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Identification of the Inducing Agent of the 2,4-Dichlorophenoxyacetic Acid Pathway Encoded by Plasmid pJP4

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The inducing agent of the 2,4-dichlorophenoxyacetic acid (2,4-D) pathway of *Alcaligenes eutrophus* JMP134 (pJP4) was determined through the analysis of promoterless *lacZ* transcriptional fusions with *tfd* structural genes. β-Galactosidase activity was measured in the presence and absence of 2,4-D. Fusions of the individual genes act both as reporters and disrupters of gene expression. Increases in reporter activity were expected in fusions occurring in genes which encode enzymes which function after the production of the inducing intermediate. This analysis indicates that dichloromuconate is the inducing intermediate.

Chlorinated derivatives of phenoxyacetic acid have been released to the environment as herbicides over the last 50 years. One widely used herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), is rapidly degraded by soil bacteria (16). Several strains of multiple genera are capable of degrading 2,4-D (1, 7). The bacterium *Alcaligenes eutrophus* JMP134 carries the catabolic plasmid pJP4 (5) (Fig. 1), which encodes resistance to mercury; the degradation of 2,4-D, 3-chlorobenzoate (3CBA), and phenoxyacetic acid; and the cometabolic degradation of trichloroethylene (4, 5, 9, 10). The plasmid pJP4 is an 80-kilobase pair (kbp), broad-host-range, P1 incompatibility group plasmid (4). The catabolic genes *tfdA*, *tfdB*, and *tfdCDEF* have been localized (5, 6), sequenced, and characterized (15, 22, 25). Two regulatory genes have been identified and designated *tfdR* and *tfdS* (9, 13, 14). The products of these genes are LysR family proteins that are thought to act as transcriptional activators, as well as negative regulators, of the pathway (9, 11, 14, 18).

Early studies of these regulatory elements (9, 13, 14) indicated that the inducing effector of the 2,4-D pathway was an unspecified pathway intermediate. The presence of pJP4 allows *A. eutrophus* JMP134 to grow at the expense of 3CBA. Although this metabolic process only uses the products of the *tfdCDEF* operon (chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, *cis*-2-chlorodiene lactone hydrolase, and chloromaleylacetate reductase, respectively) (Fig. 1), activities related to *tfdA* (2,4-D dioxygenase) and *tfdB* (2,4-dichlorophenol hydroxylase) are detected at induced levels in the presence of 3CBA. Induction of the entire 2,4-D pathway by 3CBA or its metabolic products indicates that 2,4-D or 2,4-dichlorophenol are not required to induce the expression of 2,4-D pathway structural genes.

There are published examples of intermediates and end products acting as inducers of aromatic catabolic pathways (2, 21, 26). It has been proposed, by extension, that chloromaleylacetate or dichloromuconate may serve as the inducer of the 2,4-D pathway (13, 14). These intermediates are, however, unstable and not commercially available. Additionally, cells are not readily permeable to these compounds (13). Such obstacles have made the experimental determination of an effector and the direct measurement of its influence on transcription difficult at best.

A genetic approach was developed to determine the inducing agent of the 2,4-D pathway when *A. eutrophus* is grown in the presence of 2,4-D. *lacZ* fusions within 2,4-D structural genes were used to monitor the levels of transcription from pathway genes. When the *lacZ* transposon inserts into and disrupts a gene whose expression is required for the production of the inducer, no additional transcriptional activation will occur. This will be reflected by levels of β-galactosidase activity that are the same whether 2,4-D is present or absent. However, if the *lacZ* transposon insertion is in a gene not required for synthesis of the inducing intermediate, elevated levels of activity will be observed in the presence of 2,4-D. This experiment is possible because the *tfd* genes are either independently transcribed or are encoded within an operon in the same order they appear in the pathway.

