JOURNAL OF BACTERIOLOGY, July 1987, p. 2950–2955
0021-9193/87/072950-06$02.00/0
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Vol. 169, No. 7

Analysis, Cloning, and High-Level Expression of 2,4-Dichlorophenoxyacetate Monooxygenase Gene *tfdA* of *Alcaligenes eutrophus* JMP134

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Plasmid pJP4 of *Alcaligenes eutrophus* JMP134 contains all genes for the degradation of 2,4-dichlorophenoxyacetic acid (2,4-D). Five of these genes, *tfdB*, *tfdC*, *tfdD*, *tfdE*, and *tfdF*, have recently been localized and cloned (R. H. Don, A. J. Weightman, H.-J. Knackmuss, and K. N. Timmis, J. Bacteriol. 161:85–90, 1985). Gene *tfdA*, which codes for the 2,4-D monooxygenase, has now been found by mutagenesis with transposon Tn*5*. A 3-kilobase fragment of pJP4 cloned in a broad-host-range vector could complement the 2,4-D-negative phenotype of two mutants which lacked 2,4-D monooxygenase activity. The cloned *tfdA* gene was also transferred to *A. eutrophus* JMP222, which is a cured derivative of JMP134. The recombinant strain could utilize phenoxyacetic acid as a sole source of carbon and energy. *Pseudomonas* sp. strain B13, containing the cloned *tfdA*, was able to degrade phenoxyacetic acid and 4-chlorophenoxyacetic acid. Gene *tfdA* was subcloned and analyzed by deletions. Expression of 2,4-D monooxygenase in *Escherichia coli* containing a 1.4-kilobase subfragment was demonstrated by radioisotopic enzyme assay, and a protein of 32,000-dalton molecular mass was detected by labeling experiments. A 2-kilobase subfragment containing *tfdA* has been sequenced. Sequence analysis revealed an open reading frame of 861 bases which was identified as the coding region of *tfdA* by insertion mutagenesis.

Bacteria which degrade halogenated aromatic compounds are of general importance for the detoxification of pesticides and herbicides in nature (17). The extensive use of chlorinated phenoxyalcanoic acid herbicides in agriculture has induced the rapid evolution and dissemination of specific degradative pathways for these compounds in soil and water bacteria. 2,4-Dichlorophenoxyacetic acid (2,4-D) especially is known to be metabolized by organisms belonging to a variety of different bacterial genera, such as *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Corynebacterium*, and *Pseudomonas* (4, 5, 7, 11–13, 16, 33, 35–37).

Alcaligenes eutrophus JMP134 is one of the best-characterized organisms among this group. It harbors a conjugative plasmid, pJP4, of about 80 kilobase (kb) size, which carries genes essential for the degradation of 2,4-D, 2-methyl-4-chlorophenoxyacetic acid, and 3-chlorobenzoic acid (3-CB) (7). Recently this plasmid has been isolated and characterized physically (8). Five genes involved in the degradation of 2,4-D and 3-CB have already been localized by transposon mutagenesis and cloned in *Escherichia coli*. Functions have been assigned to four of them by biochemical studies (9): *tfdB*, *tfdC*, *tfdD*, and *tfdE* encode 2,4-dichlorophenol hydroxylase, dichlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, and chlorodienelactone hydrolase, respectively. Transposon insertion mutation of gene *tfdA* encoding the first enzyme in 2,4-D degradation, which is generally designated as a monooxygenase (9, 35), could not be found in this experiment. Another research group, however, reported the expression of the ability to remove the acetate side chain from 2,4-D by an *E. coli* strain containing a cloned 21-kb *Hin*dIII fragment from pJP4 (1). Characterization of gene *tfdA* would complete our knowledge about the degradative functions in *A. eutrophus* JMP134 and would make it available for genetic engineering studies involving manipulation of microbial organisms to broaden their degrading capacity, or involving the potential use of *tfdA* as a gene specifying herbicide resistance for plants.

We have now isolated transposon mutants of *A. eutrophus* JMP134 inactivated in 2,4-D monooxygenase and have localized gene *tfdA* by cloning and deletion analysis. We have shown the expression of the cloned 2,4-D monooxygenase in different strains, detected the corresponding protein by specific labeling, and determined the nucleotide sequence of a 2-kb fragment containing the *tfdA* coding region.

