7-1996

Characterization of a Chromosomally Encoded 2,4-Dichlorophenoxyacetic Acid/a-Ketoglutarate Dioxygenase from *Burkholderia* sp. Strain RASC

Yuichi Suwa
Michigan State University

Alice D. Wright
Michigan State University

Fumitasu Fukimori
Michigan State University

Kathy A. Nummy
Michigan State University

Robert P. Hausinger
Michigan State University

Characterization of a chromosomally encoded 2,4-dichlorophenoxyacetic acid/alpha-ketoglutarate dioxygenase from Burkholderia sp. strain RASC.

Y Suwa, A D Wright, F Fukimori, K A Nummy, R P Hausinger, W E Holben and L J Forney

Appl. Environ. Microbiol. **1996, 62(7):2464.**

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1996, p. 2464–2469 Vol. 62, No. 7
0099-2240/96/$04.00+0

Characterization of a Chromosomally Encoded 2,4-Dichlorophenoxyacetic Acid/α-Ketoglutarate Dioxygenase from *Burkholderia* sp. Strain RASC

YUICHI SUWA,[1]† ALICE D. WRIGHT,[1] FUMIYASU FUKIMORI,[1]‡ KATHY A. NUMMY,[1] ROBERT P. HAUSINGER,[1,2] WILLIAM E. HOLBEN,[3]§ AND LARRY J. FORNEY[1]*

Center for Microbial Ecology and Departments of Microbiology,[1] Biochemistry,[2] and Crop and Soil Science,[3] Michigan State University, East Lansing, Michigan 48824

Received 7 December 1995/Accepted 19 April 1996

The findings of previous studies indicate that the genes required for metabolism of the pesticide 2,4-dichlorophenoxyacetic acid (2,4-D) are typically encoded on broad-host-range plasmids. However, characterization of plasmid-cured strains of *Burkholderia* sp. strain RASC, as well as mutants obtained by transposon mutagenesis, suggested that the 2,4-D catabolic genes were located on the chromosome of this strain. Mutants of *Burkholderia* strain RASC unable to degrade 2,4-D (2,4-D$^-$ strains) were obtained by insertional inactivation with Tn*5*. One such mutant (d1) was shown to have Tn*5* inserted in *tfdA*$_{\mathbf{RASC}}$, which encodes 2,4-D/α-ketoglutarate dioxygenase. This is the first reported example of a chromosomally encoded *tfdA*. The *tfdA*$_{\mathbf{RASC}}$ gene was cloned from a library of wild-type *Burkholderia* strain RASC DNA and shown to express 2,4-D/α-ketoglutarate dioxygenase activity in *Escherichia coli*. The DNA sequence of the gene was determined and shown to be similar, although not identical, to those of isofunctional genes from other bacteria. Moreover, the gene product (TfdA$_{\mathbf{RASC}}$) was purified and shown to be similar in molecular weight, amino-terminal sequence, and reaction mechanism to the canonical TfdA of *Alcaligenes eutrophus* JMP134. The data presented here indicate that *tfdA* genes can be found on the chromosome of some bacterial species and suggest that these catabolic genes are rather mobile and may be transferred by means other than conjugation.

Synthetic chlorinated organic compounds have been used extensively as herbicides and pesticides over the last five decades. Certain of these compounds, such as 2,4-dichlorophenoxyacetic acid (2,4-D), are subject to rapid biological degradation in natural environments (26, 38, 42, 43), suggesting that catabolic pathways specific for their degradation have evolved and been disseminated among various microbial populations.

The best-described pathway for 2,4-D degradation, both genetically and biochemically, is the plasmid-encoded pathway of the saprophytic soil bacterium *Alcaligenes eutrophus* JMP134 that was isolated in Australia (39). This organism harbors an 88-kb self-conjugable plasmid (pJP4), which encodes all of the structural and regulatory genes needed to convert 2,4-D to 2-chloromaleylacetic acid, namely, *tfdA* through *tfdF*, *tfdR*, and *tfdS* (11, 12, 20, 28–30, 37). This intermediate is subsequently reduced by the chromosomal *mar* gene product to β-ketoadipate, which is further metabolized by chromosomally encoded gene products to ultimately yield CO_2 (32).

Members of other bacterial genera, such as *Pseudomonas*, *Sphingomonas*, *Bordetella*, *Acinetobacter*, *Arthrobacter*, *Xanthobacter*, and *Corynebacterium*, have been shown to mineralize 2,4-D (9, 10, 14, 18, 24–26, 31, 34). The diversity of 2,4-D-degrading bacteria has recently been assessed by polyphasic taxonomic characterization of isolated bacteria (46), by hybridization of 2,4-D catabolic genes from isolated bacteria (3, 4, 17, 46), and by hybridization analysis of total soil bacterial community DNA (22, 25, 26). These studies have shown that there is substantial phylogenetic diversity among 2,4-D-degrading populations and significant genetic diversity among the 2,4-D-catabolic genes they carry. Studies in our group have focused on determining the genetic and functional diversity of TfdA (2,4-D/α-ketoglutarate dioxygenase) and assessing the biogeographical and among-species distribution of this enzymatic activity (15–17, 24, 27, 36, 46, 47).

Earlier experiments in this laboratory showed that *Burkholderia* strain RASC contained a single 11-kb plasmid, pRASC, and that this plasmid had no detectable homology to the pJP4 plasmid (46). The lack of homology and the small size of pRASC suggested that at least some genes known to be involved in 2,4-D degradation might be located on the chromosome. Here, we show that none of the genes involved in 2,4-D degradation by *Burkholderia* strain RASC are encoded on pRASC. We also report the cloning and DNA sequence analysis of *tfdA*$_{\mathrm{RASC}}$, show that the gene is located on the chromosome of strain RASC, and biochemically characterize its product, 2,4-D/α-ketoglutarate dioxygenase.

