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

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The findings of previous studies indicate that the genes required for metabolism of the pesticide 2,4dichlorophenoxyacetic 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 Tn5. One such mutant (d1) was shown to have Tn5 inserted in fdA_{RASC} , which encodes 2,4-D/ α ketoglutarate dioxygenase. This is the first reported example of a chromosomally encoded fdA. The $tfdA_{RASC}$ 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_{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 CO₂ (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-Ddegrading 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-Dcatabolic 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*_{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 MgSO₄ · 7H₂O, and 0.035 g of CaCl₂. 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

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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.

Tn5 mutagenesis. Kanamycin-resistant Tn5 transposon mutants of strain RASC were selected following conjugation with the Tn5 suicide vector pRL1062a (48), which can replicate only in *E. coli*. Triparental matings between strain RASC, the donor strain *E. coli* DH5 α (pRL1062a), and *E. coli* DH5 α (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 Tn5-containing derivatives of strain RASC.

To obtain insertion mutants bearing Tn5 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::Tn5 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::Tn5. 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 Tn5 insertion derivative of pRASC (pRASC::Tn5) to facilitate monitoring of this cryptic plasmid. Electrocompetent cells of strain RASC/pRASC::Tn5 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 ³⁵S-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 μ m), and the concentration of 2,4-D was determined by high-pressure liquid chromatography as described previously (22). When Tn5 insertion mutants were being analyzed, kanamycin (50 μ g/ml) was added to the culture and test media.

To quantitate the ability to completely mineralize 2,4-D, a single drop (ca. 40 μ l) 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*-¹⁴C]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 ¹⁴CO₂ 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 Tn5 insertions for the inability to incorporate [U-*ring*-¹⁴C]2,4-D into cell biomass on solid media (13). Briefly, dilutions of bacterial suspensions subjected to Tn5 mutagenesis were spread onto nitrocellulose filters resting on PIA/2 supplemented with 50 μ g 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 μ g of Irgasan per ml, 50 μ g of kanamycin per ml, and 500 μ g of 2,4-D per ml including 0.5 mCi of [U-*ring*-¹⁴C]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 ¹⁴C into their cell biomass. These colonies were presumptive 2,4-D⁻ mutants. Presumptive evidence that Tn5 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

fragment with the *tfdA* gene from pJP4 that had been subcloned from pUS311 (15).

Cloning of *tfd4*_{RASC}. The modified Tn5 transposon used for mutagenesis of strain RASC has the *oriV* origin of replication (48). Consequently, DNA containing *tfd4*_{RASC}::Tn5 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 Tn5 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 *Xba1-Pst1* DNA fragment from pS18 that flanked the transposon hybridized at low stringency with *tfd4*. This fragment was subcloned into pGEM3Zf(+) (Promega, Madison, Wis.) to generate pYS15 and was used as a probe to identify the wild-type *tfd4*_{RASC} gene.

Genomic DNA from a plasmid-cured derivative of RASC was purified (5) and completely digested with EcoRI. Since pYS15 hybridized to a single 7.2-kb EcoRI 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 DH5a by electroporation to create a library of subclones, which were then screened by colony hybridization (40) with the 1.0-kb XbaI-PstI 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 EcoRI. One plasmid, pYC6, which contained a 7.2-kb EcoRI fragment was selected for further analysis. Templates for DNA sequencing were obtained by using exonuclease III (Erasea-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 \times 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 $100,000 \times g$ for 30 min at 4°C. The resulting cell extracts were adjusted to 20% glycerol and 0.1 mM dithiothreitol; then, (NH₄)₂SO₄ was added to 50% saturation. Precipitated proteins were collected by centrifugation (10,000 \times g for 10 min at 4°C), dissolved in buffer A (20 mM Tris-HCI [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 diffuoride 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). Tn5 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 EcoRI confirmed that Tn5 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 Tn5 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 ¹⁴CO₂ 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 Tn5 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 Tn5 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 XbaI-PstI 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/\alpha-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 HindIII-ScaI 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 PstI subclone (Fig. 1), had no measurable activity, suggesting that one or both PstI sites interrupt the $tfdA_{RASC}$ gene or that the gene was entirely contained on the *HindIII-PstI* fragment. The pYB232 subclone was selected for DNA sequence analysis

DNA and amino acid sequence analysis of tfdA_{RASC}. The HindIII-PstI, PstI-PstI, and PstI-ScaI DNA fragments, which make up the 2.1-kb HindIII-ScaI 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 tfdAbegins 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 PstI 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 ter-

