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Capture of a Catabolic Plasmid That Encodes Only 2,4-Dichlorophenoxyacetic Acid:α-Ketoglutaric Acid Dioxygenase (TfdA) by Genetic Complementation

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The modular pathway for the metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D) encoded on plasmid pJP4 of *Alcaligenes eutrophus* JMP134 appears to be an example in which two genes, *tfdA* and *tfdB*, have been recruited during the evolution of a catabolic pathway. The products of these genes act to convert 2,4-D to a chloro-substituted catechol that can be further metabolized by enzymes of a modified *ortho*-cleavage pathway encoded by *tfdCDEF*. Given that modified *ortho*-cleavage pathways are comparatively common and widely distributed among bacteria, we sought to determine if microbial populations in soil carry *tfdA* on plasmid vectors that lack *tfdCDEF* or *tfdB*. To capture such plasmids from soil populations, we used a recipient strain of *A. eutrophus* that was rifampin resistant and carried a derivative of plasmid pJP4 (called pBH501aE) in which the *tfdA* had been deleted. Upon mating with mixed bacterial populations from soil treated with 2,4-D, transconjugants that were resistant to rifampin yet able to grow on 2,4-D were obtained. Among the transconjugants obtained were clones that contained a ca. 75-kb plasmid, pEMT8. Bacterial hosts that carried this plasmid in addition to pBH501aE metabolized 2,4-D, whereas strains with only pEMT8 did not. Southern hybridization showed that pEMT8 encoded a gene with a low level of similarity to the *tfdA* gene from plasmid pJP4. Using oligonucleotide primers based on known *tfdA* sequences, we amplified a 330-bp fragment of the gene and determined that it was 77% similar to the *tfdA* gene of plasmid pJP4 and 94% similar to *tfdA* from *Burkholderia* sp. strain RASC. Plasmid pEMT8 lacked genes that exhibited significant levels of homology to *tfdB* and *tfdCDEF*. Moreover, cell extracts from *A. eutrophus*(pEMT8) cultures did not exhibit TfdB, TfdC, TfdD, and TfdE activities, whereas cell extracts from *A. eutrophus*(pEMT8)(pBH501aE) cultures did. These data suggest that pEMT8 encodes only *tfdA* and that this gene can effectively complement the *tfdA* deletion mutation of pBH501aE.

Previous studies have shown that the ability to metabolize 2,4-dichlorophenoxyacetic acid (2,4-D) is widely distributed among phylogenetically diverse bacteria and that there is genetic and biochemical diversity in the pathways used for 2,4-D degradation (1, 9, 17, 19, 45–47). This suggests that distinct pathways for metabolism of 2,4-D may have evolved independently of one another. One of these pathways, the pathway found on plasmid pJP4 from *Alcaligenes eutrophus*, has been characterized most extensively (10, 24–26, 32, 35, 44). On this plasmid, the genes required for the metabolism of 2,4-D are organized in three transcriptional units. The *tfdA* and *tfdB* genes are transcribed separately and encode enzymes that convert 2,4-D into 3,5-dichlorocatechol, which is then further metabolized by a modified *ortho*-cleavage pathway. The *tfdCDEF* genes comprise the third transcriptional unit and encode the enzymes of the modified *ortho*-cleavage pathway. These genes exhibit moderate levels of DNA sequence similarity to *clcABC* and *tcbCDEF*, which are involved in the metabolism of 3-chlorobenzoic acid and 1,2,4-trichlorobenzene, respectively (40, 53). Likewise, the *tfdB* gene encodes a monooxygenase that converts 2,4-dichlorophenol (2,4-DCP) to 3,5-dichlorocatechol and exhibits sequence similarity with other bacterial genes that encode phenol hydroxylases (34). Thus, it appears that homologs of *tfdCDEF* and *tfdB* are comparatively common. In contrast, the *tfdA* gene does not exhibit a significant level of similarity with any other known gene and may be a unique gene that is required for initiation of 2,4-D metabolism (16). The *tfd* genes required for the conversion of 2,4-D to 3-oxoadipate are often (although not always [31, 45]) encoded on self-transmissible broad-host-range plasmids (9, 14, 50) and so are transferred together and can confer the ability to use 2,4-D as a sole carbon source. However, it is also conceivable that hosts with homologs of *tfdCDEF* and *tfdB* could metabolize 2,4-D if they were to acquire *tfdA*. The purpose of this study was to determine if certain plasmids in soil bacterial populations encode only *tfdA* and lack homologs of the other *tfd* genes found on plasmid pJP4. We found this to be the case for one of the three plasmids captured by genetic complementation.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *A. eutrophus* JMP228(pBH501aE) was the recipient strain used as the genetic sink in the plasmid capture experiments (50); plasmid pBH501aE is a derivative of plasmid pJP4 (9) that was obtained through site-specific deletion of the 566-bp *Nru*I fragment of the *tfdA* gene into which an *nptII* (kanamycin resistance) cassette was inserted. This strain was used in experiments designed to capture plasmids from soil. It was found that these plasmids had to carry only a *tfdA*-like gene since the rest of the 2,4-D catabolic pathway is located on pBH501aE and the chromosome of *A. eutrophus* JMP228 (50). *A. eutrophus* BH501aE is JMP134 (9) in which plasmid pJP4 is replaced by pBH501aE. *A. eutrophus* JMP228n is a nalidixic acid-resistant mutant of JMP134