The strains and plasmids used are summarized in Table 1. *lac* fusions were constructed by using a mini-Tn*5* transposon (3) carried by the donor strain *Escherichia coli* S17 pUT mini-Tn*5* *lacZ1* (3). Insertion in the proper orientation produces a transcriptional fusion. The β-galactosidase gene *lacZ1* is promoterless in this construct. *lacZ1* is otherwise complete. Transcription terminates after *lacZ1* eliminating any expression of residual sequence of genes in which the transposon has inserted. Plasmid pUT will not replicate in *A. eutrophus*. *A. eutrophus* AEK101, used as the recipient, is a rifampin-resistant derivative of *A. eutrophus* AEO106 (9), a pJP4-cured derivative of JMP134 (4). The plasmid pJP4 was introduced into AEK101 by electroporation. Plasmid integrity was determined by restriction mapping and growth of AEK101 pJP4 on 0.05% 2,4-D as sole carbon source. AEK101 pJP4 was grown overnight at 30°C in 5 ml of brain heart infusion agar medium (BHI; Difco Laboratories, Detroit, Mich.) with 25 μg of $HgCl_2$ per ml. The donor strain *E. coli* S17 pUT mini-Tn*5* *lacZ1* was grown overnight at 37°C in 5 ml of LB medium (17) containing 100 μg of ampicillin and 50 μg of kanamycin per ml. After overnight growth the cultures were mixed and centrifuged at 1,500 × *g* for 10 min. The pellet was resuspended in 0.05 ml of phosphate-buffered saline (PBS) (17). The suspension was spread on a total nutrient agar (TNA) plate (20). The plate was incubated at 30°C for 6 to 8 h. After incubation, the cells were scraped off the plates into 1 ml of PBS. One-tenth of a milliliter of the cell resuspension was plated on BHI agar plates containing 150 μg of rifampin, 25 μg of mercury, and 50 μg of

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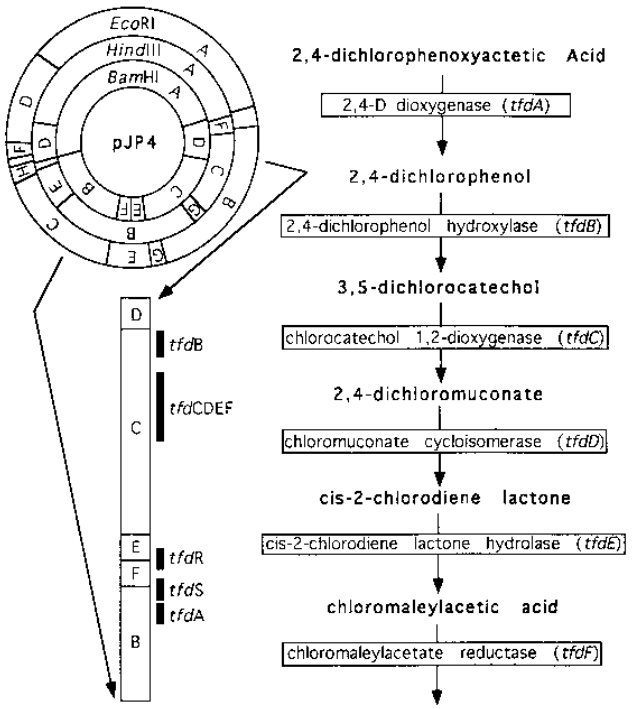

FIG. 1. Restriction digest map of the plasmid pJP4. The positions of the fragments and the relative positions of the genes are from data reported elsewhere (5, 9, 17, 21). The linear map represents the region encompassing the *tfd* genes. Intermediates produced and the respective genes and gene products responsible are shown on the right.

kanamycin per ml. Growth indicated the presence of *A. eutrophus* AEK101 pJP4::Tn*5*-*lacZ1*.

Transconjugants were tested for the ability to grow on phosphate-buffered minimal medium (MMO) (24) with 2,4-D as a sole carbon source. 2,4-D was added at a concentration of 0.05%. Colonies were picked from the BHI plates and replica plated to MMO-2,4-D agar plates. Cultures that were unable to grow on 2,4-D were then tested for the ability to grow on 2,4-D in a liquid culture (MMO, 0.05% 2,4-D). 2,4-D concentrations were monitored by UV absorption at 293 nm. Cultures unable to completely metabolize 2,4-D were conjugated to *Pseudomonas putida* PP0300 (ATCC 17514). Successful conjugation and marker transfer to *P. putida* indicated the Tfd$^-$ phenotype was due to transposon insertion in the plasmid *tfd* genes and not a pleiotropic effect from an insertion into the chromosome of *A. eutrophus.*

