

# Constitutive Expression of the Cloned Phenol Hydroxylase Gene(s) from *Alcaligenes eutrophus* JMP134 and Concomitant Trichloroethylene Oxidation

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Given the demonstrated phenol-dependent trichloroethylene (TCE) degradation in *Alcaligenes eutrophus* JMP134 (A. R. Harker and Y. Kim, *Appl. Environ. Microbiol.* 56:1179-1181, 1990), this work represents a purposeful effort to create a constitutive degrader of TCE. Genes responsible for phenol hydroxylase activity were identified by Tn5 transposon mutagenesis. Mutants lacked both phenol hydroxylase and catechol 2,3-dioxygenase activities. Southern blot analysis of total DNA showed that all mutants contained a single copy of Tn5 inserted in the same 11.5-kb *EcoRI* fragment. Complementation with a cosmid-based gene bank constructed from *A. eutrophus* AEK101 allowed the isolation of three recombinant cosmids carrying a common 16.8-kb *HindIII* fragment. Deletion and subcloning analysis localized the genes involved in phenol hydroxylase and catechol 2,3-dioxygenase activities. Partial sequence analysis of regions within the cloned phenol hydroxylase-expressing fragment shows significant homology to the oxygenase and oxidoreductase subunits of toluene-3-monooxygenase from *Pseudomonas pickettii*. The Tn5-induced *phl* mutant, carrying a recombinant plasmid expressing the phenol hydroxylase activity, degrades TCE in the absence of induction. Complete removal of TCE (50  $\mu$ M) within 24 h was observed in minimal medium containing only 0.05% ethanol as a carbon source. The bacterium removed 200  $\mu$ M TCE to below detectable levels within 2 days under noninducing and nonselective conditions.

The pathways involved in bacterial degradation of aromatic compounds have been extensively studied, and many represent good models for the degradation of xenobiotics. *Alcaligenes eutrophus* JMP134(pJP4) is a soil bacterium with diverse metabolic capabilities, including the use of 2,4-dichlorophenoxyacetic acid and phenol (10, 32, 33) as the sole carbon source. Trichloroethylene (TCE) is likewise cometabolized by two separate and distinct pathways in *A. eutrophus*: a chromosomally encoded phenol-dependent pathway, and the plasmid-encoded 2,4-dichlorophenoxyacetic acid pathway (16). A variety of aerobic microorganisms have been shown to degrade TCE when grown under conditions in which specific substrates induce relevant activities. These inducers include various aromatic and aliphatic hydrocarbons such as toluene (26, 27), phenol (13, 16), isopropylbenzene (8), propane (44), and methane (23, 43).

Although a number of studies have been performed to elucidate the genetic and molecular architecture of the plasmid-encoded 2,4-dichlorophenoxyacetic acid pathway (11, 12, 17, 19, 25, 30-32, 42), little is known about the chromosomally encoded phenol pathway in *A. eutrophus* JMP134. Constitutive expression of these genes would have tremendous potential for the remediation of TCE-contaminated sites. Such strains have been previously engineered by using toluene-4-monooxygenase genes from *Pseudomonas mendocina* (47) and toluene-2-monooxygenase from *Burkholderia cepacia* G4 (38).

In this paper, the isolation of mutants defective in phenol degradation is described. The genes for phenol hydroxylase and catechol 2,3-dioxygenase activity were cloned and ex-

pressed independently and constitutively under various conditions in *A. eutrophus*. Expression of the gene(s) encoding phenol hydroxylase activity effects the oxidation and removal of TCE in the absence of aromatic induction and under conditions of limited carbon.

## MATERIALS AND METHODS

**Strains, plasmids, media, growth conditions, and chemicals.** The bacterial strains and plasmids used in this study are listed in Table 1. Strains of *Escherichia coli* were grown on Luria-Bertani (LB) medium (24). Cultures of *Alcaligenes eutrophus* were maintained on tryptone-yeast extract-glucose medium (TNA) (29). All strains for the determination of enzyme activities were grown on minimal salts medium (MMO) (41) supplemented with the appropriate carbon sources (indicated amount of phenol and/or benzoate, 0.1 or 0.05% ethanol, and 0.3% Casamino Acids). Antibiotics were used at the following concentrations (micrograms per milliliter): ampicillin, 100 (*E. coli*); carbenicillin, 100 (*A. eutrophus*); kanamycin, 50 (*E. coli*) and 100 (*A. eutrophus*); rifampin, 150 (*A. eutrophus*); and tetracycline, 20 (*E. coli*) and 25 (*A. eutrophus*). Typically, 40-ml cultures in 250-ml Erlenmeyer flasks were grown with shaking at 180 rpm at 30 and 37°C for *A. eutrophus* and *E. coli*, respectively. *Pseudomonas aeruginosa* was cultured as for *A. eutrophus*. Phenol, catechol, and benzoate were purchased from Aldrich Chemical Co. Casamino Acids and all antibiotics were purchased from Difco and Sigma, respectively.