1* TTTTCAGCCG TTGTGAACGG CAATGCAACA T 51* STGAGCGTCG TCGCAAATCC CCTTCATCCT C 101* AGACATCGAC CTTCGAGAGG CCTTGGTTC C 101* AGACATCGAC CTTCGAGAGG CACTAAGTCC C 151* AACGGCTAAT GGACGAGAAG TCGGTGCTGG T 151* AAAACGAGAT GGACCAAAG GCCGTCTTGG T 151* AAAACGAGAT GGACCAAAG GCCGTCTTGG T 201* AGTCAAGACC AGCAGATCC CTTGCCGCGC Z 210* AGTCAAGACC AGCAAATCC CTTGCCGCGC Z 210* AGTCAAGACC AGCAAATCC CTTGCCGCGC Z 210* GATCAAGACC AGCAAATCC CTTGCCGCGC Z 210* GATCAAGACC AGCAAATCC CTTGCCGCGC Z 251* CGGTTTCATC AAAGTCAACC AGAGCCGTC C 251* CGGTTTCATC AAAGTCAACC AGAGCCGTC C 301* TGGCGGACAT CTCAACACC AGGGCGCAA 301* TGGCGGCGAG TCGTGGGAA CTTCCGCGAA C 351* GCCCGCGCGC GCACCCGCGC CCCGCATACTC Z 401* CTCCTTTCAG CAACCCGCGCAA ACCGAGTTC T 401* TTCCCTTTCAG CAACCCGCGCAA ACCGAGTTC T 451* TACCACCGTC GCGCGCGAA ACCGAGTTC T 501* GACGCCGCTC CCCGGCGCAA ACCGAGTTC T 51* GCACTACCA CTGAACTCC GCTTCATTC T 51* GCACTACGCA CTGAACTCCC GCTTCATTC T	CTTAGAAAAG	GAGCAAAAAA
51' FTCAGEGETEG TEGECAAATEC CETTEATECT C 51' ATGRECATCA ATTECGAATAC CETTEATECT C 101' AGACATCGAC ATTECGAAGGE CETTEGGETEC C 101' CAACETTEGE CTACAAGGEG CACTAAGTEC C 151' AACGGETAAT GGACGAAAG TEGETGEGEG 151' AAACGAGATE GACCAAAAG GECGTETEG G 151' AAACGGETAAT GGACGAAAAG GECGTETEG G 201' AGTCAGGATE AGCACGATAC CACGGETEC G 201' AGTCAGGATE AGCACGATACC CAGGACTEC C 2101' GATCAAGACE AGCAGATECE CTTTGCGECGE A 251' CGGTTTCATE AAGTCAACC AGAGGECGTE G 251' CGGTTTCATE AAAGTCAACC AGAGGECGTE G 251' CGGTTTCATE AAAGTCAACC AGAGGECGTE G 301' TGGECGGACAT CTCGAACGTE AGTGTEGAAG G 351' GCCCGCGAGG TEGTTGGGAA CTTCGCGAA C 351' GCCCGCGAGG TEGTTGGGAA CTTCGCGAA C 401' CTCCTTTCAG CAACCTGETG CTCGCTATTE A 451' TTCCGCGTC GGGCGCGAA CTTCGCGAA C 451' TTCCTTCAG CAACCTGETG CCCGCTATTE A 401' CTCCTTTCAG CAACCTGCTG CCGCGTAA C 451' TTCCCTTTCAG CAACCTGCTG CCGCGTAT C 451' TTCCCTTTCAG CAACCTGCTG CCGCGTAT C 51' GCCCCGCGAGG TEGTTGGGAA CTTCCGCGAA CTTCCGAGGA C <td>TAAATTCAAA</td> <td>GGAGATAAGT</td>	TAAATTCAAA	GGAGATAAGT
51* ATCAGECATCA ATTECEDATA CETTEATECA C 101* AGACATEGAC CTTEGAGAGG CETTEGETTE C 101* CAACETTEGE CTACAAGEGE CATTAGTEC C 151* AACGETTAT GEACAAGEGE CATTAGTEC C 151* AAAACGAGAT GGACCAAAAG GECGTETTEG T 201* AGTEAAGATE AGCAGATEGE CTTEGEGEGE A 201* GATEAAGAE AGCAGATEGE CTTEGEGEGE A 201* GATEAAGAE AGCAGATEGE CTTEGEGEGE A 251* CGGTTTCATE AAGGTCAACE AGAGGECGTE C 251* CGGTTTCATE AAAGTCAACE AGAGGECGTE C 251* CGGTTTCATE AAAGTCAACE AGAGGECGTE C 301* TGGEGGACAT CTEGAACGTE AGTGTEGATE C 351* GECEGECAGE TEGTEGGGAA CTECEGAAC C 351* GECEGEGAGE TEGTEGGGAA CTECEGAAC C 401* CTECETTECAA CAGECTGETE CECEGTATE C 451* TTECEGEGTE GEGEGEGGAA ACTEGEGATTE C 451* TTECEGETE GGEGEGEGAA ACTEGEGATTE C 451* TTECEGEGTE CTEGGGACET CCAATECEGAAC 551* GACGACEGE CECEGAGACT CCAATECEGAAC 551* GACGACEGE CECEGAGACT 551* GACCACEGE CECEGAGACT 551* GACATACEGE CATACECATE CECEGATECE 551* GACACACEGE CAATECEATE CECEGEGAA 551* <td>CTTTTCGCCG</td> <td>CAGGGGTCGA</td>	CTTTTCGCCG	CAGGGGTCGA