cured of plasmid pJP4 (38, 50). *Escherichia coli* CM120(RP4) is a *trpA229-tonB* mutant of W3110 (2) and harbors the IncP plasmid RP4 (Tra^+ Tc^r Km^r Ap^r) (6, 48). *E. coli* HB101(pRK2013) (13) was used as a helper strain to mobilize pSP329. Plasmid pSP329 (Tc^r) is a broad-host-range IncP cloning vector that was constructed and kindly provided by T. Tsoi. *E. coli* S17-1λpir(pUTmini-Tn*5Km1*) (8) and DH5α (12) were used in mating experiments performed to tag pEMT8 with a kanamycin resistance gene.

Media and culture conditions. The media and culture conditions used in the mating experiments and for maintenance of the strains were the same as the media described by us previously (50). All cultures to be used for plasmid DNA extraction and mating experiments were grown in Luria-Bertani (LB) broth containing the appropriate antibiotics when required. Cultures to be used for measurements of growth and 2,4-D disappearance and for the enzyme assays were grown in MMO mineral medium (43) supplemented with pyruvate (500 mg/liter) and 2,4-D (250 mg/liter).

Plasmid capture from soil via conjugation. The soil sample used in this study was obtained from an untreated control plot at the National Science Foundation long-term ecological research site at the Kellogg Biological Station in Hickory Corners, Mich.; this plot was adjacent to plots that had been regularly treated with 2,4-D since 1988 (20). The sample was collected in the fall of 1993 and was also used in our previous plasmid capture experiments (50). The sample was stored at 4°C until it was used and was preincubated at room temperature (23 ± 2°C) for 2 to 4 weeks prior to the start of the mating experiments. A 100-g soil sample in a 200-ml beaker was treated with 2,4-D in phosphate buffer at a final concentration of 100 mg/kg. One 100-g control sample received only phosphate buffer. The soil samples were incubated at room temperature (23 ± 2°C), and 18 days after 2,4-D was added, the plasmid capture experiment was performed as described previously (50) by using a 5-g subsample.

The total number of heterotrophic bacteria and the most probable number of 2,4-D-degrading bacteria in soil samples were determined as described previously (50). To show that the putative transconjugants were derived from the recipient, the Rep-PCR fingerprints (7, 57) were compared.

Plasmid DNA isolation and Southern hybridization. Plasmid DNA was isolated by using a modified method of Kado and Liu (21) with modifications that have been described previously (49, 50). Plasmid DNA digested with restriction enzymes was analyzed by Southern hybridization (37) by using DNA probes from internal regions of the six structural genes for 2,4-D metabolism (*tfdA*, *tfdB*, *tfdC*, *tfdD*, *tfdE*, and *tfdF*) encoded on plasmid pJP4 (19, 50). Hybridization experiments under high-, medium-, and low-stringency conditions were performed as described previously (17, 50).

Tagging of plasmid pEMT8 with a kanamycin resistance gene. To facilitate characterization of pEMT8, a kanamycin resistance gene was introduced into the plasmid. To do this, *A. eutrophus* JMP228(pEMT8)(pBH501aE) was first cured of plasmid pBH501aE through mobilization of pSP329 (Tc^r) into the strain by means of helper plasmid pRK2013. This yielded *A. eutrophus* JMP228 (pEMT8)(pSP329) transconjugants, which were then mated with *E. coli* S17-1λpir(pUTmini-Tn*5Km1*) (8). The mating mixture was resuspended in LB broth containing 100 μg of rifampin per ml and 50 μg of kanamycin per ml. After three transfers in the same broth, the cells were pelleted, resuspended in LB broth, and mated with *E. coli* DH5α. DH5α(pEMT8::mini-Tn*5Km1*) transconjugants were selected on LB agar containing 50 μg of kanamycin per ml that was incubated at 42°C, a temperature that is nonpermissive for the growth of *A. eutrophus* JMP228. The presence of pEMT8::mini-Tn*5Km1* was confirmed by the results of an analysis of plasmid DNA restricted with *Eco*RI.