**Transposon mutagenesis.** Plasmid pUW964 was used to generate Tn5-induced mutants of *A. eutrophus* AEK101 defective in phenol metabolism. pUW964 has the ability to transfer into *A. eutrophus* strains but is unable to replicate in this host because of the limited host range of the ColE1 origin (46). The recipient, *A. eutrophus* AEK101, was isolated by selection of the parental strain, *A. eutrophus* AEO106, on TNA containing rifampin. TNA agar plates containing kanamycin and rifampin effectively select for *A. eutrophus* AEK101::Tn5 exconjugants and do not permit growth of the donor cells. *A. eutrophus* AEK101 was grown overnight in TNA broth plus rifampin. *E. coli* S17(pSUP2021) and *E. coli* HB101(pUW964) were grown to exponential phase in LB medium supplemented with kanamycin. A 1-ml portion of each donor strain was mixed with 0.5 ml of the recipient strain in a 1.5-ml Eppendorf tube. The mixture was centrifuged for 1 min in a microcentrifuge, and the pellet was suspended in 100  $\mu$ l of TNA broth. The cell mixture was spread onto nitrocellulose filters (Millipore Co.), which were placed on TNA agar plates and incubated for 4 to 6 h at 30°C. Each filter was then washed with 2 ml of MMO. Samples (0.1 ml) of the suspension were spread on TNA plates containing rifampin and kanamycin. The plates were incubated at 30°C. Km<sup>r</sup> Rif<sup>r</sup> colonies of *A. eutrophus* were visible after 48 h.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) <sup>a</sup>	Reference or source
<i>E. coli</i>		
HB101	F <sup>-</sup> <i>hsdS20 recA13 arg-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44</i> λ <sup>-</sup> Thi <sup>-</sup> Leu <sup>-</sup>	5
LE392	F <sup>-</sup> <i>hsdS574 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55</i> λ <sup>-</sup>	Promega Co.
S17	<i>hsdR hasM<sup>+</sup> recA</i> Thi Pro	39
<i>P. aeruginosa</i>		
PAO1c	Prototroph	29
<i>A. eutrophus</i>		
JMP134	Prototroph, Phl <sup>+</sup> Tfd <sup>+</sup> Hg <sup>f</sup>	10
AEO106	Prototroph, Phl <sup>+</sup> Tfd <sup>-</sup> Hg <sup>f</sup>	17
AEK101	Rif <sup>r</sup> derivative of AEO106	This study
AEK301	AEK101::Tn5, Phl <sup>-</sup> Km <sup>f</sup>	This study
AEK311	AEK301 revertant, Phl <sup>+</sup> Km <sup>-</sup>	This study
Plasmids		
pMMB67EH	Tac expression vector, cloning sites of pUC18, Ap <sup>r</sup>	14
pSUP2021	pBR325- <i>mob</i> ::Tn5, Km <sup>f</sup> , Ap <sup>r</sup> , Cm <sup>r</sup>	39
pRK2013	Km <sup>r</sup> Tra <sup>+</sup> ; ColE1 replicon	9
pUW964	TraRK2 <sup>+</sup> D( <i>repRK2</i> ) <i>repE1</i> <sup>+</sup> Tn5, Tn7, Km <sup>f</sup> , Sm <sup>f</sup> , Sp <sup>r</sup>	46
pVK102	IncP, <i>cos</i> <sup>+</sup> , Km <sup>f</sup> , Tc <sup>r</sup>	20
pYK301	16.8-kb <i>Hind</i> III fragment of AEO106 DNA in pVK102	This study
pBSIIKS+	Ap <sup>r</sup> , commercial cloning vector	Stratagene
Clones in pMMB67EH		
pYK3011	11.2-kb <i>Hind</i> III- <i>Bam</i> HI fragment from pYK301, phlH <sup>+</sup> , C23O <sup>+</sup> , Ap <sup>r</sup>	This study
pYK3017	6.8-kb <i>Eco</i> RI- <i>Bam</i> HI fragment from pYK3011, Ap <sup>r</sup>	This study
pYK3018	7.3-kb <i>Xho</i> I- <i>Bam</i> HI fragment from pYK3011, Ap <sup>r</sup>	This study
pYK3019	7.9-kb <i>Xho</i> I- <i>Bam</i> HI fragment from pYK3011, Ap <sup>r</sup>	This study
pYK3020	8.5-kb <i>Xho</i> I- <i>Bam</i> HI fragment from pYK3011, Ap <sup>r</sup>	This study
pYK3021	9.1-kb <i>Xho</i> I- <i>Bam</i> HI fragment from pYK3011, phlH <sup>+</sup> , Ap <sup>r</sup>	This study
pYK3022	9.3-kb <i>Hind</i> III- <i>Pst</i> I fragment from pYK3011, C23O <sup>+</sup> , Ap <sup>r</sup>	This study
pYK3023	6.4-kb <i>Hind</i> III- <i>Eco</i> RI fragment from pYK3011, C23O <sup>+</sup> , Ap <sup>r</sup>	This study
pYK3024	4.1-kb <i>Hind</i> III- <i>Eco</i> RI fragment from pYK3011, C23O <sup>+</sup> , Ap <sup>r</sup>	This study
pYK3025	3.1-kb <i>Hind</i> III- <i>Pst</i> I fragment from pYK3024, C23O <sup>+</sup> , Ap <sup>r</sup>	This study

