modified procedure of Hegeman (46). Cells prepared as the above was resuspended in phosphate-acetone buffer (0.05 M potassium phosphate [pH 7.5] and 10% acetone) for catechol 2,3-dioxygenase and in Tris buffer (20 mM Tris.Cl and 400 µM EDTA) for catechol 1,2-dioxygenase. The cell was suspended in an appropriate buffer in an eppendorf tube (approximately 200 µg/ml) and disrupted by sonication (Branson Sonifier) while the cell was kept on ice. The cell debris was then removed by centrifugation at 13,000 x g for 15 min in microfuge (Beckman). The supernatant was immediately used to measure enzyme activities. Catechol 2,3-dioxygenase was assayed by measuring the increase in optical density at 375 nm due to the formation of the reaction product, 2-hydroxymuconic semialdehyde. Catechol 1,2-dioxygenase was assayed by measuring the increase in optical density at 256 nm due to the formation of cis-cis muconic acid. The reaction was performed at room temperature in 1.0 ml quartz cuvettes with 1 cm light path. The final volume of 1 ml contained 970 µl of phosphate-acetone buffer for catechol 2,3-dioxygenase or 970 µl of Tris buffer for catechol 1,2-dioxygenase, 10 µl of 0.01 M catechol, and 20 µl of cell extact. The reaction was initiated by the addition of enzyme. One unit of enzyme is defined as the amount that oxidizes 1 µmole of catechol per minute. Specific activity is expressed as units per milligram of protein. Protein

concentration was determined by the Bradford procedure (14) with bovine serum albumin as the standard. UV absorbance was measured on a Shimadzu UV-160 spectrophotometer.

CHAPTER IV

RESULTS

Cloning of the Structural Genes for Phenol Hydroxylase and Catechol 2,3-dioxygenase from *A. eutrophus* AEK106

Isolation and Characterization of *Tn*5-Induced Mutants

The plasmid pUW964 was used to generate *Tn*5-induced *A. eutrophus* AEK101 mutants defective in phenol metabolism. The plasmid pUW964 has a ColE1 origin of replication and the broad-host-range conjugation genes from RK2, so it can be transferred by itself into *A. eutrophus*. This plasmid is able to replicate in *E. coli*, but not in *A. eutrophus* due to the limited host range for ColE1 origin. The recipient *A. eutrophus* AEK101 was isolated after growth of the parental strain, *A. eutrophus* AEO106, on TNA containing rifampicin (150 μg/ml), hence TNA agar plates containing kanamycin and rifampicin effectively selected *A. eutrophus* AEK101*Tn*5 exconjugants and did not permit growth of the donor cells. Replica plating methods enabled the isolation of independent exconjugants from the selective medium. Over 6,000 Km^r Rif^r exconjugants was obtained by conjugation. These exconjugants were tested for their growth on phenol as a sole carbon and energy source. The resulting colonies were screened for their growth on benzoate to isolate mutants deficient in phenol hydroxylase which is the first enzyme

metabolizing phenol into catechol. This screening procedure was adopted because benzoate and phenol are catabolized via catechol, a common intermediate. Results of enzyme assays using these two metabolites demonstrated that both of catechol 1,2-dioxygenase for *ortho*-ring cleavage of catechol and catechol 2,3-dioxygenase for *meta*-ring cleavage pathway were induced in *A. eutrophus* AEO106 and AEK101 by phenol. Only catechol 1,2-dioxygenase activity was detected during growth on benzoate, indicating that benzoate is metabolized through *ortho*-cleavage pathway (Table 5). In light of these results, it was presumed that a mutation which allows growth on benzoate, but not on phenol, should be deficient either in the phenol hydroxylase structural gene or in its regulatory gene(s).

Five mutants which showed growth on benzoate, but not on phenol, were isolated. All the mutants appeared to be sensitive to streptomycin which is encoded on pUW964, and no plasmid was detactable in these mutant strains.

Some enzymes are subject to severe catabolite repression. Several carbon sources were screened for repression in order to find a primary carbon source which would minimally affect the enzyme activities (Table 5). The activity of phenol hydroxylase was slightly reduced by either ethanol (0.1%) or benzoate (2.5 mM). Addition of 5.0 mM benzoate decreased this enzyme activity about three fold. Other carbon sources such as casamino acid or glucose also repressed the phenol hydroxylase activity to a level similar to that of 5.0 mM benzoate (data not shown). Catechol 2,3-dioxygenase was severely affected by the presence of benzoate. 5.0 mM benzoate repressed this enzyme activity to levels over five hundred times less than that

of AEO106 grown on phenol only. Ethanol was less repressive. In contrast, catechol 1,2-dioxygenase was not affected at all. AEK101 appeared to be slightly more sensitive to catabolite repression than the wild type, AEO106.

Enzyme activities in mutant AEK301 were analyzed (Table 5). Ethanol (0.1%) or benzoate (2.5 mM) was used as a carbon source to reduce the catabolite repression. AEK301 grown on ethanol expressed negligible amounts of three enzyme activities tested. But when grown on benzoate, catechol 1,2-dioxygenase was expressed in the same level as the wild type. Other mutants (AEK302 to AEK305) also showed similar results to AEK301 (data not shown). The loss of three enzyme activities caused by single site mutation in these mutants would imply that the genes specifying these enzymes lie in an operon or these genes are controlled by a single disrupted regulatory mechanism.

Southern analysis for *Tn*5 insertion.

The physical characterization of the sites of *Tn*5 insertion was carried out by Southern blot analysis of the total genomic DNA isolated from the mutants. The genomic DNA was digested with *Eco*RI, which does not cleave within *Tn*5, and hybridized with ^{32}P-labeled internal *Hind*III fragment of *Tn*5. The hybridization pattern showed that all mutants contained *Tn*5 in the same 11.5 kb *Eco*RI fragment (Fig. 6). Further digestion of the genomic DNA with *Bam*HI also showed two bands (7.8 and 8.2 kb each) from all five mutants, suggesting that insertion of *Tn*5 occured at the same site in all mutants.

Cosmid Library of A. eutrophus DNA

A. eutrophus AEO106 DNA was partially digested with *Hind*III. A total of 10 μg DNA (~20 to 30kb) was ligated with 5 μg of *Hind*III-digested and phosphatase-treated pVK102. A portion of ligated DNA was then packaged into lambda particles in *vitro* and the particles were transduced into *E. coli* LE392. The transductants were selected on tetracycline-containing LB plates. Approximately 1 μg of packaged DNA yielded 0.5 to 1.0 x 10^4 independent Tc^r clones. These transductants were analyzed for their resistance to kanamycin. Only a few of these (2 or 3 out of 100 clones) were found to be kanamycin resistant, implying that most clones have chromosomal DNA inserts. A portion of clones were analyzed for the sizes of the inserted DNA. These were ranged from 17 to 35 kb, with an average of 25 kb.

Identification of Cosmid Clones Complementing Mutant Strains

Triparental replica plating method was employed to identify cosmid clones complementing phenol hydroxylase-defective mutants from *E. coli* LE392 gene bank. Complementing mutants were successfully identified on the selective medium which contained MMO plus 2.5 mM phenol in the presence of tetracycline (50 μg/ml) and rifampicin (150 μg/ml). Each positive clone was isolated from master plate and analyzed for the presence of cosmid. Three kinds of cosmids were isolated from all positive clones. These are designated pYK301, pYK302, and pYK303 after analysis of their restriction

enzyme cleavage patterns (Fig. 9). Both pYK302 and pYK303 were found to contain a common 16.8-kb *Hind*III fragment of pYK301.

The three enzyme activities in the mutant strains were analyzed after transfer of the positive cosmids by triparental mating (Table 6). AEK301 harboring any one of three cosmids (pYK301 to pYK303) expressed both phenol hydroxylase and catechol 2,3-dioxygenase activities. The activity of catechol 1,2-dioxygenase still remained repressed when grown on ethanol, but in the presence of benzoate this enzyme activity was expressed. Similar results were obtained with the other mutant strains (data not shown). Different induction mode of catechol 1,2-dioxygenase implies the existence of two isofunctional enzymes in AEK101. Bayly et al. (49) also reported that two or more isofunctional enzymes were present at various steps during the metabolism of phenol, p-cresol, and toluate in *A. eutrophus* 345.

Although the mutants harboring these cosmids were initially identified for their growth on the selective media, it was found later that no cosmid would allow AEK301 to grow on phenol as a sole carbon and energy source. Growth on selective media is probably due to the presence of ethanol which was used for the preparation of antibiotics (Tc and Rif). Under this condition, complementing mutants were distinguished by the thick brown-yellowish growth, indicating accumulation of 2-hydroxymuconic semialdehyde or photo-oxidation of catechol. No detectable growth on phenol as a sole carbon source implies that intermediate genes responsible for the conversion of 2-hydroxymuconic semialdehyde are missing or not expressed enough to support the growth on phenol.

Though the activity of phenol hydroxylase in AEK301 carrying pYK301 is expressed in the similar level to that of AEK101 (24.15 vs. 28.56), catechol formed by this enzyme does not seem to be channeled into the *ortho*-cleavage pathway. This indicates that catechol 1,2-dioxygenase on benzoate pathway is not induced by its substrate, catechol.

Subcloning and Localization of the Genes for Phenol Hydroxylase and Catechol 2,3-dioxygenase

To localize the genes for phenol hydroxylase and catechol 2,3-dioxygenase, cosmid pYK301 was first cut with *Hind*III. From the resulting 16.8 kb *Hind*III fragment a series of BamH1 deletions were made and subcloned into tac expression vector pMMB67EH (Fig. 12). Only plasmid pYK3011, a 11.2 kb *Hind*III-*Bam*HI fragment (coordinates 0 to 11.2 kb), allowed AEK301 to express the activities of phenol hydroxylase and catechol 2,3-dioxygenase. To further localize the gene specifying phenol hydroxylase, various deletion and subclones were prepared from pYK3011 after mapping for restriction endonuclease sites (Fig. 13). Plasmid pYK3021, a 9.1 kb *Xho*I-*Bam*HI fragment (coordinates to 2.1 to 11.2 kb) expressed phenol hydroxylase activity, but catechol 2,3-dioxygenase activity was not detected in this plasmid. Neither plasmid pYK3022 (coordinates 0 to 9.3 kb) nor pYK3020 (coordinates 2.6 to 11.2 kb) allowed AEK301 to restore phenol hydroxylase activity, indicating that more than 6.4 kb are needed for the activity. A large sized phenol hydroxylase gene complex (5.5 kb) has been reported in *Pseudomonas* CF600 strain by Shingler et al. (76, 86). They have demonstrated that this fragment

encoded six distinct proteins and all components were required for the growth on phenol in other *Pseudomonas* strains which lack this enzyme. A multi-component phenol hydroxylase enzyme similar to that of *Pseudomonas* CF600 might be operational in *A. eutrophus* AEO106. Plasmid pYK3022, though it failed to express phenol hydroxylase activity, allowed AEK301 to express catechol 2,3-dioxygenase activity. Further deletion and subcloning of this plasmid resulted in plasmid pYK3024, which carries 4.1 kb *Hind*III-*Eco*R I fragment. Because *Xho*I site (coordinates 2.6 kb) is needed for phenol hydroxylase activity as shown in pYK3020 and pYK3021, the gene specifying catechol 2,3-dioxygenase should be present within a 2.6 kb *Hind*III-*Xho*I fragment.

Plasmid pYK3024 expressed high levels of catechol 2,3-dioxygenase activity, comparable with those of AEK101 grown on phenol (Table 6). Generally as the size of DNA insert was decreased, higher activity of catechol 2,3-dioxygenase was observed. Plasmid pYK3021 also exhibited higher phenol hydroxylase activity than pYK3011 which contains 2.1kb more DNA. However, phenol hydroxylase activity was shown to be higher in cosmid pYK301 than in both subcloned plasmids (about two fold increase than pYK3011), even though the pYK301 is twice as large and much lower in copy number. This result implies that either promoter region for the binding of positive regulator is deleted or *trans*-acting activator is inactivated by deletion of 5.6 kb *Bam*HI-*Hind*III fragment from pYK301. It seemed that catechol 2,3-dioxygenase was not affected by the deletion of this portion, since little difference of this enzyme activity was observed in either plasmid.

Expression of Phenol Hydroxylase in *Pseudomonas aeruginosa* PAO1c

Early attempts to isolate the phenol hydroxylase gene by direct insertion of chromosomal DNA of *A. eutrophus* AEO106 into *P. aeruginosa* PAO1c through the construction of a plasmid library failed. PAO1c cannot grow on phenol due to the lack of the phenol hydroxylase gene, but it possesses an *ortho*-cleavage pathway for catechol metabolism. It was thought that upon the addition of external source of active phenol hydroxylase, this strain would grow on phenol as a sole carbon and energy source. Olsen et al. (59) succeeded in isolating phenol hydroxylase gene from the chromosome of *P. pickettii* PKO1 by using this simple strategy. Even the cosmid library did not allow us to isolate a positive transformant. Three cosmids (pYK301 to pYK303) which complemented mutant strains AEK301 were inserted into PAO1c by transformation and each transformant was analyzed for their growth on phenol. It was at first very surprising that none of the cosmids allowed PAO1c to grow on phenol. Phenol hydroxylase activity was also not detected in any cosmid in PAO1c. However, this enzyme activity was detected in pYK3011 and pYK3021 at a comparable level to that of AEK301 (Table 7). The activity was detected only in the presence of phenol, indicating the gene is transcribed from its own promoter. These facts have led us to suspect the existence of negative regulator which was inactivated by deletion of 5.6kb *Bam*HI-*Hind*III fragment.

PAO1c containing pYK3011 was able to barely grow on phenol

owing to the presence of catechol 1,2-dioxygenase. Very small colonies were visible on phenol containing MMO plate only after four or five days of incubation, but no growth was observed in liquid medium. An extraordinary fragment (over 6.5kb) was needed for the expression of phenol hydroxylase activity in PAO1c, but catechol 2,3-dioxygenase activity was not detected in any plasmids in PAO1c (Fig. 13, and Table 7). This indicates that the catechol 2,3-dioxygenase gene is under different regulatory control from that of phenol hydroxylase gene. Separation of structural genes encoding phenol hydroxylase activity and the catechol *meta* pathway enzymes into different regulatory regions has been proposed by Wigmore et al. in *Pseudomonas putida* U (8, 11, 116) and by Bayly et al. in *Alcaligenes eutrophus* 335 (48).

Regulation of Phenol Degradation Pathway in *A. eutrophus* AEO106

Isolation and characterization of Tn5-Induced Mutants

A suicide plasmid pSUP2021 was used to generate *Tn*5-induced *A. eutrophus* AEK101 mutants defective in phenol metabolism. This plasmid is a derivative of pBR325 that carries a kanamycin resistance gene in *Tn*5 and a ColE1 origin of replication, so it is able to replicate in *E. coli*, but not in *A. eutrophus* due to the limited host range for ColE1 origin. The pSUP2021 was introduced into *A. eutrophus* AEK101 by plate matings with the donor strains *E. coli* S17 (pSUP2021) on TNA plates for 4 to 6 hours. *E. coli* S17 has *tra* genes

integrated in its chromosome, so it can mobilize the plasmid pSUP2021 which carries the IncP1 *mop* site (96). Over 4,000 Km^r Rif^r exconjugants were obtained by conjugation. These exconjugants were tested for their growth on phenol as a sole carbon and energy source. Among thirty six colonies which failed to grow on phenol, one mutant, AEK201 was further isolated due to its ability to grow on benzoate according to the screening procedure described before. This mutant did not carry any plasmid, but it was resistant to ampicillin and chloramphenicol which are encoded on the plasmid pSUP2021. This mutant could have arisen by *Tn*5-promoted insertion of the entire plasmid through replicon fusion into the chromosomal DNA of *A. eutrophus* AEK101, leading two copies of *Tn*5. The physical characterization of the site of *Tn*5 insertion was carried out by Southern blot analysis of the chromosomal DNA isolated from AEK201. The genomic DNA was digested with *Eco*RI, which does not cleave within *Tn*5, and hybridized with ^{32}P-labeled internal *Hind*III fragment (3.3 kb) of *Tn*5. As expected, two *Eco*RI fragments (6.5 and 14.5 kb each) were detected (Fig.7). Further hybridization test with *Bam*HI-digested DNA and the same probe exhibited three different fragments (3.4, 5.5, and 12.0 kb each). Because plasmid pSUP2021 contains two *Bam*HI sites (one in the transposon *Tn*5 and the other near *Tn*5), four fragments should be hybridized. The reason for this discrepancy could be due to one *Bam*HI site near *Tn*5 being disrupted during transposition or two *Bam*HI fragments being the same in size. The results of cloning and mapping of the *Tn*5 flanking DNA with colony hybridization test suggested that the latter would be correct.

The results of the enzyme assays revealed that none of enzyme activities tested was detected in AEK201 grown on phenol plus ethanol (Table 5). Growth on benzoate is mediated through *ortho*-cleavage pathway, evidenced from the presence of catechol 1,2-dioxygenase. This enzyme might be different from the phenol-induced catechol 1,2-dioxygenase. The existence of isofunctonal enzymes in aromatic degradation pathway is also discussed by Bayly et al. (49).