MATERIALS AND METHODS

Strains and plasmids. *A. eutrophus* JMP134 (7) contains plasmid pJP4 and degrades 2,4-D (Tfd$^+$) and 3-CB (3cb$^+$). Strain JMP222 is a streptomycin-resistant, pJP4-negative derivative of strain JMP134 and is Tfd$^-$ and 3cb$^-$ (7). *Pseudomonas* sp. strain B13 (10) degrades 3-CB and 4-chlorophenol (29). *E. coli* LE392 (27) was used for cloning experiments. *E. coli* HB101 (6) was the recipient for the conjugative transfer of mutated pJP4 derivatives. *E. coli* S17-1 (32) contains a chromosomally integrated RP4 derivative which is able to mobilize broad-host-range plasmids. Strain K38 (=C600)(2) containing plasmid pGP1-2 (34) can express T7 RNA polymerase controlled by a heat-sensitive lambda repressor, and plasmids pT7-5 and pT7-6 (S. Tabor, unpublished data) carry the corresponding T7 promotor in front of a multiple cloning site. Plasmids pVK101 (22), pGSS33 (30) and pKT231 (3) are mobilizable broad-host-range vectors. pSUP2021 is a mobilizable plasmid carrying transposon Tn*5* (specifies kanamycin resistance). For sequencing we used M13tg130 and M13tg131 (21). pDOC37 (D.

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O'Connor, unpublished data) was the source of the omega fragment (15).

Media and cultural conditions. *A. eutrophus* strains were grown with aeration at 30°C. PYE (7) was used as complete medium; liquid PYE medium was supplemented with fructose (10 mM) when cells were grown for preparative plasmid isolation. Minimal medium contained (per liter): K_2HPO_4, 1.6 g; KH_2PO_4, 0.4 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; and $FeSO_4 \cdot 7H_2O$, 0.01 g. Carbon sources were added to the following final concentrations: 2,4-D, 1 mM; 3-CB, 2 mM; 4-chlorophenoxyacetic acid, 1 mM; phenoxyacetic acid (PAA), 4 mM; fructose, 10 mM; and sodium pyruvate, 15 mM. Matings on solid medium were done on PYE agar plates (7). *E. coli* strains were grown in LB medium (25) at 37°C if not otherwise specified. Antibiotics were incorporated at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; streptomycin, 25 μg/ml; and spectinomycin, 80 μg/ml.

Conjugative crosses. For transposon mutagenesis, donor and recipient cells were grown in liquid culture to the late exponential phase and mixed in a 1:1 ratio relative to their optical density. Samples of 1.5 ml of the mixture were spread onto PYE plates and incubated overnight at 30°C. Plate surfaces were then washed with 0.7% NaCl solution, the cell suspension was diluted, and samples were spread onto selective plates.

For complementation tests, mutant strains were grown overnight in liquid culture and spread onto 2,4-D minimal plates, and donor strains were streaked onto plate surfaces. Conjugation and selection were simultaneously done by incubation at 30°C for 14 days.

For all other conjugations, cells were grown in liquid culture overnight, mixed, and spotted onto PYE plates. After incubation at 30°C for 4 to 6 h, cells were suspended in 0.7% NaCl solution, diluted, and spread onto selective plates.

Transposon mutagenesis. The mobilizable transposon carrier plasmid pSUP2021 (32) was transferred by conjugation from the mobilizing donor strain *E. coli* S17-1 (32) to *A. eutrophus* JMP134. Transconjugants were selected from mating mixture on fructose minimal medium containing 380 μg of kanamycin per ml and subsequently screened for loss of 2,4-D degradative ability by replica plating on minimal medium containing 2,4-D as the single carbon source. The absence of degradative functions was also verified by testing on 2,4-D indicator medium (24).

Oxygen uptake assays. *A. eutrophus* wild-type or mutant strains were grown overnight in 250 ml of minimal medium containing 15 mM sodium pyruvate. For induction of 2,4-D-degradative genes, the same medium was used with addition of 3-CB to a final concentration of 1 mM (9). Cells were harvested by centrifugation at 4°C, washed three times in cold minimal medium without carbon source, and finally suspended in a portion of minimal medium to an optical density at 420 nm (A_{420}) of 30.

For assay of 2,4-D monooxygenase or 2,4-dichlorophenol hydroxylase activity, 1 volume of cell suspension was mixed with 9 volumes of minimal medium saturated with oxygen to yield an A_{420} of 3, and background level of oxygen uptake was determined for 10 min. Then substrate was added to a final concentration of 1 mM 2,4-D, 1 mM PAA, or 0.2 mM 2,4-dichlorophenol, and linear decrease of oxygen concentration was measured over 20 min.

Preparative plasmid isolation. Plasmid pJP4 and transposon Tn*5* containing derivatives of pJP4 were isolated from *A. eutrophus* by the procedure of Hansen and Olsen (18). Plasmids from *E. coli* were prepared by the alkaline lysis method described by Maniatis et al. (25).