Plasmid mating experiments. Plasmids were transferred among various bacterial strains by conjugation on LB agar plates incubated at 28°C overnight as described previously (28).

Cloning and sequence analysis of the *tfdA*-like gene of pEMT8. A 10.5-kb *Sac*I fragment of pEMT8 was ligated (37) into the *Sac*I site of pGEM3Zf(+) (Promega Corp., Madison, Wis.) and transformed by electroporation into *E. coli* XL1Blue (Stratagene) by using the instructions of the manufacturer (Bio-Rad Laboratories, Richmond, Calif.).

Oligonucleotide primers internal to *tfdA* (33, 51) were used to amplify a region of the *tfdA*-like gene of pEMT8 by PCR. The amplified DNA was electrophoretically separated on a 1.5% agarose gel, purified by using a GeneClean kit (Bio 101, Inc., La Jolla, Calif.), and sequenced by using an Applied Biosystems model 373A automated sequencer.

Dynamics of 2,4-D degradation. To measure rates of 2,4-D metabolism, *A. eutrophus* strains were grown in MMO mineral medium supplemented with 500 mg of pyruvate per liter and 250 mg of 2,4-D per liter. At 3-h intervals, the optical densities at 550 nm of the cultures were determined, and loss of 2,4-D and potential formation of 2,4-DCP were determined by high-performance liquid chromatography (HPLC) as described previously (19). After 24 h of incubation the cells were harvested, washed with 50 mM Tris-HCl buffer (pH 8.0), and used for enzyme assays.

Enzyme assays. 2,4-D dioxygenase (TfdA) activity was assayed by using a modification of a procedure described by Pieper et al. (36). Freshly harvested cells were resuspended to an optical density at 550 nm of about 4 and incubated with 1 mM 2,4-D at 30°C. The 2,4-D concentration was measured by HPLC (19).

2,4-Dichlorophenol hydroxylase (TfdB) activity was determined by the modified procedure of Beadle and Smith (3, 22). Freshly harvested bacterial cells were resuspended in 100 mM KH_2PO_4-Na_2HPO_4 (pH 7.6) containing 0.03 mM flavin adenine dinucleotide, 0.1 mM EDTA, and 1 mM dithiothreitol (buffer A), broken with a French pressure cell, and centrifuged at 50,000 × *g* for 40 min. The cell extract was added to reaction mixtures containing 0.15 mM NADPH in buffer A. After nonspecific NADPH oxidation had been recorded at 340 nm, 0.025 mM 2,4-dichlorophenol was added, and oxidation of NADPH was further monitored.

The cell extracts used to measure chlorocatechol 1,2-dioxygenase (TfdC), chloromuconate cycloisomerase (TfdD), and dienelactone hydrolase (TfdE) activities were prepared by resuspending cells in 50 mM Tris-HCl buffer (pH 8.0) containing 4 mM $MnSO_4$ and 0.1 mM dithiothreitol, breaking the cells with a French pressure cell, and centrifuging the preparations at 50,000 × *g* for 40 min. Chlorocatechol 1,2-dioxygenase (TfdC) activity was measured spectrophotometrically by the procedure of Dorn and Knackmuss (11). Chloromuconate cycloisomerase (TfdD) activity was determined as described previously (41) by using 2,4-dichloro-*cis*,*cis*-muconate (0.1 mM) which had been prepared by incubating 3,5-dichlorocatechol with partially purified chlorocatechol 1,2-dioxygenase (27) as the substrate. Dienelactone hydrolase (TfdE) activity was measured in the presence of 50 mM Tris-HCl buffer (pH 8.0) and *cis*-4-carboxymethylenebut-2-en-4-olide (*cis*-dienelactone) by using a modification of the procedure of Schmidt and Knackmuss (42).

Specific activities are expressed below as nanomoles of substrate converted or product formed per minute per milligram of protein; the values given are means of the values obtained in duplicate assays. Values similar to those presented in this paper were obtained in independent experiments. Protein contents were determined by using Bio-Rad Protein Assay Dye Reagent Concentrate and bovine serum albumin as the standard.