101* AGACATEGAC CTTEGAGAGG CETTEGAGTE C 101* CAACETTEGE CTTEGAGAGG CACTAGETE C 101* CAACETTEGE CTTEGAGAGG CACTAGETE C 151* AAACGGETAAT GGACGAGAAG TEGEGEGEG Z 151* AAAACGAGAT AGCAGAGATE G CTTEGEGEGE Z 201* AGTEAGGATE AGCAGAATEGE CTTEGEGEGE Z 201* GATEAAGACE AGAGGATATE AAAGACCATE C 201* GATEAAGACE AGAGGAATEGE CTTEGEGEGE Z 201* GATEAAGACE AAAGGTEAATE AAAGACCTEC C 251* CGGTTTEATE AAAGGTEAATE AAAGGACCTE C 251* CGGTTTEATE AAAGTEGAACE AGAGGCCGTE C 301* TGGCGGACAT CTEGAAACGTE AGTEGACGATE C 351* GCCCCCGAGG TGGTGTGGGAA CTTEGCGAAG 401* TTECCTTTECA CAACETTECE C 401* TTECCTTTECA CAACETGETE CCCGATATE 451* TACCACCGTE GACGACGTAT CCGAGATTE 501* GACGACTACE CCACTACCGA CAACECACTAE 51	CTTTTCGTCG	GACAGGTAGA
101* CAACCTTGCG CTACAAGGCG CACTAAGTCC C 151* AAACGGCTAAT GGACGAGAAG TCGGTGCTGG T 151* AAAACGAGAT GGACCAAAAG GCCGTCTTGG T 201* AGTCAAGACC AGCAAATCGC CTTGCGCGC Z 201* GATCAAGACC AGCAAATCGC CTTGCGCGC Z 201* GATCAAGACC AGCAAATCGC CTTGCGCGC Z 201* GATCAAGACC AGCAAATCGC CTTGCGCGC Z 251* CGGTTTCATC AAGGTCAATC AAAGACCTTC Q 251* CGGTTTCATC AAAGTCAACC AGAGGCCGTC Q 301* TGGCGGACAT CTCAAACGTC AGTGTCGAGA Q 351* GCCCGCGAGG TCGTGGGAA CTTCGCGAAT Q 351* GCTCGCGGAGG TCGTGGGAA CTTCGCGAAT Q 401* CTCCTTTCAG CAACCTGCTG CTCGCTATTC Q 401* CTCCTTTCAA CAGCCGCGAC ACGAGTTCT Q 451* TACCACCGTC GGCGGCGAA ACGAGTTCT Q 451* TACCACCGTC CGGGCGCAA ACGAGTTCT Q 501* GACGATCTTC CCGAGGATT CAAGAAGGAA Q 551* GCACTACGCA CTGAACTCCC GCTTCTCTGCT Q 551* GCACTACGCA CTGAACCCAA GTTTCTTTTT Z 601* AAAGTCAACG CAATGCCAA GTTTCTTCT Z 551* GCACTACGCA CTGAACTCCC GCTTCCGGTG Q 551* GCACTACGCA CTGAACTCCC GCTTCCTGCT Z 651* CACGCCGGCT CCGGACGAA GTTTCTTTTTT Z <td>GACCGAGGTC</td> <td>CGAGAGATCG</td>	GACCGAGGTC	CGAGAGATCG
151' AAACGGCTAAT GGACGAGAAG TCGGTGCTGG 1 151' AAAACGAGAT GGACGAAAAG GCCGCTTTGG 1 201' AGTCAGGATC AGCAGATCGC CTTCGCGCGC 2 201' GATCAAGACC AGGACAAAAG GCCGTTTGCCCGC 2 201' GATCAAGACC AGGACAAATCG CTTCGCGCGC 2 201' GATCAAGACC AAGGTCAATC AAAACGGCTC 0 251' CGGTTTCATC AAGGTCAACA AAGAGCCTC 0 301' TGGCGGGACAT CTCGAACGTC AGTGTCGAGG 0 301' TGGCGGGAGAT CTCGAACGTC AGTGTCGAGG 0 301' TGGCGGGAGAT CTCGAACGTC AGTGTCGAGA CTTCGCGATG 0 301' TGGCGGCGAGG TCGCTTCAG AGTGTCGGAA CTTCGCGATG 0	GGCCGAGGTC	CGTGACGTCG
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201 AGTCAGGATC AGCAGATCGC CTTCGCGCGC A 201 GATCAAGACC AGCAAATCGC CTTTGCGCGCC A 211 CGGTTTCATC AAGGTCAATC AAAGACCTTC C 251 CGGTTTCATC AAAGTCAACC AGAGGCCGTC C 201 TGGCGGACAT CTCGAACGT AGTGTCGAGG C 201 TGGCGGACAT CTCGAACGTC AGTGTCGAGG C 201 TGGCGGACAT CTCGAACGTC AGTGTCGAGG C 201 TGGCGGACAT CTCGAACGTC AGTGTCGACG C 201 TGGCGGACAT CTCGAACGTC AGTGTCGAAG C 201 TGGCGGACAT CTCGAACGTC AGTGTCGAAG C 201 GCCCCGCGAG TGGTCGGGAA CTTCGCGAAT C 201 CTCCTTTCAG CAACCTGCTG CCCGCTACTC C 201 TTCCTTTCAA CAGCCTGCTG CTCGCGTATTC A 201 TTCCGTTTCAA CAGCCGGCGAA ACCGACTTCT C 201 CTCCTTTCAA CAGCCGCGCAA ACCGACTTCT C 201 TTCCGTTTCAA CAGCCGCGCAA ACCGACTTCT C 201 TTCCGCCGTC GCGCGCGAA ACCGACTTCT C 201 GACGACTTCT CCGAGGACCAT CCAATCCCAGAA C 201 GACGACTTCT CCGAGGACAT ACCGACTTCT C 201 GACGACTCTC CCGAGGACAA CTTCACCAGAA C 201 GACGACACCA CTGAACTCCC GCTCCACTGCA A 201 GACGACACG CAATCCCAGA GTTTCTTTTT A 201 GACGACCACC CAATCCCAA GTTTCTTTTTT A	TGTTTCGTGG	ACAACCTCTG