MATERIALS AND METHODS

Bacterial strains, media, and reagents. *Burkholderia* strain RASC was originally isolated in Oregon from return activated sludge on the basis of its ability to degrade 2,4-D (2) and was kindly provided by P. Amy. Strains of *Escherichia coli* were grown on Luria-Bertani (LB) broth or LB agar medium (40) at 37°C except when otherwise specified. Full-strength peptone-tryptone-yeast extract-glucose (PTYG) medium contained (per liter) 2.5 g of peptone (Difco Laboratories, Detroit, Mich.), 2.5 g of tryptone (Difco), 5.0 g of yeast extract (Difco), 5.0 g of glucose, 0.3 g of $MgSO_4 \cdot 7H_2O$, and 0.035 g of $CaCl_2$. PTYG was used at 1/10 strength (PTYG/10) throughout these experiments. MMO basal salts medium (MMO) was used as described previously (44), except that nitrilotriacetic acid was omitted since it appeared to serve as a carbon source for the strain and the medium was supplemented with 2,4-D and/or Casamino Acids (Difco) as indicated below. For solid media, 1.5% (wt/vol) Bacto Agar (Difco) was added. PIA/2 medium consists of half-strength (22.5 g/liter) *Pseudomonas* isolation agar (Difco) supplemented with 6.8 g of Bacto Agar per liter (to account for halving

* Corresponding author.
† Present address: National Institute for Resources and Environment, Tsukuba, Japan.
‡ Present address: Department of Microbiology, Monash University, Clayton, Victoria, Australia.
§ Present address: Division of Biological Sciences, The University of Montana, Missoula, MT 59812.

the recipe) and 20 ml of glycerol per liter. All cultures of strain RASC were incubated at 30°C. Broth cultures were incubated with shaking at 150 rpm on a rotary platform shaker.

Restriction endonucleases were purchased from GIBCO-BRL, Gaithersburg, Md., and used as specified by the manufacturer.

Tn*5* mutagenesis. Kanamycin-resistant Tn*5* transposon mutants of strain RASC were selected following conjugation with the Tn*5* suicide vector pRL1062a (48), which can replicate only in *E. coli*. Triparental matings between strain RASC, the donor strain *E. coli* DH5α(pRL1062a), and *E. coli* DH5α(pRK2013), which carried a helper plasmid, were performed as described previously (8), and the strains were plated onto PIA/2 agar containing 50 μg of kanamycin per ml and incubated for 4 days at 30°C. The PIA/2 agar contains Irgasan (Ciba-Geigy, Basel, Switzerland), which selects against both *E. coli* strains, while the kanamycin selects for Tn*5*-containing derivatives of strain RASC.

To obtain insertion mutants bearing Tn*5* specifically on pRASC and not the chromosome, a strategy involving both conjugation and transformation techniques was used. As before, the mating suspensions were plated on PIA/2 agar containing 50 μg of kanamycin per ml. Following incubation, all of the kanamycin-resistant colonies on the plates were collected by suspension and subsequent washing with TE buffer (10 mM Tris [pH 7.0], 1 mM EDTA). Plasmids were extracted from this suspension by the method of Hirsch et al. (21). A fresh preparation of strain RASC was transformed with this plasmid preparation by electroporation at 2.5 kV for 5 ms with a gene pulser (Bio-Rad, Hercules, Calif.) as specified by the manufacturer and subsequently plated on PIA/2 agar containing 50 μg of kanamycin per ml. Since wild-type pRASC and pRASC::Tn*5* derivative plasmids have the same origin of replication, they are incompatible with each other. Hence, growth on this medium forces segregation of wild-type pRASC and selects for cells harboring pRASC::Tn*5*. Kanamycin-resistant colonies were purified twice on this selective medium to ensure segregation of wild-type pRASC.

Plasmid curing. Strain RASC was cured of its plasmid (pRASC) by physical loss via electroporation. This was accomplished by using a Tn*5* insertion derivative of pRASC (pRASC::Tn*5*) to facilitate monitoring of this cryptic plasmid. Electrocompetent cells of strain RASC/pRASC::Tn*5* were prepared as specified by the manufacturer and then diluted to about 10^5 cells per ml with sterile distilled water. This cell suspension was subjected to electroporation at 2.5 kV for about 5.5 ms and then plated onto PTYG/10 agar. Several hundred colonies were screened, and several clones that were sensitive to kanamycin and presumably cured of the plasmid were identified. The loss of pRASC from these strains was confirmed by Southern blot hybridization of *Eco*RI-digested total DNA preparation of strain RASC as described previously (40), with ^{35}S-labeled pRASC DNA (Genius; Boeringer Mannheim, Indianapolis, Ind.) as a probe.

2,4-D biodegradation assays. Cultures to be tested were incubated overnight in PTYG/10 broth; then 0.1 ml was transferred to 3.5 ml of MMO supplemented with 500 mg of 2,4-D per ml and 0.3% (wt/vol) Casamino Acids and incubated with shaking at 30°C. After 3 days, a 1-ml sample of culture was taken and the cells were pelleted by centrifugation in a microcentrifuge (Sorvall, MC 12V; Dupont) at 14,000 rpm for 3 min. The supernatant was filtered (pore size, 0.45 μm), and the concentration of 2,4-D was determined by high-pressure liquid chromatography as described previously (22). When Tn*5* insertion mutants were being analyzed, kanamycin (50 μg/ml) was added to the culture and test media.

To quantitate the ability to completely mineralize 2,4-D, a single drop (ca. 40 μl) of overnight culture in PTYG/10 broth was transferred to 5 ml of MMO supplemented with 100 mg of 2,4-D per ml, 0.1% (wt/vol) Casamino Acids, and 0.1 mCi of [U-*ring*-^{14}C]2,4-D (Sigma, St. Louis, Mo.) in a 25-ml glass bottle. The bottle was tightly closed with a double-layer rubber stopper from which a dipper containing a filter paper wick was hung. After 1 week of incubation at room temperature, 0.5 ml of 2 N KOH was added to the filter paper wick and 0.5 ml of 2 N HCl was added to the culture fluid by injection through the stopper. The amount of $^{14}CO_2$ absorbed on the KOH-saturated wick was determined by liquid scintillation counting.

Screening for 2,4-D$^-$ mutants. 2,4-D$^-$ mutants were obtained by screening clones with chromosomal Tn*5* insertions for the inability to incorporate [U-*ring*-^{14}C]2,4-D into cell biomass on solid media (13). Briefly, dilutions of bacterial suspensions subjected to Tn*5* mutagenesis were spread onto nitrocellulose filters resting on PIA/2 supplemented with 50 μg of kanamycin per ml and incubated overnight at 30°C. Replica filters were made from these master filters, laid onto PTYG/10 agar supplemented with 125 μg of Irgasan per ml, 50 μg of kanamycin per ml, and 500 μg of 2,4-D per ml including 0.5 mCi of [U-*ring*-^{14}C]2,4-D, and incubated for 2 days. The filters were transferred onto an agar plate, containing only 40 mM sodium phosphate buffer (pH 6.8), for 1 h to diffuse unincorporated label from the filter and then allowed to dry. Dried filters were exposed to X-ray film, which was subsequently developed and then matched to the master filters to identify colonies that had failed to incorporate ^{14}C into their cell biomass. These colonies were presumptive 2,4-D$^-$ mutants. Presumptive evidence that Tn*5* had inserted into $tfdA_{RASC}$ was obtained by demonstrating that the transformation of a mutant with pYS11 complemented the mutation and restored the ability of mutants to metabolize 2,4-D. This plasmid (pYS11) contains a 1.2-kb *Sph*I fragment with the *tfdA* gene from pJP4 that had been subcloned from pUS311 (15).