<sup>a</sup> Abbreviations: phlH, phenol hydroxylase; C23O, catechol 2,3-dioxygenase; Phl<sup>-</sup>, deficient in phenol degradation. Antibiotics: Ap, Cb, Cm, Gm, Km, Rif, Sm, Sp, Tc, and Tp refer to ampicillin, carbenicillin, chloramphenicol, gentamicin, kanamycin, rifampin, streptomycin, spectinomycin, tetracycline, and trimethoprim, respectively. Hg<sup>f</sup>, mercury resistant.

**Screening for phenol hydroxylase-deficient mutants.** *A. eutrophus* transconjugants grown on TNA agar plates containing rifampin and kanamycin were replica plated on the same medium and MMO agar plates containing rifampin and kanamycin with one of the following carbon sources: phenol (0.05%), benzoate (0.05%), or phenol (0.05%) plus Casamino Acids (0.3%). Casamino Acids supported the growth of the cells on the phenol-containing medium. The phenol-utilizing cells turn dark brown on this medium because of the formation and autooxidation of catechol. After 2 to 3 days of incubation at 30°C, mutants showing no growth on phenol but growth on benzoate were selected for further study. This screening procedure was adopted because benzoate and phenol are catabolized via catechol, a common intermediate. It is presumed that a mutation which allows growth on benzoate but not on phenol is deficient in either a structural gene(s) or a regulatory gene(s) required for the direct hydroxylation of phenol.

**Construction of a genomic library.** *A. eutrophus* AEO106 DNA was partially digested with *Hind*III and size fractionated on a 10 to 40% sucrose gradient by standard procedures (24). A total of 10 μg of DNA (~20- to 30-kb fragments) was ligated with 5 μg of *Hind*III-digested and phosphatase-treated pVK102 (20). Ligated DNA was packaged with lambda DNA packaging extracts (Promega Co.), and phage particles were used to transduce competent *E. coli* LE392 cells. Transductants were selected on LB plates containing tetracycline.

**Identification of complementing clones.** A triparental replica-plating method described by Andersen and Douglas (2) was used to screen the gene bank for complementation of *A. eutrophus* mutants. An appropriate titer of the gene bank (~1,000 to 3,000 colonies per plate) was spread on LB plates containing tetracycline. 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 the *A. eutrophus* mutant to be complemented. The cells used for preparing the lawn were from cultures grown overnight on TNA plates containing kanamycin plus rifampin for *A. eutrophus* mutants and on LB plates containing kanamycin for *E. coli*. A loopful of each strain was mixed into 200 μl of TNA, and 100 μl of the mixture was used to prepare the lawn. The mating plates were incubated for 4 to 6 h at 30°C. Cells from the mating plates

were then transferred by replica plating to MMO plates containing 0.03% phenol, kanamycin, rifampin, and tetracycline. *A. eutrophus* colonies resulting from complementation appeared after incubation for 2 to 3 days at 30°C. The complementing *E. coli* gene bank clones were then identified and purified for further study.

**Southern hybridization.** Nick translation was performed with a kit purchased from Promega. The probe DNA was prepared from plasmid pSUP2021, which was isolated from *E. coli* S17. Nucleic acids were transferred from the agarose gel to a blotting membrane (Zeta-Probe GT blotting membranes; Bio-Rad Ltd.) with a vacuum blotter (MilliBlot-V; Millipore) for 30 min. DNA was hybridized in 0.25 M Na<sub>2</sub>HPO<sub>4</sub> (pH 7.2)-7% sodium dodecyl sulfate (SDS) for 20 h at 65°C with gentle agitation as recommended by the manufacturer (Bio-Rad). Washing solutions contained 20 mM Na<sub>2</sub>HPO<sub>4</sub> and 5% SDS. The SDS concentration was 1% for the final step at 65°C.