Radioisotopic 2,4-D assay. For the assay of 2,4-D monooxygenase in *E. coli*, cells of strain K38 containing both pGP1-2 and the pT7 recombinant plasmid were induced for overproduction of enzyme. They were grown at 30°C in 20 ml of LB medium under ampicillin and kanamycin selection. At an A_{590} of 1.0 the temperature was raised to 42°C for 25 min, rifampin was added to a final concentration of 100 μg/ml, and cells were shaken for 2 h at 37°C. Harvesting, washing, and incubation followed the protocol given by Amy et al. (1) with the following modifications: 20 ml of cell suspension was incubated with 0.1 μCi of ^{14}C-labeled 2,4-D in a 250-ml Warburg flask, which was closed with a tapered glass stopper and a serum stopper. A 0.5-ml volume of beta-phenylethylamine was placed in the central vial, and 2 ml of 1 N H_2SO_4 was injected into the surrounding cell suspension. After 1 h of gentle shaking, the trapped $^{14}CO_2$ was eluted from the central vial with 5 ml of Rotiszint 22 (Roth GmbH & Co. Chemische Fabrik, Karlsruhe, Federal Republic of Germany) and counted for radioactivity.

Radioactive labeling of plasmid-encoded proteins. The following protocol was received from Stan Tabor (Harvard Medical School, Boston, Mass.). Cells of *E. coli* K38 containing both pGP1-2 and the pT7-recombinant plasmid were grown in LB medium under ampicillin and kanamycin selection. At an A_{590} of 0.5, 0.2 ml of cells was centrifuged, washed in 5 ml of M9 medium (25), recentrifuged, and suspended in 1.0 ml of M9 medium supplemented with 20 μg of thiamine per ml and 18 proteinogenic amino acids (each 0.01%, without cysteine and methionine). The cells were shaken at 30°C for 60 min, and then the temperature was increased to 42°C. After 15 min, 10 μl of a rifampin stock solution (20 mg/ml in methanol) was added to a final concentration of 200 μg/ml, and the cells were left at 42°C for 10 min. Thereafter the temperature was decreased to 30°C for 20 min, and samples were then pulsed with 10 μCi of L-[^{35}S]methionine (Amersham; cell labeling grade) for 5 min at room temperature and centrifuged. The cell pellet was suspended in 120 μl of cracking buffer (60 mM Tris hydrochloride, pH 6.8, 1% sodium dodecyl sulfate [SDS], 1% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue). The samples were heated to 95°C for 3 min and loaded onto an SDS-polyacrylamide gel as described by Laemmli (23).

RESULTS

Transposon mutants of pJP4. Of 1,000 kanamycin-resistant clones which had Tn*5* stably integrated in their genome, we isolated 8 mutants that were unable to grow on 2,4-D. All of these strains carried the transposon on plasmid pJP4, as determined by conjugative transfer of kanamycin resistance to *E. coli* HB101 and *A. eutrophus* JMP222. Four 2,4-D$^-$ mutants were still able to grow on 3-CB, thus belonging to mutant class A, B, or F, as described by Don et al. (9). To determine the specific enzyme defects, oxygen uptake of these strains was measured during incubation of whole cells with 2,4-D and 2,4-dichlorophenol as substrates. Two mutant strains were deficient in 2,4-D monooxygenase activity, whereas their 2,4-dichlorophenol hydroxylase was still active when 3-CB was used as an inducer. This fact assigns these strains to mutant class A.

Restriction mapping of pJP4 derivatives of *tfdA* mutants showed that in addition to insertion of Tn*5*, large deletions or rearrangements had occurred which involved *Eco*RI fragments C, E, G, H, and I. Because of their complexity they could not be physically characterized further.

Cloning of *tfdA*. We first cloned the 21-kilobase *Hin*dIII fragment of pJP4 in pVK101. A restriction map of the fragment is shown in Fig. 1. The recombinant plasmid pVJH21 was transferred by direct conjugation on selective medium to *tfdA*-defective transposon mutants, using *E. coli* S17-1 as a mobilizing donor strain. Colonies appeared in mating experiments on 2,4-D medium after 14 days and showed wild-type growth. The system was further used to test subcloned fragments for complementation or marker rescue of Tn*5* mutants. *Sac*I fragments from pVJH21 were shotgun-ligated with the broad-host-range vector plasmid pGSS33, and *E. coli* LE392 was transformed with the DNA. Recombinant plasmids were isolated, identified, brought into strain S17-1 by transformation, and subsequently transferred to the transposon mutants. Plasmids such as pGJS3 containing a 3-kilobase *Sac*I fragment of pJP4/pVJH21 were able to restore 2,4-D metabolism in both mutants.