Cloning of $tfdA_{RASC}$. The modified Tn*5* transposon used for mutagenesis of strain RASC has the *oriV* origin of replication (48). Consequently, DNA containing $tfdA_{RASC}$::Tn*5* could be cloned by partial digestion of mutant strain d1 genomic DNA with *Sau*3AI followed by self-ligation and transformation into *E. coli*. The circularized fragments with Tn*5* were propagated in *E. coli* cells as plasmids and conferred kanamycin resistance. A 12.3-kb plasmid (pS18) from one of the transformants was chosen for further study. A 1.0-kb *Xba*I-*Pst*I DNA fragment from pS18 that flanked the transposon hybridized at low stringency with *tfdA*. This fragment was subcloned into pGEM3Zf(+) (Promega, Madison, Wis.) to generate pYS15 and was used as a probe to identify the wild-type $tfdA_{RASC}$ gene.

Genomic DNA from a plasmid-cured derivative of RASC was purified (5) and completely digested with *Eco*RI. Since pYS15 hybridized to a single 7.2-kb *Eco*RI fragment of genomic DNA of wild-type strain RASC, DNA fragments of about 7 kb were gel purified on a 1% low-melting-temperature agarose gel (SeaPlaque agarose; FMC, Rockland, Maine) and ligated into pGEM3Zf(+). The ligated DNA was transformed into *E. coli* DH5α by electroporation to create a library of subclones, which were then screened by colony hybridization (40) with the 1.0-kb *Xba*I-*Pst*I fragment as a probe. Eight colonies hybridized strongly with the probe (data not shown), and plasmid DNA was purified from these and analyzed by restriction digestion with *Eco*RI. One plasmid, pYC6, which contained a 7.2-kb *Eco*RI fragment was selected for further analysis. Templates for DNA sequencing were obtained by using exonuclease III (Erase-a-Base; Promega) to produce nested deletions of pYC6 DNA that were subcloned into plasmid pBluescriptII KS(+) or SK(+) (Stratagene, La Jolla, Calif.). DNA sequencing was performed at the Michigan State University DNA Sequencing Facility with an ABI Robotic Catalyst and 373A DNA sequencer.

Purification of $TfdA_{RASC}$. The $TfdA_{RASC}$ enzyme was purified essentially as described for TfdA (16) with minor modifications. Cultures (2 liters) of strain RASC in PTYG/10 supplemented with 2,4-D (2.5 mM) were incubated with shaking to near the end of the exponential growth phase and then harvested by centrifugation at 6,000 × *g* for 10 min at 4°C. The cell pellets were resuspended in 25 ml of 20 mM phosphate buffer (pH 7.2) and stored at −20°C prior to use. The cell suspension was adjusted to a final concentration of 1 mM phenylmethylsulfonyl fluoride and 0.1 mM EDTA, and the cells were disrupted by passage through a precooled (4°C) French pressure cell at 120 MPa. The cell lysate was cleared of debris by centrifugation at 100,000 × *g* for 30 min at 4°C. The resulting cell extracts were adjusted to 20% glycerol and 0.1 mM dithiothreitol; then, $(NH_4)_2SO_4$ was added to 50% saturation. Precipitated proteins were collected by centrifugation (10,000 × *g* for 10 min at 4°C), dissolved in buffer A (20 mM Tris-HCl [pH 7.26], 20% glycerol, 0.1 mM dithiothreitol, 0.1 mM EDTA), and dialyzed against buffer A at 4°C. The dialyzed sample was applied to a column of DEAE-Sepharose (2.5 by 30 cm, equilibrated in buffer A) and eluted with a 200-ml linear salt gradient to 0.5 M NaCl in buffer A with a flow rate of 2.5 ml min^{-1} at 4°C. The fractions containing enzyme activity (eluting at approximately 0.1 M NaCl) were pooled, dialyzed at 4°C against buffer A, and applied to a MonoQ HR 10/10 column (Pharmacia-LKB Biotechnology Inc., Piscataway, N.J.) equilibrated in buffer A at room temperature. A 100-ml linear salt gradient to 0.2 M NaCl in buffer A was used to elute the enzyme at 1 ml min^{-1}. Active fractions were combined, and the protein solution was concentrated with a Centricon 30 microconcentrator (Amicon Corp., Danvers, Mass.). This sample was chromatographed on a Superose 12 column (1.0 by 30 cm) in buffer A at 1 ml min^{-1} at room temperature.

$TfdA_{RASC}$ enzyme assay and characterization. The 2,4-D/α-ketoglutarate dioxygenase activity of the $TfdA_{RASC}$ enzyme was assayed at 30°C as previously described for TfdA (15, 16). One unit of activity is defined as the amount of enzyme that forms 1 μmol of 2,4-dichlorophenol per min under these conditions. Kinetic parameters were calculated as previously described (16), and protein determinations were made by the method of Lowry et al. (35) with bovine serum albumin as a standard.

The subunit molecular weight of $TfdA_{RASC}$ was determined by denaturing gel electrophoresis by the method of Laemmli (33) with a 4.5% acrylamide stacking gel and a 12% running gel. To visualize the polypeptides, gels were stained with Coomassie brilliant blue (7). The amino-terminal sequence of the enzyme was determined by automated Edman degradation with a model 477A sequencer (Applied Biosystems, Foster City, Calif.) once the denatured proteins were transferred to a polyvinylidene difluoride membrane.

Preparation of cell extracts of subclones. *E. coli* cells carrying plasmid subclones were cultured in LB broth containing ampicillin (100 μg/ml) at 37°C with shaking. At the end of the exponential growth phase, the cells were harvested by centrifugation (6,000 × *g* for 10 min at 4°C), resuspended in 25 ml of 20 mM phosphate buffer (pH 7.2), and stored at −20°C prior to use. Suspended cells were disrupted by passage through a precooled (4°C) French pressure cell at 120 MPa, and the lysates were cleared by centrifugation at 100,000 × *g* for 30 min at 4°C. $TfdA_{RASC}$ activity was assayed as described above.