Cloning of the Tn5-carrying *Eco*RI Fragments

DNA isolated from AEK102 was digested with *Eco*RI, and ligated to *Eco*RI-cleaved pGEM vector DNA. The recombinant plasmids were transformed into *E. coli* JM109, and the transformants were selected on LB plates containing ampicillin and kanamycin. Two different plasmids from positive clones were isolated. When cutting with *Eco*RI, two insert *Eco*RI fragments, 6.5 kb and 14.5 kb each, were detected. These fragments were shown to be the same in size as those appearing on the Southern hybridization analysis performed earlier. pAEK201, which contains 14.5 kb insert DNA, was used for colony hybridization analysis.

Identification of Cosmid Clones Complementing the mutant AEK201

Both colony hybridization and triparental replica plating methods were employed to identify cosmid clones complementing AEK201 from cosmid library. Using the 14.5 kb *Tn*5-containing

*Eco*RI fragment from pAEK201 as a probe in colony hybridization experiment, a number of positive clones were identified from master plates. These clones are divided into five groups according to their restriction patterns and designated pYK201, pYK202, pYK203, pYK204, and pYK205 respectively (Fig. 8). All these cosmid clones are over 30 kb in size and share a common 6.0 kb *Hind*III-*Xho*I fragment which contains 4.3 kb EcoR1 fragment. Approximately 4.2 kb DNA out of 4.3 kb *Eco*RI fragment was found to be the same as that on pAEK201. The remaining 4.6 kb extra DNA, which is calculated by subtracting 5.7 kb (For *Tn*5) plus 4.2 kb from 14.5 kb insert DNA of pAEK201 was shown to share the same restriction sites as those of pSUP2021.

The cosmids pYK201, pYK203, and pYK205 have incomplete *Hind*III cut at an end of the insert DNA. Two other cosmids, pYK202 and pYK204, were found to carry different *Hind*III insert DNA and different restriction enzyme cleavage patterns except a common 6.0 kb *Hind*III-*Xho*I fragment, indicating the duplication of this fragment on the chromosome. When each cosmid was inserted into the mutant AEK201 by triparental mating using helper plasmid pRK2013, all cosmids allowed AEK201 to grow on phenol as a sole carbon source.

Triparental replica plating method was also used to evaluate the efficiency for the identification of cosmid clones which complement AEK201. This method was found to be very efficient. It allowed us to directly isolate five cosmid clones from master plates, which are the same as those identified by colony hybridization.

The enzyme activities in complementing mutant strains were

measured (Table 6). AEK102 harboring any one of five cosmids (pYK201 to pYK205) restored all three enzyme activities tested in the same levels as those of AEK101. Simultaneous induction and repression modes imply that the genes specifying these enzymes lie in an operon or are under the same regulatory control Previously we described the cloning of phenol hydroxylase and catechol 2,3-dioxygenase structural genes from different source of chromosomal DNA of AEO106. Therefore, *Tn*5-interrupted region in AEK201 might encode a regulatory gene (s) rather than the structural genes.

Subcloning and Localization of
the Regulatory gene, *phlR*1

To localize the region complemeting AEK201, various fragments of cosmid DNA (pYK201 to pYK205) were subcloned into tac expression vector pMMB67EH (Fig. 10). Two common fragments, 6.0 kb *Xho*I-*Hind*III and 4.3 kb *Xho*I-*Bam*HI each, were cloned from pYK201 into multiple cloning site of the pMMB67 vector. The resulting plasmids pYK2013 and pYK2010 were transferred into AEK201 by triparental mating method. They allowed AEK201 to grow on phenol as a sole carbon source. Plasmid pYK2010 was further subcloned by digesting with various restriction enzymes in order to find the smallest fragment (Fig. 11). Plasmid pYK2020, a 2.4 kb *Sal*I-*PstI* fragment, was finally isolated. When placed in AEK201, this plasmid allowed the mutant to grow on phenol while restoring all three enzyme activities to wild type levels. The 4.3 kb *Xho*I-*Bam*HI fragments from other cosmids were also subcloned and tested for the enzyme activities. The same results as pYK2010 were

obtained with these cloned plasmids. We designated the gene encoding this *trans*-acting regulator *phlR*1.

Regulation of Phenol Hydroxylase and Localization of its Regulatory Gene, *phlR*2

In the preceding section, it was demonstrated that cosmid pYK301 did not express phenol hydroxylase activity when placed in *P. aeruginosa* PAO1c, but plasmid pYK3011, which is subcloned from pYK301 by deleting 5.6 kb *Bam*HI-*Hind*III fragment, permitted this strain to grow on phenol, expressing this enzyme activity. When pYK301 was *in trans* with plasmid pYK3011, the enzyme activity was not detected, indicating the existence of *trans*-acting factor which negatively functions on the expression of phenol hydroxylase in PAO1c. To determine which region carries this function, a series of deletions was made from cosmid pYK301 and subcloned into *Pseudomonas* vector pRO1727, pRO2321, and pRO1769 (Fig. 14). Plasmids pYK3026 and pYK3029 completely inhibited the activity when in *trans* with pYK3011, whereas pYK3028 and pYK3031 did not affect at all, indicating the regulatory gene(s) spans over a 3.1kb *Bam*HI-*Eco*RI fragment. Although this fragment completely inhibited phenol hydroxylase activity in PAO1c (Table 7), the presence of this region fully induced this enzyme activity in *A. eutrophus* AEK301 (Table 6). Cosmid pYK301, which contains the intact regulatory region, expressed about twice as much of the activity as pYK3011 did, even though the copy number of pYK301 is much lower than that of pYK3011. But the regulatory gene does not seem to act on catechol 2,3-dioxygenase. This gene(s) is, therefore, likely to encode

an activator-repressor protein specific for phenol hydroxylase in *A. eutrophus* AEK101. We have designated this regulatory gene as *phlR*2. It seems that some effector molecule is required for *phlR*2 to act as an activator in AEK101, which is absent in PAO1c. This could be either one of the downstream metabolites of the *meta*-pathway or other gene product(s). In other phenol-degrading bacteria such as *Pseudomoans putida* U (8, 116), *P. pickettii PKO*1 (59, 60), and *Alcaligenes eutrophus* 335 (48), it is suggested that the gene specifying phenol hydroxylase is under positive transcriptional control. In *A. eutrophus* AEK101, other regulatory gene *phlR*1, which is derived from cosmid pYK201, was also found to positively control whole phenol-degrading pathway. In order to find whether or not *phlR*1 has any effect on the expression of *phlR*2 and structural genes, plasmid pYK2027 was introduced *in trans* into PAO1c containing various plasmids. This plasmid was constructed by cloning a 2.4 kb *Hind*III-*Bam*HI fragment from the multicloning sites of pYK2020, which contains *phlR*1, into pRO2321. Neither the phenol hydroxylase activity nor the catechol 2,3-dioxygenase activity was affected by the presence of pYK2027, indicating that two regulatory genes, *phlR*1 and *phlR*2 function independently on the induction of phenol catabolic pathway enzymes in *A. eutrophus* AEK101 (Table 7).

TCE Degradation

TCE Degradation in *A. eutrophus* JMP134

The ability of *Alcaligenes eutrophus* JMP134 (pJP4) to degrade TCE was first observed as substrate-dependent oxygen uptake. Cells

induced for the expression of the 2,4-D pathway were placed in the electrode chamber and either 2,4-D (500 ppm) or TCE (3 ppm) was added. Both exhibited an immediate substrate-dependent response. Uninduced cells grown on casamino acids did not respond to the addition of 2,4-D or TCE.

JMP134 expresses two monooxygenases which have activities analogous to those of enzymes from other organisms which have been demonstrated to remove TCE. The two JMP134 monooxygenases are the phenol hydroxylase encoded on the chromosome and the 2,4-dichlorophenol hydroxylase encoded as part of the 2,4-D pathway on plasmid pJP4. The response of each pathway to induction and aromatic-substrate activation was unique, indicating the absence of shared regulation or common substrates (Table 8). 2,4-D induced JMP134 (pJP4) exhibited substrate-dependent oxygen responses to 2,4-D and 2,4-dichlorophenol but not to phenol. Phenol-induced AEO106 (plasmid-cured JMP134) exhibited a positive response to phenol but no response to 2,4-D or 2,4-dichlorophenol.

The results of direct measurement of TCE removal are shown in Figure 15. The initial rate of removal (0.2 nmol per min per mg of protein) for phenol-induced AEO106 is comparable to rates at similar concentrations obtained for *Pseudomonas putida* F1 (109). Phenol-induced AEO106 removed TCE to below detectable levels at a rate higher than that observed for 2,4-D induced JMP134. 2,4-D induced JMP134 removed about 60% of the TCE, with apparent cessation of activity at a rate approximately one-third that of the phenol-dependent pathway. This is consistent with the results we obtained

in initial batch culture experiments analyzed by headspace GC analysis, in which only 40% of the TCE was consistently removed. Similar observations have been attributed to the action of a toxic intermediate which inactivates the monooxygenase responsible for activity (109, 111). Others have reported complete removal of TCE as we observed for the phenol-induced chromosomally encoded enzyme (70, 71).

Identification of the Chromosomally Encoded Gene Responsible for TCE Degradation

Various subclones constructed from cosmid pYK301 were analyzed for the capability of degrading TCE in *A. eutrophus* AEK301 and in *P. aeruginosa* PAO1c (Fig. 16). Three plasmids including pYK301 were found to effect the degradation of TCE in AEK301 and only two plasmids, pYK3011 and pYK3021, endowed PAO1c with the ability to degrade TCE. All strains expressing the active phenol hydroxylase have been shown to remove TCE. PAO1c harboring pYK301 does not express phenol hydroxylase activity due to the presence of the regulatory gene *phlR2*, and therefore failed to remove TCE. Neither catechol 2,3-dioxygenase nor catechol 1,2-dioxygenase degrades TCE. These results clearly demonstrate that only phenol hydroxylase is responsible for TCE degradation in AEK101.

Comparison of TCE Degradation by AEK101 and AEK301 Containing Various Plasmids

The extent of TCE degradation was observed in wild type and in complementing AEK301 under various culture conditions (Table 9). AEK301 harboring pYK301 degraded TCE with the same pattern as that of AEK101. These strains completely removed TCE added (25 μM) in the presence of 2.5mM of phenol and benzoate. The presence of ethanol or casamino acid reduced the rate of TCE degradation, implying the existence of catabolite repression by these metabolites. TCE was not removed under low concentration of phenol (0.5 mM) in these strains. The opposite results was obtained when plasmid pYK3011 or pYK3021 was introduced into AEK301. These plasmids were made by deleting *phlR*2 from pYK301. AEK301 harboring these plasmids (pYK3021 and pYK3011) removed TCE only at low concentration of phenol (0.5 mM). Furthermore these strains did not require any aromatic inducer for the TCE removal, suggesting the constitutive expression of phenol hydroxylase in the absence of *phlR*2. However phenol hydroxylase activity in AEK301 containing these plasmids was expressed much higher when phenol was added into the medium than in the absence of phenol. This result demonstrates that phenol is still a strong inducer even in the absence of *phlR*2, implying secondary regulatory system might be operational.

The extent of TCE degradation by AEK301 appeared to be different, depending on the plasmids and substrates employed. Plasmid pYK3021 enabled AEK301 to remove TCE with better

capability than pYK3011. This might be due to the different sizes of insert DNA, with higher activity in shorter DNA than in longer one. TCE degradation was somewhat reduced in enriched media such as LB or TNB. But minimal medium containing less than 0.05% ethanol allowed AEK301(pYK3021) to degrade TCE completely.

TCE degradation by *Pseudomonas aeruginosa* PAO1c harboring pYK3021 is also shown in Table 9. This strain removed only restricted amounts of TCE and phenol is needed for TCE removal which is unlike to AEK301, suggesting a different induction mechanism for the phenol hydroxylase gene is functioning in this strain.

The degree of AEK301 (pYK3021) to remove TCE was measured in the presence of various concentrations of TCE (Fig. 17). The cells were cultured in MMO plus 0.1% casamino acid without any antibiotics. 200 μM of TCE was completely removed within two days. When the concentration of TCE increased up to 400 μM, TCE degradation had been continued until two days, then ceased with removal of 70% of detectable TCE. The same results were observed when the cells were cultured under selective pressure where carbenicillin (100 μg/ml) was added into the medium.

Plasmid pJP4 Pathway of TCE degradation

The *tfdB* gene which encodes 2,4-dichlorophenol hydroxylase was cloned from pJP4. 8.0 kb *Hind*III fragment containing *tfdB* was first cloned into *E. coli* expression vector pVJ256, then 2.3 kb SalI fragment which contains *tfdB* was isolated from the resulting plasmid, pHK101 and cloned into pMMB67EH. A schematic diagram

is shown in Figure 18. This plasmid (pYK101) was transferred into *A. eutrophus* AEK101 by triparantal conjugation. AEK101 containing pYK101 expressed *tfdB* gene activity in the presence of inducer, but failed to degrade TCE. This enzyme activity was not detected in the absence of inducer. This result indicates that either TfdB is not responsible for TCE removal or the activity is not expressed enough. The latter would be more plausible considering the following aspects. JMP134 does not exhibit any TCE degrading ability even when grown on 2,4-D in the presence of other carbon source such as casamino acid. The activity of TfdB in such medium was also observed to be higher in JMP134 than that of AEK (pYK101). TfdB has been known to be a likely candidate due to its similarity to chromosomally-encoded phenol hydroxylase. Both enzymes are monooxygenases which hydroxylate a substituted aromatic ring. Hausinger et al. (37) have reported that the *tfdA* gene product, which is generally known to be a monooxygenase and thus considered to be one of the likely candidates for TCE removal in 2,4-D pathway, is α–ketoglutarate dependent dioxygenase. *P. aeruginosa* and *E. coli* expressing activity of the cloned *tfdA* gene were also ineffective in TCE removal. These facts further support the hypothesis of the involvement of TfdB in TCE degradation.

CHAPTER V

DISCUSSION

Transposon Mutagenesis and Cloning of the Structural Genes for Phenol Hydroxylase and Catechol 2,3-dioxygenase from *Alcaligenes eutrophus* AEO106

Although many papers have described the regulation of chromosomally encoded phenol metabolism, very little is known about the molecular genetics of phenol pathway in *Alcaligenes eutrophus* strains. In this study, we have isolated the chromosomally encoded phenol hydroxylase gene(s) and catechol 2,3-dioxygenase gene(s) through transposon mutagenesis and complemetation test.

As a first step to isolate phenol hydroxylase deficient mutants, transposon mutagenesis was performed in *A. eutrophus* AEK101, a Rif^r derivative of AEO106, using plasmid pUW964. Replica plating methods used for the isolation of Rif^r Km^r exconjugants in this study was found to be very convenient and efficient, enabling us to isolate more than 100 independent exconjugants per a plate. Over 6,000 Rif^r Km^r exconjugants, approximately 3% of these exconjugants were unable to grow on phenol. Most of these did not show the growth on benzoate, either. Five independent mutants obtained from this study were analyzed for the restriction cleavage patterns of *Tn*5 tagged chromosomal DNA by Southern blot hybridization. The results

showed that all mutants have the same general sites interrupted by *Tn*5, implying that there would be a preferential site on the AEK101 chromosome for *Tn*5 insertion. However, it could not be ruled out that proximal locations of *Tn*5 insertion sites would make it difficult to interpret the results without extensive detailed mapping. Cloning and further restriction analysis of *Tn*5 flanking DNA from each mutant will solve this problem.

Most phenol-degrading bacteria are known to catabolize phenol through the *meta*-cleavage pathway (48, 59, 94, 95). In these bacteria, the enzymes for the *ortho*-cleavage pathway are shown to be strictly repressed when they grow on phenol. In this study *A. eutrophus* AEO106 was found to express both activities of catechol 2,3-dioxygenase and catechol 1,2-dioxygenase when grown on phenol, suggesting that catechol formed by phenol hydroxylase is metabolized through both pathways. Pieper et al. (84) also reported that *A. eutrophus* JMP134 expressed high enzyme activities for the *ortho*-cleavage pathway as well as the activities for the *meta*-cleavage enzymes from the phenol-grown cells. However, complete repression of catechol 2,3-dioxygenase activity was observed when grown on benzoate in AEO106, a plasmid cured derivative of JMP134. Repression of the *meta*-cleavage pathway enzymes was also demonstrated during the metabolism of the substituted phenol such as 2,4-dichloro- or 4-chloro-2-methyl phenol in JMP134 (85).

Simultaneous induction of both catechol cleavage pathways by phenol has led us to employ a simple screening procedure for isolating mutants defective in phenol hydroxylase in AEK101. If the mutant can grow on benzoate but not on phenol, then this mutant

should be deficient in phenol hydroxylase structural gene or its regulatory gene, because *Tn*5-induced mutation impaired on either catechol *ortho* cleavage pathway or *meta* cleavage pathway would allow the mutant to grow on phenol. Five mutants isolated according to this screening procedure were also found to be defective in all three enzymes tested, phenol hydroxylase, catechol 1,2-dioxygenase, and catechol 2,3-dioxygenase. Both the simultaneous induction and repression indicates that the genes specifying these enzymes are coordinately regulated. If the mutation occured on the phenol hydroxylase gene, the genes encoding these enzymes could comprise an operon. But the mode of catabolite repression of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase appears to be different, strongly contradicting this assumption. The former was not affected by any catabolites, whereas the latter was severely affected by various catabolites (Table 5). So it is likely that mutants are impaired on the regulatory gene rather than on the structural gene of phenol hydroxylase. Further cloning and hybridization experiments confirmed this assumption.