The transposon-induced *A. eutrophus* mutants were then examined to determine the exact location of transposon insertion. Plasmid DNA preparations, transformations, and other DNA manipulations were carried out following published procedures (17). The point of insertion carried on *Bam*HI restriction fragments was cloned into pBluescript (Stratagene) using transposon-encoded kanamycin resistance as the selectable marker. *E. coli* DH5α (BRL) was used as the recipient strain for cloning experiments. Cloned inserts were sequenced with an Applied Biosystems automated sequencer. A 17-bp oligonucleotide primer was synthesized with the sequence from the 3′ end of the kanamycin gene in the mini-Tn*5* transposon (3). This allowed sequencing through the end of the transposon into the gene where the transposon was inserted. Sequence data were aligned with MacVector 4.1. (IBI) and compared to published sequences available for each of the *tfd* genes. Sequence data show insertions in each of the *tfd* structural genes: *tfdA*, pJP4::Tn*5*-2; *tfdB*, pJP4::Tn*5*-6; *tfdC*, pJP4::Tn*5*-1; *tfdD* pJP4::Tn*5*-3; and *tfdE*, pJP4::Tn*5*-5.

The success of the proposed analysis relies on a single transpositional insertion within a single gene. If two transposons insert independently into the plasmid, the activity of a given mutant could be misinterpreted. The possibility of multiple insertions would probably not be detected by sequence analysis because the individual insertion points were cloned independently. Southern hybridization experiments were used to demonstrate that only a single insertion had occurred in each of the mutants isolated (data not shown). Plasmid DNA from pJP4 mini-Tn*5* insertion mutants was digested separately with *Bam*HI and *Eco*RI. The fragments were separated by electrophoresis on a 0.7% agarose gel. The DNA was depurinated and neutralized. DNA was transferred to nylon membrane (Zeta probe GT; Bio-Rad) with a vacuum blotter (Milliblot-V; Millipore). An internal *Hin*dIII fragment of the kanamycin resistance gene from the mini-Tn*5* transposon was used as a probe. The probe was labelled with [^{32}P]dATP by nick translation (17). Hybridization was performed according to procedures supplied by the manufacturer of the membrane at high stringency. The membrane was then exposed to X-ray film (Kodak Omat) and developed according to the manufacturer's protocols.

Five classes of *A. eutrophus* mutants were isolated from mini-Tn*5* transposition experiments that were kanamycin, rifampin, and mercury resistant. Each strain was grown on MMO (0.3% Casamino Acids). Casamino Acids act as the primary carbon and energy source but do not repress the expression of the *tfd* pathway (9). Each experimental culture was grown to an optical density of 1.0 at 425 nm and considered to be comparable. It was assumed, for purposes of these experiments, that since the *tfd* pathway was blocked in all strains, the presence of 2,4-D made no significant contribution to the growth or protein content of the cells.

The inducing condition in these experiments is defined as an initial concentration of 0.05% 2,4-D in the culture medium.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Bacterial strains		
Alcaligenes eutrophus		
JMP134	Prototroph, Tfd$^+$ Hg^r	4
AEO106	Cured derivative of JMP134	9
AEK101	Rif^r, derivative of AEO106	Kim & Harker
Escherichia coli		
S17	*hsdR*$^-$ *hasM*$^+$ *recA* thi pro	3
DH5α	F$^-$ *supE44 hsdR17 recA1 gyrA96 end A1 thi-1 relA1 deoR* λ$^-$	Bethesda Research Labs
Pseudomonas putida PPO300	Prototroph, Nal^r	ATCC 17514
Plasmids		
pJP4	Isolated from JMP134, Tfd$^+$ Hg^r	4
pUT	Ap^r, Km^r, delivery plasmid for mini Tn-*5* lacZ1	12

[a] Hg^r, mercury resistant; Tfd$^+$, growth on 2,4-D. Ap, Km, Rif, and Nal refer to ampicillin, kanamycin, rifampin, and naladixic acid, respectively.