**Other DNA protocols.** Total genomic DNA from *A. eutrophus* wild-type and mutant strains was prepared as described by Ausubel et al. (3). DNA was further purified by centrifugation on CsCl gradients (24). Plasmid DNA was isolated from *Alcaligenes* strains by the method of Birnboim and Doly (4). Plasmid DNA from *E. coli* was isolated by standard methods (24). Restriction enzyme mapping, agarose gel electrophoresis, isolation and purification of DNA fragments from agarose gels, and preparation of competent *E. coli* cells were performed by the standard procedures (24). Digestion of DNA with restriction endonucleases, ligation of DNA with T4 DNA ligase, and calf intestinal alkaline phosphatase treatment were done as specified by the manufacturer (Promega Co.).

**Nucleotide sequence determination and analysis.** Various restriction fragments from pYK3021 were isolated by gel electrophoresis and electroelution and subcloned into the multiple-cloning site of the pBSIIKS+ sequencing vector (Stratagene). The nucleotide sequence was determined by the dideoxy method of Sanger et al. (36) facilitated by the use of an Applied Biosystems automated sequencer. The sequence data were compared with GenBank and EMBL databases by using the Blastx program for similarity to the putative amino acid sequences in all six reading frames (1, 15).

**Plasmid mobilization.** A triparental mating method (9) was used to transfer

TABLE 2. Enzyme activities in *A. eutrophus* AEO106 and its derivatives

Strain	Culture conditions	Enzyme activity <sup>a</sup> of:	
		Phenol hydroxylase	Catechol 2,3-dioxygenase
AEO106	Phenol (2.5 mM)	52.2	0.132
	Ethanol (0.1%)	ND <sup>b</sup>	ND
	Phenol (2.5 mM)-ethanol (0.1%)	32.6	0.023
AEK101	Phenol (2.5 mM)	51.1	0.125
	Ethanol (0.01%)	ND	ND
	Phenol (2.5 mM)-ethanol (0.1%)	28.6	0.017
AEK301	Phenol (2.5 mM)	ND	ND
	Phenol (2.5 mM)-ethanol (0.1%)	ND	0.001
AEK301 with:			
pYK301	Phenol (2.5 mM)-ethanol (0.1%)	24.2	0.024
pYK3011	Phenol (2.5 mM)-ethanol (0.1%)	13.0	0.021
pYK3021	Phenol (2.5 mM)-ethanol (0.1%)	16.7	ND
pYK3023	Phenol (2.5 mM)-ethanol (0.1%)	ND	0.081
pYK3024	Phenol (2.5 mM)-ethanol (0.1%)	ND	0.103

<sup>a</sup> Phenol hydroxylase is expressed as the increase above basal levels of nanomoles of oxygen consumed per minute. Cultures were normalized to an optical density of 1.0 at 425 nm. Catechol 2,3-dioxygenase activity is expressed as micromoles of product formed per minute per milligram of protein.

<sup>b</sup> ND, not detected (<0.03 nmol of O<sub>2</sub> per min for phenol hydroxylase; <0.0006 μmol/min/mg for catechol 2,3-dioxygenase).

the recombinant pVK102 or pMMB67 from *E. coli* to *A. eutrophus* in the presence of helper plasmid pRK2013. Loopfuls of parental cells which were grown overnight on the appropriate plates were mixed in TNA at a ratio of 1:1:1. An aliquot was spread on TNA and then incubated for 3 to 5 h at 30°C. Transconjugants were selected by streaking or plating dilutions of the mating mixture onto the appropriate selective media.

**Enzyme assays.** Phenol hydroxylase activity was assayed with resting cells. Oxygen consumption was measured with a Clark oxygen electrode (Yellow Springs Instrument Co.) in the presence and absence of 0.5 mM phenol as described by Sala-Trepal et al. (35). Enzyme activity is expressed as the increase above basal levels of oxygen consumption upon addition of phenol in nanomoles per hour at a culture density (measured as optical density at 425 nm) of 1.0. Catechol 2,3-dioxygenase was assayed by the method of Nozaki (28). Catechol 1,2-dioxygenase was measured by the modified procedure of Hegeman (18). Cells were harvested during the late exponential phase and suspended in phosphate-acetone buffer (50 mM potassium phosphate [pH 7.5], 10% acetone) for catechol 2,3-dioxygenase determination and in Tris buffer (20 mM [pH 8.0], 400 μM EDTA) for catechol 1,2-dioxygenase determination. The cell suspension was disrupted by sonication, and the cell debris was removed by centrifugation at 13,000 × *g* for 10 min in a microcentrifuge. The supernatant was immediately used to measure enzyme activities. The protein concentration was determined by the procedure of Bradford (6).

