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Toluene Degradation by *Pseudomonas putida* F1: Genetic Organization of the *tod* Operon

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***Pseudomonas putida* PpF1 degrades toluene through *cis*-toluene dihydrodiol to 3-methylcatechol. The latter compound is metabolized through the well-established *meta* pathway for catechol degradation. The first four steps in the pathway involve the sequential action of toluene dioxygenase (*todABC1C2*), *cis*-toluene dihydrodiol dehydrogenase (*todD*), 3-methylcatechol 2,3-dioxygenase (*todE*), and 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase (*todF*). The genes for these enzymes form part of the *tod* operon which is responsible for the degradation of toluene by this organism. A combination of transposon mutagenesis of the PpF1 chromosome, as well as analysis of cloned chromosomal fragments, was used to determine the physical order of the genes in the *tod* operon. The genes were determined to be transcribed in the order *todF*, *todC1*, *todC2*, *todB*, *todA*, *todD*, *todE*.**

Pseudomonas putida F1 (PpF1) initiates the oxidation of toluene by incorporating both atoms of molecular oxygen into the aromatic nucleus to form (+)-*cis*-1(*S*),2(*R*)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol; 13, 18, 30). This reaction is catalyzed by a multicomponent enzyme system which has been designated toluene dioxygenase (28). The individual components of toluene dioxygenase have been purified and are organized as shown in Fig. 1. A flavoprotein, ferredoxin$_{TOL}$ reductase (25), accepts electrons from NADH and transfers them to a small iron-sulfur protein, ferredoxin$_{TOL}$ (26). Ferredoxin$_{TOL}$ then reduces the terminal oxygenase which is a large iron-sulfur protein that has been designated ISP$_{TOL}$ (15, 24). The reduced oxygenase catalyzes the oxidation of toluene to *cis*-toluene dihydrodiol. The structural genes for the three protein components of toluene dioxygenase have been termed *todA*, *todB*, and *todC*, and mutants defective in each of the genes have been isolated (11).

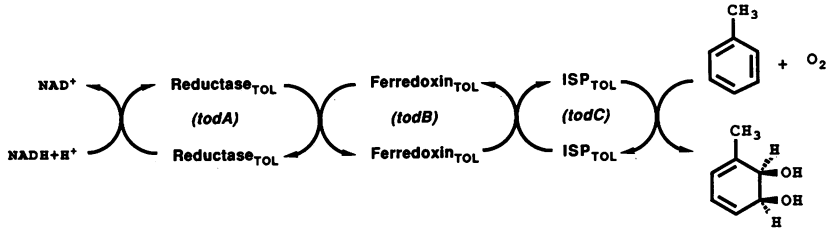

FIG. 1. Oxidation of toluene to *cis*-toluene dihydrodiol by toluene dioxygenase. Structural genes for each component are given the prefix *tod*.

The further metabolism of *cis*-toluene dihydrodiol involves an NAD$^+$-dependent dehydrogenation reaction to form 3-methylcatechol (21). Extradiol cleavage at the 2,3 position by 3-methylcatechol 2,3-dioxygenase yields 2-hydroxy-6-oxo-2,4-heptadienoate, which is further metabolized to 2-hydroxypenta-2,4-dienoate and acetate. These reactions and the gene designations for the individual enzymes involved are shown in Fig. 2.

Although the biochemistry of the dihydrodiol pathway shown in Fig. 2 is reasonably well understood, little is known about the genetic organization and regulation of the reactions that are involved. Our previous studies have shown that the genes involved in toluene metabolism (*tod*) are coordinately induced (B. A. Finette and D. T. Gibson, submitted for publication). This suite of genes has been designated as the *tod* operon. This operon has been cloned into the cosmid cloning vector pLAFR1 by our laboratory (W. R. McCombie, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, K53, p. 155). The resulting plasmid, designated pDTG301, contains two presumably contiguous chromosomal *Eco*RI fragments. Each of these *Eco*RI fragments has been cloned separately into the broad-host-range vector pKT230 (2), and one fragment has been shown to encode for the *todABC1C2DE* genes. This plasmid has been designated pDTG351.