201* GATCAAGACC AGCAAATCGC CTTTGCGCGC A 251* CGGTTTCATC AAAGTCAACC AAAGACCTTC A 251* CGGTTTCATC AAAGTCAACC AGAGGCCGTC A 301* TGGCGGACAT CTCGAACGTC AGTGTCGAGA A 301* TGGCGGACAT CTCCAAACGTC AGTGTCGAGA C 301* TGGCGGCAGG TGGTCGGGAA CTTCGCGAAC C 351* GCTCGCGGAGG TGGTCGGGAA CTTCGCGAAC C 401* CTCCTTTCAG CAACCTGCTG CCCGATATTC A 401* TTCCGTTCAA CAGCGGGCGAA ACCGAGTTC C 451* TACCACCGTC AGGGGGCGAA ACCGAGTTC C 451* TACCACCGTC AGGGGGCGAT ACGGAGTTC C 501* GACGACTCTC CCACACGCGAC CCACACGCAGA CTTCTCTTCA 501* GACGACTCTC CCACAGGACAT CCACACGCGACA CCACAGCGAGA CCACACGCGACA 501* GACGACTCTC CCACACACGCA CCACACGCGACA CCACAGCGACA CCACACGCGACA CCACCAGGCACA 501* GACGACACGC CAACTGCCACC GCACTACGCA CCACCAGCGACA CCTCCTTCTTCT CACCGACGCACA CCTCCCGTCG CCAC	AATTTCGGGC	CACTCGAAGG
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251* CGGTTTCATC AAAGTCAACC AGAGGCCGTC 301* TGGCGGACAT CTCGAACGTC AGTGTCGCAGAG 301* TGGCGGACAT CTCAAACGTC AGTGTCGCAGAG 301* TGGCGGACAT CTCAAACGTC AGTGTCGCAGAG 351* GCCCGCGAGG TGGTCGGGAA CTTCGCGAGAG CTTCGCGAAC 401* CTCCTTTCAG CAACCTGCTG CCCGCTATC A 401* TTCCTTTCAA CAGCCTGCTG CCCGCTATC A 451* TTCCGTTCAA CAGCCTGCTG CCCGCAGAGTTC CA 451* TTCCGCGTC GGGCGGCGAT ACGGAGTTCT C 501* GACGACCTCC CAGGCGGCGAT ACGGAGTTCT C 501* GACGACTCTC CCGAGAGTT CAAGAAGAAA C 501* GACGACTACCGC CTCGAGGAGTT CAAGAAGAAA C 51* GCACTACCGC CTGAAGAACT CCCCGGGTA ACGAGAGTCT 51* GCACTACCGC CATGCACATC CCCCCGGTGA C 51* GCACTACCGC CATGCCATG CCCCCGCGGTA A 601* AAGCGCAACG CAA	GAGATTCAAG	TACGCGGAGT
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301* TGGCGGACAT CTCAAACGTC AGTGTCGATG 351* GCCCGCGAGG TGGTCGGAAA CTTCGCGAAC CTTCGCGAAC 351* GCTCCTTTCAG CAACCTGCTG CCCGCTACTC C 401* TTCCTTTCAA CAGCCGGCGAA ACCGACTTC A 401* TTCCGTTTCAA CAGCCGGCGAA ACCGACTTC A 451* TTCCGCCGTC GGGCGGCGAA ACCGAGTTC C 451* TACCACCGTC CAGGCGGCGAA ACCGAGTTC C 501* GACGACTCTC CCGAGGAGAA CCACACGACG CAAACTGCAA C 501* GACGACTCTC CCGAGGACT CCACTACGCA CTTCCTTCT C 51* GCACTACGCA CTGAAACTCCC GCTTTATTCT T 51* GCACTACGCA CTGCAGCACTG CCACCGCGTCA C 51* GCACTACGCA CTGCAGCACTG CCACCGCGTCA C 51* GCACTACGCA CTGCACTACCC GCTTTATTCT T 601* AAAGCCAACG CAATGCCATG CCTCCGGTGAA C 61* AAAGGCCACGC CATCCGAGCAA GTTTCTTTTT	GCAAGGTCGC	GCAACGCGAT
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351* GCTCGCGAGG TCGTTGGGAA CTTCGCGAAT C 401 CTCCTTTCAG CAACCTGCTG CCCGCTACTC C 401* TTCCTTTCAA CAGCCTGCTG CCCGCTACTC C 401* TTCCGTTCAA CAGCCTGCTG CTCGCTATTC A 451* TTCCGCCGTC GGCGCGCGAC ACCGAGTTCT C 451* TACCACCGTC AGGCGGCGAT ACGGAGTTCT C 501 GACGCGCTGC CTCGGGACCT CCAATCCGAG T 501* GACGACTCTC CCGAGGATTT CAAGAAGAAGA C 551* GCACTACGCG CTGCATTCCC GCTTCTGCT C 601* AAGCCAACG CTGCATTCCC GCTTCATTCT T 601* AAGCCAACG CAATGCCATG CCCCCGGTGA A ************************************	CAGCTCTGGC	ACAGCGACAG