Complementation test allowed us to isolate three different cosmid clones. AEK301 harboring pYK301 restored the activities of phenol hydroxylase and catechol 2,3-dioxygenase. But incapability to grow on phenol as a sole carbon source and the formation of yellow color on the plate by this complementing strain suggest that the structural gene responsible for the conversion of 2-hydroxymuconic semialdehyde is repressed. Catechol 1,2-dioxygenase was also not induced in this strain. These observations also support that the regulatory gene, which controls the genes for

phenol pathway, would be interrupted by *Tn*5 in these mutant strains.

Even though catechol 1,2-dioxygenase was not induced in AEK301 containing pYK301, this enzyme was expressed during growth on benzoate, indicating that a common intermediate, catechol does not act as an inducer. Independent induction of this enzyme by these two metabolites implies that two isofunctional enzymes may be operational in AEK101. The existence of isofunctional enzymes for the degradation of aromatic compounds has also been describeed in many bacteria (49, 65, 83, 113). Bayly et al. (49) has reported that more than two isofunctional enzymes (including three catechol 2,3-dioxygenase) are involved in phenol metabolism in *A. eutrophus* 345.

Subcloning of pYK301 has led us to isolate a 9.0 kb *Xho*I-*Bam*HI fragment (coordinates 2.0 to 11.0 kb) which encodes phenol hydroxylase. More than 6.5 kb *Xho*I-*Pst*I fragment (coordinates 2.6 to 9.1 kb) was shown to be required for complete phenol hydroxylase activity. This is confirmed under heterogenetic background experiment using *Pseudomonas aeruginosa* PAO1c. This strain, which lacks functional phenol hydroxylase gene, acquired the ability to grow on phenol in the presence of pYK3021 (coordinates 2.1 to 11.2 kb), but both pYK3020 (coordinates 2.6 to 11.2 kb) and pYK3022 (coordinates 0 to 9.3 kb) do not allow PAO1c to grow on phenol. The detection of substantial amount of catechol 1,2-dioxygenase activity in PAO1c harboring either pYK3011 or pYK3021 when grown on phenol has proved that these plasmids contain a phenol hydroxylase gene(s), metabolizing catechol through *ortho*-cleavage pathway on the chromosome. Shingler et al. (76, 86) has

reported a multicomponent phenol hydroxylase in *Pseudomonas* sp. CF600. 5.5 kb DNA region encoding six different polypeptides for this enzyme were cloned and sequenced from megaplasmid pVI150 in this strain. This enzyme is different from typical phenol hydroxylases or other bacterial hydroxylases, which are simple flavoproteins. Gibson et al. (98) suggested that phenol is degraded by toluene dioxygenase, a multicomponent enzyme, through an alternative monohydroxylation in *Pseudomoans putida* F1. Phenol hydroxylase in *A. eutrophus* might could be a multicomponent enzyme, judging from the large fragment required for complementation. It will be interesting to see whether this enzyme has a function toward other aromatic compounds such as toluene.

Most phenol-degrading bacteria are known to degrade phenol through the *meta*-cleavage pathway and the genes specifying this pathway are often clustered. Plasmid pVI150 in *Pseudomonas* CF600 and the chromosomal genes encoding phenol pathway enzymes in *P. pickettii* PKO1 are also found to metabolize phenol through the *meta* cleavage pathway and the genes for this pathway lie in a cluster. Likewise it is conceivable that the genes for the phenol catabolic pathway of *A. eutrophus* AEK101 are clustered. In this study we found that the genes for phenol hydroxylase and catechol 2,3-dioxygenase lie adjacent to each other in *A. eutrophus* AEO106.

The gene encoding catechol 2,3-dioxygenase (C23O) was localized in 4.1 kb *Hind*III-*Eco*RI (coordinates 0 to 4.1 kb) fragment from pYK301. Because 1.6 kb *Xho*I-*Eco*RI (coordinates 2.5 to 4.1 kb) fragment is required for phenol hydroxylase activity, less than 2.5

kb (*Hind*III-*Xho*I) is sufficient for this gene activity. It will be interesting to investigate whether pYK302 encodes the subsequent *meta* pathway genes, because this plasmid carries more than 9.0 kb extra DNA beyond this *Hind*III site. However, the cosmid pYK302 does not allow AEK301 to grow on phenol as a sole carbon source, either. This result implies that either the extra DNA has no these genes or the enzymes are not induced enough for AEK301 to grow on phenol. Further cloning and the analysis of enzyme activities will solve this problem.

Overproduction of the C23O activity was observed in pYK3023 and pYK3024 in which part of phenol hydroxylase gene was deleted. Phenol strongly induced the activity of C23O, implying this gene utilizes its own promoter for transcription. However, PAO1c harboring these plasmids did not express C23O activity. Identical results were observed with pYK3011, which carries both genes for phenol hydroxylase and C23O. PAO1c harboring pYK3011 expressed only phenol hydroxylase activity, indicating that the gene for C23O is not cotranscribed with phenol hydroxylase gene. Different regulatory system, therefore, should be present for the induction of C23O activity. Bayly et al. (48) has proposed that the genes encoding phenol hydroxylase is in a separate operon from the genes encoding catechol *meta*-cleavage pathway in *Alcaligenes eutrophus* 335, with the former under positive control and the latter under negative control. They also proposed that at least one regulatory gene exerts a controlling effect over the expression of all phenol pathway enzymes. Wigmore et al. (8, 116) has reported similar observation from *P. putida* U, suggesting the presence of two separate operons for

phenol catabolic pathway. Molecular analysis of phenol-degrading pathway in *P. pickettii* PKO1 suggested the existence of two different regulatory genes, *tbuR*, a positive regulator for the expression of phenol hydroxylase and *tbuS*, an activator-repessor protein for *meta*-cleavage of catechol (60). In the next section, we described the cloning and characterization of two putative regulatory genes involved in phenol metabolism in *A. eutrophus* AEK101. One regulatory gene, *phlR1*, positively controls the whole phenol pathway, whereas *phlR2* acts as both an activator and a repressor specific for only phenol hydroxylase. Data obtained from this study also imply that the third regulatory gene is operational in phenol metabolism, placing the induction mechanism of phenol pathway under the complex regulatory circuit in this strain. Cloning and analysis of *Tn*5 tagged DNA from the mutants should be performed to characterize this regulatory mechnism.

Fortnagel et al. (53, 54) has reported and sequenced two distinct genes encoding catechol 2,3-dioxygenase from *A. eutrophus* JMP222, a pJP4 cured derivative of *A. eutrophus* JMP134. These two catechol 2,3-dioxygenase genes were found not to share any sequence homology with each other. The genes are also different from the one isolated in this study, indicating that at least three different catechol 2,3-dioxygenases may exist on the chromosome of this strain. Though it has been well known that such isofunctional enzymes commonly appear for the degradation of structurally related aromatic compounds, the exact reason for the occurrence of these enzymes are not clear. These enzymes probably have significant impact on the total enzyme activity. Isofunctional

enzymes in *A. eutrophus* may also play a role in the global control of the phenol catabolic pathway by providing effector by-products.

Regulation of Phenol Degradation Pathway in *A. eutrophus* AEK101

In this study, we isolated two regulatory genes involved in phenol metabolism. *phlR1*, which is localized on a 2.4 kb *Sal*I-*Pst*I fragment from cosmid DNA complementing mutant AEK201, was found to positively control the whole phenol pathway. Restriction enzyme analysis of cosmid DNA revealed that this regulatory region is duplicated, evidenced from two different *Hind*III fragments which share the same regulatory region. The gene(s) cloned from each *Hind*III fragment complemented AEK201. Duplicates of regulatory genes were also identified on TFD degrading plasmid pJP4 in *A. eutrophus* JMP134 (65). In this plasmid, the *tfdS* was known to be an identical copy of *tfdR* by sequencing analysis, indicating the copy of regulatory gene is not uncommon in this strain. At present we do not know whether *Tn*5 is inserted at both sites. Simultaneous insertion of *Tn*5 resulted from homologous recombination between two fragments could be conceivable. If only one site is interrupted, then there should be certain control mechanism by which the other intact fragment remains to be repressed. It is interesting that the extra copy of the genes or gene clusters seem to be unexpressed in many bacteria (9).

Although the mutant AEK201 is very stable with reversion rate of approximately 1.0×10^{-8} on TNA plates, sudden acclimation of the mutant strain to growth on phenol was often observed during growth

on benzoate. All these revertants remained Km resistant, indicating *Tn5* insertion site was not changed. This result implies that regulatory system acting on benzoate metabolism may compensate for this mutation by some alternative mechanism. *TcbR*, a regulatory protein involved in chlorobenzene degradation pathway in *Pseudomonas sp.* strain P51, is known to cross-react with the promoter of *tfdCDEF* on pJP4 (106). The *xylCAB* operon, which is responsible for the 'upper' pathway of toluene degradation on the TOL plasmid in *P. putida* mt-2, has been shown to be activated by the heterologous regulatory genes *ntrC* or *nifA*, which are involved in nitrogen metabolism (28). It is very plausible that homologous regulatory protein working on one aromatic ring cleavage pathway could be operational on triggering the other. Sequencing of *phlR1* should be undertaken to compare any similarity of this protein with other regulatory proteins.

phlR2, a regulatory gene specific for phenol hydroxylase, is localized on a 5.0 kb *Pst*I-*Eco*RI fragment, which is just adjacent to the phenol hydroxylase gene. The modes of gene expression in two different strains, AEK301 and PAO1c, were found to be opposite. AEK301 with pYK301, which contains a regulatory gene *phlR2*, expressed twice as much phenol hydroxylase activity as that in AEK301 with pYK3011, but the presence of *phlR2* completely repressed the enzyme activity in PAO1c. Higher activity of phenol hydroxylase in AEK301 harboring pYK301 was confirmed by repeated measurement. The pYK301 was constructed in a low copy number vector (pVK102), whereas a high copy number vector (pMMB67) was used for pYK3011. pYK301 is also larger in size (40

kb vs. 20 kb). These results further support the idea that the presence of *phlR2* increases the activity of phenol hydroxylase in AEK301, implying that *phlR2* functions as an activator in AEK301, but as a repressor in PAO1c..

A single regulatory gene acting as both an activator and a repressor has been shown for *merR* (79), *araC* (92), *oxyR* (100), *tbuS* (60), and *tfdS* (56), which are involved in mercury ion resistance, arabinose operon, the expression of genes induced by oxidative stress in *Salmonella typhimurium*, catechol *meta* cleavage pathway in *Pseudomonas pickettii* PKO, and the expression of *tfdB* on TFD pathway in *A. eutrophus* JMP134, respectively. These regulators are generally known to function at the transcriptional level, acting as activators or repressors according to the availability of effectors.

It is interesting that *tfdB* gene shares some similarities with *phlH*. Two genes encode monooxygenases which hydroxylate a substituted aromatic ring in JMP134, one encoding phenol hydroxylase on the chromosome and the other encoding 2,4-dichlorophenol hydroxylase (DCPH) on plasmid pJP4 as part of TFD pathway. The *tfdS* was reported to require one of the downstream metabolites as an effector to activate the *tfdB* gene. Similar regulatory mode to that of *tfdS* on plasmid would be imaginable for *phlR2* on the chromosome, requiring one of the downstream metabolites of the *meta*-cleavage pathway as an effector for the full induced activity of phenol hydroxylase. As predicted by Olsen et al. (59), under such a regulatory system, the absence of regulator would make the structural gene no longer fully repressed nor be induced, expecting constitutive expression of the gene in the basal level. This

model seems to be effective for *tfdS*. AEK301 containing plasmid pYK3021 and pYK3011, which have no *phlR2*, expresses phenol hydroxylase activity very weakly under non-inducing conditions, but phenol still acts as a strong inducer, eliciting ten times more enzyme activity. By the definition of Collins et al. (17), this enzyme activity is expressed 'semiconstitutively' in this strain. Pieper et al. (84) also used this terminology for the expression of *tfdB* in a mutant of *A. eutrophus* JMP134, JMP134-1. This mutant constitutively expressed DCPH activity, but at a significantly higher level when induced with TFD.

The phenol hydroxylase activity, however, was not detected in *P. aeruginosa* PAO1c harboring pYK3011 when the cell was grown in the absence of phenol. This phenomenon is clearly unlike the results with *tfdS* that the basal level of DCPH activity was detected in PAO1c containing *tfdB* gene regardless of the previous culture conditions in the absence of *tfdS*. Phenol still acts as an inducer in PAO1c which contains *phlH* but not *phlR2*. It is predictable that a 9.1 kb *Xho*I-*Bam*HI of pYK3011 fragment contains its own promoter and possible regulatory region on which secondary regulatory system exerts in such a way that phenol still acts as an inducer.

Genetic analysis of promoter region of the *dmpKLMNOP*, the multicomponent phenol hydroxylase gene in *Pseudomonas sp* CF600, has revealed that upstream of this region contains the invariant -24 GG -12 GC sequence which has been shown to require the *rpoN* gene product, a sigma factor in *E. coli*, for the expression from this promoter in *P. putida* (76). Another striking finding of this promoter is that upstream region also contains an inverted repeat,

which is similar to the symmetrical recognition sequence proposed to be involved in the binding of *E. coli* repressors and activators. It will be very interesting to see whether this kind of DNA sequence is also present in the promoter of *phlH of A. eutrophus* AEK101, considering the presence of the presumed repressor-activator gene, *PhlR2*.

From this study it has been shown that expression of the genes involved in phenol pathway are tightly regulated by multiple systems in *A. eutrophus* AEK101. One regulatory gene, *phlR1* appears to control the whole phenol pathway, whereas the other regulatory gene, *phlR2* is implicated in the expression of phenol hydroxylase gene itself. Cloning analysis of catechol 2,3-dioxygenase gene revealed that other regulatory gene(s) exists for the expression of this gene and possibly the genes for the subsequent *meta* -pathway enzymes.

Previously isolated *Tn*5-induced mutant strains (AEK301 to AEK305) are found to be deficient in three enzyme activities tested, phenol hydroxylase, catechol 2,3-dioxygenase and catechol 1,2-dioxygenase. The pleiotropic effect of *Tn*5 insertion on the expression of phenol pathway enzymes implies that either a common positive regulator is mutated or the genes for this pathway lie in an operon. Southern blot analysis using a 5.2 kb *Eco*RI fragment (coordinates 6.3 to 11.5 kb of pYK301) as a probe revealed that neither the structural gene of phenol hydroxylase nor the regulatory gene *phlR2* was interrupted by *Tn*5, implying that *Tn*5 insertion might be occurred on other regulatory gene which positively controls the expression of the whole phenol pathway enzymes in addition to *phlR1* (data not shown). The existence of another regulatory gene for

whole phenol pathway can also be deduced from the fact that mutant AEK301 harboring pYK301 does not grow on phenol as a sole carbon source, even though an adequate amount of enzyme activities of phenol hydroxylase and catechol 2,3-dioxygenase are detected. Both the repression of catechol 1,2-dioxygenase activity which is induced by phenol in wild type and the formation of yellow color on the phenol-containing plate due to the accumulation of 2-hydroxymuconic acid semialdehyde strongly suggest this hypothesis. Cloning of *Tn*5 flanking DNA in these mutants along with the analysis of enzyme activities should be performed to elucidate the complex regulatory mechanisms involved in phenol degradation pathway in *A. eutrophus* strain.

TCE Degradation by Phenol Hydroxylase

Initially we demonstrated that phenol-metabolizing pathway induces TCE-degrading activity in *A. eutrophus* AEO106 and phenol hydroxylase was suspected as a likely candidate. Nelson et al. (71) have proposed that a single enzyme can accept molecular oxygen and oxidize TCE. This would be sufficient for the complete dechlorination of TCE due to the instability of TCE-oxide intermediates. Shields et al. (94) demonstrated that a single enzyme is involved in the hydroxylation of toluene, cresol, and phenol, and in TCE degradation in *P. cepacia* G4.

In this study, we isolated the phenol hydroxylase gene(s) and confirmed this single enzyme is responsible for TCE degradation. AEK301, a *Tn*5-induced mutant defective in phenol metabolism, degrades TCE very efficiently in the absence of aromatic inducer

when this mutant harbors pYK3011 or pYK3021. These plasmids were constructed by deleting a DNA fragment which contains a *trans*-acting regulatory gene, *phlR2* . This gene was found to have a repressor-activator function specific for the expression of phenol hydroxylase in *A. eutrophus* AEO106. The mode of TCE degradation of AEK301 harboring pYK301 is almost the same as that of the wild type AEK101, but totally different from that of AEK301 harboring pYK3021. The presence of aromatic inducer is prerequisite for TCE removal by the wild type and the mutant AEK301 harboring pYK301, whereas pYK3011 enables AEK301 to degrade TCE without any inducer.