Nucleotide sequence accession number. The sequence of the $tfdA_{RASC}$ gene was deposited with GenBank under accession number U25717.

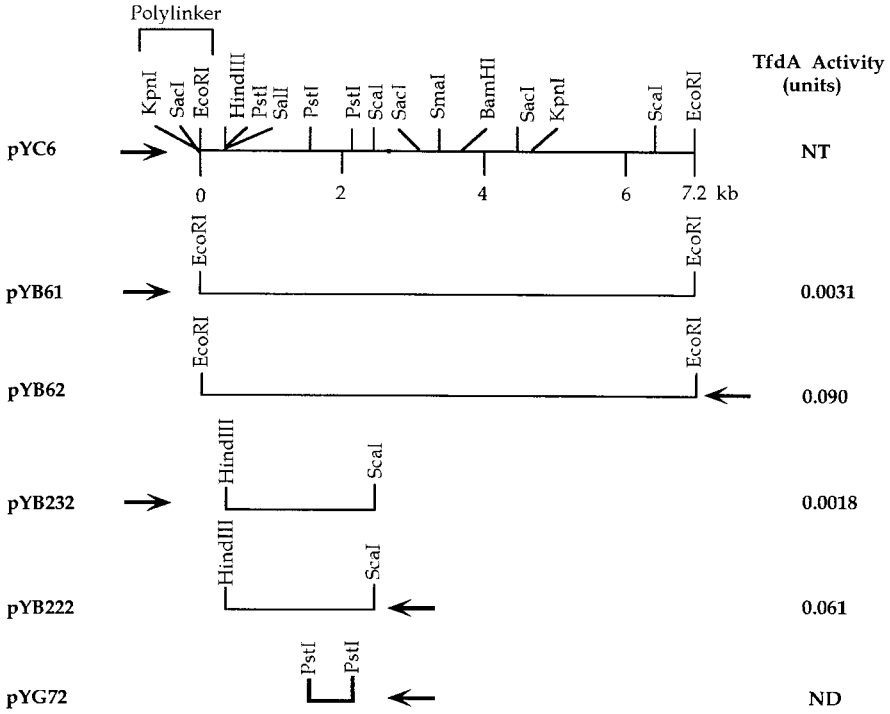

FIG. 1. Subcloning of the 2,4-D/α-ketoglutarate dioxygenase gene of *Burkholderia* sp. strain RASC. The location of the *lac* promoter from the vector in each recombinant plasmid is indicated by an arrow. The 2,4-D/α-ketoglutarate dioxygenase specific activities in *E. coli* JM109 containing the indicated plasmids were measured as described in Materials and Methods. NT, not tested; ND, not done.

RESULTS AND DISCUSSION

2,4-D degradation by plasmid insertion mutants and plasmid-cured derivatives of strain RASC. Strain RASC harbors an 11-kb plasmid (pRASC) which has no apparent homology to the 88-kb self-transmissible plasmid pJP4 encoding the 2,4-D degradative genes in *A. eutrophus* JMP134 (46). Tn*5* insertion derivatives of this plasmid were examined to determine whether pRASC encodes genes involved in 2,4-D degradation. Fifty independent pRASC insertion mutants were cultivated at 30°C for 3 days in MMO minimal salts medium supplemented with 0.3% (wt/vol) Casamino Acids and 500 μg of 2,4-D per ml, and the concentration of 2,4-D remaining in the culture fluid was determined. Each of the 50 mutants completely degraded the 2,4-D. Restriction analyses of the mutant plasmids with *Eco*RI confirmed that Tn*5* insertion into pRASC was not site specific. Each of the 28 plasmids examined was 19 kb, which was consistent with insertion of an 8-kb fragment with the transposon into the plasmid, and there were 25 clearly different restriction patterns, indicating that Tn*5* had inserted at random locations in pRASC (data not shown).

Further evidence that the genes responsible for 2,4-D degradation in strain RASC are not plasmid encoded was obtained by analysis of plasmid-free derivatives of this strain. Five pRASC-free isolates were obtained as described above, and all were able to degrade 2,4-D as evidenced by the evolution of $^{14}CO_2$ from radiolabelled 2,4-D at rates comparable to that observed with the wild-type strain (data not shown).

Several alternative plasmid isolation protocols, including those specialized for isolation of large plasmids (6, 19, 23) and pulsed-field gel electrophoresis following in-well lysis (41), were performed in attempts to detect large plasmids or episomes that might reside in strain RASC. Despite repeated attempts, we were never able to obtain evidence for the existence of extrachromosomal replicons other than pRASC. These results, the plasmid insertion data, and the data from plasmid-cured strains indicate that no genes essential for 2,4-D degradation are plasmid encoded in *Burkholderia* sp. strain RASC.

Isolation and characterization of 2,4-D$^-$ mutants. Approximately 4,000 Tn*5* insertion mutants were screened for the inability to incorporate [^{14}C]2,4-D into biomass (data not shown). Of these, two putative 2,4-D$^-$ mutants were obtained and further characterized. The ability of mutant d1 to degrade 2,4-D was restored when it was transformed with pYS11, which contains the entire *tfdA* gene from pJP4 (data not shown). This result suggests that the Tn*5* insertion in d1 interrupted a gene that was isofunctional to *tfdA*. These data indicate that the $tfdA_{RASC}$ gene is encoded on the chromosome. Preliminary data (27, 49) suggest that other genes required for the catabolism of 2,4-D, including *tfdB* and *tfdC*, are also chromosomally encoded in this strain. This is in contrast to the plasmid-encoded degradative pathways of *A. eutrophus* JMP134 and other strains (46).