401 CTCCTTTCAG CAACCTGCTG CCCGCTACTC C 401 TTCCTTTCAA CAACCTGCTG CCCGCTACTC C 451 TTCCGCCGTC GGCGGCGCAA ACGGAGTTCT C 451 TTCCGCCGTC GGCGGCGCAT ACGGAGTTCT C 501 GACGACGCTGC CTCGGGAACT CCAATCCGAG T 501 GACGACTTC CCGAGGAGTT CAAGAAGAAA C 501 GACGATTCTC CCGAGGATT CAAGAAGAAA C 501 GACGATACGCA CTGAGAATTC CAAGCAGGAA C 501 GACGATACGCA CTGAGAATTC CAAGAAGAAA C 501 GACGATACGCA CTGAGAATTC CACGCCGGTA CTTCTTCTTC C 501 GACGACTACGCA CTAATCCATC CCCCCGGTCA C C C C C C C C C C C CCCCCGGTCA C C C C C C CCCCCGGTCA C C C C C C C C C C C C C	CAACTCTGGC	ATAGCGACAG
401* TTCCTTTCAA CAGCCTGCTG CTCGCTATTC A 451* TTCCGCCGTC GGGCGGCGAA ACCGAGTTCT 451* TACCACCGTC CAGGCGGCGAA ACCGAGTTCT 501* GACGCGCTGC CTCGGGCACA CCAATCCCAGA 501* GACGCGCTGC CTCGGGCACA CCAATCCCAGA 501* GACGCATCTC CCGAGGAGTTT CAAGAAGGAA 551* GCACTACGCA CTGGAACTCCC GCTTCCTGTCT 601* AAGCGCAACG CAATGCCATG CCCCCGGTCA 601* AAAGTCAACG CAATGCCAAG GTTTCTTTT 601* AAAGTCAACG CAATGCCAAG GTTTCTTTT 601* AAAGTCAACG CAATGCCAAG GTTTCTTTT 651* CACGCCGGGAT CTTCCGGTGG CCCCAGGGAG 651* CACGCCGGGAT CTTCCGAGCGAC GTTTCTTTT 701* CGTCGAAGGC CTTCCGGTGG CCAAGGCGC 751* TCGAGCACGC GACGCACGC GAACTTGT CATCGAAGCAC 751* TCGAGCACGC GACGACGCGACACC AAGTTGTGCGA CAACCGCTCC 751* TCGAGCACGC GAACTGTGGGA CAACCGCC	GATGCTCTCC	GCGGTGGTGG
451' TTCCGCCGTC GGGCGGCGAC ACCGAGTTET ACCGACTTET 451' TACCACCGTC AGGCGGCGAC ACCGAGTTET ACCGACGACT 501' GACGACTGC CTGGGGACT CCAATCCGAG T 501' GACGACTTC CCGAGGACTT CCAAGAAGGAA C 501' GACGACTTC CCGAGGACTT CCAAGAAGGAA C 551' GCACTACGCA CTGAATCCCC GCTTCCTGTC C 551' GCACTACGCA CTGAATCCCC GCTTTATTCT T 601' AAGCGCAACG CAATGCCATC CCTCCGGTGA C 601' AAGCGCCAGCT CCGGGGCGCAA GTTTCTTCT A 601' AAAGTCAACG CAATGCCATC CCTCCGGTGA GTTCTTTTT 601' AAAGTCAACG CAATGCCATC CCTCCGGTGA GTTTCTTTTT 61' CACGCCGGCT CCGGGGCGCAA GTTTCTTTTTT A 651' CACGCCGGCT CCGGGCGCAA GTTTCTTTTT A 701' CGTCGAAGGC GACACACGC GAATTCGTGT A 751' TCGAGCACGC GACGACACG GAACTTGT A	AATGCTTTCA	GCAATCGTGC
451* TACCACCGTC AGGCGGGGAT ACGGAGTTET C 501* GACGCGCTGC CTCGGGGACCT CCAATCCGAG T 501* GACGATTET CCGGGGATT CAAGAAGAGA C 551* GCACTACGCA CTGAACTCCC GCTTCCTGET C 551* GCACTACGCG CTGCATTCCC GCTTCATTCT T 601* AAGCGCAACG CAATGCCATG CCGCCGGTCA A ************************************	GCGACATGCG	TGCGGCATAC
501' GACGCGCTGC CTCGGGACCT CCAATCCGAG T 501' GACGACGCTGC CTCGGGGATT CAAGAAGAAA 551' GCACTACGCA CTGAACTCCC GCTTCTTGTCT 551' GCACTACGCA CTGAACTCCC GCTTCTTGTCT 601' AAGCGCAACG CAATGCCATG CCGCCGGTCA A ************************************	GCGACATGCG	CGCAGCGTAC
501* GACGATETTE CEGAGGATTT CAAGAAGGAA C 551* GCACTACGCA CTGAACTCCC GETTECTGET C 551* GCACTACGCG CTGCATTCCC GETTECTGET C 601* AAGGCCAACG CAATGCCATG CCGCCGGTCA P 601* AAAGTCAACG CAATGCCATG CCCCCGGTCA P 601* AAAGTCAACG CAATGCGATG CCTCCGGTGA G 651* CACGCCGGCT CCGGGCGCAA GTTTETETTE P 651* CACGCCGGGAT CTGGACGCAA GTTTETETTT P 701* CGTCGAAGGC CTTCCGGTGG CCGAAGGTCG C 751* TCGAGCACGC GACACAGCG GAATTCGTGT P 751* TCGAGCACGC GACGCAACCG AAGTTTGTET P 801* GGAGATCTGG TGAGTGGGA CAACCGCTGC G 801* GGGGATCTTG TGATGTGGGA CAACCGCTGC G 851* GTACGACATE TCGGCCAGGC GTGAGCTGC G 851* GTACGACATE TCGGCCAGGC GTGAACTTCG G 851* GTACGACGC CACCGCAGCG GTGAACTTCG G 851* GTACGACGCC CACGCCAGCC GTGAACTTCG C	TTGGAAGGGC	TGCGTGCCGA