Phenol concentration in medium also affects the degree of TCE degradation. Only under higher amount of phenol (2.5 mM) TCE degradation was observed in the wild type AEK101 and AEK301 (pYK301). When phenol concentration was reduced into 0.5 mM, these strains did not degrade TCE any more. This observation is well correlated with the enzyme assay in which the activity of phenol hydroxylase was expressed only at 2.5 mM of phenol but repressed at 0.5 mM of phenol. When AEK301 (pYK3021) grows on phenol, catechol is accumulated, which in turn causes toxic effect on the growth. To prevent the toxic effects of catechol, benzoate was also added into medium so that catechol produced by phenol hydroxylase could be channeled through benzoate-induced *ortho*-cleavage pathway. Under this condition, AEK301 (pYK3021) degrades TCE at lower concentration of phenol (0.5 mM), but most of TCE remained unchanged at higher (2.5 mM). This phenomenon is obviously contradictory to that of the wild type or AEK301 (pYK301). This

result could be partly due to the inhibition of growth by catechol, because even in the presence of benzoate, the extent of dark brown color formation, which indicates the accumulation of catechol, was more obvious at higher phenol concentration (2.5 mM). Inhibition of TCE degradation by higher concentration of phenol in AEK301 (pYK3021) could also be explained from possible competitive inhibition between the natural substrate, phenol, and the fortuitous substrate, TCE as described by Folsom et al. (34).. It is possible that the enzyme attack on TCE can be retarded under the circumstance where phenol hydroxylase activity is relatively low, which is the case in AEK301 (pYK3021), and the concentration of phenol is high. Further analysis should be performed to elucidate kinetics and interactions between phenol and TCE in AEK301 (pYK3021).

Even though AEK301 (pYK3021) degrades TCE without phenol, indicating constitutive expression of phenol hydroxylase activity, this activity was observed to be further inducible by phenol to levels a full order of magnitudes higher. According to the definition of Collins et at. (17) this enzyme activity is semiconstitutively expressed. Considering the multiple regulatory mechanisms of phenol catabolic pathway operating in AEK101, it is conceivable that a secondary regulatory system would be functional in such a way that phenol still acts as an inducer in the absence of *phlR2*.

The activity of phenol hydroxylase was also observed to be significantly higher when AEK301 (pYK3021) grows on casamino acid (0.1%) in the presence of TCE than without TCE. It is possible that a secondary regulatory system may be arisen with the broad substrate (inducer) specificity for the induction of phenol hydroxylase gene so

that TCE acts like a gratuitous inducer under the environment where a strictly regulatory gene is not expressed. *P. aeruginosa* PAO1c harboring pYK3021 expressed phenol hydroxylase activity only in the presence of phenol and TCE was degraded only under this condition. This results demonstrate that a heterogenetic regulatory system is operational in *P. aeruginosa* PAO1c, supporting the hypothesis of the existence of a secondary regulatory system in AEK301, which is totally different from that of PAO1c. Analysis of promotor region with deletion and protein fusion will clearly elucidate these regulatory mechanisms.

This secondary regulation model, once proved, then will provide invaluable information for the understanding how regulatory proteins have evolved to adjust new inducers, which will in turn be utilized for developing microorganisms that have increased substrate and/or inducer ranges for the degradation of more complex and toxic chemicals. In conjunction with this view and , it is also valuable to investigate the degradation by AEK301 (pYK3021) of other chlorinated ethylenes and phenolic compounds.

The onset of TCE degradation by AEK301 (pYK3021) is also different from that in wild type. TCE degradation was observed to occur sometime after the growth of AEK301(pYK3021), whereas it immediately follows the cell growth in AEK101. This phenomenon is consistent with the observation of higher phenol hydroxylase activity in the stationary phase than in the exponential phase in AEK301 (pYK3021), which is the opposite in AEK101 (data not shown). Enriched media such as TNB partially reduced the degree of TCE removal, but minimal medium containing less than 0.05% ethanol as

a sole carbon and energy source enables AEK301 (pYK3021) to degrade TCE completely. It is conceivable that catabolite repression exists or certain regulatory mechanism related to carbon limited induction is operating.

AEK301 (pYK3021) removed 200 μM of the TCE to below detectable level within two days under non-selective pressure. When the concentration increased up to 400 μM, apparent cessation was observed after about 70% removal of added TCE in two days. This cessation would be related with toxic effect induced by TCE during its metabolism. Toxicity has been known to occur through covalent modification of cellular molecules by toxic intermediates produced during TCE mineralization in many TCE degrading bacteria (87, 111). The diminution in biodegradation rate would be severe if the enzyme responsible for TCE removal is sensitive to toxic intermediates. Phenol hydroxylase in AEK101 seems to be less sensitive to toxic intermediates, so the cessation might be caused by general cytotoxicity during the mineralization of high concentration of TCE. It should not be ruled out the possibility that this cessation could be partly due to the loss of enzyme activity resulted from homologous recombination between the plasmid-borne phenol hydroxylase gene and its chromosomal counterpart. Because phenol hydroxylase activity was observed to decrease with the prolonged culture of AEK301 harboring these plasmids.

Although this soil recombinant bacterium should be tested under various physico-chemical conditions similar to nature and approved for the release into environment before its application in *situ* bioremediation of contaminant sites, it is likely to be an excellent

candidate owing to the following merits. TCE mineralization does not require any aromatic inducers or antibiotics and is performed even under carbon-limited circumstance which is frequently encountered in natural environment. The capacity of TCE removal is very high with limited sensitivity to TCE-mediated toxicity.

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APPENDIXES

APPENDIX A

TABLES

TABLE I

PHENOL DEGRADATION BY MICROORGANISMS

Microorganism	genetic organization	aromatic degradation pathway	reference
Bacteria			
Alcaligenes eutrophus 335	chromosome	*meta*-pathway	48
A. eutrophus 345	chromosome	*meta*-pathway	49
A. eutrophus JMP134	chromosome (*phlH*)	*meta*-pathway *ortho*-pathway	84, 85
Bacillus stearothermophilus	unknown	unknown	42
Pseudomonas aeruginosa T1	unknown	*meta*-pathway	89
P. cepacia G4	chromosome	*meta*-pathway	94
P.pickettii PKO1	chromosone (t*buD*)	*meta*-pathway	59, 60
P. putida F1	chromosome (*todABC*)	*meta*-pathway	98, 118
P. putida U.	unknown	*meta*-pathway	7, 30
P. sp. CF600	plasmid (*dmpKLMNOP*)	*meta*-pathway	6, 76, 95
P. sp. EST1001	plasmid (*pheA*)	*ortho*-pathway	78
Rhodococcus sp. P1	unknown	*meta*-pathway	102
Streptomyces setonii	unknown	*meta*-pathway	3
Yeast			
Candida tropicalis	unknown	*ortho*-pathway	75
Trichosporon cutaneum	unknown	*ortho*-pathway	74

TABLE 2

AEROBIC DEGRADATION OF TCE BY MICROORGANISMS

Microorganism	Inducer	Enzyme	Source or Reference
Aromatic pathway			
Pseudomoans putida F1	Toluene	Toluene dioxygenase	70, 109, 111
P. mendocina	Toluene	p-toluene monooxygenase	117
P. cepacia G4	Toluene, cresol, phenol	o-toluene monooxygenase	34, 93, 94
P. pickettii PKO1	Toluene	m-toluene monooxygenase	55
Pseudomonas spec. JR1	Isopropylbenzene, toluene	Isopropylbenzene dioxygenase	20
Rhodococcus erythropolis BD1	Isopropylbenzene, toluene	Isopropylbenzene dioxygenase	20
Alipathic pathway			
Mycobacterium vaccae JOB5	Propane	Propane monooxygenase	110
Alcaligenes denitrificans	Isoprene	Propene monooxygenase	29
Rhodococcus erythropolis JE77	Isoprene		
Methanotrophs			
Methylosinus trichosporium OB3b	Methane	Methane monooxygenase	104
Strain 46-1	Methane, methanol	Methane monooxygenase	61
Nitrosomonas europaea	Ammonia	Ammonia monooxygenase	4, 87

TABLE 3

BACTERIAL STRAINS AND PLASMIDS

Strain or Plasmid	Relevant Characteristics	Reference
E.coli		
C600	thi thr leu tonA lacY supE	64
HB101	F^-, hsdS20 recA13 arg14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl -1 supE44 λ^- thi^- leu^-	13
JM109	(pro-lac) recA1 thi-1 supE endA gyrA96 hsdR relA1(F' traD36 proAB $lacI^q$ laczΔM15)	U.S.B. Co.
LS392	F^-, hsdS574 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55 λ^-	Promega Co.
S17	thi pro $hsdR^-$ $hasM^+$ recA	96
Alcaligenes eutrophus		
JMP134	Prototroph, Phl^+ Tfd^+ Hg^r	25
AEO106	Prototroph, Phl^+ Tfd^-	44
AEK101	Rif^+, derivative of AEO106	This study
AEK201	AEK101::Tn5, Phl^- Km^r	This study
AEK211	AEK201 revertant, Phl^+ Km^-	This study
AEK301	AEK101::Tn5, Phl^- Km^r	This study
AEK302	AEK101::Tn5, Phl^- Km^r	This study
AEK303	AEK101::Tn5, Phl^- Km^r	This study
AEK304	AEK101::Tn5, Phl^- Km^r	This study
AEK305	AEK101::Tn5, Phl^- Km^r	This study
AEK311	AEK301 revertant, Phl^+ Km^-	This study
Pseudomonas aeruginosa		
PAO1c	Prototroph	47
Plasmids		
pJP4	TFD^+ Hg^r	25
pMMB67EH	Tac expression cloning vector with cloning sites of pUC18,Ap^r	38
PMMB67HE	Tac expression cloning vector with opposite cloning sites of pUC18, Ap^r	38

TABLE 3 (Continued)

Strains or Plasmids	Relevant Characteristics	Reference
pGEM7zf(+)	Blue screening cloning vector, Ap^r	Promega Co.
pRK2013	Km^r Tra^+; ColE1 replicon	24
pRO1727	Cloning vector, Tc^r, Cb^r	18
pRO1769	Cloning vector, Sm^r, Gm^r	19
pRO2321	Cloning vector, Tc^r, Tp^r	119
pSUP2021	pBR325-mob::Tn5, Km^r, Ap^r, Cm^r	96
pUW964	TraRK2$^+$ Δ(repRK2) repE1$^+$ Tn5, Tn7 Km^r, Sm^r, Sp^r, Tp^r	112
pVJ256	Cloning vector, Ap^r	Dr. Vijayakumar
pVK102	IncP, cos^+, Km^r, Tc^r	58
pTZ18R	Blue screening cloning vector, Ap^r	U.S.B. Co.
pTZ19R	Blue screening cloning vector, Ap^r	U.S.B. Co.

Phl^-: deficient in phenol degradation.
Tfd^-: deficient in 2,4-dichlorophenoxyacetic acid degradation.
Antibiotics: Ap, Cb, Cm, Gm, Km, Rif, Sm, Sp,Tc, Tp refer to ampicillin, carbenicillin, chloramphenicol, gentamycin, kanamycin, rifampicin, streptomycin, spectinomycin, tetracycline, and trimethoprim respectively.
Hg^r: mercury resistant.

TABLE 4

RECOMBINANT COSMIDS AND PLASMIDS

Recombinant Plasmids	Relevant Characteristics
pYK201	35-kb HindIII digest of AEO106 DNA cloned into pVK102, *phlR*1$^+$, Tc^r
pYK202	HindIII digest of AEO106 DNA cloned into pVK102, *phlR*1$^+$, Tc^r
pYK203	HindIII digest of AEO106 DNA cloned into pVK102, *phlR*1$^+$, Tc^r
pYK204	HindIII digest of AEO106 DNA cloned into pVK102, *phlR*1$^+$, Tc^r
pYK205	HindIII digest of AEO106 DNA cloned into pVK102, *phlR*1$^+$, Tc^r
pYK301	16.8-kb HindIII digest of AEO106 DNA cloned into pVK102, *phlR*2$^+$, *phlH* $^+$, *c23O*$^+$, Tc^r
pYK302	HindIII digest of AEO106 DNA cloned into pVK102, *phlR*2$^+$, *phlH* $^+$, *c23O*$^+$, Tc^r
pYK303	HindIII digest of AEO106 DNA cloned into pVK102, *phlR*2$^+$, *phlH* $^+$, *c23O*$^+$, Tc^r
pAEK201	pGEM carrying 14.5-kb EcoRI fragment from AEK201, Km^r Ap^r
pYK101	8.0-kb HindIII fragment of pJP4 cloned into pVJ256, *tfdB*$^+$, Ap^r
pYK102	2.3-kb SalI fragment from pYK101 cloned into pGEM, *tfdB*$^+$, Ap^r
pYK103	2.3-kb XhoI-HindIII fragment from pYK102 cloned into pMMB67EH, *tfdB*$^+$, Ap^r
pYK2010	4.3-kb XhoI-BamHI fragment from pYK201 cloned into pMMB67EH *phlR*1$^+$, Ap^r
pYK2011	4.3-kb XhoI-BamHI fragment from pYK203 cloned into pMMB67EH *phlR*1$^+$, Ap^r
pYK2012	4.3-kb XhoI-BamHI fragment from pYK205 cloned into pMMB67EH *phlR*1$^+$, Ap^r
pYK2013	6.0-kb XhoI-HindIII fragment from pYK201 cloned into pMMB67EH *phlR*1$^+$, Ap^r
pYK2014	6.5-kb BamHI-XhoI fragment from pYK201 cloned into pMMB67EH
pYK2015	8.1-kb HindIII-BamHI fragment from pYK201 cloned into pMMB67EH, *phlR*1$^-$, Ap^r
pYK2016	7.9-kb BamHI-HindIII fragment from pYK201 cloned into pMMB67EH, *phlR*1$^-$, Ap^r
pYK2017	4.5-kb XhoI-BamHI fragment from pYK201 cloned into pMMB67EH
pYK2018	3.1-kb SalI fragment from pYK2024 cloned into pMMB67EH
pYK2019	2.2-kb PstI-SalI fragment from pYK2024 cloned into pMMB67EH
pYK2020	2.4-kb SalI-PstI fragment from pYK2024 cloned into pMMB67EH *phlR*1$^+$, Ap^r
pYK2021	1.8-kb SalI-PstI fragment from pYK2024 cloned into pMMB67EH
pYK2022	1.5-kb PstI fragment from pYK2017 cloned into pMMB67EH

TABLE 4 (Continued)

Recombinant Plasmids	Relevant Characteristics
pYK2023	1.7-kb EcoRI-SalI fragment from pYK201 cloned into pMMB67EH
pYK2024	4.3-kb XhoI-BamHI fragment from pYK201 cloned into pTZ18R *phlR*1$^+$, Ap^r
pYK2025	3.1-kb SalI fragment from pYK2023 cloned into pTZ18R, *phlR*1$^+$, Ap^r
pYK2026	4.3-kb EcoRI fragment from pYK2013 cloned into pMMB67EH
pYK2027	2.4-kb BamHI-HindIII fragment from pYK2020 cloned into pRO2321, *phlR*1$^+$, Tp^r
pYK3010	14.0-kb PstI-HindIII fragment from pYK301cloned into pMMB67EH, *phlR*2$^+$, Ap^r
pYK3011	11.2-kb HindIII-BamHI fragment from pYK301cloned into pMMB67EH, *phlH* $^+$, *phlR*2$^+$, *c23O* $^+$, Ap^r
pYK3012	11.4-kb BamHI-HindIII fragment from pYK301 cloned into pMMB67EH,*phlR*2$^+$, Ap^r
pYK3013	5.8-kb BamHI fragment from pYK301 cloned into pMMB67EH, Ap^r
pYK3014	5.4-kb HindIII-BamHI fragment from pYK301 cloned into pMMB67EH,*c23O* $^+$, Ap^r
pYK3015	5.6-kb BamHI-HindIII fragment from pYK301 cloned into pMMB67EH
pYK3016	4.6-kb EcoRI-BamHI fragment from pYK3011 cloned into pMMB67EH
pYK3017	6.8-kb EcoRI-BamHI fragment from pYK3011 cloned into pMMB67EH
pYK3018	7.3-kb XhoI-BamHI fragment from pYK3011 cloned into pMMB67EH
pYK3019	7.9-kb XhoI-BamHI fragment from pYK3011 cloned into pMMB67EH
pYK3020	8.5-kb XhoI-BamHI fragment from pYK3011 cloned into pMMB67EH
pYK3021	9.1-kb XhoI-BamHI fragment from pYK3011 cloned into pMMB67EH, *phlH* $^+$, Ap^r
pYK3022	9.3-kb HindIII-PstI fragment from pYK3011 cloned into pMMB67EH, *c23O* $^+$, Ap^r
pYK3023	6.4-kb HindIII-EcoRI fragment from pYK3011 cloned into pMMB67EH, *c23O* $^+$, Ap^r
pYK3024	4.1-kb HindIII-EcoRI fragment from pYK3011 cloned into pMMB67EH, *c23O* $^+$, Ap^r
pYK3025	3.1-kb HindIII-PstI fragment from pYK3024 cloned into pMMB67EH, *c23O* $^+$, Ap^r
pYK3026	7.5-kb PstI-HindIII fragment from pYK3012 cloned into pRO1727, *phlR*2$^+$, Tc^r
pYK3027	7.5-kb PstI-HindIII fragment from pYK3012 cloned into pTZ18R, *phlR*2$^+$, Ap^r
pYK3028	5.6-kb BamHI-HindIII fragment from pYK3027 cloned into pRO2321,*phlR*2$^-$, Tp^r

TABLE 4 (Continued)