Cloning of $tfdA_{RASC}$. The wild-type $tfdA_{RASC}$ gene was cloned from a library of genomic DNAs of strain RASC by using as a probe a 1.0-kb *Xba*I-*Pst*I DNA fragment from the transposon-gene junction composed mostly of DNA that flanked the transposon insertion site in mutant d1. Eight colonies that hybridized strongly with the probe (data not shown) were obtained, and one of these contained a plasmid (pYC6) with a 7.2-kb *Eco*RI fragment. Several subclones of this DNA were made in pBluescriptII KS(+) (Fig. 1). Plasmids pYB61 and pYB62 were subclones of the entire 7.2-kb *Eco*RI fragment that differed in orientation with respect to the *lac* promoter of pBluescriptII KS(+). Both of these plasmids expressed 2,4-D/α-ketoglutarate dioxygenase activity in *E. coli*

JM109, suggesting that the cloned fragment encodes the native promoter region for the $tfdA_{RASC}$ gene and that this promoter functions in *E. coli*. However, *E. coli* containing pYB62 had nearly 30-fold-greater activity than did *E. coli* containing pYB61 (Fig. 1), indicating that the native promoter is much weaker than the *lac* promoter in *E. coli*. Additional smaller subclones were constructed in each orientation and assayed for 2,4-D/α-ketoglutarate dioxygenase activity, ultimately resulting in a 2.1-kb *Hin*dIII-*Sca*I fragment that still retained activity (Fig. 1). The forward orientation of this subclone, pYB222, also had approximately 30-fold more activity than did the reverse orientation, pYB232. Plasmid pYG72, containing a 1-kb *Pst*I subclone (Fig. 1), had no measurable activity, suggesting that one or both *Pst*I sites interrupt the $tfdA_{RASC}$ gene or that the gene was entirely contained on the *Hin*dIII-*Pst*I fragment. The pYB232 subclone was selected for DNA sequence analysis.

DNA and amino acid sequence analysis of $tfdA_{RASC}$. The *Hin*dIII-*Pst*I, *Pst*I-*Pst*I, and *Pst*I-*Sca*I DNA fragments, which make up the 2.1-kb *Hin*dIII-*Sca*I DNA fragment of pYB232 (Fig. 1), were further subcloned into pBluescriptII KS(+) or SK(+) and used to create a series of nested deletions for sequencing. Appropriate-sized deletion derivatives were subjected to DNA sequence analysis. An open reading frame corresponding to the $tfdA_{RASC}$ gene contains 891 bases of coding sequence (45) aligned with the *tfdA* gene of *A. eutrophus* JMP134 (Fig. 2). The initiation codons differ for the two genes, with $tfdA_{RASC}$ encoding the traditional ATG while *tfdA* begins with GTG. Both genes terminate with a TAG stop codon; however, $tfdA_{RASC}$ is larger than *tfdA* by 30 nucleotides. Additional evidence that the open reading frame encodes the $tfdA_{RASC}$ gene is that it contained a *Pst*I site that, when cleaved, resulted in loss of dioxygenase activity (described above); also, the amino-terminal sequence was identical to that of purified $TfdA_{RASC}$ (described below). In all, there were 185 differences in the aligned nucleotide sequences (excluding the additional 30 bases of $tfdA_{RASC}$). Thus, the overall DNA sequence similarity for the two genes was 78.5%, which was close to that expected from Southern hybridization experiments. Analysis of the sequence showed that 110 of the 185 nucleotide differences occur in the third (or wobble) position of the codon or in another position that preserves the identity of the amino acid. This suggests that structural and functional requirements of the enzyme constrain the degree of sequence difference allowed. The degree of identity in the amino acid sequence was higher than that in the DNA sequence. BLAST searches (1) of existing gene and protein databases using both DNA and amino acid sequences of *tfdA* and $tfdA_{RASC}$ were performed, but no evidence of another highly related gene or gene product was found.

In contrast to the coding regions, the promoter regions of $tfdA_{RASC}$ and *tfdA* are not highly conserved. For example, the putative −10 and −35 regions involved in RNA polymerase binding possess only three identical positions in each region (Fig. 2). The weak homology to the consensus sequences for these regions in RASC is consistent with the observation that the cloned $tfdA_{RASC}$ gene is poorly expressed in *E. coli* unless it is under the control of a heterologous promoter. In contrast, these regions in *tfdA* from *A. eutrophus* JMP134 more nearly match the sequences for RNA polymerase-binding sites in *E. coli* (45).

The translated amino acid sequences of $TfdA_{RASC}$ and TfdA were aligned and compared (Fig. 3). The $TfdA_{RASC}$ enzyme is composed of 297 amino acids, while TfdA contains 287. As expected from the above DNA sequence comparison, all of the additional protein residues are at the carboxyl terminus. The data indicate 80.5% identity of amino acids for the first 287 residues. Of the 56 differences observed when the sequences of $TfdA_{RASC}$ and TfdA were compared, 42 represented conservative substitutions (as determined by analysis with Genetyx-Mac [Software Development Co. Ltd., Tokyo, Japan]). Allowing conservative substitutions, the two enzymes have 95.5% similarity for the first 287 residues. The positions of histidine residues, shown to be important for enzymatic function (16), are also highly conserved, with all nine histidine positions of TfdA being matched in $TfdA_{RASC}$ (Fig. 3). In