551' GCACTACGCA CTGAACTCCC GCTTCCTGCT C 551' GCACTACGCG CTGCATTCCC GCTTTATTCT T 601' AAGCGCAACG CAATGCCATG CCCCCGGTGA G 601' AAAGCCCACG CAATGCCATG CCCCCGGTGA G 601' AAAGCCCGCGCT CCGGGCGCAA GTTTCTTCT A 601' AAAGCCCGGCT CCGGGCGCAA GTTTCTCTTC A 651' CACGCCGGGAT CTGGACGCAA GTTTCTTTT A 651' CACGCCGGGAT CTGGACGCAA GTTTCTTTT A 701' CGTCGAAGGC CTTCCGGTGG CCGAAGGCCG G 701' CGTCGAAGGC CTTCCGGTGG CCGAAGGTCG C 701' CATCGAAGGC CGTCCCGTCG CCGAAGGTCG C 751' TCGAGCACGC GACCAACCG AAGTTTGTCT A 751' TCGAGCATCG GACGCAACCG AAGTTTGTCT A 801' GGGGATCTTG TGATGTGGGA CAACCGCTCC G 801' GGGGATCTTG TGATGTGGGA CAACCGCTCC G 851' GTACGACATC TCGGCCAGGC GTGAACTGC G 851' GTACGACGTC CACGCCAGGC GTGAACTTCC G 851' GTACGACGTC CCGCCGCG GTGAACTTCC G	CTGCAGGGAT	TGCGTGCAGA
551* GCACTACGCG CTGCATTCCC GCTTTATTCT T 601* AAGCGCAACG CAATGCCATG CCGCCGGTCA A 601* AAAGTCAACG CAATGCCATG CCCCCGGTGA A 601* AAAGTCAACG CAATGCCATG CCTCCGGTGA A 651* CACGCCGGCT CCGGGCGCAA GTTTCTTTC A 651* CACGCCGGAT CTGGACGCAA GTTTCTTTT A 701* CGTCGAAGGC CTTCCGGTGG CCGAAGGTCG C 701* CATCGAAGGC CGTCCGTCG CCGAAGGTCG C 751* TCGAGCACGC GACACAGCG AAGTTTCGTT A 751* TCGAGCATCG TGATGTGGGA CAACCGCTCC G 801* GGGGATCTTG TGATGTGGGA CAACCGCTCC G 801* GGGGATCTTG TGATGTGGGA CAACCGCTGC G 851' GTACGACATC TCGGCCAGGC GTGAGCTGCG C 851' GTACGACGTC CACGCCAGGC GTGAACTTCG C 851' GTACGACGCT CCGGCCAGGC GTGAACTTCG C 851' GTACGACGCT CCGCCAGGC GTGAACTTCG C	CGGCGACACC	GACTATTCGG
601' AAGCGCAACG CAATGCCATG CCGCCGGTCA A 601' AAAGTCAACG CAATGCGATG CCCCCGGTGA A 601' AAAGTCAACG CAATGCGATG CCTCCGGTGA G 651' CACGCCGGCT CCGGGCGCAA GTTTCTTTT A 651' CACGCCGGGAT CTGGACGCAA GTTTCTTTT A 701' CGTCGAAGGC CTTCCGGTGG CCGAAGGCCG A 701' CGTCGAAGGC GCCCCGTCG CCGAAGGCCG A 701' CGTCGAGCACG GACACAGCGG GAAGTTGGTG C 751' TCGAGCACGC GACACAGCGG AAGTTTGTGT A 751' TCGAGCATGG GACGCAACGG AAGTTTGTGT A 801' GGAGATCTGG GAACCCCTGC GAACCCCTGC G 851' GTACGACATC TCGGCCAGGC GTGAGCTGCG GTGAGCTGCGC GTGAGCTGCG G	TGGCGACACA	GAGTATTCTG
601* AAAGTCAACG CAATGCGATG CCTCCGGTGA G 651* CACGCCGGCT CCGGGCGCAA GTTTCTCTTC A 651* CACGCCGGAT CTGGACGCAA GTTTCTTTT A 701* CGTCGAAGGC CTTCCGGTGG CCGAAGGCCG G 701* CATCGAAGGC CTTCCGGTGG CCGAAGGTCG C 751* TCGAGCACGC GACACAGCGG GAATTCGTGT A 751* TCGAGCATCG GACGCAACCG AAGTTTGTCT A 801* GGAGATCTGG TGAGTGGGA CAACCGCTGC G 801* GGGGATCTTG TGATGTGGGA CAACCGCTGC G 851* GTACGACATC TCGGCCAGGC GTGAGCTGC G 851* GTACGACGTC CACGCCAGGC GTGAACTTCG C 851* GTACGACGTC CACGCCAGGC GTGAACTTCG C	ACTGGCCGCT	GGTTCGAACC
651' CACGCCGGCT CCGGGCGCAA GTTTCTCTTC 651' CACGCGGGAT CTGGACGCA GTTTCTTTTT 701' CGTCGAAGGC CTTCCGGTGG CCGAAGGCC 701' CATCGAAGGC CGTCCGACGTGG CCGAAGGTCG 701' CATCGAAGGC GGCCCGTCG CCGAAGGTCG 751' TCGAGCACGC GACGCAACCG AAGTTTGTGTF 751' TCGAGCATGC GACGCAACCG AAGTTTGTCT 801' GGGGATCTG TGATGTGGGA CAACCGCTGC 801' GGGGATCTTG TGATGTGGGA CAACCGCTGC 851' GTACGACATC TCGGCCAGGC GTGAGCTGC GGGCAACTC 851' GTACGACGTC ACGGCCAGGC GTGAACTTCG GGGCAACTC 851' GTACGACGTC ACGGCCAGGC GTGAACTTCG GGGCAACTC 851' GTACGACGTC ACGCCAGGC GTGAACTTCG GGGAACTTCG	GCTGGCCGCT	GATCCGCACC