**TCE degradation assay.** Cells were grown on TNA agar plates and suspended in MMO. Suspensions were diluted to an optical density at 425 nm of 0.1. A 20-ml portion of this cell suspension was dispensed into a 100-ml serum bottle and sealed with a Teflon-lined stopper and an aluminum crimp seal. Various concentrations of TCE from a stock solution in pentane were added by injection through the septum with a gas-tight syringe (Hamilton, Reno, Nev.). The cultures were incubated at 30°C with shaking at 180 rpm. A 1-ml portion of culture after overnight incubation was extracted with an equal volume of pentane, and 1 μl of the pentane phase was removed for gas chromatography analysis.

**Gas chromatography.** Gas chromatography analysis was performed with a Hewlett-Packard 5890 gas chromatograph equipped with a 25-m cross-linked methyl silicone gum capillary column (Hewlett-Packard) and an electron capture detection system. A 1-μl portion of each sample was injected through a 10-μl 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°C/min; and nitrogen carrier gas flow, 6 ml/min. Under these conditions, TCE had a retention time of 2.2 min.

## RESULTS AND DISCUSSION

**Isolation and characterization of Tn5-induced mutants.** Over 6,500 Km<sup>r</sup> Rif<sup>r</sup> exconjugants were screened for the ab-

sence of growth on phenol as a sole carbon source and the presence of growth on benzoate. Five mutants (AEK301 to AEK305) were isolated by this screening method, and all were sensitive to streptomycin; resistance to streptomycin is encoded on the carrier plasmid pUW964. No plasmids were recoverable from the mutant strains.

Because phenol is often a poor growth substrate, several carbon sources were screened to find a primary carbon source which would minimally affect the enzyme activities of interest (Table 2). The activity of phenol hydroxylase was slightly reduced by ethanol (0.1%). Addition of 5.0 mM benzoate or Casamino Acids decreased this enzyme activity about threefold. Benzoate at 5.0 mM repressed catechol 2,3-dioxygenase activity to 0.002% of that expressed by AEO106 grown on phenol alone.

Enzyme activities in mutant AEK301 were determined (Table 2). AEK301 grown in the presence of phenol and ethanol expressed no detectable activity for the two enzymes tested. Other mutants (AEK302 to AEK305) showed results identical to those shown by AEK301. The loss of two enzyme activities caused by a single insertional mutation implies that the genes specifying these enzymes lie in an operon or that these genes are controlled by a single disrupted regulatory gene.

**Southern analysis for Tn5 insertion.** Physical characterization of the sites of Tn5 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 Tn5, and hybridized with a <sup>32</sup>P-labeled internal *Hind*III fragment of Tn5. The hybridization pattern showed that all mutants contained Tn5 in the same 11.5-kb *Eco*RI fragment (Fig. 1). Digestion of the genomic DNA with *Bam*HI showed two bands (7.9 and 8.8 kb each) from all five mutants, suggesting that insertion of Tn5 occurred at the same site in all mutants.

**Cosmid library of *A. eutrophus* DNA.** *Hind*III fragments of chromosomal DNA from *A. eutrophus* were ligated with the cosmid vector pVK102. Approximately 1 μg of packaged DNA yielded 0.5 × 10<sup>4</sup> to 1.0 × 10<sup>4</sup> independent Tc<sup>r</sup> clones. These transductants were analyzed for their resistance to kanamycin. Only 3 of 100 clones were kanamycin resistant, implying that most clones have chromosomal DNA inserts. A number of

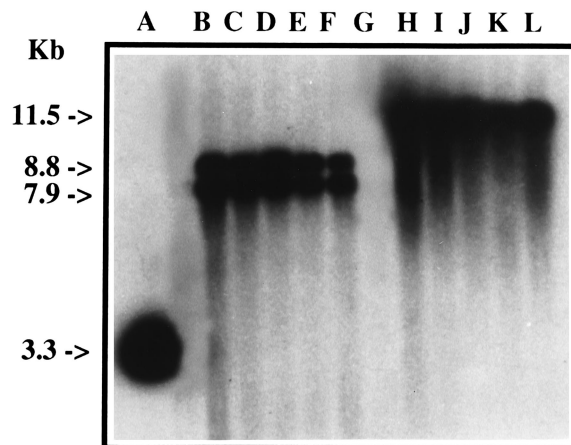


FIG. 1. Southern blot of DNA from AEK301 to AEK305. Lanes: A, 3.3-kb *Hind*III restriction digest of Tn5; B to F, *Bam*HI restriction digests of DNA from AEK301 to AEK305, respectively; G, *Eco*RI restriction digest of DNA from AEK101; H to L, *Eco*RI restriction digests of DNA from AEK301 to AEK305, respectively. The hybridization probe was the 3.3-kb *Hind*III fragment of Tn5 labeled with <sup>32</sup>P.