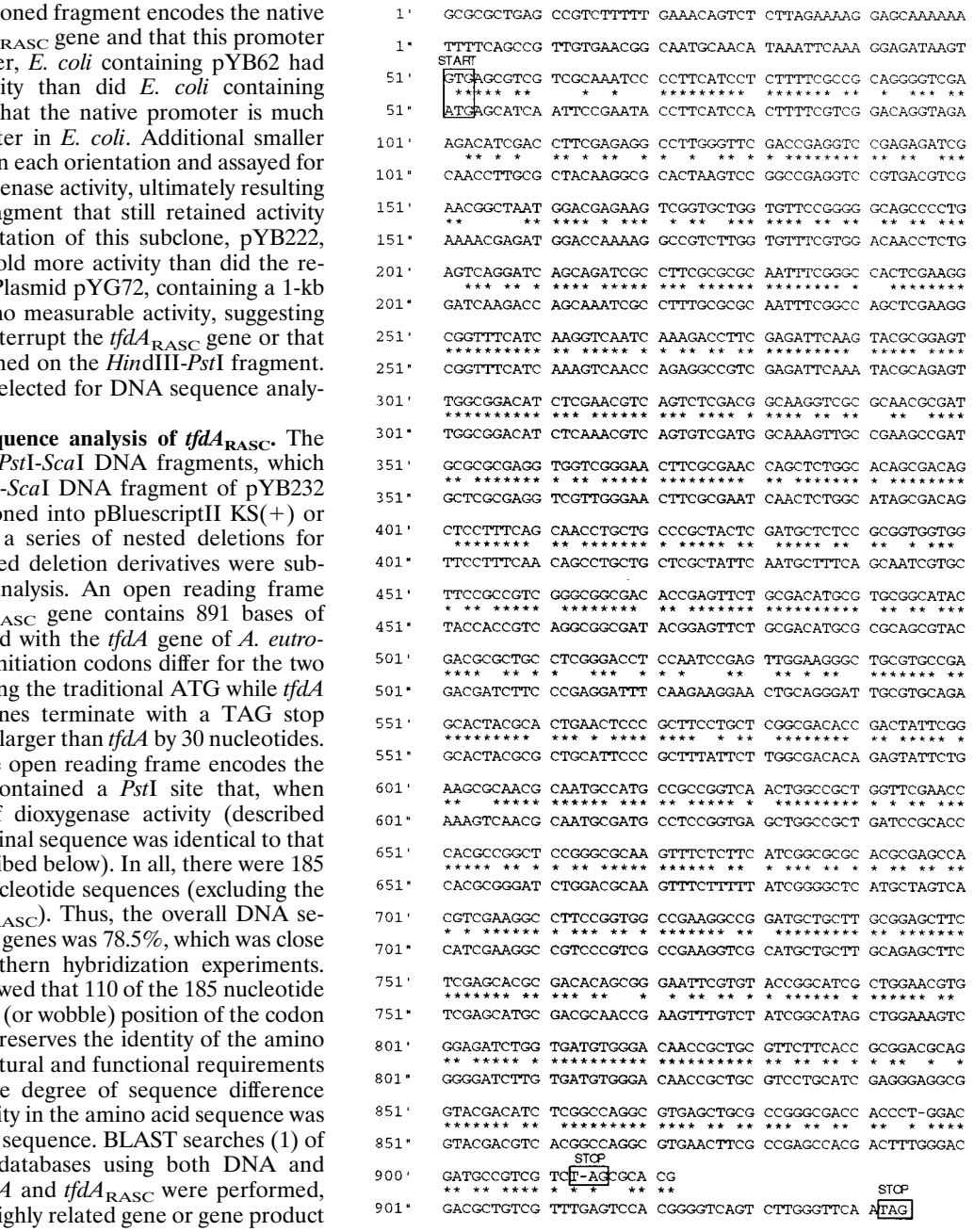

FIG. 2. Comparison of nucleotide sequences of the 2,4-D/α-ketoglutarate dioxygenase gene of *Burkholderia* sp. strain RASC ($tfdA_{RASC}$; bottom row) with that of *A. eutrophus* JMP134 (*tfdA*; top row). Identical nucleotides are indicated by asterisks. The initiation and termination codons and the putative RNA polymerase-binding sites are boxed.

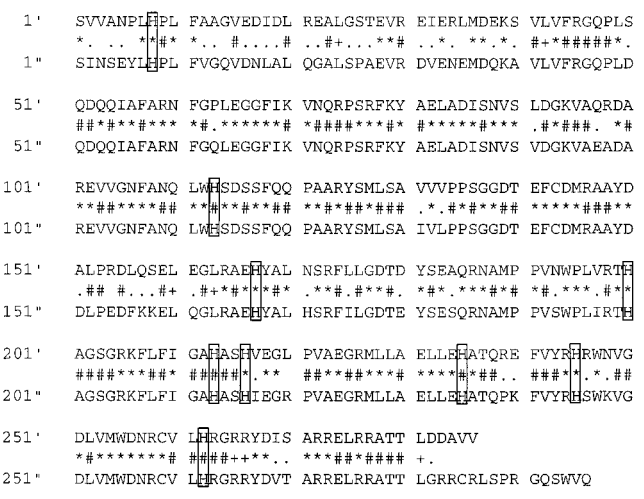

FIG. 3. Comparison of the amino acid sequences of 2,4-D/α-ketoglutarate dioxygenase from *Burkholderia* sp. strain RASC (bottom row) and *A. eutrophus* JMP134 (top row). Identical amino acids encoded by the identical codon are indicated by asterisks; degenerate codons differing at the wobble position or the first nucleotide are indicated by # or +, respectively. Amino acids which are related by conservative replacement are indicated by •. Histidine residues are boxed.

addition, $TfdA_{RASC}$ contains a 10th histidine at position 172, in contrast to TfdA, which contains an asparagine residue at that position.

Examination of Fig. 3 shows that the distribution of nonidentical residues in these enzymes was apparently not random. For example, 18 of 56 mismatches were found in the amino-terminal 41 residues of the enzyme. An additional pocket of mismatches (6 of 11 residues) exists between positions 151 and 162 of the enzyme. Finally, a string of five consecutive mismatches plus the additional 10 residues unique to $TfdA_{RASC}$ were found at the COOH terminus. Histidines were generally present in conserved regions, except for two that were present in the C terminus of the protein.

Purification and characterization of $TfdA_{RASC}$. $TfdA_{RASC}$ was purified, and typical results are summarized in Table 1. The dioxygenase activity of $TfdA_{RASC}$ appeared to be more labile than that of TfdA of *A. eutrophus* JMP134 (16) but was stabilized by the inclusion of glycerol and dithiothreitol in all buffers during the purification. Despite the lability of the enzyme, it was purified 117-fold to apparent homogeneity to give a specific activity of 10.5 U/mg of protein. This value compares well with that (16.9 U/mg) found for recombinant *A. eutrophus* TfdA isolated from *E. coli* (16).

Preliminary data indicate that cell extracts of strain RASC degraded 2,4-D by a ferrous ion- and α-ketoglutarate-dependent mechanism similar to that previously characterized in *A. eutrophus* JMP134 (15, 16). No 2,4-D/α-ketoglutarate dioxygenase activity was detected in cell extracts of strain RASC grown in the absence of 2,4-D. Moreover, maximal dioxygenase activity was found in cell extracts that were harvested during the exponential growth phase before 2,4-D was exhausted. As found for TfdA (16), the $TfdA_{RASC}$ enzyme was capable of using several phenoxyacetate compounds as substrates, including phenoxyacetate without chlorine substituents and the chlorinated compound 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). The enzyme had greatest affinity for 2,4-D (K_m, approximately 2 μM), whereas the K_m values for phenoxyacetate and 2,4,5-T were approximately 310 and 42 μM, respectively. The two enzymes behaved similarly in all measured parameters, including cosubstrates, cofactor requirements, activity, preferred substrate, and substrate range.

TABLE 1. Purification of $TfdA_{RASC}$ from *Burkholderia* sp. strain RASC

Purification step	Total amt of protein (mg)	Activity (U)	Recovery (%)	Sp Act[a]	Fold purification
Cell extract	1,710	1,730	100	0.09	1
$(NH_4)_2SO_4$	1,020	2,400	139	0.21	2.3
DEAE-Sepharose	90	900	52	0.9	10
MonoQ	10	680	39	6.8	75.6
Superose 12	6.0	630	36	10.3	117

[a] Specific activity is expressed as units of activity per milligram of protein, where 1 U is the amount of enzyme that forms 1 μmol of 2,4-DCP per min.