651* CACGCGGGAT CTGGACGCAA GTTTCTTTT A 701* CGTCGAAGGC CTTCCGGTGG CCGAAGGCCG G 701* CATCGAAGGC CGTCCGTCG CCGAAGGTCG C 751* TCGAGCAGCG GACACAGCGG GAATTCGTGT A ******** ******** 751* TCGAGCATCG GACGCAACCG AAGTTTGTCT A 801* GGAGATCTGG TGATGTGGGA CAACCGCTGC G 801* GGGGATCTTG TGATGTGGGA CAACCGCTGC G 851* GTACGACATC TCGGCCAGGC GTGAGCTGCG G 851* GTACGACGTC CACGCCAGGC GTGAACTTCG C 851* GTACGACGTC CACGCCAGGC GTGAACTTCG C STGP STGP	ATCGGCGCGC	ACGCGAGCCA
701' CGTCGAAGGC CTTCCGGTGG CCGAAGGCCG G 701' CATCGAAGGC CGTCCCGTCG CCGAAGGTCG G 751' TCGAGCACGC GACCACGGG GAATTCGTGT A 751' TCGAGCATGC GACCACCG AAGTTTGTCT A 801' GGAGATCTGG TGATGTGGGA CAACCGCTGC G 801' GGAGATCTG TGATGTGGGA CAACCGCTGC G 801' GGAGATCTG TGATGTGGGA CAACCGCTGC G 851' GTACGACATC TCGGCCAGGC GTGACCTGC G 851' GTACGACATC TCGGCCAGGC GTGACCTCC G 851' GTACGACGTC ACGCCAGGC GTGAACTTCG C 851' GTACGACGTC CCGGCCAGGC GTGAACTTCG C	ATCGGGGCTC	ATGCTAGTCA
701* CATCGAAGGE CGTCCCGTCG CCGAAGGTCG C 751* TCGAGCACGE GACACAGCGG GAATTCGTGT A 751* TCGAGCATGE GACGCAACCG AAGTTTGTCT A 801* GGAGATCTG TGATGTGGGA CAACCGCTGE G 801* GGGGATCTG TGATGTGGGA CAACCGCTGE G 851* GTACGACATE TCGGCCAGGE GTGAGCTGE G 851* GTACGACATE CCGGCCAGGE GTGAACTTCG C 851* GTACGACGTE CACGGCCAGGE GTGAACTTCG C 851* GTACGACGTE CACGGCCAGGE GTGAACTTCG C	GATGCTGCTT	GCGGAGCTTC
751' TCGAGCACGC GACACAGCGG GAATTCGTGT A ********** ******** 751* TCGAGCATGC GACGCAACCG AAGTTTGTGT A 801' GGAGATCTGG TGATGTGGGA CAACCGCTGC G 801* GGGGATCTTG TGATGTGGGA CAACCGCTGC G 851' GTACGACATC TCGGCCAGGC GTGAGCTGCG G 851' GTACGACGC CAGGC GTGAACTTC G 851' GTACGACGC CAGGC GTGAACTTCG C S10* GTGACGCCAGGC GTGAACTTCG C	CATGCTGCTT	GCAGAGCTTC
751* TCGAGCATGE GACGEAACCG AAGTTTGTET A 801' GGAGATCTGG TGATGTGGGA CAACCGETGE G 801* GGGGATCTTG TGATGTGGGA CAACCGETGE G 851' GTACGACATE TEGGECAGGE GTGAGETGE G 851* GTACGACGE ACCGECAGGE GTGAACTTEG C 851* GTACGACGE ACCGECAGGE GTGAACTTEG C 851* GTACGACGE ACCGECAGGE GTGAACTTEG C	ACCGGCATCG	CTGGAACGTG
801' GGAGATCTGG TGATGTGGGA CAACCGCTGC G 801' GGGGATCTTG TGATGTGGGA CAACCGCTGC G 851' GTACGACATC TCGGCCAGGC GTGAGCTGCG G 851' GTACGACGTC ACGGCCAGGC GTGAACTTCG G 851' GTACGACGTC ACGGCCAGGC GTGAACTTCG G 851' GTACGACGTC CAGGCCAGGC GTGAACTTCG G	ATCGGCATAG	CTGGAAAGTC
801* GGGGATCTTG TGATGTGGGA CAACCGCTGC G 851* GTACGACATC TCGGCCAGGC GTGAGCTGCG C 851* GTACGACGCC ACGGCCAGGC GTGAACTTCG C STOP STOP	GTTCTTCACC	GCGGACGCAG
851' GTACGACATC TCGGCCAGGC GTGAGCTGCG C ******* ** ********* ***** 851' GTACGACGTC ACGGCCAGGC GTGAACTTCG C <u>STCP</u>	GTCCTGCATC	GAGGGAGGCG
851" GTACGACGTC ACGGCCAGGC GTGAACTTCG C	CCGGGCGACC	ACCCT-GGAC
	CCGAGCCACG	ACTTTGGGAC
900' GATGCCGTCG TC <u>T-AG</u> CGCA CG		STOP
901* GACGCTGTCG TTTGAGTCCA CGGGGTCAGT C	CTTGGGTTCA	ATAG

