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A Three-component Dicamba *O*-Demethylase from *Pseudomonas maltophilia*, Strain DI-6

GENE ISOLATION, CHARACTERIZATION, AND HETEROLOGOUS EXPRESSION*

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Dicamba *O*-demethylase is a multicomponent enzyme from *Pseudomonas maltophilia*, strain DI-6, that catalyzes the conversion of the widely used herbicide dicamba (2-methoxy-3,6-dichlorobenzoic acid) to DCSA (3,6-dichlorosalicylic acid). We recently described the biochemical characteristics of the three components of this enzyme (*i.e.* reductase$_{DIC}$, ferredoxin$_{DIC}$, and oxygenase$_{DIC}$) and classified the oxygenase component of dicamba *O*-demethylase as a member of the Rieske non-heme iron family of oxygenases. In the current study, we used N-terminal and internal amino acid sequence information from the purified proteins to clone the genes that encode dicamba *O*-demethylase. Two reductase genes (*ddmA1* and *ddmA2*) with predicted amino acid sequences of 408 and 409 residues were identified. The open reading frames encode 43.7- and 43.9-kDa proteins that are 99.3% identical to each other and homologous to members of the FAD-dependent pyridine nucleotide reductase family. The ferredoxin coding sequence (*ddmB*) specifies an 11.4-kDa protein composed of 105 residues with similarity to the adrenodoxin family of [2Fe-2S] bacterial ferredoxins. The oxygenase gene (*ddmC*) encodes a 37.3-kDa protein composed of 339 amino acids that is homologous to members of the Phthalate family of Rieske non-heme iron oxygenases that function as monooxygenases. Southern analysis localized the oxygenase gene to a megaplasmid in cells of *P. maltophilia*. Mixtures of the three highly purified recombinant dicamba *O*-demethylase components overexpressed in *Escherichia coli* converted dicamba to DCSA with an efficiency similar to that of the native enzyme, suggesting that all of the components required for optimal enzymatic activity have been identified. Computer modeling suggests that oxygenase$_{DIC}$ has strong similarities with the core α subunits of naphthalene 1,2-dioxygenase. Nonetheless, the present studies point to dicamba *O*-demethylase as an enzyme system with its own unique combination of characteristics.

The herbicide dicamba (2-methoxy-3,6-dichlorobenzoic acid) has been used to effectively control broadleaf weeds in crops such as corn and wheat for almost 40 years. Like a number of other chlorinated organic compounds, dicamba does not persist in the soil because it is efficiently metabolized by a consortium of soil bacteria under both aerobic and anaerobic conditions (1–4). Studies with different soil types treated with dicamba have demonstrated that 3,6-dichlorosalicylic acid (DCSA),[1] a compound without herbicidal activity, is a major product of the microbial degradation process (2, 3, 5). Soil samples taken from a single site exposed to dicamba for several years yielded a number of bacterial species capable of utilizing dicamba as a sole carbon source (6). These soil microorganisms could completely mineralize dicamba to carbon dioxide, water, and chloride ion (7). Studies on the metabolism of dicamba in the cells of one of these bacteria, the DI-6 strain of *Pseudomonas maltophilia*, showed that DCSA is a major degradation product (7, 8).

We have been investigating dicamba *O*-demethylase, the enzyme involved in the first step of the dicamba degradation pathway in *P. maltophilia*, strain DI-6. We previously demonstrated that cell lysates contain an *O*-demethylase that catalyzes the rapid conversion of dicamba to DCSA (9). We also partially purified the enzyme and found that at least three separate components are required for activity (9). Recently, we provided a detailed description of the purification and characterization of the reductase$_{DIC}$, ferredoxin$_{DIC}$, and oxygenase$_{DIC}$ components of dicamba *O*-demethylase (10). Oxygenase$_{DIC}$ is a homotrimer $(\alpha)_3$ with a subunit molecular mass of ~40 kDa and contains a single Rieske [2Fe-2S] cluster. Ferredoxin$_{DIC}$ is a monomer with an estimated molecular mass of 14 kDa and has a single [2Fe-2S] cluster resembling those found in adrenodoxin and putidaredoxin. Reductase$_{DIC}$, a monomer with a molecular mass of ~45 kDa, has the typical yellow color and UV fluorescence indicative of a flavin-containing molecule. All of the biochemical and physical data suggest that oxygenase$_{DIC}$ can be classified as a member of the family of Rieske non-heme iron oxygenases (11).