RESULTS

Isolation of 2,4-D-degradative plasmids from agricultural soil amended with 2,4-D. Plasmid-encoded *tfdA* genes were captured from microbial populations in an agricultural soil by using *A. eutrophus* JMP228 (Rif^r) that carried pBH501aE, a derivative of plasmid pJP4 in which the *tfdA* gene had been insertionally inactivated (50). Acquisition of a *tfdA* gene through horizontal plasmid transfer produced transconjugants that were able to use 2,4-D as a sole carbon source. Mating experiments done with the 2,4-D-amended soil gave 3.3×10^5 CFU of *A. eutrophus* JMP228(pBH501aE) transconjugants per ml of mating mixture. As in previous studies (50), the number of transconjugants was the same on medium/liter containing 250 mg of 2,4-D per liter and media containing 1,000 mg of 2,4-D per liter. When unamended soil was used, no 2,4-D-degrading transconjugants were found. The most probable number of 2,4-D-degrading organisms in the unamended soil was 1.1×10^2 organisms per g of soil, a value which was very low compared with the 3.2×10^7 organisms per g of soil found in soil that had been amended with 2,4-D.

A total of 32 *A. eutrophus* JMP228(pBH501aE) transconjugants from plates containing 1,000 mg of 2,4-D per liter were examined. Of these transconjugants, 28 contained pBH501aE and plasmid pEMT1, which has been described previously (50). One transconjugant lacked pBH501aE but had acquired a different plasmid, pEMT9. The remaining three transconjugants contained a previously undescribed plasmid, pEMT8, in addition to pBH501aE. Plasmid pEMT8 was ca. 75 kb long and had an *Eco*RI restriction pattern that clearly differed from the restriction patterns of pEMT1 and pEMT9 (Fig. 1).

Characterization of pEMT8. Two observations suggest that plasmids pEMT8 and pBH501aE were compatible with one another and were required for the metabolism of 2,4-D. First, cells that contained pEMT8 always contained pBH501aE, suggesting that these plasmids could be stably maintained within the same host and that pEMT8 was not an IncP plasmid. Second, efforts to transfer pEMT8 from *A. eutrophus* JMP228 (pBH501aE)(pEMT8) to *A. eutrophus* JMP228n always resulted in transconjugants which carried both pBH501aE and pEMT8 (data not shown). This suggests that both plasmids are required for 2,4-D degradation and that pEMT8 alone may not encode the entire complement of *tfd* genes. This was con-

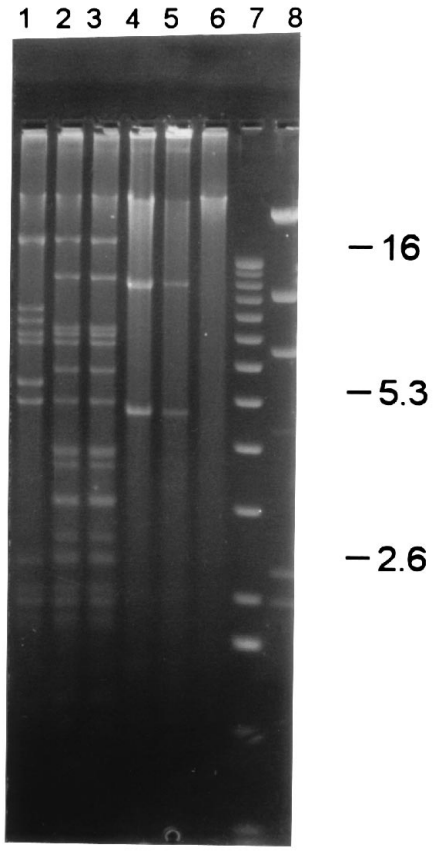

FIG. 1. Agarose gel electrophoresis of plasmid DNA after digestion with *Eco*RI. Lane 1, pEMT1 and RP4; lanes 2 and 3, pEMT8 and RP4; lanes 4 and 5, pEMT9 and RP4; lane 6, RP4; lane 7, 1-kb ladder; lane 8, λ *Hin*dIII. Plasmid band sizes (in kilobases) are indicated on the right. *Eco*RI digestion of RP4 yields one 60-kb fragment.

firmed by curing *A. eutrophus* JMP228(pBH501aE)(pEMT8) of pBH501aE by introducing IncPα plasmid RP4 by conjugation with *E. coli* CM120(RP4). Since RP4 and pBH501aE belong to the same incompatibility group (IncP), selection for RP4 (Tc^r) resulted in a loss of pBH501aE (Fig. 1). The resulting clones were unable to grow in mineral salts medium with 2,4-D as a sole carbon source, indicating that pEMT8 alone is not able to confer the ability to metabolize 2,4-D. In a control experiment, the same procedure was followed with *A. eutrophus* JMP228 (pBH501aE)(pEMT1). Previous studies had shown that pEMT1 alone confers the ability to metabolize 2,4-D (50). As expected, clones cured of plasmid pBH501aE retained the ability to metabolize 2,4-D.