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. europhus* JMP134 (*tfdA*; top row). Identical nucleotides are indicated by asterisks. The initiation and termination codons and the putative RNA polymerase-binding sites are boxed.

minus. 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

```
1 '
  1.
         SINSEYLHPL FVGQVDNLAL QGALSPAEVR DVENEMDQKA VLVFRGQPLD
        QDQQIAFARN FGPLEGGFIK VNQRPSRFKY AELADISNVS LDGKVAQRDA
##*#**#*** *#.*****# *####***#* #****#*** #**** #**** #****
 51'
 51
         QDQQIAFARN FGQLEGGFIK VNQRPSRFKY AELADISNVS VDGKVAEADA
         REVVGNFANQ LWHSDSSFQQ PAARYSMLSA VVVPPSGGDT EFCDMRAAYD
**##****## **#**## **#*## .*.#*##*##
REVVGNFANQ LWHSDSSFQQ PAARYSMLSA IVLPPSGGDT EFCDMRAAYD
101
101"
        151'
151"
        AGSGRKFLFI GAFASFVEGL PVAEGRMLLA ELLEFATQRE FVYRFRWNVG
####***##* ######*.** ##**##***# ****#**#
AGSGRKFLFI GAHASHIEGR PVAEGRMLLA ELLEHATQPK FVYRHSWKVG
201
201
         DLVMWDNRCV LHRGRRYDIS ARRELRRATT LDDAVV
251
        H******** H###++**... ***##*#### +.
DLVMWDNRCV LERGRRYDVT ARRELRRATT LGRRCRLSPR GQSWVQ
251 "
```

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, Tfd A_{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

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. 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/a-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 μ M), 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.

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 crossreacted 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 aminoterminal 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.

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