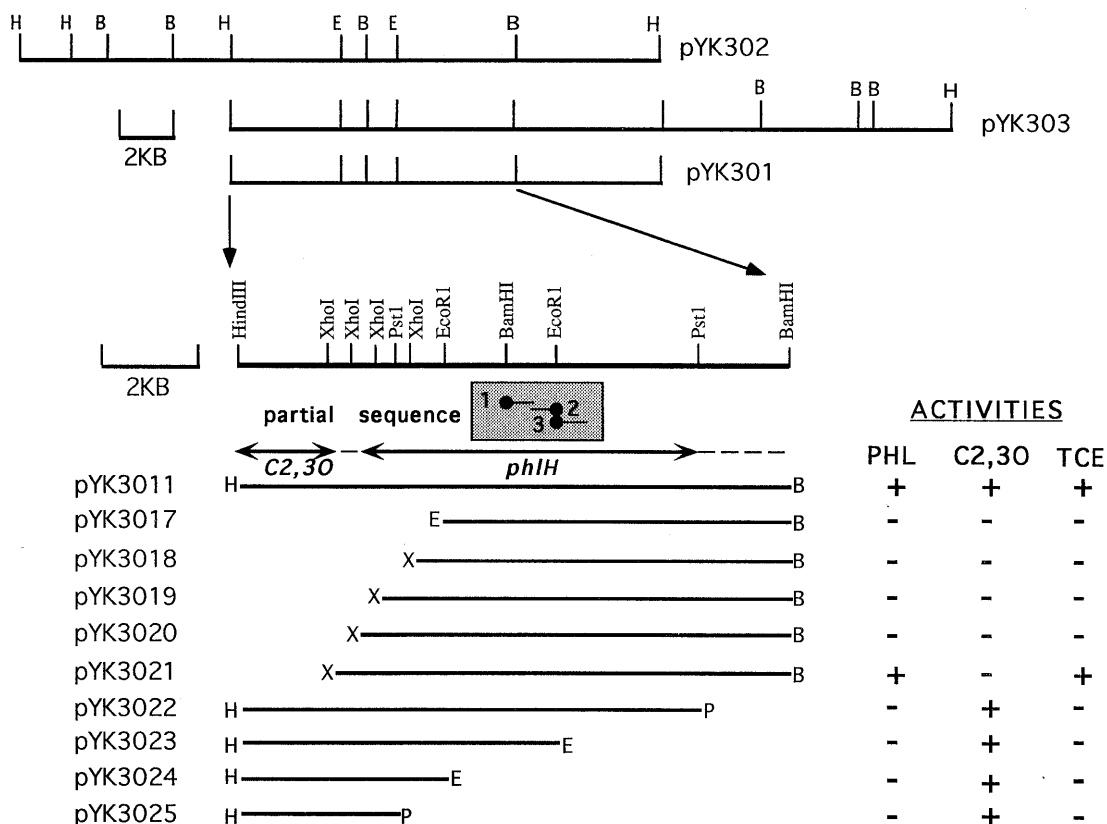


FIG. 2. Partial restriction map and subcloning of the cosmids pYK301, pYK302, and pYK303. Restriction enzyme abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; X, *Xho*I. Activity designations: PHL, phenol hydroxylase, C2,3O, catechol 2,3-dioxygenase; and TCE, TCE degradation.

these clones were analyzed for the sizes of the inserted fragments of DNA. These ranged from 17 to 35 kb, with a mean of 25 kb.

**Identification of cosmid clones complementing mutant strains.** A triparental replica-plating method (2) was used to identify cosmid clones from the *E. coli* LE392 gene bank which complement phenol hydroxylase-defective mutants. Complementation was successfully demonstrated on the selective medium. Each positive clone was isolated from the master plate and analyzed for the presence of a cosmid. Three classes of cosmids were isolated from all positive clones and are designated pYK301, pYK302, and pYK303 after analysis of their restriction enzyme cleavage patterns (Fig. 2). Both pYK302 and pYK303 contain the 16.8-kb *Hind*III fragment of pYK301.

The two enzyme activities were analyzed in the mutant strains after transfer of the positive cosmids by triparental mating (Table 2). AEK301 harboring any one of three cosmids (pYK301 to pYK303) expressed both phenol hydroxylase and catechol 2,3-dioxygenase activities. The activity of phenol hydroxylase in AEK301 carrying pYK301 was expressed at a level similar to that of the parent strain AEK101 (24.2 versus 28.6 nmol of O<sub>2</sub> per min).

**Subcloning and localization of the genes for phenol hydroxylase.** To localize the genes which express phenol hydroxylase and catechol 2,3-dioxygenase activity, the 16.8-kb *Hind*III fragment of pYK301 was subjected to a series of *Bam*HI deletions. These were subcloned (Fig. 2) into the *tac* expression vector pMMB67EH. All plasmids constructed with this vector were first transferred into *E. coli* by transformation and then mobilized into *A. eutrophus* mutant strains by triparental mating.