Recombinant Plasmids	Relevant Characteristics
pYK3029	5.0-kb PstI-EcoRI fragment from pYK3027 cloned into pRO1727, *phlR2*$^+$, Tc^r
pYK3030	5.0-kb PstI-EcoRI fragment from pYK3027 cloned into pTZ18R, *phlR2*$^+$,Ap^r
pYK3031	4.1-kb PstI fragment from pYK3030 cloned into pRO1727, Tc^r
pYK3032	3.0-kb PstI fragment from pYK3030 cloned into pRO1727, Tc^r
pYK3033	2.6-kb EcoRI fragment from pYK3030 cloned into pRO1769, Gm^r
pYK3034	1.9-kb PstI-BamHI fragment from pYK3030 cloned into pTZ18R, Ap^r
pYK3035	11.2-kb HindIII-BamHI fragment from pYK3011 into pRO1727, *phlH* $^+$, *c23O* $^+$, Cb^r

Gene designations: *phlH*, phenol hydroxylase; *c23O*, catechol 2,3-dioxygenase; *phlR1*, regulatory gene 1; *phlR2*, regulatory gene 2

Antibiotics: Ap, Cb, Gm, Tc, and Tp refer to ampicillin, carbenicillin, gentamycin, tetracycline, and trimethoprim respectively

TABLE 5

ENZYME ACTIVITIES IN *A. eutrophus* AEO106 AND ITS DERIVATIVES

Strains	Culture Conditions	Enzyme Activities[a]		
		Phenol Hydroxylase	Catechol 2,3-Dioxygenase	Catechol 1,2-Dioxygenase
AEO106	Phenol (2.5 mM)	52.19	0.1323	1.5233
	Benzoate (5.0 mM)	ND[b]	ND	3.6266
	Ethanol (0.1%)	ND	ND	ND
	PHL (2.5 mM)/BA (2.5 mM)	43.19	0.0021	2.2590
	PHL (2.5 mM)/BA (5.0 mM)	19.78	0.0002	3.0217
	PHL (2.5 mM)/EtOH (0.1%)	32.61	0.0229	1.8964
AEK101	Phenol (2.5 mM)	51.13	0.1253	1.7046
	Benzoate (5.0 mM)	ND	ND	3.4951
	Ethanol (0.01%)	ND	ND	ND
	PHL (2.5 mM)/BA (2.5 mM)	34.87	0.0012	2.2542
	PHL (2.5 mM)/BA (5.0 mM)	12.84	0.0002	2.7573
	PHL (2.5 mM)/EtOH (0.1%)	28.56	0.0165	1.7538
AEK201	PHL (2.5 mM)/BA (2.5 mM)	ND	ND	2.1214
	PHL (2.5 mM)/EtOH (0.1%)	ND	ND	ND
AEK301	PHL (2.5 mM)/BA (2.5 mM)	0.04	0.0002	2.8237
	PHL (2.5 mM)/EtOH (0.1%)	0.03	0.0012	0.0035

[a] Phenol hydroxylase is expressed as increase above basal levels of nanomoles of oxygen consumed per minute, normalized to an optical density of 1.0 at 425nm. Other enzyme activities are expressed as specific activities (μmol of product formed/min mg protein).

[b] ND is not detected.

TABLE 6

ENZYME ACTIVITIES IN *A. eutrophus* AEK201 AND AEK301 CARRYING VARIOUS PLASMIDS

Plasmid	Culture Conditions[b]	Enzyme Activities[a] When the Plasmid is in AEK201			in AEK301		
		PHL	C2,3O	C1,2O	PHL	C2,30	C1,2O
None	PHL/EtOH	ND[c]	ND	ND	<0.03	<0.001	<0.007
pYK201	PHL	53.45	0.1397	2.1496	<0.03	<0.001	<0.007
	PHL/EtOH	30.55	0.0161	1.8037	<0.03	<0.001	<0.007
	PHL/BA	31.32	0.0018	2.5296	<0.03	<0.001	2.1098
pYK301	PHL/EtOH	ND	ND	ND	24.15	0.0237	0.0291
	PHL/BA	ND	ND	2.7573	27.11	0.0210	2.7856
pYK3011	PHL/EtOH	ND	ND	ND	12.95	0.0213	0.0228
	PHL/BA	ND	ND	2.3808	12.00	0.0195	2.5503
pYK3021	PHL/EtOH	ND	ND	ND	16.65	<0.001	0.0353
	PHL/BA	ND	ND	ND	18.58	<0.001	0.0166
pYK3023	PHL/EtOH	ND	ND	ND	<0.03	0.0810	0.0346
pYK3024	PHL/EtOH	ND	ND	ND	<0.03	0.1032	0.0678

[a] Phenol hydroxylase is expressed as increase above basal levels of nanomoles of oxygen consumed per minute, normalized to an optical density of 1.0 at 425nm. Other enzyme activities are expressed as specific activities (μmol of product formed/min mg protein). PHL, phenol hydroxylase; C1,2O, catechol 1,2-dioxygenase; C2,3O, catechol 2,3-dioxygenase.

[b] concentration of each carbon source is follows; ethanol (EtOH), 0.1%; PHL (phenol), 2.5 mM; benzoate (BA), 2.5 mM.

[c] ND, not detected

TABLE 7

ENZYME ACTIVITIES IN *Pseudomonas aeruginosa* PAO1c CARRYING VARIOUS PLASMIDS

Plasmids	Culture Conditions[b]	Enzyme Activities[a] in PAO1c (AEK301) Phenol Hydroxylase	Catechol 2,3-Dioxygenase	Catechol 1,2-Dioxygenase
None	Ethanol	ND[c] (ND)	ND (ND)	ND (ND)
	PHL/EtOH	ND (<0.03)	ND (<0.0006)	ND (<0.007)
pYK201	Ethanol	ND (ND)	ND (ND)	ND (ND)
	PHL/EtOH	2.04 (<0.03)	ND (<0.0006)	ND (<0.007)
pYK2019	PHL/EtOH	ND (<0.03)	ND (<0.0006)	ND (<0.007)
pYK301	Ethanol	ND (ND)	ND (ND)	ND (ND)
	PHL/EtOH	ND (24.15)	ND (0.024)	ND (0.029)
pYK3011	Ethanol	ND (0.0053)	ND (ND)	ND (ND)
	PHL/EtOH	10.60 (12.95)	ND (0.0213)	0.6605 (0.0228)
pYK3024	Ethanol	ND (ND)	ND (ND)	ND (ND)
	PHL/EtOH	ND (<0.03)	ND (0.1032)	ND (0.0678)
pYK301 pYK3011	PHL/EtOH	ND	ND	ND
pYK201 pYK3011	PHL/EtOH	10.38	ND	0.8363
pYK201 pYK3023	PHL/EtOH	ND	ND	ND
pYK2019 pYK301	PHL/EtOH	ND	ND	ND
pYK2019 pYK3011	PHL/EtOH	11.66	ND	1.0727

[a] Phenol hydroxylase is expressed as increase above basal levels of nanomoles of oxygen consumed per minute, normalized to an optical density of 1.0 at 425nm. Other enzyme activities are expressed as specific activities (μmol of product formed/min mg protein). PHL, phenol hydroxylase; C1,2O, catechol 1,2-dioxygenase; C2,3O, catechol 2,3-dioxygenase.

[b] concentration of each carbon source is follows; ethanol (EtOH), 0.1%; PHL (phenol), 2.5 mM.

[c] ND, not detected

TABLE 8

INDUCTION OF SUBSTRATE-DEPENDENT OXYGEN CONSUMPTION

Strain	Inducer	Oxygen Consumption[a] in the Presence of		
		2,4-D	Phenol	2,4-DCP
AEO106	None	37.5	0.0	16.3
	2,4-D	35.5	0.0	8.8
	Phenol	25.0	338.0	26.3
JMP134	None	38.0	0.0	37.0
	2,4-D	270.0	0.0	313.0
	Phenol	88.0	275.0	42.5

[a] Reported as nanomoles of oxygen consumed per minute, normalized to an optical density of 1.0 at 425nm. 2,4-D, 2,4-Dichloroacetic acid. 2,4-DCP, 2,4-Dichlorophenol.

TABLE 9

TCE DEGRADATION BY THE PLASMIDS
pYK301 AND pYK3021

Substrate	TCE Degradation*			
	AEK101	AEK301 (pYK301)	AEK301 (pYK3021)	PAO1c (pYK3021)
Phenol (2.5 mM)/ Benzoate (2.5 mM)	99	99	6	54
Phenol (0.5 mM)/ Benzoate (2.5 mM)	5	2	99	38
Phenol (2.5 mM)/ Ethanol (0.1%)	74	33	0	31
Phenol (2.5 mM/ Casamino acid (0.3%)	46	39	0	12
Benzoate (2.5 mM)	0	0	99	0
Ethanol (0.05%)	0	0	99	0
Casamino acid (0.3%)	0	0	99	0
TNB broth	0	0	65	0

* TCE concentration was expressed as percent decrease after overnignt growth as the average of duplicate determination.

APPENDIX B

FIGURES

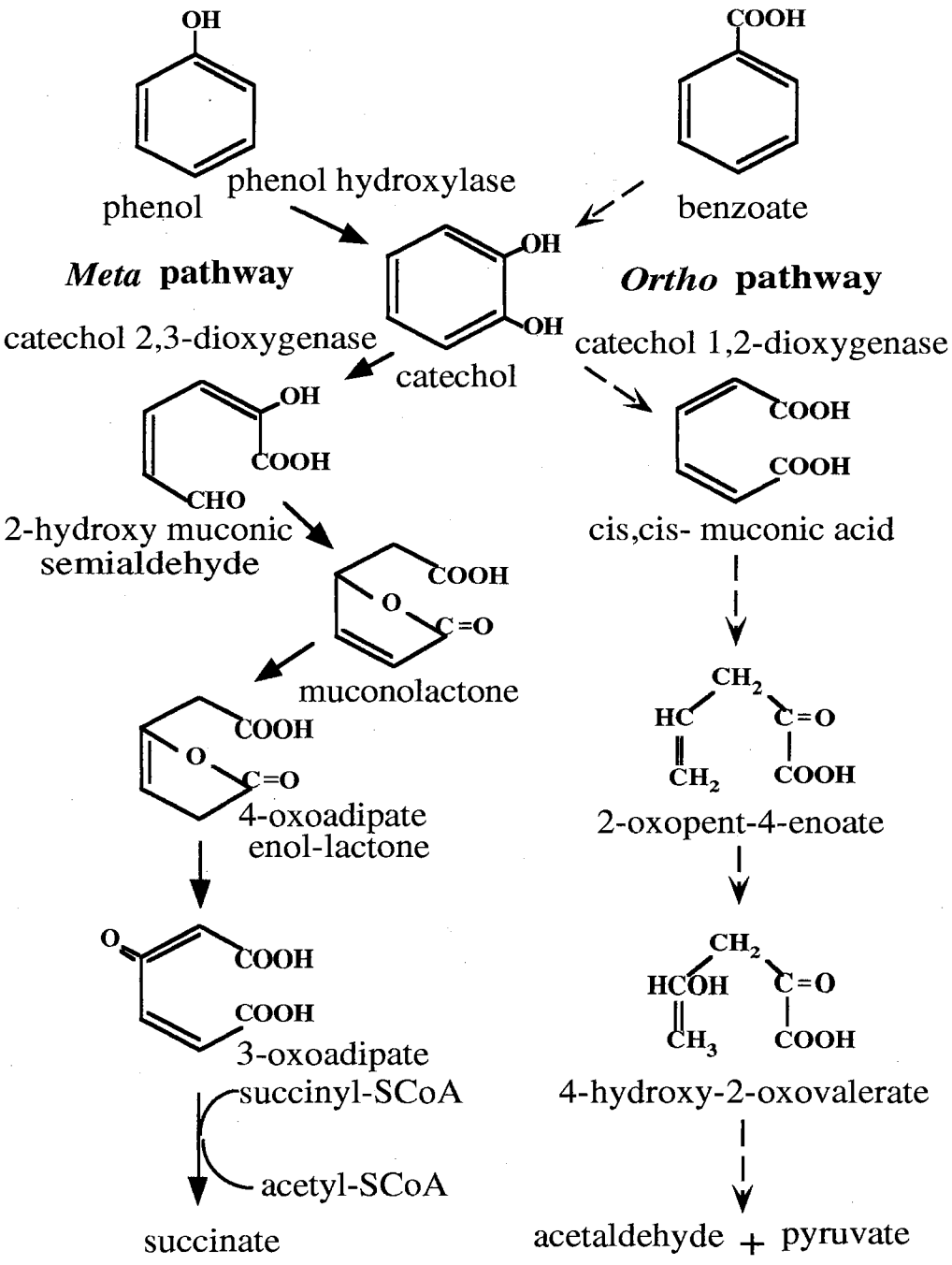

Figure 1. Phenol and Benzoate Metabolism

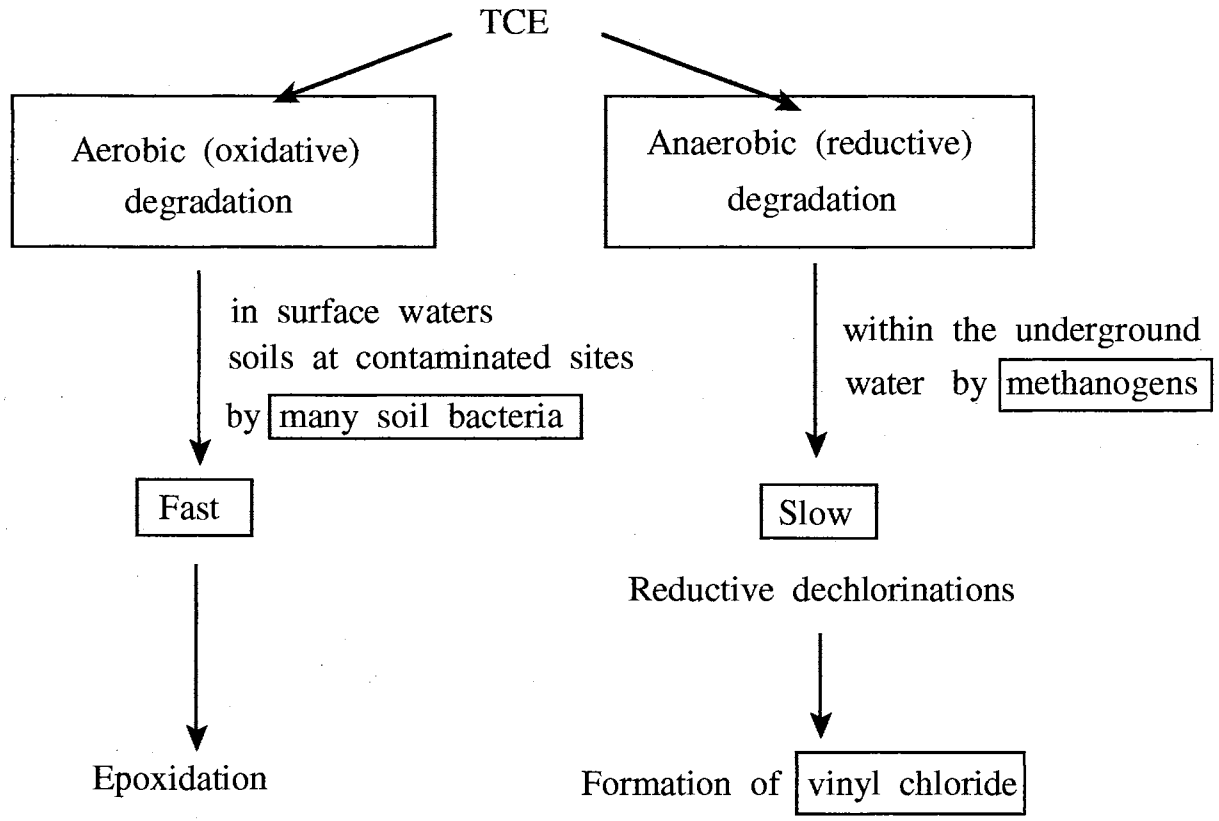

Figure 2. Biodegradation of TCE

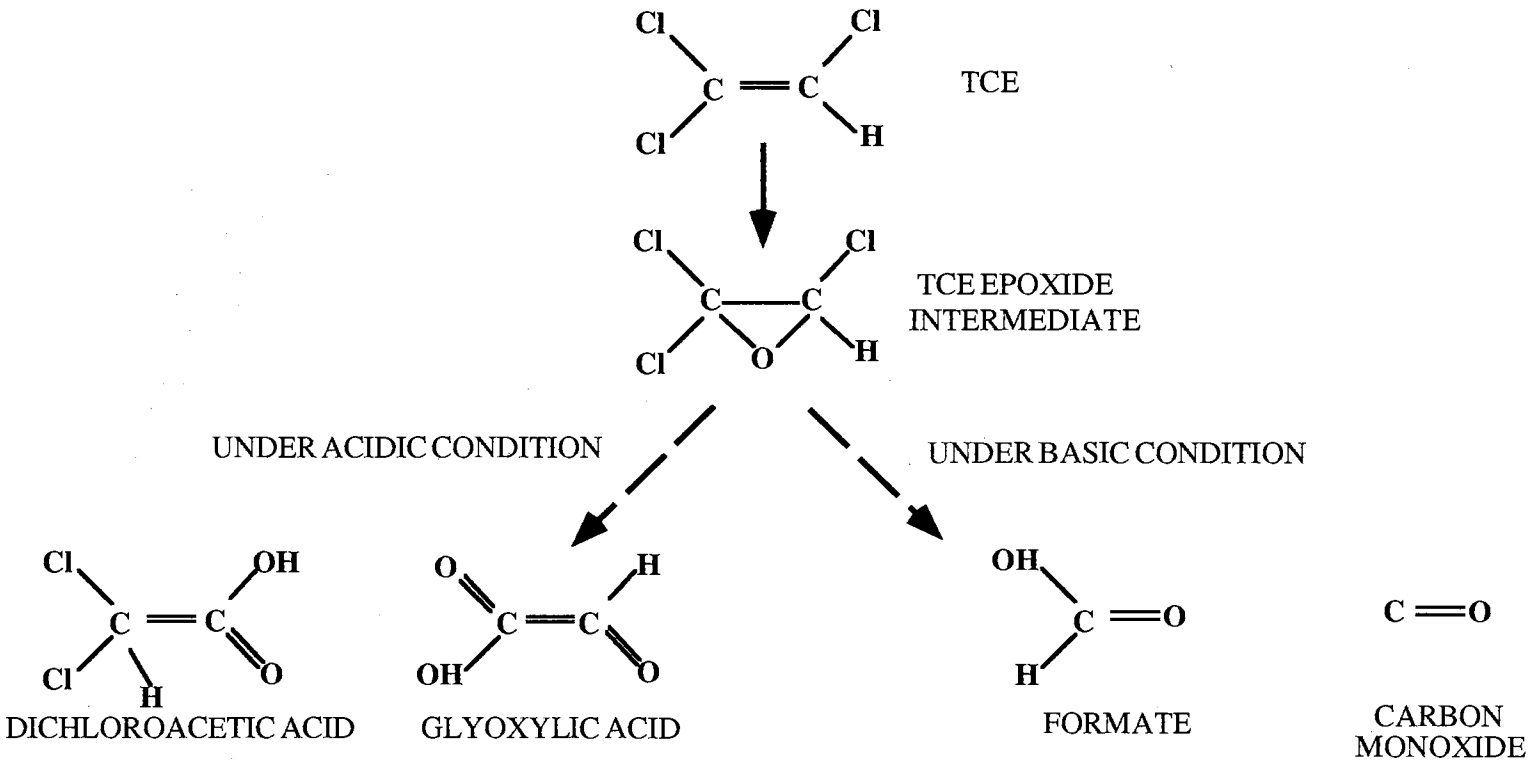

Figure. 3. Proposed Mechanism of Aerobic TCE Degradation in Aqueous Systems.