Expression in *A. eutrophus* JMP222. Strain JMP222 is able to grow on phenol as a carbon source by using chromosomally located genes for a meta-cleavage pathway, but cannot grow on PAA. On the other hand, we know from oxygen uptake assays with induced cultures of strain JMP134 that the *tfdA*-encoded 2,4-D monooxygenase can also convert unsubstituted PAA to phenol. Thus, growth on PAA was chosen as a test for expression of *tfdA* in strain JMP222. The 3-kb *Sac*I fragment was subcloned by using the broad-host-range vector pKT231, and hybrid plasmids pKJS31 and pKJS32 (inserts in opposite orientations) were subsequently mobilized from *E. coli* S17-1 to *A. eutrophus* JMP222. Colonies growing on PAA (Paa$^+$) appeared after 3 to 4 days; no difference was observed in the behavior of clones carrying the different hybrid plasmids. All tested Paa$^+$ colonies were kanamycin resistant, whereas from colonies selected on kanamycin several had lost the ability to grow on PAA.

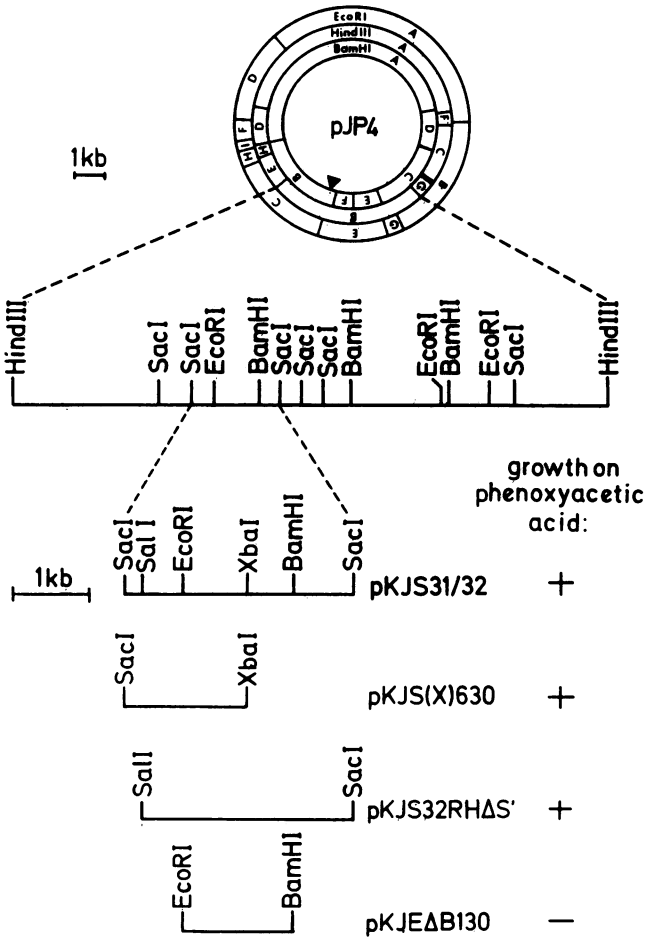

FIG. 1. Cloning and deletion analysis of *tfdA*. Hybrid plasmids containing fragments of pJP4 cloned in broad-host-range vector pKT231 were transferred from *E. coli* S17-1 to *A. eutrophus* JMP222 by conjugation. Transconjugants carrying an intact *tfdA* gene were able to grow on PAA as a carbon source. The localization of *tfdA* on pJP4 is indicated by the solid triangle.