We now describe the use of a hybrid suicide plasmid, pRKTV14, for obtaining Tn*5* insertional mutations in the chromosome of PpF1. Biochemical characterization of *tod*:: Tn*5* mutants and complementation studies with cloned DNA fragments from pDTG351 have enabled us to determine the

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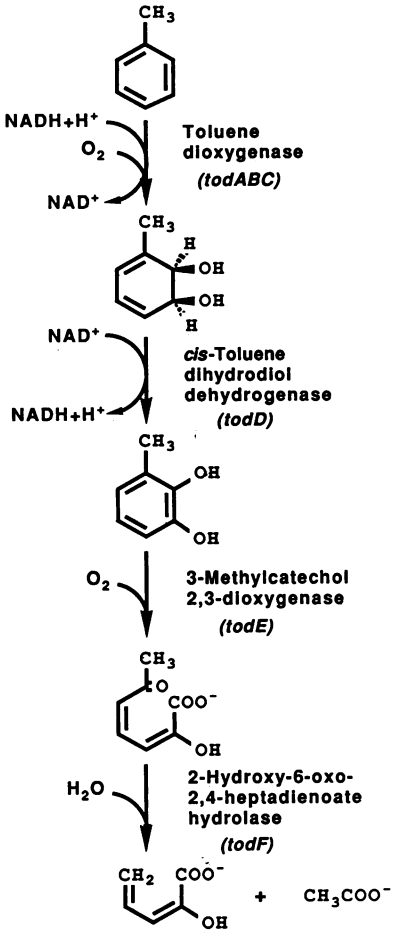

FIG. 2. Reactions used by PpF1 to degrade toluene to 2-hydroxypenta-2,4-dienoate and acetate.

TABLE 2. Plasmids

Plasmid	Relevant properties[a]	Reference or source
RK2	Km^r Tc^r Cb^r	16
pRK2013	Km^r	10
pRKTV14	Km^r Sm^r Sp^r Tp^r (pRK2013::Tn*7*::Tn*5*)	—[b]
pKT230	Km^r Sm^r	2
pDTG301	Tc^r *todABCDEF*	—[c]
pDTG351	Km^r *todABCDE*	—[c]

[a] Abbreviations: Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Cb^r, carbenicillin resistance; Sm^r, streptomycin resistance; Sp^r, spectinomycin resistance; Tp^r, trimethoprim resistance.

[b] —, Received from N. J. Panopoulos. This plasmid was constructed by first transposing Tn*7* into the kanamycin resistance gene of pRK2013 and then transposing Tn*5* into a nonessential region of Tn*7*.

[c] —, McCombie, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984.

genetic organization and direction of transcription of the *tod* genes coding for the catabolism of toluene to 2-hydroxypenta-2,4-dienoate and acetate.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used are listed in Tables 1 and 2. The transpositional mutants isolated during this study are also listed (see Tables 4 and 5), and the recombinant plasmids constructed are shown (see Fig. 4). Mineral salts base medium (MSB) was prepared as described previously (23). Toluene was introduced to cultures in the vapor phase. Nitro Blue Tetrazolium/2,3,5-triphenyl-2H-tetrazolium chloride indicator medium (NBT-TTC) for mutant screening was previously described (11). L broth (tryptone [10 g/liter], yeast extract [5 g/liter], NaCl [5 g/liter]) was supplemented with 0.1% glucose and served as complete medium. Solid media contained 2% agar. Antibiotics, when needed to select for plasmids, were added to the media at the following concentrations: kanamycin, 25 μg/ml for *P. putida* and 50 μg/ml for *Escherichia coli*; streptomycin, 25 μg/ml for both *E. coli* and *P. putida*. *P. putida* was grown at 30°C, and *E. coli* was grown at 37°C.