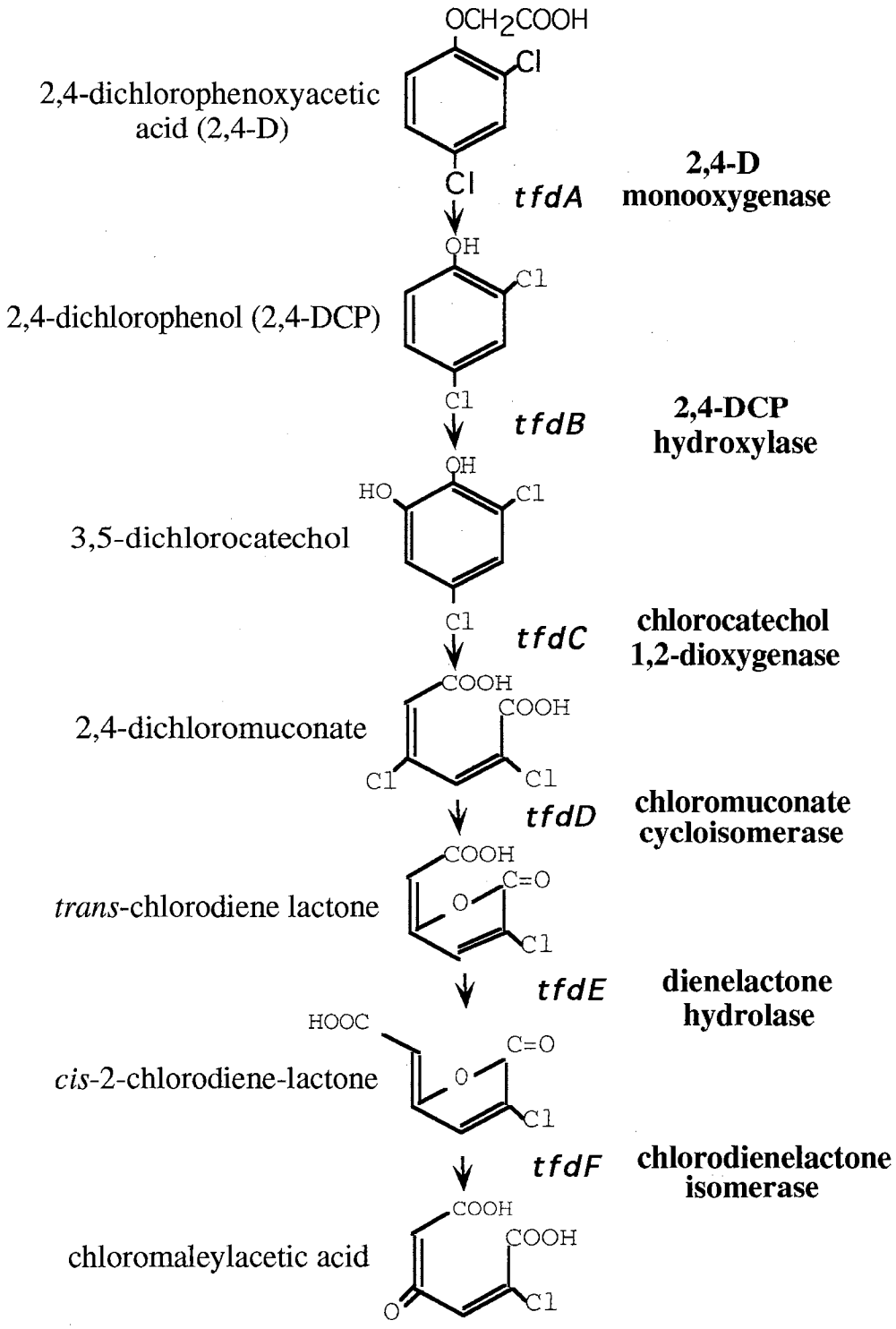

Figure 4. TFD Pathway on the Plasmid pJP4.

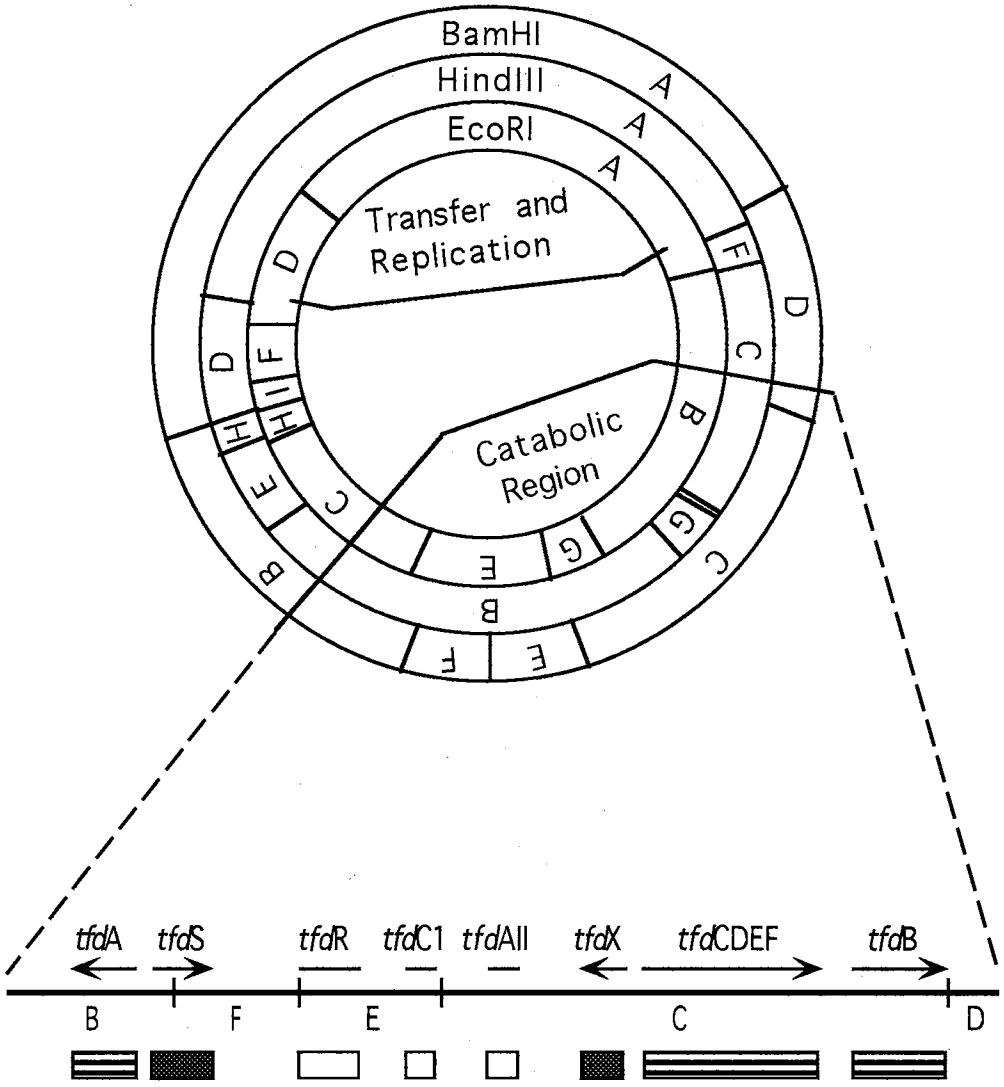

Figure 5. Physical Map of Plasmid of pJP4 and Positions of the TFD Genes on BamHI Fragments.

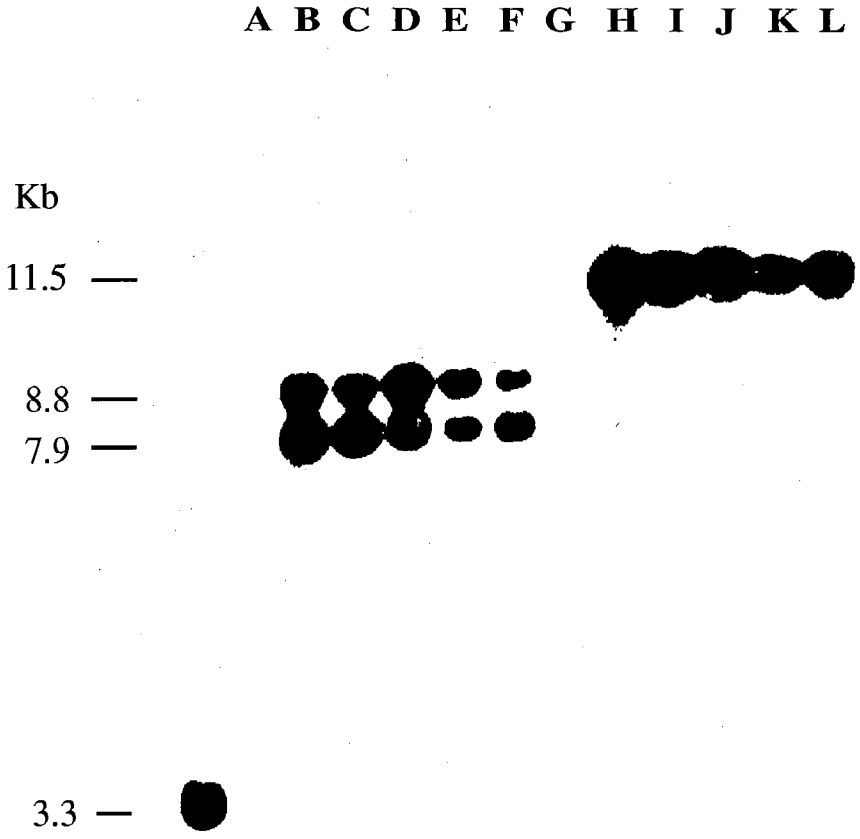

Figure 6. Southern Blot of DNA from AEK301 to AEK305. Lane A, 3.3kb HindIII cut of *Tn*5; B to F, BamHI cut DNA from AEK301 to AEK305; G, EcoRI cut of AEK101; H to L, EcoRI cut DNA from AEK301 to AEK305 respectively. The probe was ^{32}P-labeled 3.3 kb HindIII fragment of *Tn*5

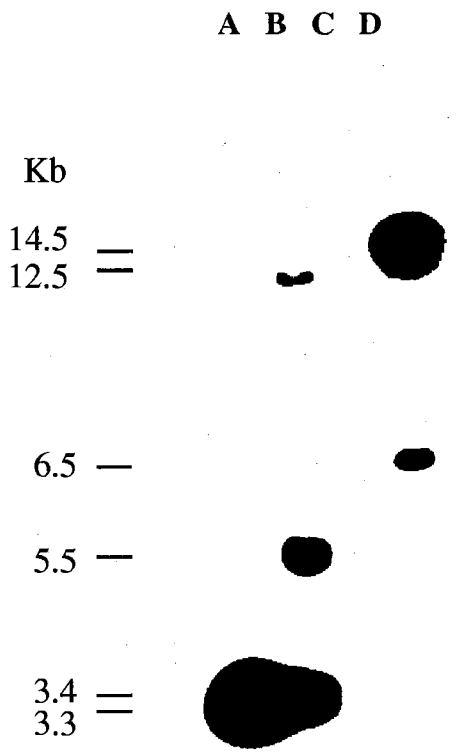

Figure 7. Southern Blot of DNA from AEK201. Lane A, 3.3kb HindIII cut of *Tn*5; B, AEK201 DNA cut with BamHI; C, AEK101 DNA cut with EcoRI; D, AEK201 DNA cut with EcoRI. The probe was ^{32}P-labeled 3.3kb HindIII fragment from *Tn*5.

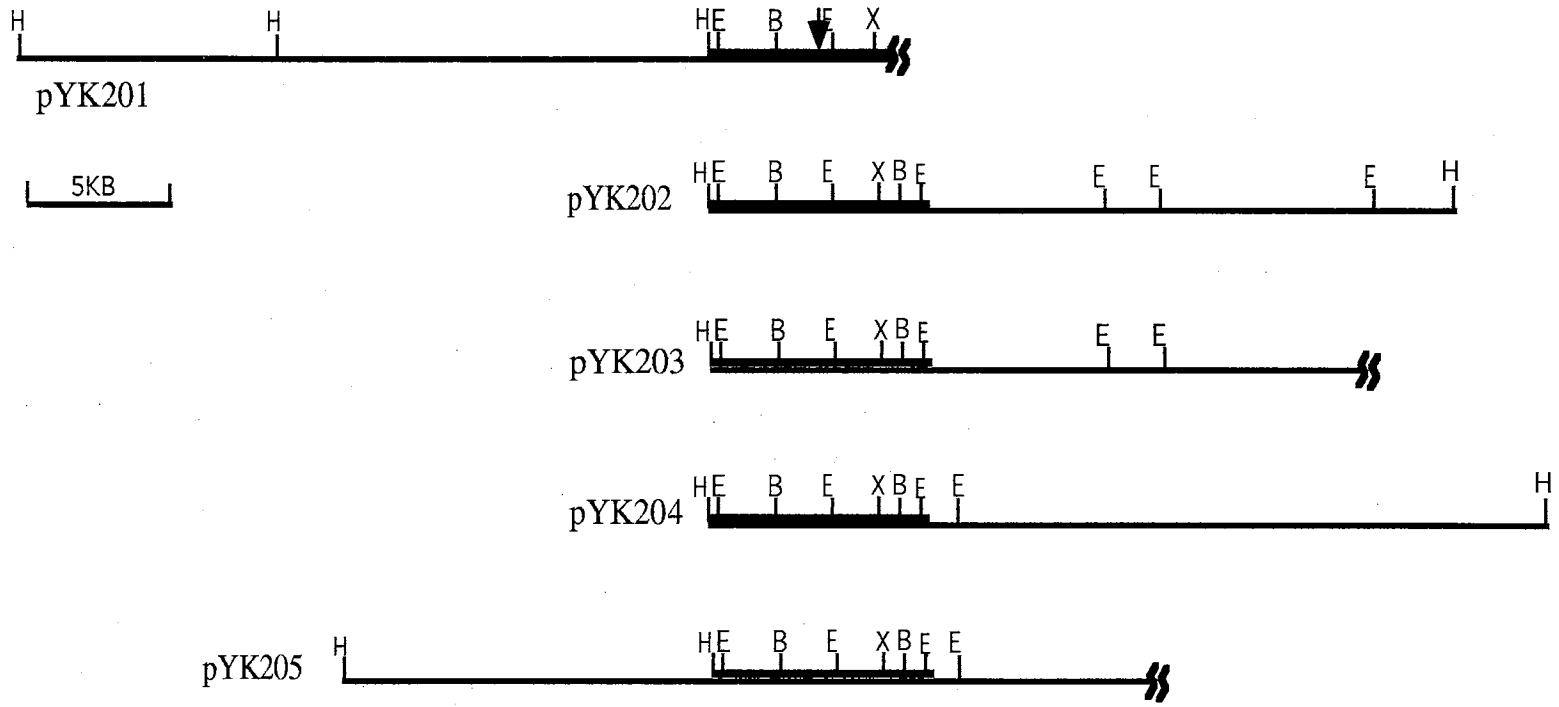

Figure 8. Partial Physical Map of Cosmids pYK201 to pYK205. Simbols: ▼, Location of *Tn*5 insertion; ≋, incomplete HindIII cut; ▬, common fragment; E, EcoR1; H, HindIII; B, BamHI; X, XbaI.