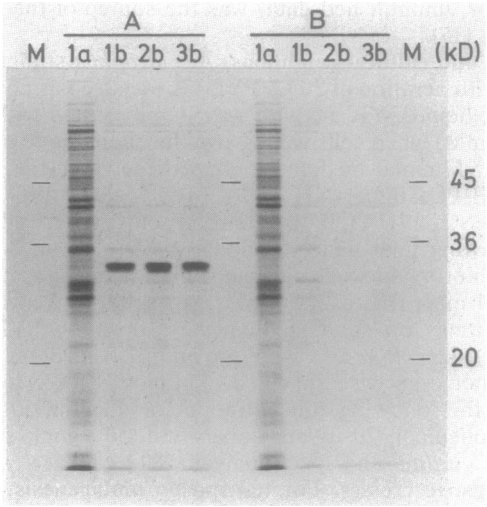

FIG. 2. Expression of gene *tfdA* by T7 RNA polymerase. Three DNA fragments able to confer PAA degradation on *A. eutrophus* JMP222 (1, 2.8-kb *Sac*I-*Sal*I; 2, 2.1-kb *Bam*HI-*Sal*I; 3, 1.4-kb *Xba*I-*Sal*I) were cloned in T7 promoter plasmids pT7-5 (A) and pT7-6 (B), yielding constructions with both orientations of the insert in relation to the T7 promoter. Strains of *E.coli* K38 carrying a heat-inducible T7 RNA polymerase gene on plasmid pGP1-2 and one of the hybrid pT7 plasmids were used for specific labeling of plasmid-encoded proteins: gene expression was induced by heat, the host RNA polymerase was inhibited with rifampin, and cells were pulsed with L-[^{35}S]methionine as described in Materials and Methods (lanes b). For labeling of total cell protein, samples were treated omitting both heat induction and rifampin addition (lanes a). Proteins were separated by electrophoresis on a 12.5% SDS-polyacrylamide gel and revealed by autoradiography. Transcription from the *Xba*I to the *Sal*I site resulted in expression of a 32,000-dalton protein (panel A), whereas no protein was expressed from opposite orientations (panel B). M, Molecular weight markers (transferred from Coomassie blue-stained gel).

Expression in *Pseudomonas* sp. strain B13. As the side-chain cleavage enzyme encoded by *tfdA* accepts differently substituted and unsubstituted PAAs as substrates, we supposed that introduction of *tfdA* into *Pseudomonas* sp. strain B13, which is able to degrade 4-chlorophenol (29), could extend the degradative capacity of strain B13 to 4-chlorophenoxyacetic acid. Plasmids pKJS31 and pKJS32 were transferred by conjugation from *E. coli* S17-1 to *Pseudomonas* sp. strain B13. As expected, transconjugants were able to grow on 4-chlorophenoxyacetic acid, and, in addition, they could also use PAA as a growth substrate.

Deletion analysis. We used the growth of strain JMP222 on PAA as an appropriate system to test constructions with several deletions for the expression of *tfdA*. Small DNA fragments were deleted between unique restriction sites on pKT231 and the *Sac*I insert. Up to the *Xba*I site from one

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                    30                  60
GGATCCTGTCTCAGCTGGCGCGCAATGCTCGAACCCGCTGCGATATACAGCCGTTCGTAG

                    90                 120
TGCAGGTGCTCCACCGTGATTCCAGGCTCCTGGGGGTAGAAGCGGCCGACACCGAGATGG

                   150                 180
ATGGTGCCGGCACGCAGGGCCTCGATCTGCCGCACCTTGGGCATCAGGGCCAGAGACAGC

                   210                 240
GTCGCCCCCGGGACCGCCTGCGTGAACGCATGGAGCAATGCCGGGACGGTCTGGTAGATC

                   270                 300
GCCGTGCCGAGGTAGCCGATATCGAGTTGGCCGATCTCGCCCCGGCTGGCGGCGCGGGAC

                   330                 360
CGGTCCACGGAAGTCCGACCCAGTTCGAGCATGCGCCGTGCATCTTCGAGAAACGCGGCC

                   390                 420
CCGGCGGGCGTGAGCTGCACGCCGCGCGCGCTGCGCTCGAACAACAACACGCCCAGATGC

                   450                 480
TGTTCGAGCGCGTGAATCTGTCGCGTGACCGGGGGCTGGGAAATATGCAGCCGCCGCGCG

                   510                 540
GCGGCACCGACGTTGCCCTCCTCCGCGGCAGCAACGAAATAGCGAAGCTGTCGAAACTCC

                   570                 600
ATTCTTCACTCCTGGTGGCTGGCTCCGGCTGCCGGAGAGCCATACCGATCCCGTATCGCT

                   630                 660
                                XbaI
CGCGCTGATGGAAGGTATTAGACCATATGGCCCGGCATTTCTAGACTACCGCCATGATAA

                   690                 720
AACTCGGCTGCTCTCTCGTCTGCTGGAACATCTTCAGGCGCGCTGAGCCGTCTTTTTGAA

                   750                 780
ACAGTCTCTTAGAAAAGGAGCAAAAAAGTGAGCGTCGTCGCAAATCCCCTTCATCCTCTT
                                SerValValAlaAsnProLeuHisProLeu

                   810                 840
TTCGCCGCAGGGGTCGAAGACATCGACCTTCGAGAGGCCTTGGGTTCGACCGAGGTCCGA
PheAlaAlaGlyValGluAspIleAspLeuArgGluAlaLeuGlySerThrGluValArg

                   870                 900
GAGATCGAACGGCTAATGGACGAGAAGTCGGTGCTGGTGTTCCGGGGGCAGCCCCTGAGT
GluIleGluArgLeuMetAspGluLysSerValLeuValPheArgGlyGlnProLeuSer

                   930                 960
CAGGATCAGCAGATCGCCTTCGCGCGCAATTTCGGGCCACTCGAAGGCGGTTTCATCAAG
GlnAspGlnGlnIleAlaPheAlaArgAsnPheGlyProLeuGluGlyGlyPheIleLys