TABLE 1. Bacterial strains

Strain	Relevant properties[a]	Reference or source
Pseudomonas putida		
PpF1	Tol$^+$ prototroph	14
PpF401	Tol$^+$ *trp*-1	—[b]
PpF3	Tol$^-$ *todB*	11
PpF4	Tol$^-$ *todC1*	11
PpF12	Tol$^-$ *todA*	11
PpF39/D	Tol$^-$ *todD*	13
PpF106	Tol$^-$ *todC2*	11
PpF107	Tol$^-$ *todE*	—[c]
Escherichia coli		
QSRO	F$^-$ *thi lacY leuB hsdR hsdM nalA supE44* Rif^r	R. J. Meyer
NECO100	Rif^r *thi leu trp recA*	N. J. Panopoulos
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI^{q}ZΔM15*]	27

[a] Gene designations are those of Bachmann (1) except for *tod* (toluene degradation): *todA*, ferredoxin$_{TOL}$ reductase; *todB*, ferredoxin$_{TOL}$; *todC1*, large subunit of terminal oxygenase; *todC2*, small subunit of terminal oxygenase; *todD*, *cis*-toluene dihydrodiol dehydrogenase; *todE*, 3-methylcatechol 2,3-dioxygenase; *todF*, 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase.

[b] —, B. A. Finette, Ph.D. thesis, University of Texas at Austin, Austin, 1984.

[c] —, Finette and Gibson, manuscript submitted.

TABLE 3. Transfer of kanamycin resistance from RK2, pRK2013, and pRKTV14 to *E. coli* QSRO and *P. putida* PpF1[a]

Plasmid	Transfer frequency[b] to:	
	E. coli QSRO	*P. putida* PpF1
RK2	8.7×10^{-1}	4.9×10^{-2}
pRK2013	4.7×10^{-1}	1×10^{-11}
pRKTV14	8.6×10^{-1}	2.9×10^{-8}

[a] All plasmids were selected for kanamycin resistance.
[b] Transfer frequencies are expressed as the number of exconjugants per donor.

Generation and screening of Tn5 insertional mutations. PpF1 and PpF401 were each filter mated with *E. coli* NECO(pRKTV14). Equal volumes of an exponential-phase donor culture and a stationary-phase recipient culture were pushed onto a sterile 0.45-μm-pore-size filter. Membranes were placed on L agar plates and incubated for 12 to 16 h at 30°C. The mating mixtures were suspended in 50 mM KH_2PO_4 buffer (pH 7.2) and plated on selective media. Auxotrophic Tn*5* insertional mutants were obtained by plating the mating mixture onto L agar supplemented with kanamycin and chloramphenicol. Chloramphenicol (50 μg/ml) was used to prevent growth of the donor NECO-100(pRKTV14) while still allowing growth of the recipient PpF1. Kanamycin-resistant colonies were subsequently tested for their ability to grow on MSB medium supplemented with 0.2% glucose. Strains unable to grow on this medium were characterized by using the auxanography procedures described by Davis et al. (4). Selection and screening of *tod*::Tn*5* insertional mutants were performed by plating the mating mixture onto NBT-TTC indicator medium supplemented with kanamycin and chloramphenicol. These plates were incubated in the presence of toluene vapors for 48 h at 30°C. The screening of mutant strains on this indicator medium has been previously described (11). The particular enzymes missing in these mutant strains were determined by enzyme assays of crude cell extracts as described previously for mutants generated by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Finette and Gibson, submitted). The reversion frequencies of Tn*5*-generated mutants were determined by growing cells in L broth to stationary phase (10^{11} cells per ml) and plating onto MSB glucose or MSB toluene medium.

DNA techniques. DNA was prepared by a modification of the alkaline-sodium dodecyl sulfate procedure of Birnboim and Doly (3) as described by Ish-Horowitz and Burke (17) and was purified by centrifugation in a cesium chloride-ethidium bromide density gradient. DNA was stored at −20°C in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]). DNA cleavage with restriction enzymes and ligation with T4 ligase was performed as recommended by the supplier (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Cleavage products were visualized by 1.0% agarose gel electrophoresis in TBE buffer (0.1 M Tris, 0.089 M boric acid, 0.002 M disodium EDTA). Restriction fragments were sized by using the length versus reciprocal of mobility method of Southern (6, 22). Plasmid DNA was transformed into *E. coli* by using a calcium chloride-thymidine-glycerol procedure (19). Plasmid DNA was introduced into *P. putida* PpF1 by a triparental mating, using pRK2013 as a mobilizing plasmid as described previously (5).