The ability of the *tfdA* homolog on pEMT8 to complement a *tfdA* mutant was demonstrated in several ways. First, *A. eutrophus* JMP228(pEMT8)(RP4) (Rif^r) was mated with *A. eutrophus* BH501aE (carrying pBH501aE) (Rif^s), and Rif^r transconjugants able to grow with 2,4-D as a sole carbon source were obtained. These clones were shown to harbor plasmids pBH501aE and pEMT8. Second, JMP228(pEMT8)(RP4) was used as a donor in a mating experiment performed with *Burkholderia* sp. strain TFD6-1b, a mutant of the 2,4-D-degrading strain TFD6, which has a Tn*5* insertion in a chromosomally encoded *tfdA* gene (31). Transconjugants of *Burkholderia* sp. strain TFD6-1b able to metabolize 2,4-D were found to harbor pEMT8 (data not shown). Third, transfer of the tagged plasmid, pEMT8::mini-Tn*5Km1*, from *E. coli* DH5α to *A. eutrophus* JMP228(pBH501aE) yielded *A. eutrophus* transconjugants that metabolized 2,4-D. These results indicate that pEMT8 is a self-transmissible broad-host-range plasmid that encodes at least a TfdA-like function that is able to complement the *tfdA* mutations found on plasmid pBH501aE and in the chromosome of *Burkholderia* sp. strain TFD6-1b.

Characterization of the *tfdA* homolog of pEMT8. Southern hybridization analyses were done to determine if plasmid pEMT8 encoded genes that were homologs of the *tfd* genes of plasmid pJP4. The *tfdA* gene of plasmid pJP4 did not hybridize to restricted pEMT8 DNA under high-stringency conditions. However, under medium-stringency conditions, the *tfdA* gene

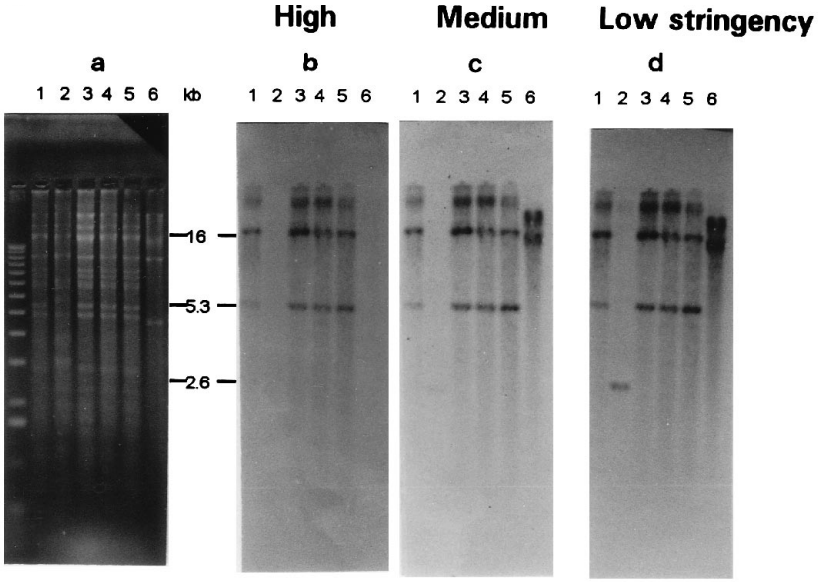

FIG. 2. (a) Agarose gel electrophoresis of plasmid DNA after digestion with *Eco*RI. (b through d) Southern blot hybridized with the *tfdA* gene probe of pJP4 under high-, medium-, and low-stringency conditions. Lanes 1, 3, 4, and 5, pEMT1 and pBH501aE; lanes 2, pEMT8 and pBH501aE; lanes 6, pEMT9. Plasmid band sizes (in kilobases) are indicated between panels a and b.

TABLE 1. Hybridization of *A. eutrophus* strains carrying different plasmids with probes specific for the *tfd* genes of plasmid pJP4 from *A. eutrophus* JMP134

Probe	Hybridization[a]				
	JMP134	JMP228 (pEMT8::mini-Tn*5Km1*)	JMP228 (pBH501aE) (pEMT8)	JMP228 (pBH501aE)	JMP228
tfdA	+++	+	+	NH	NH
tfdB	+++	NH	+++	+++	NH
tfdC	+++	NH	+++	+++	NH
tfdD	+++	NH	+++	+++	NH
tfdE	+++	NH	+++	+++	NH
tfdF	+++	NH	+++	+++	NH

[a] +++, hybridization occurred under high-stringency conditions, as described in Materials and Methods; +, hybridization occurred under low-stringency conditions, as described in Materials and Methods; NH, no hybridization occurred under low-stringency conditions.

of plasmid pJP4 hybridized weakly to a 2.3-kb *Eco*RI fragment, while under low-stringency conditions the gene hybridized to two *Eco*RI fragments (2.3 and 5.9 kb) (Fig. 2 and data not shown). This suggests there was an *Eco*RI site in the *tfdA* gene of pEMT8, as there is in the *tfdA* gene of pJP4. In contrast to pEMT8, restricted pEMT1 and pEMT9 hybridized to *tfdA* under high- and medium-stringency conditions (Fig. 2). Unlike the *tfdA* gene, the *tfdB*, *tfdC*, *tfdD*, *tfdE*, and *tfdF* genes of plasmid pJP4 did not hybridize to pEMT8, even under low-stringency conditions (Table 1).