Only plasmid pYK3011, an 11.2-kb *Hind*III-*Bam*HI fragment, allowed AEK301 to express the activities of phenol hydroxylase and catechol 2,3-dioxygenase. Preliminary experiments indicate that the DNA segments deleted from pYK301 to produce pYK3011 contain a *trans*-acting phenol hydroxylase regulatory gene (data not shown).

Various deletion subclones were prepared from pYK3011 after the region was mapped for common restriction sites (Fig. 2). Plasmid pYK3021, a 9.1-kb *Xho*I-*Bam*HI fragment, expressed phenol hydroxylase activity, but catechol 2,3-dioxygenase activity was not detected. Neither plasmid pYK3022 nor pYK3020 allowed AEK301 to express phenol hydroxylase activity, indicating that more than 6.5 kb is needed for expression of this activity. Although plasmid pYK3022 failed to express phenol hydroxylase activity, it allowed AEK301 to express catechol 2,3-dioxygenase activity. Further deletion analysis of this plasmid resulted in plasmids pYK3024 and pYK3025, both of which express catechol 2,3-dioxygenase activity.

**Identification of the gene(s) responsible for TCE degradation.** Various strains carrying the subclone-derived plasmids were analyzed for the ability to degrade TCE in *A. eutrophus* AEK301 (Fig. 2). Of the plasmids tested, only three, pYK301, pYK3011, and pYK3021, caused the degradation of TCE. The strains harboring these plasmids expressed phenol hydroxylase activity. Catechol 1,2-dioxygenase was expressed (2.8 μmol/min/mg of protein) when these strains were grown on benzoate. TCE was not oxidized in the presence of active catechol 1,2-dioxygenase (data not shown) or catechol 2,3-dioxygenase (Table 3; Fig. 2). The evidence indicates that the enzyme

TABLE 3. Enzyme activities in *A. eutrophus* AEK301 carrying various plasmids

Plasmid	Enzyme activity <sup>a</sup> of:		TCE degradation
	Phenol hydroxylase	Catechol 2,3 dioxygenase	
None	ND <sup>b</sup>	ND	-
pYK301	27.11	0.0210	+
pYK3011	12.00	0.0195	+
pYK3021	18.58	ND	+
pYK3024	ND	0.1032	-

<sup>a</sup> Phenol hydroxylase is expressed as the increase above basal levels of nanomoles of oxygen consumed per minute. Cultures were normalized to an optical density of 1.0 at 425 nm. Catechol 2,3-dioxygenase activity is expressed as micromoles of product formed per minute per milligram of protein. TCE degradation is expressed as the presence (+) or absence (-) of TCE removal beyond the abiotic control.

<sup>b</sup> ND, not detected (<0.03 nmol of O<sub>2</sub> per min for phenol hydroxylase; <0.0006 μmol/min/mg for catechol 2,3 dioxygenase).

complex which expresses phenol hydroxylase activity is responsible for TCE oxidation.

**TCE degradation by *A. eutrophus* AEK301 containing various plasmids.** The extent of TCE degradation was observed in *A. eutrophus* AEK301 under various culture conditions (Table 4). Under conditions of phenol induction, AEK301 harboring pYK301 degraded TCE with the same pattern as that of the wild type, *A. eutrophus* AEK101. This strain completely removed added TCE (25 μM) in the presence of 2.5 mM phenol. The presence of ethanol reduced the effectiveness of TCE degradation. AEK301 harboring either pYK3021 or pYK3011 removed TCE most efficiently in the absence of phenol. These strains did not require any aromatic inducer for TCE removal, confirming the constitutive expression of phenol hydroxylase activity.

**TCE degradation by AEK301 containing pYK3021.** The ability of AEK301(pYK3021) to remove TCE was measured in the presence of various concentrations of TCE (Fig. 3). The cells were cultured in MMO plus 0.1% Casamino Acids without antibiotic selection. TCE (200 μM) was completely removed within 2 days. The phenol concentration in the medium also affects the degree of TCE degradation. Inhibition of TCE degradation by higher concentrations of phenol in AEK301 (pYK3021) may be explained by competitive inhibition between phenol and the fortuitous substrate (TCE). Such inhibition has been described by Folsom et al. (13). Further analysis should be performed to elucidate kinetics and interactions between phenol and TCE in AEK301(pYK3021).

When the concentration of TCE was increased to 400 μM, TCE degradation continued for 48 h and then ceased after

TABLE 4. TCE degradation by enzymes encoded on plasmids pYK301 and pYK3021

Substrate	% Decrease of TCE in <sup>a</sup> :		
	AEK101	AEK301 (pYK301)	AEK301 (pYK3021)
Phenol (2.5 mM)	99	99	6
Phenol (2.5 mM)-ethanol (0.1%)	74	33	ND
Ethanol (0.05%)	ND <sup>b</sup>	ND	99
Casamino Acids (0.3%)	ND	ND	99
TNA broth	ND	ND	65

<sup>a</sup> Values are expressed as percent decrease after overnight incubation and are the means of triplicate determinations.