Materials. The following materials were obtained from the sources indicated: [methyl-^{14}C]toluene (specific activity, 26.4 mCi/mmol), Amersham Searle Corp., Arlington Heights, Ill.; NBT, kanamycin sulfate, chloramphenicol, and amino acids, Sigma Chemical Co., St. Louis, Mo.; streptomycin sulfate, Pfizer Laboratory Division, Pfizer Inc., New York, N.Y.; TTC, Eastman Kodak Co., Rochester, N.Y.; toluene, MCB Manufacturing Chemists Inc., Cincinnati, Ohio.

RESULTS

Transfer of plasmids RK2, pRK2013, and pRKTV14 to *E. coli* and *P. putida* F1. Table 3 shows that colonies resistant to kanamycin were obtained at high frequencies when RK2 was transferred to *E. coli* QSRO and PpF1. In contrast, transfer of kanamycin resistance by pRK2013 was only detected in *E. coli* QSRO. These results suggest that the ColE1 replication system of pRK2013 does not function in PpF1 as has been shown for other *Pseudomonas* strains (5, 10). Plasmid pRK TV14, which is a derivative of pRK2013, yielded stable kanamycin-resistant colonies of PpF1 at low frequencies. The exconjugants did not contain plasmids, and the results suggest that the acquisition of kanamycin resistance by PpF1 is due to transposition of Tn*5* following transfer and subsequent loss of pRKTV14.

Approximately 1.0% of the kanamycin-resistant PpF1 exconjugants were unable to grow on MSB containing 0.2% glucose as the sole source of carbon and energy. The properties of some of these auxotrophic mutants are listed in Table 4. Nine phenotypically distinct auxotrophic mutants were isolated as a result of the insertion of Tn*5* into the PpF1 chromosome. The reversion frequencies of those mutants tested ranged from 10^{-8} to less than 10^{-11}. All of the prototrophic revertants tested were kanamycin sensitive.

TABLE 4. Auxotrophic mutants of PpF1 obtained by Tn*5* mutagenesis with pRKTV14[a]

Strain	Phenotype	Genotype	Reversion frequency	Source
PpFTM1	Tol$^+$ Pro$^-$ Km^r	*pro*-3::Tn*5*	6×10^{-8}	PpF1
PpFTM13	Tol$^+$ Arg$^-$ Km^r	*arg*-3::Tn*5*	1×10^{-10}	PpF1
PpFTM16	Tol$^+$ Arg$^-$ Km^r	*arg*-4::Tn*5*	—[b]	PpF1
PpFTM2	Tol$^+$ Ura$^-$ Km^r	*ura*-1::Tn*5*	—	PpF1
PpFTM15	Tol$^+$ Trp$^-$ Km^r	*trp*-2::Tn*5*	6×10^{-9}	PpF1
PpFTM14	Tol$^+$ Met$^-$ Km^r	*met*-6::Tn*5*	3×10^{-10}	PpF1
PpFTM19	Tol$^+$ Pur$^-$ Km^r	*pur*-1::Tn*5*	—	PpF1
PpFTM7	Tol$^+$ Trp$^-$ Phe$^-$ Km^r	*trp*-1, *phe*-2::Tn*5*	1×10^{-11}	PpF401
PpFTM9	Tol$^+$ Trp$^-$ Arg$^-$ Km^r	*trp*-1, *arg*-2::Tn*5*	1×10^{-11}	PpF401
PpFTM6	Tol$^+$ Trp$^-$ His$^-$ Km^r	*trp*-1, *his*-6::Tn*5*	—	PpF401

[a] Abbreviations for genotypes are those of Bachmann (1) with the exception of toluene degradation (Tol$^+$), and kanamycin resistance (Km^r).
[b] —, Not determined.