The purified protein was composed of a single type of subunit with an apparent M_r of 34,000. This value is slightly larger than that (M_r = 32,190) reported for the *A. eutrophus*-derived enzyme (16) and is in good agreement with the value (M_r = 33,310) calculated from the sequence of the $tfdA_{RASC}$ gene. Antibodies generated against the *A. eutrophus* enzyme cross-reacted with the RASC-derived enzyme after denaturing gel electrophoresis (data not shown), which is consistent with the sequence similarity of the two proteins. In addition, the amino-terminal sequences of the two polypeptides (S-I-N-S-E-Y-G/L-H-P-L-F-V-G-Q-V for $TfdA_{RASC}$ and S-V-V-A-N-P-L-H-P-L-F-A-A-G-V for TfdA) revealed identity in 6 or 7 of 15 positions, with several additional conservative substitutions. In both cases, the N-terminal methionines were processed away.

Evolutionary considerations. The high degree of DNA sequence homology between *tfdA* and $tfdA_{RASC}$ and the similarity of the biochemical mechanisms suggest that the genes (proteins) are evolutionarily related to each other. Moreover, their unique features suggest that the proteins form a new class of α-ketoglutarate-dependent dioxygenases. The two genes differ from one another in that *tfdA* is carried on a broad-host-range plasmid whereas $tfdA_{RASC}$ is chromosomally encoded. Matheson et al. (36) have also recently shown that *tfdA* is chromosomally encoded in a bacterial strain that was independently isolated from an agricultural soil in mid-Michigan. Thus, it appears that the gene family is rather mobile and has been widely disseminated among phylogenetically diverse species.

The evolutionary origins of *tfdA* and $tfdA_{RASC}$ are unknown. However, since widespread exposure to 2,4-D has been limited to the last 50 years, it is conceivable that TfdA evolved from an ancestral protein that has activity with a naturally occurring analog of 2,4-D. The identities of the ancestral gene and the naturally occurring 2,4-D analog are unknown. We postulate that the substrate specificity of the ancestral gene has been altered through selection of variants with the ability to cleave the acetyl moiety from 2,4-D and that the gene has been recruited for 2,4-D catabolism.

ACKNOWLEDGMENTS

We thank Penny Amy for providing *Burkholderia* strain RASC, Tamara Tsoi for providing pSP329, and C. Peter Wolk for providing pRL1062a and pRK2013. We are also grateful to Cindy H. Nakatsu for helpful discussions and to Yoichi Kamagata for assaying TfdA activity.

This research was supported by National Science Foundation grant BIR 9120006 to the Center for Microbial Ecology and by financial support to Y.S. from the Japan Research Development Corporation.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.