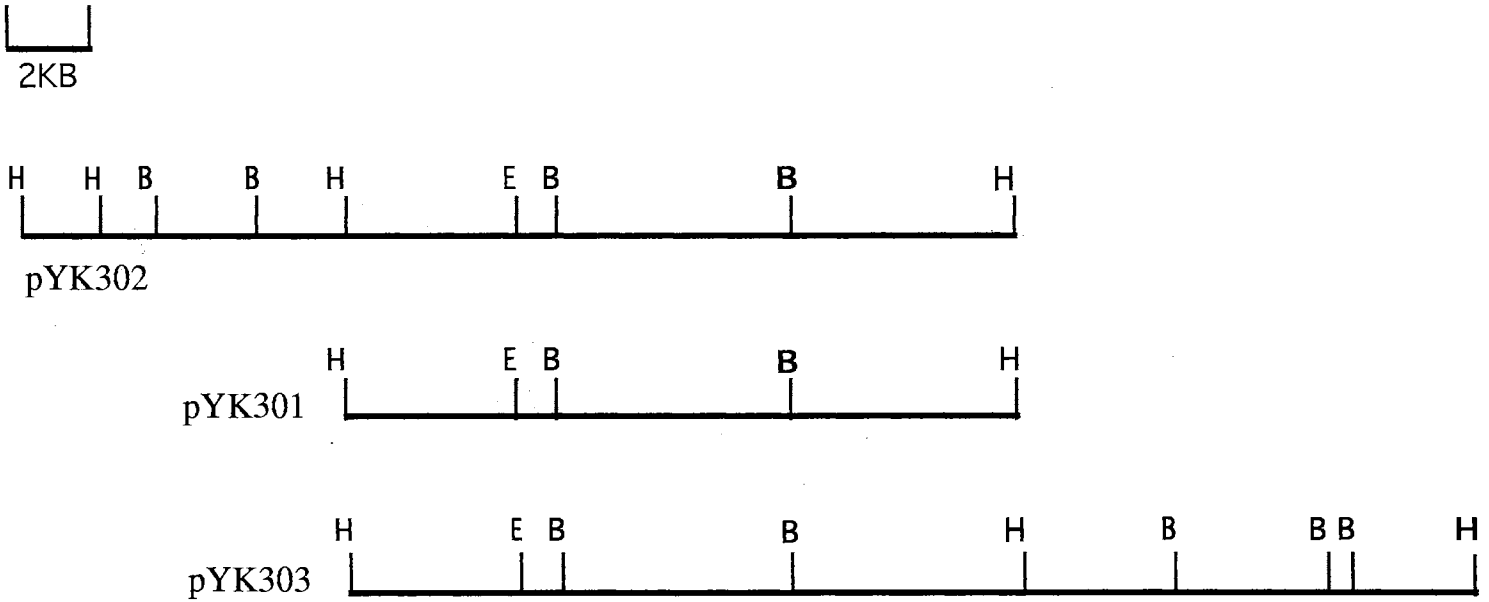

Figure 9. Partial Physical Maps of Cosmids pYK301 to pYK303
Simbols: H, HindIII; E, EcoRI; B, BamHI.

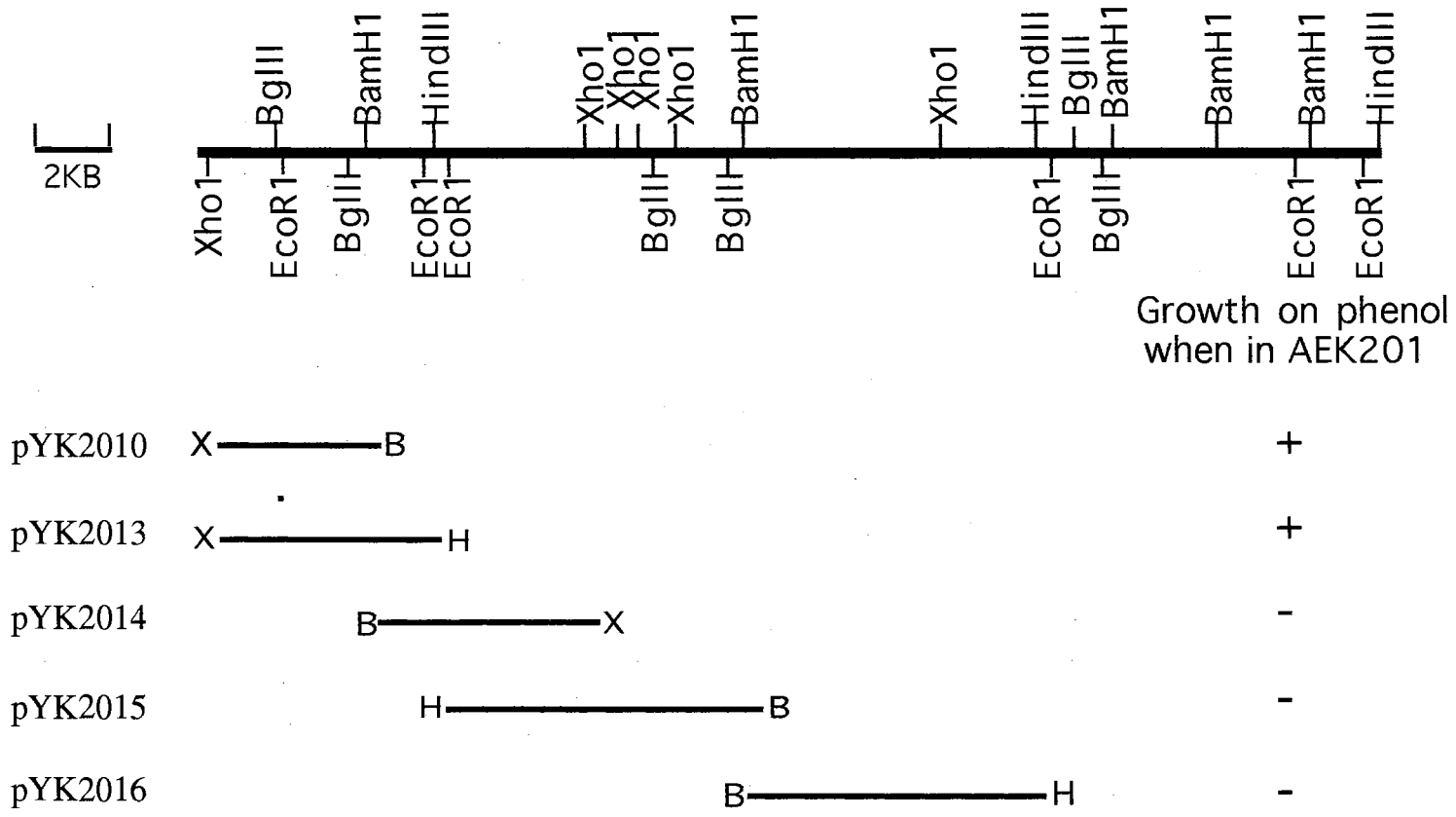

Figure 10. Physical map of Cosmid pYK201 and Subcloned DNA fragments Which Complement Mutant AEK201 to AEK205

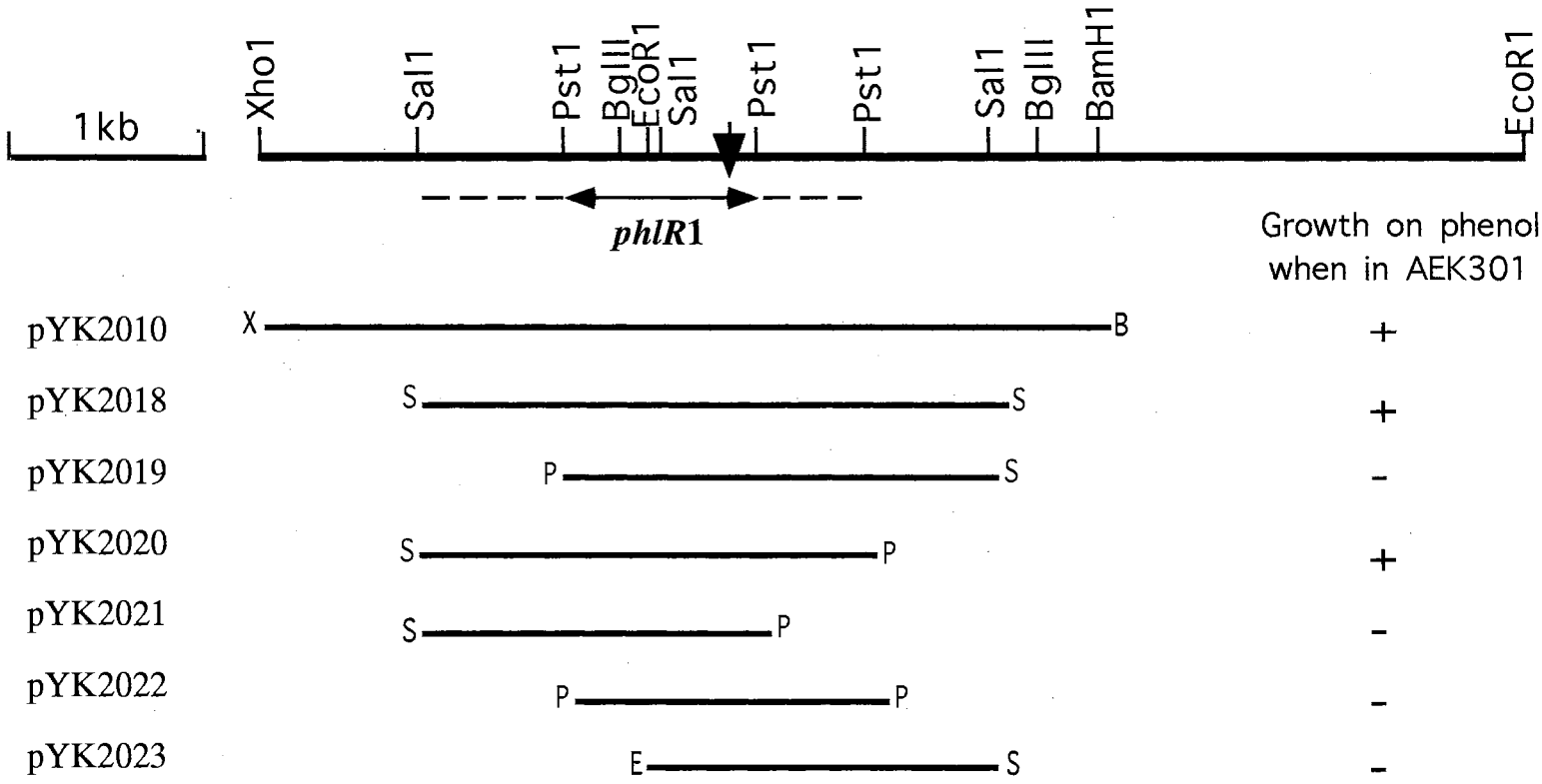

Figure 11. Subcloning and Localization of the *phl*R1.
▼, Tn5 insertion site

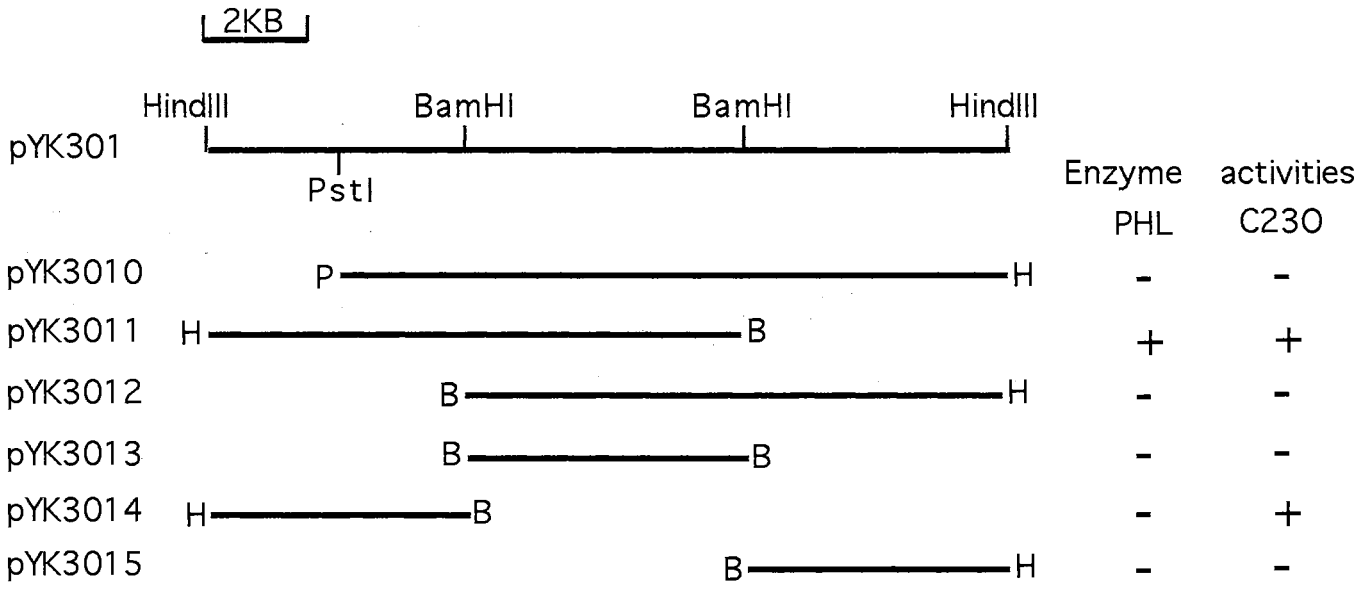

Figure 12. Subcloning and Localization of the phlH and c23O genes I
PHL; phenol hydroxylase, C23O; catechol 2,3-dioxygenase.

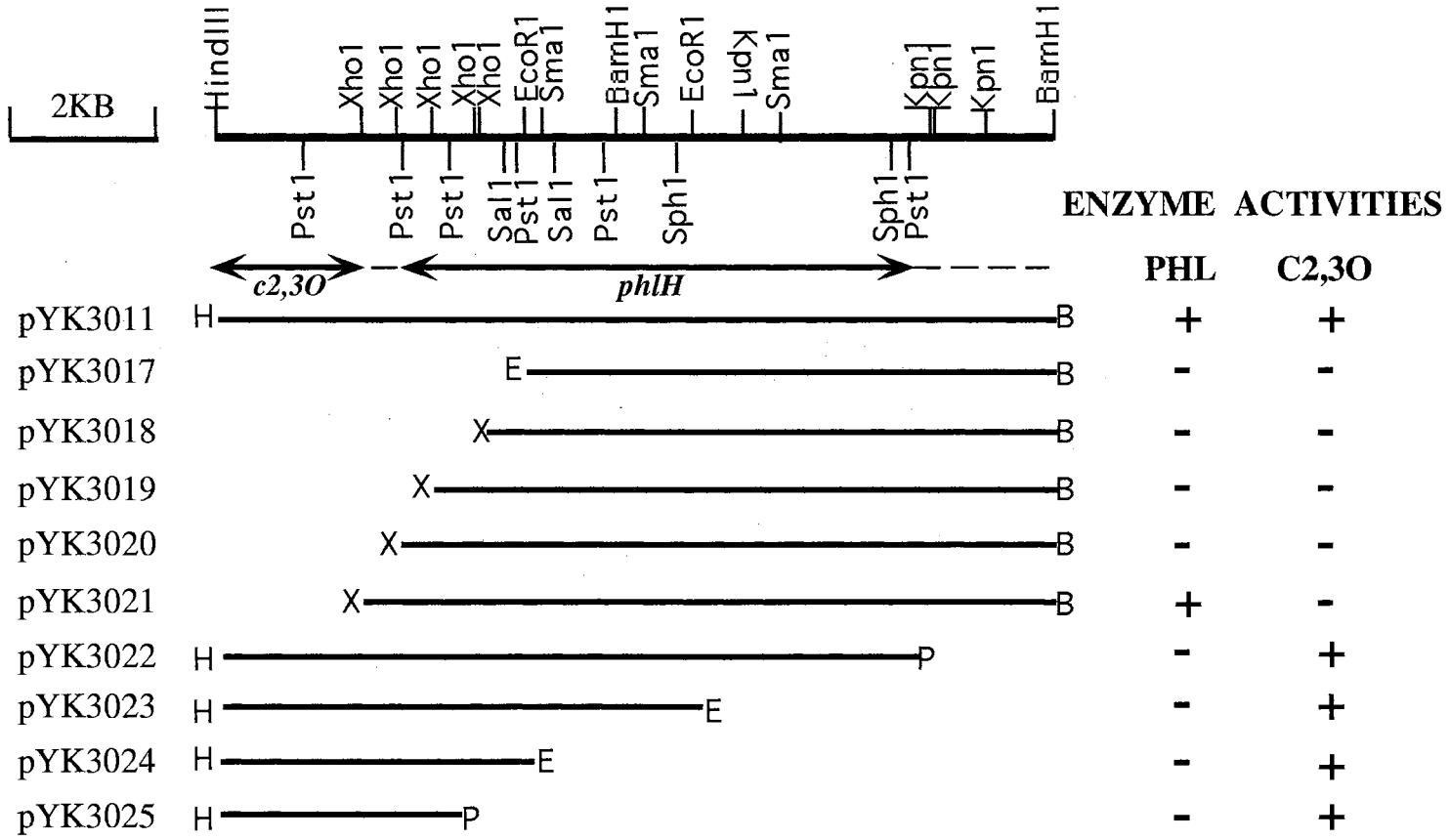

Figure 13. Subcloning and Localization of the *phl*H and *c23*O genes II
PHL; phenol hydroxylase, C23O; catechol 2,3-dioxygenase