TABLE 5. Enzymatic properties of *tod*::Tn*5* mutants derived from PpF1[a]

Strain	Toluene dioxygenase[b]							Dehydrogenase[c]	Dioxygenase[d]	Hydrolase[e]	Genotype
	Control	Rd_{TOL}	Fd_{TOL}	ISP_{TOL}	Rd_{TOL} + Fd_{TOL}	Rd_{TOL} + ISP_{TOL}	Fd_{TOL} + ISP_{TOL}				
PpF1[f]	3.4							200	280	625	WT/TOL$^+$
PpFTM11	1.4	3.2	6.2	1.3	8.0	3.0	2.5	74	—[g]	560	*todE*
PpFTM40	1.0	1.3	5.9	1.4	7.0	1.5	4.5	—	—	500	*todDE*
PpFTM38	—	—	4.5	—	6.5	—	5.0	—	—	660	*todB[A]DE*
PpFTM4	—	—	—	—	5.7	—	—	—	—	335	*todBADE*
PpFTM31	—	—	—	—	6.3	—	—	—	—	426	*todBADE*
PpFTM22	—	—	—	—	—	—	—	—	—	—	*todABCDEF*
PpFTM26	—	—	—	—	—	—	—	—	—	—	*todABCDEF*
PpFTM27	—	—	—	—	—	—	—	—	—	—	*todABCDEF*
PpFTM41	—	—	—	—	—	—	—	—	—	—	*todABCDEF*

[a] All strains except where noted were grown with arginine in the presence of toluene as described in Materials and Methods. Brackets indicate genes exhibiting low levels of expression.

[b] Nanomoles of *cis*-[^{14}C]toluene dihydrodiol formed per minute. Purified components of the toluene dioxygenase system were added to crude cell extracts as indicated in the text. Rd_{TOL}, Ferredoxin$_{TOL}$ reductase; Fd_{TOL}, ferredoxin$_{TOL}$; ISP_{TOL}, iron-sulfur protein, terminal oxygenase component.

[c] *cis*-Toluene dihydrodiol dehydrogenase, nanomoles of NAD^+ reduced per minute per milligram of protein.

[d] 3-Methylcatechol 2,3-dioxygenase, nanomoles of oxygen consumed per minute per milligram of protein.

[e] 2-Hydroxy-6-oxo-2,4-heptadienoate hydrolase, nanomoles hydrolyzed per minute per milligram of protein.

[f] Toluene-induced culture. No activity was observed with uninduced cells.

[g] —, No activity (<1.0 nmol of product formed or substrate consumed per min).

These results suggest that the introduction of pRKTV14 into PpF1 initiates random transposition and insertional inactivation of chromosomal genes by Tn*5*.

Isolation and characterization of *tod*::Tn*5* PpF1 mutants. Approximately 0.1% of the total kanamycin-resistant exconjugants were unable to grow with toluene (Tol$^-$) as the sole source of carbon and energy. Enzymatic and chemical analyses revealed that these *tod*::Tn*5* mutants showed polar effects due to the insertion of Tn*5* (Table 5). Cell extracts of PpFTM38 regained toluene dioxygenase activity when supplemented with purified ferredoxin$_{TOL}$, and this activity was stimulated by the further addition of ferredoxin$_{TOL}$ reductase. In addition, this organism also had no detectable activity for *cis*-toluene dihydrodiol dehydrogenase and 3-methylcatechol 2,3-dioxygenase. PpFTM40 did not contain detectable levels of either of these two enzymes. This strain accumulated *cis*-toluene dihydrodiol in the culture medium when grown on arginine in the presence of toluene. PpFTM11 did not contain detectable levels of 3-methylcatechol 2,3-dioxygenase and accumulated 3-methylcatechol when grown on solid or liquid media. Mutants PpFTM4 and PpFTM31 regained toluene dioxygenase activity only when cell extracts were supplemented with purified ferredoxin$_{TOL}$ reductase and ferredoxin$_{TOL}$. Both mutants were devoid of *cis*-toluene dihydrodiol dehydrogenase and 3-methylcatechol 2,3-dioxygenase activity. Several mutants, typified by PpFTM22, had no detectable activity for any of the enzymes assayed. The polar effect on gene expression caused by the insertion of Tn*5* into the *tod* operon suggests that the possible gene order and direction of transcription is *todF*, *todC*, *todB*, *todA*, *todD*, and *todE*. However, the positions of *todF* and *todC*, as well as *todB* and *todA*, could be reversed and still agree with the data presented.