2. **Amy, P. S.** 1994. Personal communication.
3. **Amy, P. S., J. W. Schulke, L. M. Frazier, and R. J. Seidler.** 1985. Characterization of aquatic bacteria and cloning of genes specifying partial degradation of 2,4-dichlorophenoxyacetic acid. Appl. Environ. Microbiol. **49:**1237–1245.
4. **Amy, P. S., M. V. Staudaher, and R. J. Seidler.** 1990. Comparison of a gene probe with classical methods for detection of 2,4-dichlorophenoxyacetic acid (2,4-D)-biodegrading bacteria in natural waters. Curr. Microbiol. **21:**95–101.
5. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, and J. G. Seidman.** 1987. Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, New York.
6. **Birnboim, H., and J. Doly.** 1979. A rapid extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1525.
7. **Cooper, T. G.** 1977. The tools of biochemistry, p. 102–103. John Wiley & Sons, Inc., New York.
8. **Ditta, G.** 1986. Tn*5* mapping of *Rhizobium* nitrogen fixation genes. Methods Enzymol. **118:**519–528.
9. **Ditzelmuller, G., M. Loidl, and F. Streichsbier.** 1989. Isolation and characterization of a 2,4-dichlorophenoxyacetic acid-degrading soil bacterium. Appl. Microbiol. Biotechnol. **31:**93–96.
10. **Don, R. H., and J. M. Pemberton.** 1981. Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. J. Bacteriol. **145:**681–686.
11. **Don, R. H., and J. M. Pemberton.** 1985. Genetic and physical map of the 2,4-dichlorophenoxyacetic acid-degradative plasmid pJP4. J. Bacteriol. **161:** 466–468.
12. **Don, R. H., A. J. Weightman, H. J. Knackmuss, and K. N. Timmis.** 1985. Transposon mutagenesis and cloning analysis of the pathways for degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP134(pJP4). J. Bacteriol. **161:**85–90.
13. **Dunbar, J., and L. J. Forney.** 1995. Unpublished data.
14. **Evans, W. C., B. S. W. Smith, H. N. Fernley, and J. I. Davies.** 1971. Bacterial metabolism of 2,4-dichlorophenoxyacetate. Biochem. J. **122:**543–551.
15. **Fukumori, F., and R. P. Hausinger.** 1993. *Alcaligenes eutrophus* JMP134 "2,4-dichlorophenoxyacetate monooxygenase" is an α-ketoglutarate-dependent dioxygenase. J. Bacteriol. **175:**2083–2086.
16. **Fukumori, F., and R. P. Hausinger.** 1993. Purification and characterization of 2,4-dichlorophenoxyacetate/α-ketoglutarate dioxygenase. J. Biol. Chem. **268:**24311–24317.
17. **Fulthorpe, R. R., C. McGowan, O. V. Maltseva, W. E. Holben, and J. M. Tiedje.** 1995. 2,4-Dichlorophenoxyacetic acid-degrading bacteria contain mosaics of catabolic genes. Appl. Environ. Microbiol. **61:**3274–3281.
18. **Greer, L. E., J. A. Robinson, and D. R. Shelton.** 1992. Kinetic comparison of seven strains of 2,4-dichlorophenoxyacetic acid-degrading bacteria. Appl. Environ. Microbiol. **58:**1027–1030.
19. **Hansen, J. B., and R. H. Olsen.** 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids PMG1 and PMG5. J. Bacteriol. **135:**227–238.
20. **Harker, A. R., R. H. Olsen, and R. J. Seidler.** 1989. Phenoxyacetic acid degradation by the 2,4-dichlorophenoxyacetic acid (TFD) pathway of plasmid pJP4: mapping and characterization of the TFD regulatory gene, *tfdR*. J. Bacteriol. **171:**314–320.
21. **Hirsch, P. R., M. Van Montagu, A. W. B. Johnston, N. J. Brewin, and J. Schell.** 1980. Physical identification of bacteriocinogenic, nodulation, and other plasmids in strains of *Rhizobium leguminosarum*. J. Gen. Microbiol. **120:**403–412.
22. **Holben, W. E., B. M. Schroeter, V. G. M. Calabrese, R. H. Olsen, J. K. Kukor, V. O. Biederbeck, A. E. Smith, and J. M. Tiedje.** 1992. Gene probe analysis of soil microbial populations selected by amendment with 2,4-dichlorophenoxyacetic acid. Appl. Environ. Microbiol. **58:**3941–3948.
23. **Holmes, D., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114:**193–197.
24. **Ka, J. O., W. E. Holben, and J. M. Tiedje.** 1994. Analysis of competition in soil among 2,4-dichlorophenoxyacetic acid-degrading bacteria. Appl. Environ. Microbiol. **60:**1121–1128.
25. **Ka, J. O., W. E. Holben, and J. M. Tiedje.** 1994. Genetic and phenotypic diversity of 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacteria isolated from 2,4-D-treated field soils. Appl. Environ. Microbiol. **60:**1106–1115.
26. **Ka, J. O., W. E. Holben, and J. M. Tiedje.** 1994. Use of gene probes to aid in recovery and identification of functionally dominant 2,4-dichlorophenoxyacetic acid-degrading populations in soil. Appl. Environ. Microbiol. **60:**1116–1120.
27. **Kamagata, Y., Y. Suwa, and L. J. Forney.** 1994. Organization of novel 2,4-D catabolic genes isofunctional to *tfdA* and *tfdB* from *Pseudomonas* sp. TFD3, abstr. Q-401. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.
28. **Kaphammer, B., J. J. Kukor, and R. H. Olsen.** 1990. Regulation of *tfdCDEF* by *tfdR* of the 2,4-dichlorophenoxyacetic acid degradation plasmid pJP4. J. Bacteriol. **172:**2280–2286.
29. **Kaphammer, B., and R. H. Olsen.** 1989. Cloning and regulation of the dichlorophenol hydroxylase gene (*tfdB*) from the 2,4-dichlorophenoxyacetic acid catabolic plasmid pJP4, abstr. K-59, p. 254. *In* Abstracts of the 89th Annual Meeting of the American Society for Microbiology 1989. American Society for Microbiology, Washington, D.C.
30. **Kaphammer, B., and R. H. Olsen.** 1990. Cloning and characterization of *tfdS*, the repressor-activator gene of *tfdB*, from the 2,4-dichlorophenoxyacetic acid catabolic plasmid pJP4. J. Bacteriol. **172:**5856–5862.
31. **Kilpi, S.** 1980. Degradation of some phenoxyacid herbicides by mixed cultures of bacteria isolated from soil treated with 2-(2-methyl-4-chloro)phenoxypropionic acid. Microb. Ecol. **6:**261–270.
32. **Kukor, J. J., R. H. Olsen, and J.-S. Siak.** 1989. Recruitment of a chromosomally encoded maleylacetate reductase for degradation of 2,4-dichlorophenoxyacetic acid by plasmid pJP4. J. Bacteriol. **171:**3385–3390.
33. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
34. **Loos, M. A., R. N. Roberts, and M. Alexander.** 1967. Phenols as intermediates in the decomposition of phenoxyacetates by an *Arthrobacter* species. Can. J. Microbiol. **13:**679–690.
35. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
36. **Matheson, V. G., L. J. Forney, Y. Suwa, C. H. Nakatsu, A. J. Sextone, and W. E. Holben.** 1996. Evidence for acquisition in nature of a chromosomal 2,4-dichlorophenoxyacetic acid/α-ketoglutarate dioxygenase gene by different species of *Burkholderia*. Appl. Environ. Microbiol. **62:**2457–2463.
37. **Matrubutham, U., and A. R. Harker.** 1994. Analysis of duplicated gene sequences associated with *tfdR* and *tfdS* in *Alcaligenes eutrophus* JMP134. J. Bacteriol. **176:**2348–2353.
38. **Parker, L. W., and K. G. Doxtader.** 1982. Kinetics of microbial decomposition of 2,4-D in soil: effects of herbicide concentration. J. Environ. Qual. **11:**679–684.
39. **Pemberton, J. M., B. Corney, and R. H. Don.** 1979. Evolution and spread of pesticide degrading ability among soil microorganisms, p. 287–299. *In* K. N. Timmis and A. Puhler (ed.), Plasmids of medical, environmental and commercial importance. Elsevier/North Holland Biomedical Press, Amsterdam.
40. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
41. **Shizuya, H., B. Birren, U.-J. Kim, V. Mancino, T. Slepak, Y. Tachiiri, and M. Simon.** 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. Proc. Natl. Acad. Sci. USA **89:**8794–8797.
42. **Sinton, G. L., L. T. Fan, L. E. Erickson, and S. M. Lee.** 1986. Biodegradation of 2,4-D and related xenobiotic compounds. Enzyme Microb. Technol. **8:**395–403.
43. **Somasundaram, L., J. R. Coats, and K. D. Racke.** 1989. Degradation of pesticides in soil as influenced by the presence of hydrolysis metabolites. J. Environ. Sci. Health. Part B **24:**457–478.
44. **Stanier, R., N. Palleroni, and M. Doudoroff.** 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43:**159–271.
45. **Streber, W. R., K. N. Timmis, and M. H. Zenk.** 1987. Analysis, cloning, and high-level expression of 2,4-dichlorophenoxyacetate monoxygenase gene *tfdA* of *Alcaligenes eutrophus* JMP134. J. Bacteriol. **169:**2950–2955.
46. **Tonso, N. L., V. G. Matheson, and W. E. Holben.** 1995. Polyphasic characterization of a suite of bacterial isolates capable of degrading 2,4-D. *Microb. Ecol.* **30:**1–22.
47. **Top, E. M., W. E. Holben, and L. J. Forney.** 1995. Characterization of diverse 2,4-dichlorophenoxyacetic acid-degradative plasmids isolated from soil by complementation. Appl. Environ. Microbiol. **61:**1691–1698.
48. **Wolk, C. P., Y. Cai, and J.-M. Panoff.** 1991. Use of a transposon with luciferase as a reporter to identify environmentally responsive genes in a cyanobacterium. Proc. Natl. Acad. Sci. USA **88:**5355–5359.
49. **Wright, A. D.** 1995. Unpublished data.