A 10.5-kb *Sac*I fragment that hybridized with the *tfdA* gene probe (data not shown) was cloned into pGEM3Zf(+) and used as a template for PCR amplification of an internal region of the *tfdA* gene. The amplification product was ca. 330 bp long, which was similar to the length of the product obtained when the *tfdA* gene of plasmid pJP4 was used as the template. The nucleotide sequence of this fragment was determined and was found to be 77% similar to the partial *tfdA* sequence of plasmid pJP4, 94% similar to the partial *tfdA* sequences of *Burkholderia* sp. strains RASC and TFD6 (31, 45), and identical to the partial *tfdA* sequence of strain I-18, a member of the *Halomonadaceae* (30) (data not shown).

Tfd enzyme activities. In order to confirm that plasmid pEMT8 encodes only 2,4-D dioxygenase (TfdA) and no other enzymes of the 2,4-D degradation pathway, the activity of TfdA in whole cells and the activities of TfdB, TfdC, TfdD, and TfdE in cell extracts of *A. eutrophus* JMP228(pEMT8::mini-Tn*5Km1*) were determined and compared with the enzyme activities of several other strains (Table 2). Cells of *A. eutrophus* JMP228(pEMT8::mini-Tn*5Km1*) were shown to have TfdA activity. In contrast, no chlorophenol hydroxylase (TfdB) activity, chlorocatechol-1,2-dioxygenase (TfdC) activity, or chloromuconate cycloisomerase (TfdD) activity was detected (Table 2). The strain examined did exhibit a very low level of dienelactone hydrolase (TfdE) activity, but comparable levels of activity were seen in *A. eutrophus* JMP228 and *A. eutrophus* JMP228(pBH501aE), which were used as negative controls (Table 2). The levels of TfdC, TfdD, and TfdE activities were very low in *A. eutrophus* JMP228(pBH501aE) cell extracts, possibly because 2,4-dichloromuconate, the putative inducer of the modified *ortho*-cleavage pathway, was not formed (5, 29). The activities of all enzymes of the 2,4-D degradation pathway in *A. eutrophus* JMP228(pBH501aE)(pEMT8) were of the same order of magnitude as the enzyme activities in *A. eutrophus* JMP134 containing pJP4, which was used as a positive control. These data confirm that plasmid pEMT8 encodes TfdA, but does not encode a phenol hydroxylase (TfdB) or the enzymes of the modified *ortho*-cleavage pathway of chlorocatechol degradation (TfdC, TfdD, and TfdE).

Dynamics of growth and 2,4-D degradation. The growth of *A. eutrophus* JMP228(pEMT8::mini-Tn*5Km1*) in mineral salts medium supplemented with 2,4-D and pyruvate was determined and compared with the growth of the same strain carrying either pBH501aE and pEMT8, pJP4, pBH501aE, or no plasmid (Fig. 3). Complementation of the *tfdA* mutation by pEMT8 in *A. eutrophus* JMP228(pEMT8)(pBH501aE) resulted in complete mineralization of 2,4-D at a rate that was comparable to the rate observed with *A. eutrophus* JMP134 (which contains pJP4). In contrast, *A. eutrophus* JMP228(pEMT8::mini-Tn*5Km1*) converted only a small percentage of 2,4-D to stoichiometric amounts of 2,4-DCP, which was not further transformed. This also suggests that pEMT8 does not contain a *tfdB* homolog that allows the strain to convert 2,4-DCP to 3,5-dichlorocatechol.