In the present study, we describe the cloning and characterization of the genes (designated as *ddmA*, *ddmB*, and *ddmC*) that encode the three components of dicamba *O*-demethylase from *P. maltophilia*, strain DI-6. We demonstrate by Southern analysis that the oxygenase gene (*ddmC*) can be localized to a megaplasmid in cells of *P. maltophilia*. Finally, we describe overexpression of each of the cloned genes in a heterologous system and demonstrate that the three purified recombinant components can be reconstituted into an active enzyme that

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY786442, AY786443, AY786444, and AY786445.

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[1] The abbreviations used are: DCSA, 3,6-dichlorosalicylic acid; DIC, dicamba; HPLC, high performance liquid chromatography; DIG, digoxigenin; ORF, open reading frame.

TABLE I
Amino acid sequences, PCR primers, and oligonucleotide used in the cloning of the genes encoding the three components of dicamba O-demethylase

The sequence of the degenerate nested PCR primers and oligonucleotide was based on the underlined amino acid residues.

Component	Sequence
Reductase	
N-terminal	SKADVVIVGAGHGGAQ(C)AIALQN
Internal	LYIRPPTFWA
Nested PCR primers	
A	5′-AARGCNGAYGTNGTNAT-3′
B	5′-ATHGTNGGNGCNGGNCA-3′
C	5′-GTNGGNGGNCKDATRTA-3′
PCR primers (probe)	5′-GGGCATGGCGGTGCACA-3′
	5′-AGGCGGTCGAAGGTCTT-3′
Ferredoxin	
N-terminal	PQITVVNQSGEESSVEASEGRTLMEVIRD
Consensus	RL(T/S/C)CQ(V/I/L)
Nested PCR primers	
A	5′-ATHACNGTNGTNAAYCA-3′
B	5′-ATGGARGTNATHMGNGA-3′
C	5′-ANYTGRCANSWNARNCG-3′
PCR primers (probe)	5′-ATGGAGGTTATTCGCGACA-3′
	5′-GCTGTCGAGCAGGTCGTTC-3′
Oxygenase	
N-terminal	TFVRNAWYVAALPEELSEKPLGRTILD
Oligonucleotide (probe)	5′-AAYGCNTGGTAYGTSGC-3′

can convert dicamba to DCSA with an efficiency similar to that observed for the native enzyme under our assay conditions.

EXPERIMENTAL PROCEDURES

Bacterial Strain and Culture Medium—*P. maltophilia*, strain DI-6, was originally isolated from a soil sample collected near a storm water retention pond at a dicamba manufacturing plant in Beaumont, TX (6). Cells were grown in reduced chloride medium (6) amended with either filter-sterilized 5 mM dicamba or with autoclaved glucose (2 mg/ml) and casamino acids (2 mg/ml) as the carbon source. Solidified medium was prepared with 1% (w/v) Gelrite.

Materials—Dicamba was a generous gift from Sandoz Agro Inc. (Des Plaines, IL). The custom oligonucleotide primers utilized in this study were commercially synthesized by Operon (Alameda, CA) and are listed in Table I.

Isolation of Genomic DNA—Genomic DNA was isolated from *P. maltophilia* according to a protocol modified from a method used for *Synechococcus* 6301 in the laboratory of Donald Bryant at Penn State University.[2] Cells were grown in 500 ml of reduced chloride medium with glucose and casamino acids at 30 °C to an A_{600} of 1.5–2.0 and harvested by centrifugation at 9,110 × *g* for 20 min. The cells in the pellet were resuspended in sucrose buffer (50 mM Tris (pH 7.5), 10 mM EDTA, 10% sucrose), incubated with lysozyme (5 mg/ml) for 30 min at 37 °C, and then lysed in 1% Sarkosyl. The lysate was centrifuged to equilibrium in a CsCl-ethidium bromide gradient in a Type 90 Ti rotor (Beckman) at 214,200 × *g* for 72 h at 20 °C. The fraction containing the genomic DNA was extracted with *n*-butanol and precipitated with 0.3 M sodium acetate and ethanol.

Isolation of Megaplasmid DNA—Cells of *P. maltophilia* were grown in 500 ml of reduced chloride medium with 5 mM dicamba for ~48 h at 30 °C with shaking (225 rpm). At this point, it was necessary to replace the culture medium because a metabolic by-product that interferes with cell growth typically accumulates in cultures of *P. maltophilia* grown with dicamba as the sole carbon source. The culture was centrifuged under sterile conditions at 5,000 × *g* for 10 min and then the pellet was resuspended in 500 ml of fresh or reduced chloride medium with 5 mM dicamba. The culture was grown for another 72 h under the same conditions and then plasmid DNA was isolated from the cells with a Qiagen-tip 100 according to a protocol recommended by the manufacturer (Qiagen) for the purification of very low-copy plasmids.