Determination of gene order in the *tod* operon by complementation analyses with cloned genes. The transposition experiments described above do not permit an unambiguous assignment of the order of the *tod* genes. Consequently, further analyses were conducted with plasmid pDTG351, which contains the *todABCDE* genes on a 10.7-kilobase fragment of PpF1 chromosomal DNA cloned into the vector pKT230. Plasmid pDTG351 DNA was prepared and subjected to single and multiple restriction endonuclease digestion. The resulting fragments were separated by agarose gel electrophoresis and sized as described in Materials and Methods. The calculated distances between restriction endonuclease sites was used to construct the restriction map shown in Fig. 3.

Construction of subclones of pDTG351 and complementation experiments with *tod* mutants of PpF1. Subclones of pDTG351 were constructed by digestion of the DNA with the appropriate restriction enzymes, ethanol precipitation, ligation, and subsequent transformation into *E. coli* JM109. Each subclone was confirmed by restriction endonuclease analysis of purified DNA. The plasmids were transferred to PpF1 *tod* mutants by the triparental mating procedure described in Materials and Methods to test the ability of each plasmid to complement individual mutations in the *tod* genes. The results of these complementation experiments

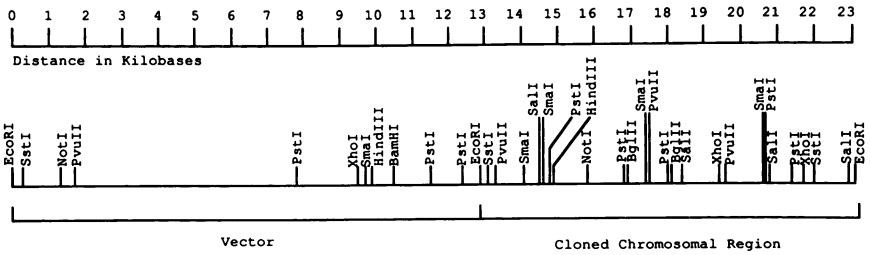

FIG. 3. Restriction map of pDTG351 DNA which contains the *todABCDE* genes.

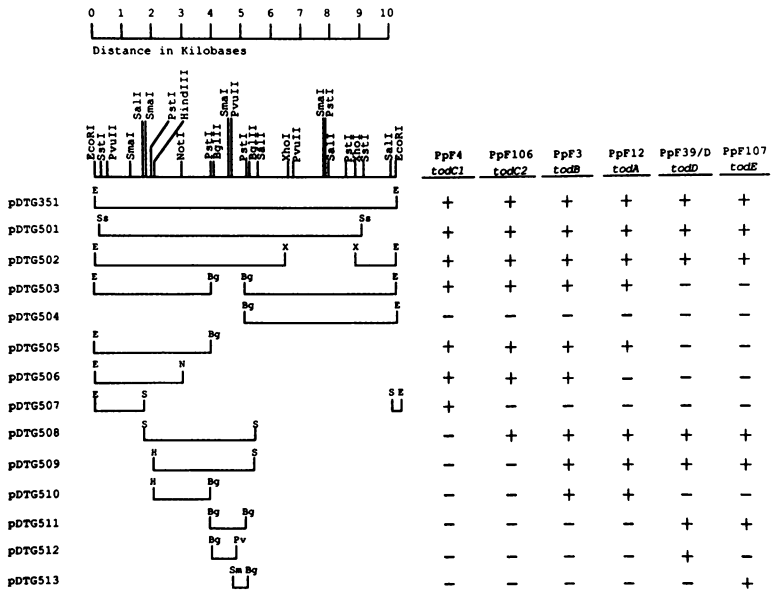

FIG. 4. Complementation of PpF1 mutants with subcloned fragments of pDTG351 DNA. Only the 10.7-kilobase cloned *Eco*RI fragment and its derivatives are shown. Abbreviations: Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; N, *Not*I; Pv, *Pvu*II; S, *Sal*I; Sm, *Sma*I; Ss, *Sst*I; X, *Xho*I.