DISCUSSION

Previous studies have shown that *tfdA*-like genes are widely distributed in phylogenetically diverse bacterial hosts from different parts of the world. These genes have been found as part of genetic mosaics with other *tfd* catabolic genes that differ in their degrees of similarity to each other and to the genes found on the canonical pathway encoded on plasmid pJP4 (17). These homologs of *tfd* genes probably underwent recombination with transcriptional units during the evolution of the catabolic genes to produce the genetic mosaics that are observed. This suggests that horizontal gene transfer, perhaps mediated in part by broad-host-range plasmids, serves to shuttle either

TABLE 2. Enzyme activities in cell extracts of *A. eutrophus* strains carrying different plasmids

Enzyme	Substrate	Enzyme activity (nmol/min/mg of protein)				
		JMP134	JMP228 (pEMT8:: mini-Tn*5Km1*)	JMP228 (pEMT8) (pBH501aE)	JMP228 (pBH501aE)	JMP228
TfdA[a]	2,4-D	38	13	36	<1	<1
TfdB	2,4-DCP	300	<5	ND[b]	<5	<5
TfdC	3,5-Dichlorocatechol	146	<2	158	<2	<2
	4-Chlorocatechol	118	<2	107	<2	<2
	Catechol	127	14	125	7	11
TfdD	2,4-Dichloromuconate	174	<2	182	<2	<2
TfdE	*cis*-Dienelactone	493	4	485	3	9

[a] TfdA activity was determined with whole cells.
[b] ND, not determined.

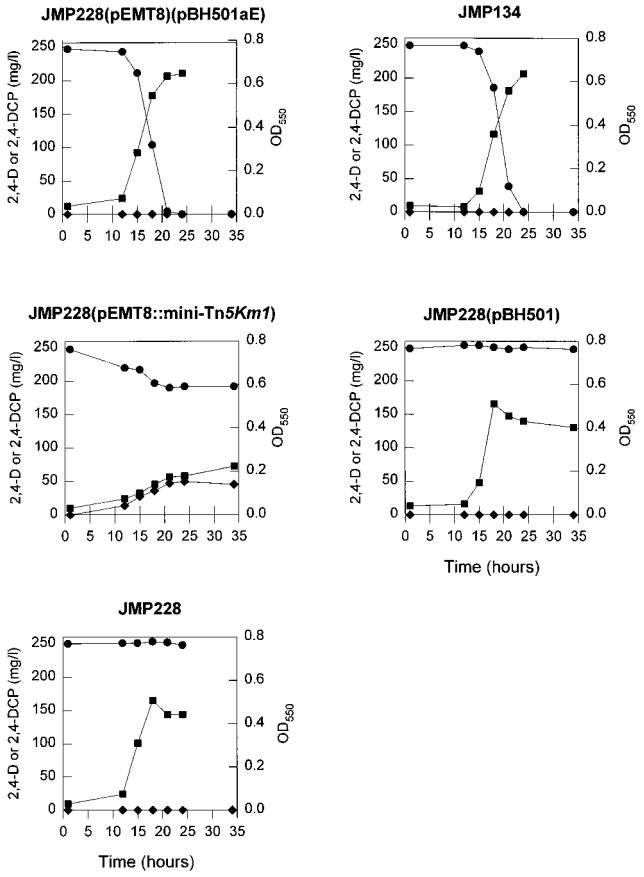

FIG. 3. Growth of different strains, disappearance of 2,4-D, and formation of 2,4-DCP in MMO mineral medium containing pyruvate (500 μg/ml) and 2,4-D (250 μg/ml). Symbols: ●, 2,4-D concentration; ■, optical density at 550 nm (OD_{550}); ◆, 2,4-DCP concentration.

single genes or more extensive genetic modules among members of the microbial gene pool. The results of our study provide direct evidence that there is a self-transferable broad-host-range vector, pEMT8, that encodes only *tfdA* and not the other *tfd* genes.

There are at least three phylogenetically distinct families of *tfdA* genes (33) that are widely distributed among strains of eubacteria (17, 47). Representatives of one gene family have high levels of DNA sequence homology to the *tfdA* gene of *A. eutrophus* JMP134 plasmid pJP4 and are often encoded on broad-host-range plasmids (50). Representatives of a second gene family have been found exclusively in strains of the genus *Burkholderia*. This gene has recently been cloned and sequenced from two distinctly different *Burkholderia* strains, strains RASC and TFD6 (31, 33, 45), and has been shown to be 77% similar to *tfdA* from pJP4 and to be chromosomally encoded. The results of analyses of partial DNA sequences of representatives of the third gene family suggest these genes are similar but not identical to the *tfdA* gene of *Burkholderia* sp. These genes have been found to be carried by strain I-18, a member of the family of *Halomonadaceae* (30), *Rhodoferax fermentans* TFD31, *R. fermentans* 6-9 (17), and *Alcaligenes paradoxus* TV1 (52). These strains represent the β and γ subgroups of the *Proteobacteria* and were isolated from geographically distinct regions, including Michigan, Oregon, Saskatchewan and Ontario in Canada, and France. The partial DNA sequence of the *tfdA* gene encoded by plasmid pEMT8, which is described in this paper, was very similar to the partial DNA sequences of members of the third gene family. These data suggest that the families of *tfdA* genes diverged from a common ancestor and have become disseminated among phylogenetically distinct species of bacteria. Often genes required for the metabolism of 2,4-D are found on conjugative broad-host-range plasmids and are horizontally transferred as a single entity (9, 14, 50). However, our data have shown that plasmid pEMT8 encodes only *tfdA* and does not contain other *tfd* genes; therefore, this plasmid represents an exception to this paradigm.