Amino Acid Sequencing—The purification of the reductase$_{DIC}$, ferredoxin$_{DIC}$, and oxygenase$_{DIC}$ components of dicamba *O*-demethylase as well as the N-terminal amino acid sequences of the purified proteins have been described (10). To obtain internal amino acid sequence information, the purified ferredoxin$_{DIC}$ and reductase$_{DIC}$ proteins were digested with trypsin and the isolated peptide fragments were sequenced by automated Edman degradation in the Protein Core Facility (Center for Biotechnology, University of Nebraska-Lincoln).

PCR Amplification and Cloning—A PerkinElmer DNA Thermal Cycler (model 480) programmed with the following profile was used for most PCR reactions: 97 °C for 5 min; 30 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and 72 °C for 7 min. Reaction mixtures (50 μl) typically contained 5 μl of 10 times buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM dNTPs, 100 pmol of each primer, 10–100 ng of template DNA, and 2.5 units of Taq polymerase (Invitrogen). *Pfu* polymerase (2.5 units) (Stratagene) was used in PCR in which new restriction sites were added to the gene coding regions to facilitate cloning into a pET expression vector. Amplified products were ligated into the vector pGEM-T Easy (Promega) or pBluescript II KS+ (Stratagene) and the mixture was transformed into competent *Escherichia coli* DH5α cells. Plasmid DNA was isolated from selected bacterial colonies with a QIAprep Spin Miniprep kit according to the manufacturer's protocol (Qiagen). Clones were screened using standard molecular techniques that included appropriate restriction digests and agarose gel electrophoresis (12). Both strands of selected clones were sequenced by the Genomics Core Research Facility (Center for Biotechnology, University of Nebraska-Lincoln) using standard sequencing primers.

Preparation and Screening of Size-fractionated Genomic Libraries—Genomic DNA from *P. maltophilia* (at least 10 μg) was digested with appropriate restriction enzymes and resolved on a 1% agarose gel using standard molecular techniques (12). Gel pieces containing restriction fragments of the desired size were excised and digested with β-agarase according to the protocol recommended by the manufacturer (New England Biolabs). The DNA fragments eluted from the gel were ligated into the vector pBluescript II KS+ (Stratagene) and the mixture was transformed into competent *E. coli* DH5α cells. Colony lifts were prepared from each library and the bacterial clones were screened with gene-specific digoxigenin (DIG)-labeled probes as described below. Plasmid DNA from positive bacterial colonies was isolated, characterized, and sequenced as described in the preceding section.

Southern Blots, and Colony Lifts, and Probes—Blots and colony lifts were prepared and hybridized with probes labeled with DIG according to the standard protocols in the DIG Application Manual (Roche). Double-stranded DNA probes were labeled with DIG-11-dUTP using either a standard PCR or a random primed method detailed in the DIG Application Manual. Oligonucleotide probes were labeled with a DIG Oligonucleotide 3′-end Labeling Kit (Roche). Nylon filters were always washed under very stringent conditions after hybridization, that is, twice for 10 min at 65 °C in 2× SSC with 0.1% SDS and then twice for 20 min at 65 °C in 0.1× SSC with 0.1% SDS. DIG-labeled DNA was detected by the chemiluminescent substrate CSPD according to the protocol recommended by the manufacturer (Roche).

Cloning of the Reductase Genes—The first 23 residues of the N-terminal sequence was determined for the purified reductase$_{DIC}$ protein (Table I) as described previously (10). A comparison of this sequence to the GenBank™ data base showed that it was 90% identical in a 20-amino acid overlap to the cytochrome P450-type reductase component of dioxin dioxygenase, a three-component enzyme previously isolated from *Sphingomonas* sp. RW1 (13). An internal sequence of 10 residues obtained from tryptic digests of the purified reductase$_{DIC}$ protein also was 80% identical to residues 61 through 70 of the same *Sphingomonas* reductase. This sequence information was used to clone the reductase gene by a two-step nested PCR approach. Three degenerate oligonucleotide primers (two sense and one antisense) were designed and synthesized (Table I). The sequence of the two sense primers was based on the N-terminal amino acid sequence of the purified reductase$_{DIC}$. Primer A (17-mer, 256 variants) was based on the sequence KADVVI and primer B (17-mer, 768 variants) was derived from the sequence IVGAGH. The sequence of the antisense primer C (17-mer, 768 variants) was based on the internal sequence YIRPPTF. Primers A and C were used in a PCR with *P. maltophilia* genomic DNA as template. An aliquot containing a mixture of the products from the first PCR was then used as the template in a second round of amplification with primers B and C. A 180-bp product was amplified in the second PCR and sequenced. The amino acid sequence predicted by this clone matched the N-terminal and internal sequence from the purified reduc-