are shown in Fig. 4. This data clearly shows that the gene order must be *todC1*, *todC2*, *todB*, *todA*, *todD*, and *todE*.

DISCUSSION

Transposons have been used for the molecular characterization of catabolic plasmids which code for the degradation of naphthalene (29), xylenes (12), and octane (8, 9). The physical organization of the *xyl* and *nah* genes are similar insofar as they both reflect the sequence of biochemical reactions of their respective catabolic pathways.

In this study, we have used a suicide plasmid (pRKTV14) for obtaining insertion of Tn*5* into the chromosome of PpF1. Plasmid pRKTV14 can easily be introduced into this strain but is not stably maintained because of its dependence on the replication genes of ColE1. A variety of auxotrophic mutants were isolated at a frequency of approximately 1.0%, which suggests that the transposition of Tn*5* is random. Reversion frequency analyses indicate that once established, Tn*5* is relatively stable in the PpF1 genome. More than 90% of the mutants isolated were also resistant to high levels of streptomycin. This is consistent with a report that Tn*5* expresses streptomycin resistance in other pseudomonads (20).

Biochemical analyses of *tod*::Tn*5* mutants revealed that insertion of Tn*5* within the *tod* operon results in the alteration of gene expression. The polar effect of Tn*5* suggests that the direction of transcription within this operon is from *todF* to *todE* in the order *todF*, *todC*, *todB*, *todA*, *todD*, *todE*. However, the assigned gene order for *todF* and *todC*, as well as for *todB* and *todA*, is not unambiguous, and the results do not distinguish between *todC1* and *todC2*, which are the genes encoding the large and small subunits of the terminal dioxygenase component (24). To determine the precise locations of the genes in the *tod* operon, subclones of a 10.7-kilobase fragment of PpF1 DNA known to contain the *todABCDE* genes were constructed. The results of complementation experiments (Fig. 4) clearly show that the gene order is *todC1*, *todC2*, *todB*, *todA*, *todD*, *todE*.

The nucleotide sequence of the *todB* gene has shown that this gene is transcribed from left to right on the restriction map shown in Fig. 4 (S.-L. Huang, G. J. Zylstra, W. R. McCombie, and D. T. Gibson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, K103, p. 223). If all of the *tod* genes are in the same operon, then the transcription of these genes must proceed in the order *todF*, *todC1*, *todC2*, *todB*, *todA*, *todD*, *todE*. This conclusion is supported by the transposon experiments (Table 4). However, the only *todF* mutant available is leaky (Finette and Gibson, submitted), and this has prevented an unambiguous assignment of the position of the *todF* gene on the cloned fragment.

It is of interest that the mutants PpFTM22, PpFTM26, PpFTM27, and PpFTM41 (Table 5) are regulatory mutants rather than Tn*5* insertions at the beginning of the *tod* operon. This is based on the fact that these mutants are not complemented by the cloned structural genes (plasmid pDTG301). In addition, revertants of PpFTM26 and PpFTM27 form blue colonies when exposed to indole after growth on L agar. This is due to the formation of indigo and is interpreted as evidence for the presence of an intact toluene dioxygenase enzyme system (7). Therefore, these reverted strains now express toluene dioxygenase constitutively. These results indicate that the *tod* genes must be under positive control since an activator protein has been eliminated by the insertion of Tn*5*. A transposon insertion in a repressor gene would result in constitutive expression of the *tod* genes, and such mutants were not isolated.

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