                   990                1020
GTCAATCAAAGACCTTCGAGATTCAAGTACGCGGAGTTGGCGGACATCTCGAACGTCAGT
ValAsnGlnArgProSerArgPheLysTyrAlaGluLeuAlaAspIleSerAsnValSer

                  1050                1080
CTCGACGGCAAGGTCGCGCAACGCGATGCGCGCGAGGTGGTCGGGAACTTCGCGAACCAG
LeuAspGlyLysValAlaGlnArgAspAlaArgGluValValGlyAsnPheAlaAsnGln

                  1110                1140
CTCTGGCACAGCGACAGCTCCTTTCAGCAACCTGCTGCCCGCTACTCGATGCTCTCCGCG
LeuTrpHisSerAspSerSerPheGlnGlnProAlaAlaArgTyrSerMetLeuSerAla

                  1170                1200
GTGGTGGTTCCGCCGTCGGGCGGCGACACCGAGTTCTGCGACATGCGTGCGGCATACGAC
ValValValProProSerGlyGlyAspThrGluPheCysAspMetArgAlaAlaTyrAsp

                  1230                1260
GCGCTGCCTCGGGACCTCCAATCCGAGTTGGAAGGGCTGCGTGCCGAGCACTACGCACTG
AlaLeuProArgAspLeuGlnSerGluLeuGluGlyLeuArgAlaGluHisTyrAlaLeu

                  1290                1320
AACTCCCGCTTCCTGCTCGGCGACACCGACTATTCGGAAGCGCAACGCAATGCCATGCCG
AsnSerArgPheLeuLeuGlyAspThrAspTyrSerGluAlaGlnArgAsnAlaMetPro

                  1350                1380
CCGGTCAACTGGCCGCTGGTTCGAACCCACGCCGGCTCCGGGCGCAAGTTTCTCTTCATC
ProValAsnTrpProLeuValArgThrHisAlaGlySerGlyArgLysPheLeuPheIle

                  1410                1440
GGCGCGCACGCGAGCCACGTCGAAGGCCTTCCGGTGGCCGAAGGCCGGATGCTGCTTGCG
GlyAlaHisAlaSerHisValGluGlyLeuProValAlaGluGlyArgMetLeuLeuAla

                                EcoRI                    BglII
GAGCTTCTCGAGCACGCGACACAGCGGGAATTCGTGTACCGGCATCGCTGGAACGTGGGA
GluLeuLeuGluHisAlaThrGlnArgGluPheValTyrArgHisArgTrpAsnValGly

                  1530                1560
GATCTGGTGATGTGGGACAACCGCTGCGTTCTTCACCGCGGACGCAGGTACGACATCTCG
AspLeuValMetTrpAspAsnArgCysValLeuHisArgGlyArgArgTyrAspIleSer

                  1590                1620
GCCAGGCGTGAGCTGCGCCGGGCGACCACCCTGGACGATGCCGTCGTCTAGCGCACGCCA
AlaArgArgGluLeuArgArgAlaThrThrLeuAspAspAlaValValEnd

                  1650                1680
TGGCGCACGCCCTTTTCGCGAAGGCCCCACAAGATGTACGCAACCCTGATCAGCGGCAGC

                  1710                1740
CGTAGCCTGGACGGCGACACCTTGGCGCAGCGCGTCCTTCGAGCGGCGGGCGGCCTGGCG

                  1770                1800
GCATGGGGATTGAGGCCCGGTGATGTCGTCGCCATCCTCATGCGCAATGACTTTCCGGTG

                  1830                1860
CTCGAAATGACGCTGGCCGCGAACCGCGCCGGCATCGTTGCGGTGCCTTTGAACTGGCAT

                  1890                1920
GCGAACCGGGACGAGATCGCCTTCATCCTCGAGGACTGCAAAGCGCGTGTGCTCGTCGCG

                  1950                1980
CACACCGATCTGCTCAAGGGCGTTGCATCCGCGGTGCCCGAGGCCTGCAAGGTGCTGGAA

                  2010                2040
GCCGCGTCGCCGCCCGAGATCCGGCAGGCCTATCGGCTGTCCGATGCGTCGTGCACGGCG

AACCCGGGCACGGTCGAC
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FIG. 3. Nucleotide sequence of the 2.06-kb *Bam*HI-*Sal*I fragment of pJP4 encoding the 2,4-D monooxygenase gene *tfdA*. The coding strand of the DNA is presented in the 5′-to-3′ direction along with the deduced amino acid sequence of the only possible open reading frame. The *Bgl*II cleavage site where the omega fragment was inserted is indicated at nucleotide 1500. A putative Shine-Dalgarno sequence in front of the coding region is enclosed in a box.

end and to the *Sal*I site from the other end, deletions could be made without loss of degradative activity (Fig. 1), whereas deletion of the smaller *Eco*RI-*Sac*I fragment resulted in inactivation of *tfdA*.