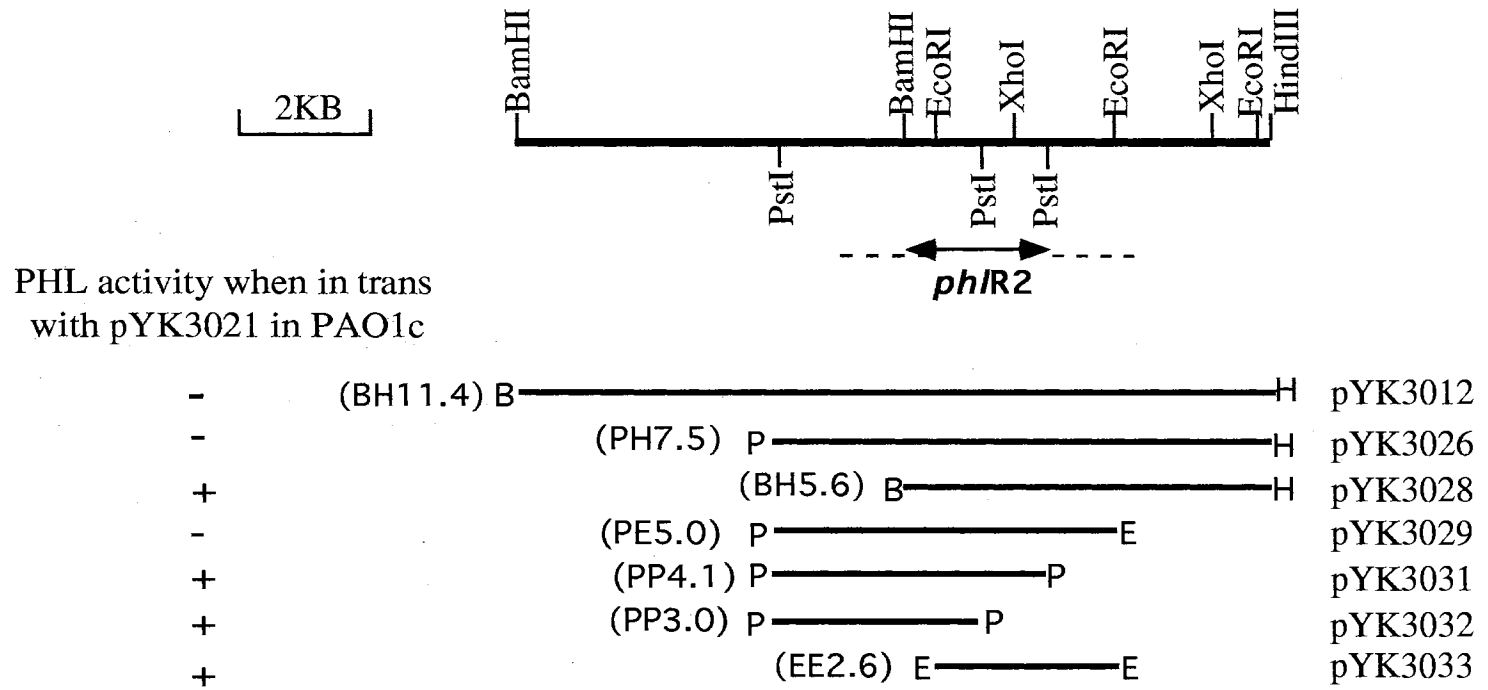

Figure 14. Subcloning and Localization of the *phl*R2.

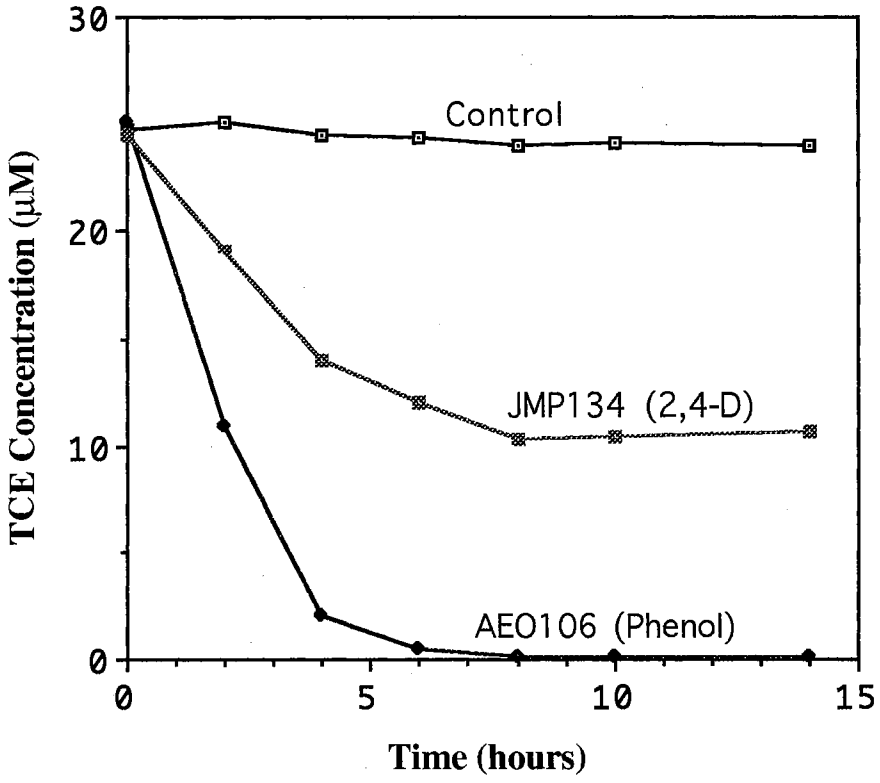

Figure 15. TCE Degradation by *Alcaligenes eutrophus* JMP134

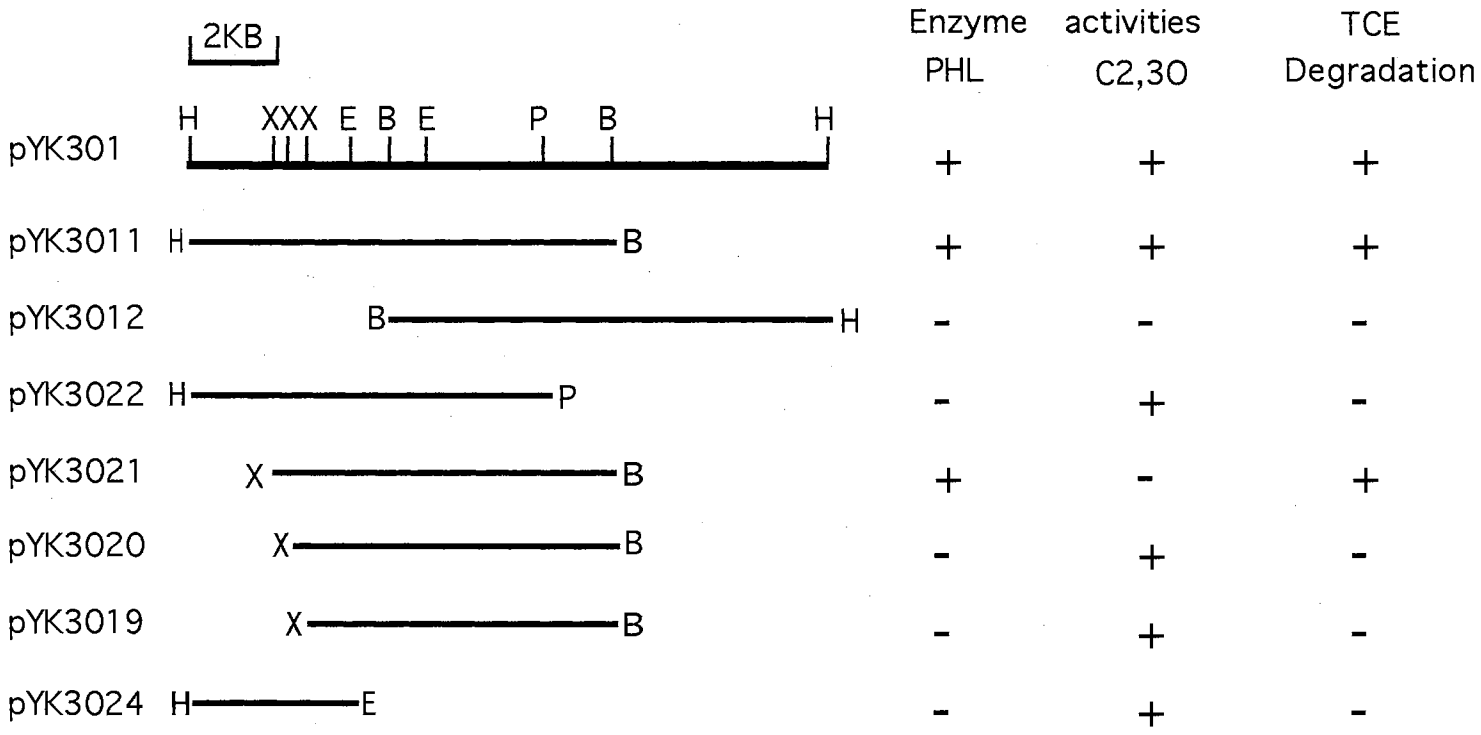

Figure 16. TCE Degradation by AEK301 Harboring Various Plasmids
E, EcoRI; B, BamHI; H, HindIII; P, PstI; X, XhoI
PHL; Phenol hydroxylase, C23O; Catechol 2,3-dioxygenase.

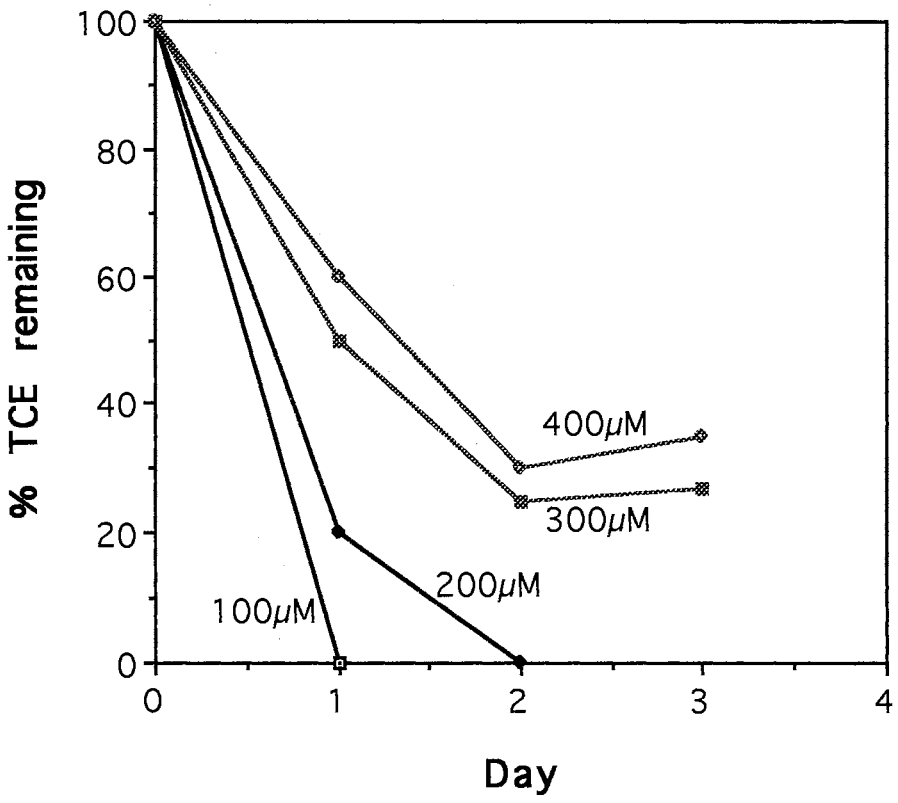

Figure 17. TCE Degradation by pYK3021 in AEK301

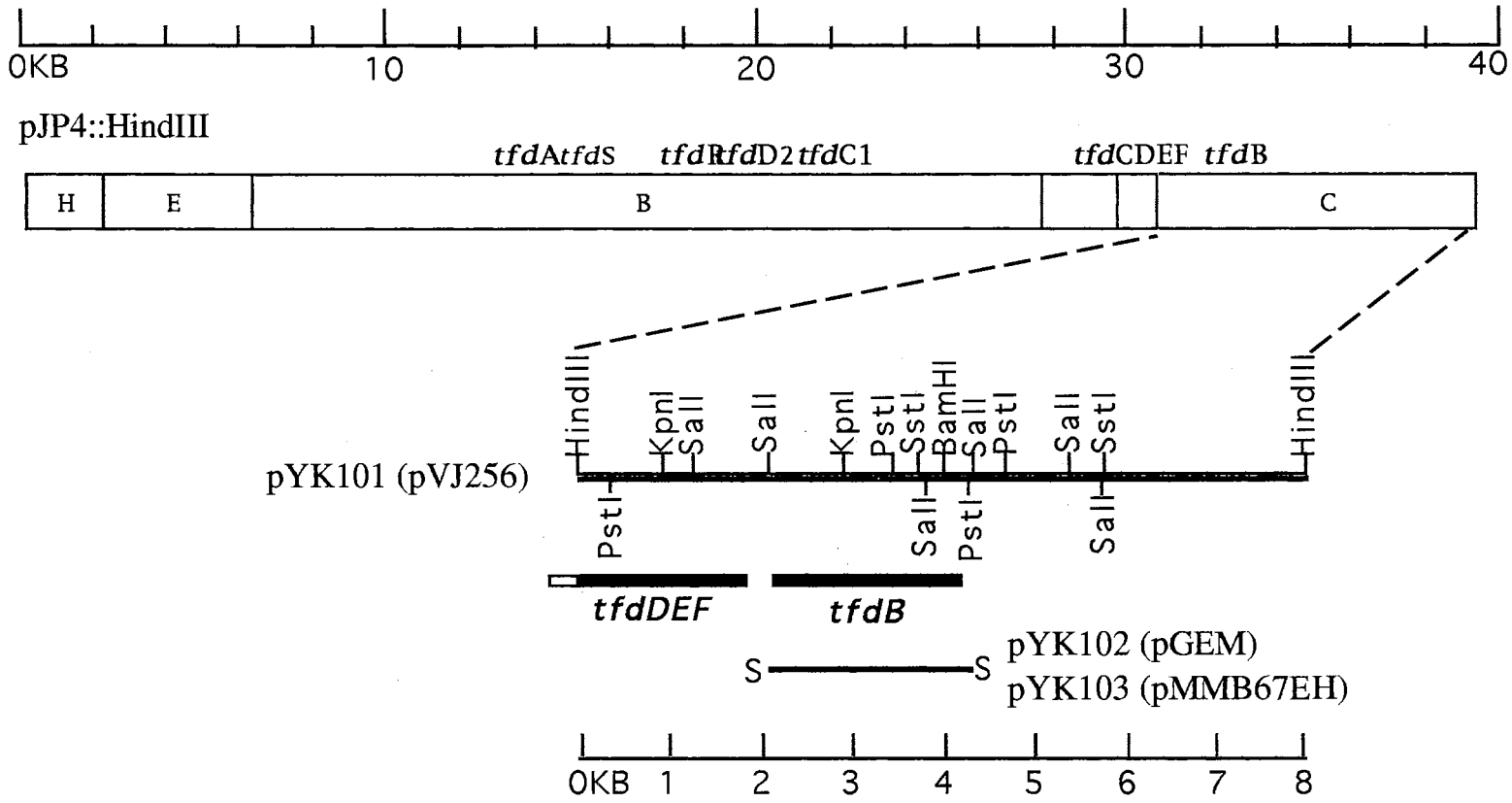

Figure 18. Cloning of the *tfdB* Gene from pJP4

2

VITA

Young-Jun Kim

Candidate for the Degree of

Doctor of Philosophy

Thesis: A RECOMBINANT APPROACH TO THE ISOLATION AND CHARACTERIZATION OF A PRIMARY DEGRADER OF TRICHLOROETHYLENE

Major Field: Microbiology

Biographical:

Personal Data: Born in Korea, October, 11, 1957, the son of Ja-Kyoung Gu and Do-Sang Kim.

Education: Graduated from Dong-Sung High School, Seoul, Korea, in January 1976; received Bachelor of Science degree in Biology from Yonsei University, Seoul, Korea, in August 1983; received Master of Science degree in Microbiology from Yousei University, Korea, in August 1985; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in July, 1993.

Professional Experience: Teaching and Research Assistant, Department of Microbiology and Molecular Genetics, Oklahoma State University, May, 1988 to July 1993; Research Assistant at International Milk Production, Korea, January, 1986 to May 1987; Military service, Korea, October, 1979 to July 1982.