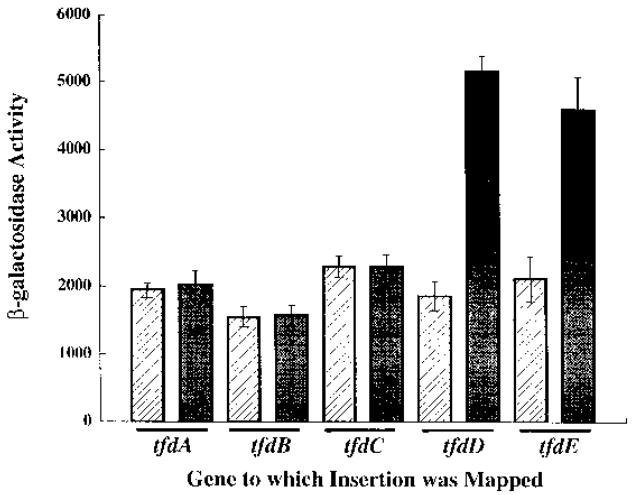

FIG. 2. β-Galactosidase activities expressed by transcriptional fusions within the *tfd* structural genes. Sequence data show insertions in each of the *tfd* structural genes: *tfdA*, pJP4::Tn*5*-2; *tfdB*, pJP4::Tn*5*-6; *tfdC*, pJP4::Tn*5*-1; *tfdD*, pJP4::Tn*5*-3; and *tfdE*, pJP4::Tn*5*-5. ▨, expression in the absence of 2,4-D; ■, expression in the presence of 2,4-D. Values reported are based on the results of triplicate experiments.

Representatives of each class of insertion mutant grown under inducing and noninducing conditions were subjected to the β-galactosidase activity assay described by Miller (19). The results of the β-galactosidase induction assays are shown in Fig. 2 and represent the averaged results of triplicate experiments. The induction assays showed that insertion isolates pJP4::Tn*5*-1, pJP4::Tn*5*-2, and pJP4::Tn*5*-6 had the same activity in the presence or absence of 2,4-D. Uninduced levels of transcription are relatively high in the *tfd* pathway. Kaphammer et al. reported high background levels of 2,4-dichlorophenol hydroxylase activities (*tfdB* gene product) which increased approximately twofold during induction (14). Critical data in this experiment are represented by the differences in transcriptional levels in comparable systems, not by any absolute value.

The isolates pJP4::Tn*5*-3 and pJP4::Tn*5*-5 showed induction (a 2.2- to 2.8-fold increase) when grown in the presence of 2,4-D. These β-galactosidase assays demonstrate that the pJP4::Tn*5*-3 and pJP4::Tn*5*-5 have transposon insertions in genes that are not involved in the production of the inducing intermediate but function only in its further metabolism. Alternatively, the pJP4::Tn*5*-1, pJP4::Tn*5*-2, and pJP4::Tn*5*-6 transposon mutants disrupt genes which are critical to the production of the inducing agent. The point of delineation between no induction and induction comes between the genes *tfdC* and *tfdD*. Dichloromuconate produced by the activity of TfdC (chlorocatechol 1,2-dioxygenase) is apparently the intermediate directly involved in the induction of the 2,4-D pathway.

Technically, these data only demonstrate that dichloromuconate is the intermediate of 2,4-D metabolism which induces transcriptional activation of the *tfdCDEF* operon. Previous studies of the regulation of the 2,4-D pathway (9, 13, 14) indicate, however, that the inducing effector of the entire 2,4-D pathway is a pathway intermediate from this portion of the pathway and effects the induction of *tfdA* and *tfdB*. It is probable that dichloromuconate or its analogs derived from alternative substrates effect the transcriptional activation of *tfdA* and *tfdB* operons as well. Muconate produced by other aromatic degradation pathways has been suggested to be the inducing agent of those pathways (2, 21, 26). The exact mechanism of the interaction between the inducer and the regulatory protein is not well characterized. It has been proposed that a protein-protein interaction between RNA polymerase and the inducer-bound activator stabilizes RNA polymerase binding to the promoter region (8, 23).

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