Specific labeling of *tfdA* gene product. All fragments able to confer PAA degradation on strain JMP222 were subcloned from pKT231 in T7 RNA polymerase-promoter plasmids pT7-5 and pT7-6, yielding constructions with both orientations of the insert in relation to the promoter. Plasmid-encoded proteins were labeled with L-[^{35}S]methionine, separated by SDS-polyacrylamide gel electrophoresis, and revealed by autoradiography. Figure 2 shows the expression of a single protein from all constructions in which the T7 RNA polymerase reads from the *Xba*I to the *Sal*I restriction site. The protein molecular weight was determined to be about 32,000 by comparison with standard protein markers.

Expression of 2,4-D monooxygenase in *E. coli*. pTJS′X535 is a hybrid plasmid of pT7-5 and the *Sal*I-*Xba*I fragment of pJP4 (Fig. 1). *E. coli* K38, containing both pGP1-2 and pTJS′X535, was able to release $^{14}CO_2$ from 2,4-D labeled in position 2 of the acetate side chain. Kinetic assays show that 70% of total trapped radioactivity is released within the first hour of incubation, thus indicating a high level of expression of 2,4-D monooxygenase from the T7 RNA promoter.

Nucleotide sequence of the cloned *tfdA* DNA. The 2.8-kb *Sac*I-*Sal*I fragment shown in Fig. 1 was subcloned in replicative forms of both phages M13tg130 and M13tg131. To create a series of deletions for primed dideoxynucleotide sequencing, unidirectional digestion with exonuclease III was carried out by the method of Henikoff (20). For this purpose, recombinants from M13tg130 were cleaved by *Sal*I and *Pst*I, whereas recombinants from M13tg131 were cleaved by *Sac*I and *Bam*HI. Clones containing plasmids with deletions were analyzed by restriction endonuclease cleavage of replicative forms, and appropriate deletions were chosen for sequencing. The nucleotide sequence was determined by the method of Sanger et al. (28).

Translation of the sequence shown in Fig. 3, from the *Xba*I to the *Sal*I site where *tfdA* gene activity is expressed, reveals one possible open reading frame which corresponds

in its length to a protein size of 32,000 as determined from electrophoresis of labeled *tfdA* gene product.

Insertion mutagenesis of cloned *tfdA*. As 2,4-D monooxygenase activity was directly correlated with expression of a protein of 32,000 molecular weight and with presence of an intact open reading frame of 861 base pairs, we assumed that insertion of transcriptional and translational stop signals at a *Bgl*II site situated within this reading frame would result in expression of a truncated protein and in inactivation of 2,4-D monooxygenase. We excised the omega fragment from pDOC37 with *Bam*HI and inserted it into a *Bgl*II-cleaved pTJS'X535. Specific labeling by the method of Tabor and Richardson (34) as described above revealed a protein of 29,000 molecular weight (Fig. 4), which matches the predicted size calculated from a shortened open reading frame of 768 base pairs. No enzymatically active 2,4-D monooxygenase was expressed from the omega-mutagenized plasmid, as shown by radioisotopic 2,4-D assay (Table 1).

DISCUSSION

In this report we describe the cloning and characterization of gene *tfdA* from *A. eutrophus* JMP134, which encodes the first enzymatic step in the degradation of 2,4-D. The identity of the cloned gene was confirmed by the following criteria: (i) complementation of *tfdA*-defective transposon mutants; (ii) expression of PAA degradative capacity in *A. eutrophus* JMP222; and (iii) expression of 2,4-D side chain cleavage in *E. coli*.

We have identified the protein encoded by *tfdA*, and we have determined the nucleotide sequence of the cloned gene. One possible open reading frame was found, which starts at base 748 with a GTG codon and ends at base 1608 in front of a TAG stop codon. The translational start was confirmed by determination of the first 16 N-terminal amino acids of the purified protein (data not shown). Upstream of base 748, a Shine-Dalgarno sequence can be identified (base 735). Estimation of a molecular weight of 31,000 to 33,000 for the *tfdA* gene product by SDS-polyacrylamide gel electrophoresis is in agreement with the molecular weight of 32,171 predicted from the 861-nucleotide open reading frame. Functional correlation of the nucleotide sequence and enzyme activity has been shown by insertion mutagenesis.