<sup>b</sup> ND, not detected (<0.5% TCE loss).

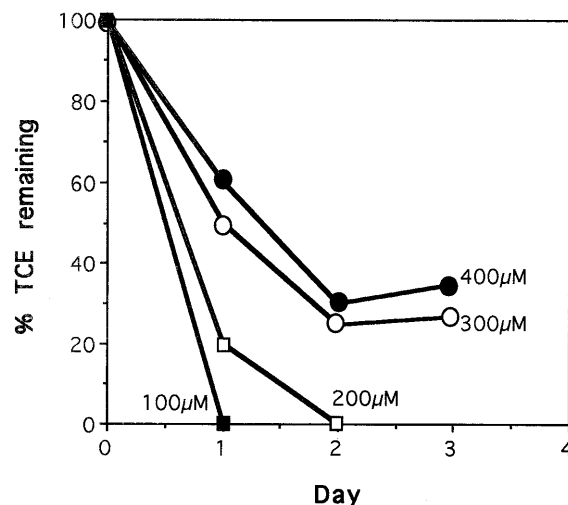


FIG. 3. TCE degradation by AEK301(pYK3021). Values indicated are initial concentrations within separate reaction vials. Degradation is reported as a percentage of the initial concentration.

removal of 70% of the detectable TCE. This cessation may be related to toxic effects induced by reactive intermediates produced during the degradation of TCE. Toxicity has been known to occur through covalent modification of cellular molecules by intermediates produced during TCE mineralization in several TCE-degrading bacteria (34, 45). The cessation of activity at 400 μM might be caused by general cytotoxicity during the mineralization of high concentrations of TCE. The possibility that cessation of activity in sealed batch cultures is a product of exhaustion of limiting nutrients cannot be overlooked.

**Identity of genes encoded on pYK3021.** There remains the possibility that pYK3021 contains regulatory gene sequences, rather than a structural gene, which when restored might enhance the expression of phenol hydroxylase activity encoded elsewhere on the chromosome. Partial sequence data were obtained for three separate sites internal to pYK3021 (Fig. 2). The sequence was analyzed in all three reading frames and both directions. Database searches of the deduced amino acid sequence of a 600-bp region (Fig. 2, sequence 1, right of the internal *Bam*HI site) revealed a 134-amino-acid segment with 91% identity to the oxygenase subunit of toluene-3-monooxygenase from *Pseudomonas pickettii* (7). Similar analysis of a 1.3-kb downstream sequence, flanking the second *Eco*RI site (Fig. 2, sequences 2 and 3), indicates a 192-bp putative amino acid segment with 70% identity to the oxidoreductase subunit from the same multicomponent enzyme (7) and lesser identity to various multicomponent phenol hydroxylases. These data indicate that these genes are transcribed from left to right as depicted in Fig. 2 (toward the vector-encoded promoter).

Plasmids pYK3011 and pYK3021 were transferred by conjugation to *Pseudomonas aeruginosa* PAO1c. This host strain does not have the ability to grow on phenol, has no detectable phenol hydroxylase activity, and is unable to effect any detectable degradation of TCE (21). PAO1c carrying pYK3011 expresses phenol hydroxylase activity at 10.6 nmol/min, a rate approximately one-third of that expressed by *A. eutrophus* AEK101 (Table 2). PAO1c carrying pYK3021 is capable of removing 46% of the available TCE in overnight cultures, a rate approximately one-half of that of its *A. eutrophus* counterpart. These data argue for the presence of structural genes which encode phenol hydroxylase activity.

Genes encoded on pYK3021 show remarkable similarities to other well-characterized multicomponent monooxygenases. The genes for toluene-3-monooxygenase from *P. pickettii* were first characterized on the basis of the cloning of phenol hydroxylase activity (21, 22). Shields et al. (37) demonstrated that a single enzyme is involved in the hydroxylation of toluene, cresol, and phenol and in TCE degradation in *Burkholderia cepacia* G4. Spain et al. (40) suggested that phenol is degraded by toluene dioxygenase, a multicomponent enzyme, through an alternative monohydroxylation in *Pseudomonas putida* F1. Despite the similarities to portions of toluene-3-monooxygenase, the exact identities of the genes from *A. eutrophus* remain to be characterized. However, phenol metabolism seems to play a critical role in TCE degradation. This is inferred by the strict concomitant expression of both activities and by the competitive inhibition by phenol of TCE degradation in AEK301 (pYK3021) (Table 4).

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