There are at least three potential explanations for why plasmid pEMT8 encodes only *tfdA*. First, it is possible that the remaining *tfd* genes were encoded on the chromosome or a second extrachromosomal element of the original host(s). Since pEMT8 was isolated only from soil treated with 2,4-D and not from untreated soil, it is likely that the original host(s) was enriched when 2,4-D was added and therefore was able to metabolize this resource. Thus, this strain(s) may have contained the whole complement of *tfd* genes encoded on more than one genetic element. Chromosomal *tfd* genes in addition to *tfdA* have been found in *Burkholderia* sp. strain RASC (23). A second possible explanation is that 2,4-D may be metabolized by microbial consortia in which the host(s) of pEMT8 converts 2,4-D to glyoxylate and 2,4-dichlorophenol, one or both of which are then metabolized by other microbial species. The results of surveys of the geographic distribution in pristine soils of bacteria able to metabolize 2,4-D suggest that such consortia may be commonly found in the environment. Fulthorpe et al. (18) used 672 soil samples collected from five continents and Hawaii as inocula for enrichment cultures and found that 2,4-D was mineralized in 63% of the cultures but that axenic cultures of 2,4-D-degrading strains were obtained from ca. 1% of these enrichments. Interestingly, an internal region of the *tfdA* gene with a nucleotide sequence nearly identical to that of plasmid pEMT8 was once amplified from a consortium by using the same PCR primers described in this study; this finding implicated homologs of the third *tfdA* gene family in the metabolism of 2,4-D by these enrichment cultures. A third potential explanation is that strains that carry plasmid pEMT8 may subsist solely through mineralization of glyoxylate that is formed upon cleavage of the 2,4-D ether bond (15).

Data from previous studies of the genetics of catabolic pathways for chlorinated aromatic compounds are consistent with the general model that peripheral enzymes are recruited for the purpose of converting the parent compounds into chloro-substituted catechols that are subsequently cleaved to produce compounds that enter bacterial intermediary metabolism (40, 53). The enzymes of various modified *ortho*-cleavage pathways are similar to one another in terms of DNA sequence, genetic organization, and the fact that they are commonly encoded on plasmids (4, 39, 40, 53). However, the peripheral enzymes of the pathways differ from one another. The evolutionary origins of these recruited genes are usually unknown, but there are a number of examples which show that they reside on transposable elements that facilitate their dissemination among bacterial populations. For example, *Pseudomonas* sp. strain P51, which metabolizes chlorobenzenes, harbors catabolic plasmid pP51, in which chlorobenzene dioxygenase (TcbA) and chlorobenzene glycol dehydrogenase (TcbB) serve as peripheral enzymes that convert chlorobenzene to a chloro-substituted catechol that is further metabolized by *ortho* cleavage catalyzed by TcbC, TcbD, TcbE, and TcbF (54, 55). The *tcbA* and *tcbB* genes are now encoded on transposon Tn*5280* (56), which suggests that the pathway evolved through the recruitment of

these genes. The evolution of the pathway used for the metabolism of 2,4-D may have evolved in an analogous manner; homologs of *tfdA* may have been recruited to the catabolic plasmids that encoded the ancestral genes of *tfdCDEF* and *tfdB*, and this may have eventually led to catabolic plasmids that encode the entire pathway for 2,4-D metabolism. This hypothesis is supported by our finding in this study that soil communities contain vectors such as pEMT8 that harbor only a *tfdA* gene and no other *tfd* genes.

The original host of plasmid pEMT8 in soil is unknown. The method which we used to examine the diversity of catabolic plasmids is dependent on the ability of the acquired plasmid to complement a genetic mutation in the *tfdA* gene of the recipient strain, thus conferring the ability to metabolize 2,4-D and providing a way to select those transconjugants that have the desired genotype. This obviates the need to cultivate the original host and may provide a more complete understanding of the genetic diversity present in the soil. Indeed, it is conceivable that pEMT8 resides in more than one bacterial host in the soil which was tested. The data presented in this paper and previously (50) show that our plasmid capture method is a useful approach for studying the genetic diversity of homologous catabolic pathways and could potentially be used to capture novel genes that could be used to broaden the substrate range or catalytic efficiency of an existing pathway or to construct a novel catabolic pathway.

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