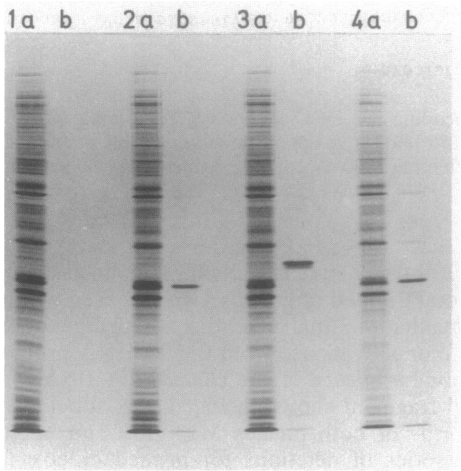

FIG. 4. Omega mutagenesis of gene *tfdA*. The omega fragment, which carries transcriptional and translational stop signals, was inserted at a *Bgl*II site situated within the coding region of *tfdA* on plasmid pTJS'X535. Gene expression was induced using the T7 RNA polymerase-promoter system. Protein was labeled with L-[^{35}S]methionine, loaded onto a 12.5% SDS-polyacrylamide gel, and revealed by autoradiography. No protein is expressed from the vector plasmid pT7-5 (lanes 1). A truncated *tfdA* gene product is expressed from both omega-mutagenized plasmids pTJS'X535omega (lanes 2 and 4), whereas a full-length protein is expressed from pTJS'X535 (lanes 3). Samples were labeled after heat induction and addition of rifampin (lanes b) or omitting induction and rifampin addition (lanes a).

TABLE 1. Correlation of 2,4-D monooxygenase activity, protein length, protein length, and open reading frames in a *tfdA*-pT7 hybrid plasmid and its insertion derivative

Plasmid	Enzyme activity[a] (kcpm)	Mol mass of protein[b] (daltons)	Open reading frame[c] (base pairs)
pT7-5	0.3		
pTJS'X535	70	32,000	861
pTJS'X535omega	0.4	29.000	768

[a] Measured release of $^{14}CO_2$.
[b] Determined by SDS-polyacrylamide gel electrophoresis.
[c] From GTG (base 748) to first in-frame stop.

It is now of interest to elucidate the regulation of 2,4-D degradation in strain JMP134. The promoter which is obviously present on the cloned *tfdA* fragment could serve as a tool for the search of a regulatory gene. Sequence analysis shows an AT-rich area upstream of the coding region, and insertion of a DNA fragment into the *Xba*I site results in poor expression of *tfdA* if a foreign promoter is not provided on the insert (data not shown). Promoter structures can therefore be expected around that area.

Interestingly, gene *tfdA* is located at a distance of 13 kb from the gene cluster encoding the hydroxylation and *meta*-cleavage of 2,4-dichlorophenol. A similar separation of catabolic genes into upper and lower pathway gene clusters has also been observed on the NAH (naphthalene degradation) plasmid (39) and the TOL (toluene degradation) plasmid (14). This finding provides additional support for the "module" theory, which postulates an evolution of degradative pathways by sequential assembly of distinct parts of the pathway (14). Together with conjugation, this provides gram-negative bacteria with an enormous flexibility for the evolution of new degradative functions.

Another trait which gene *tfdA* has in common with other catabolic genes is the broad substrate specificity of the encoded enzyme. It is able to use 2,4-D, 2-methyl-4-chlorophenoxyacetic acid, 4-chlorophenoxyacetic acid, and PAA as substrates. A variety of related compounds which have not yet been tested may perhaps be metabolized as well. Similar broad specificities have been demonstrated for xylene oxidase (19, 26). We can expect therefore that the cloned *tfdA* gene will be useful in the construction of novel pathways. Potential uses of gene *tfdA* may be seen not only in the field of bacterial degradative functions, but also in genetic engineering of plants. The metabolism of 2,4-D by some plant species has already been reported (31, 38). We achieved tolerance of a *Nicotiana silvestris* haploid cell suspension culture against 2,4-D by adapting it to higher concentrations of the synthetic growth hormone (40). As gene *tfdA* specifies the side chain cleavage of 2,4-D, the capacity of plant cells to detoxify chlorinated PAA herbicides could be increased by the transformation of cells with *tfdA*, followed by expression of an enzymatically active protein. Thus, another bacterial gene would be available, in

addition to the prevalently used neomycin phosphotransferase gene, to function as a selectable marker gene for plant genetic experiments, and perhaps transformed cells can be regenerated to whole, herbicide-resistant plants.

ACKNOWLEDGMENTS

We are grateful to R. H. Don, S. Harayama, A. Böck, and B. Friedrich for the helpful discussion of our work, to H. G. Schlegel, G. S. Sharpe, A. Pühler, and S. Tabor for kind gifts of bacterial strains and plasmids, and to F. Lottspeich for determination of the amino acid sequence.

This research was supported by grants to M.H.Z. and to H.-J. Knackmuss and K.N.T. from the Bundesministerium für Forschung und Technologie, Bonn, Federal Republic of Germany.

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