fragments of different sizes when it was hybridized at 68 °C under very stringent conditions to several restriction digests of *P. maltophilia* genomic DNA that had been blotted to a nylon membrane (data not shown). This result suggested that there are two reductase genes located at different loci in the genome of *P. maltophilia*. A map of the restriction sites surrounding the reductase genes was constructed based on the sizes of the various restriction fragments that hybridized to the probe. This restriction map suggested that full-length copies of the two reductase genes were contained on 4- and 20-kb KpnI/EcoRI fragments. To clone the first gene, a size-fractionated genomic library containing 3.0–5.0-kb KpnI/EcoRI fragments was constructed and colony lifts were prepared. The 148-bp reductase probe was hybridized to ~200 bacterial clones from the library at 68 °C and one positive clone was selected. To clone the second gene, KpnI/EcoRI fragments of *P. maltophilia* genomic DNA with a size of 15–25 kb were gel purified, digested with a number of restriction enzymes, and then hybridized by Southern blot to the same reductase probe. A second restriction map, constructed according to the sizes of restriction fragments that hybridized to the probe, suggested that a full-length reductase gene was contained on a 3.0-kb ApaI fragment. Subsequently, a size-fractionated genomic library containing 2.0–4.0-kb ApaI fragments was constructed and colony lifts were prepared. The reductase probe was hybridized to ~200 bacterial clones from the library at 68 °C and one positive clone was selected.

Cloning of the Ferredoxin Gene—The first 29 residues of the N-terminal amino acid sequence was determined for the purified ferredoxin$_{DIC}$ protein (Table I) as described previously (10). A comparison of this sequence to the GenBank data base showed that it was 35% identical in a 26-amino acid overlap to a terpredoxin from a *Pseudomonas* species, a [2Fe-2S] ferredoxin in the adrenodoxin family (14). This sequence information was used to clone the ferredoxin gene by a two-step nested PCR approach. Three degenerate oligonucleotide primers (two sense and one antisense) were designed and synthesized (Table I). The sequence of the two sense primers was based on the N-terminal amino acid sequence from the purified ferredoxin$_{DIC}$. Primer A (17-mer, 384 variants) was based on the sequence ITVVNQ and primer B (17-mer, 192 variants) was derived from the sequence MEVIRD. The sequence of the antisense primer C (17-mer, 8192 variants) was based on the amino acid sequence RL(T/S/C)CQ(V/I/L) that was part of the conserved [2Fe-2S] domain near the C-terminal end of six previously sequenced bacterial adrenodoxin-type ferredoxins (see Fig. 2). Primers A and C were used in a PCR with *P. maltophilia* genomic DNA as template. An aliquot containing a mixture of the products from the first PCR was then used as the template in a second round of amplification with primers B and C. A 191-bp product was amplified in the second PCR and sequenced. The amino acid sequence predicted by this clone matched the N-terminal and internal sequence obtained from the purified ferredoxin$_{DIC}$ protein. New sense and antisense primers based on the DNA sequence of the clone were designed (Table I) and a 149-bp probe was labeled in a PCR. The DIG-labeled probe was hybridized at 68 °C to *P. maltophilia* genomic DNA that had been digested with several restriction enzymes and blotted to a nylon membrane. A map of the restriction sites surrounding the ferredoxin gene was constructed based on the sizes of various restriction fragments that hybridized to the probe. This restriction map suggested that a full-length ferredoxin gene was contained on a 1.0-kb XhoI/PstI fragment. A size-fractionated genomic library containing 0.5–1.5-kb XhoI/PstI fragments was constructed and colony lifts were prepared. The 149-bp probe was hybridized to ~200 bacterial clones from the library at 68 °C and one positive clone was selected.

Cloning of the Oxygenase Gene—The first 27 residues of the N-terminal amino acid sequence was determined for the purified oxygenase$_{DIC}$ protein (Table I) as described previously (10). To clone the oxygenase gene, a degenerate oligonucleotide (17-mer, 32 variants) based on the sequence NAWYVA (Table I) was designed and synthesized. Genomic DNA from *P. maltophilia* was digested with several restriction enzymes, resolved on a 1% agarose gel, and blotted to a nylon membrane. The labeled oligonucleotide mixture was hybridized to the DNA on the blot at temperatures ranging from 35 to 60 °C. At 45 °C, the probe detected a single 3.5-kb XhoI/SstII fragment. A size-fractionated genomic library containing 3.0–4.0-kb XhoI/SstII fragments was constructed and colony lifts were prepared. The oligo probe was hybridized to ~200 bacterial clones from the library at 45 °C and one positive clone was selected.

Pulsed Field Gel Electrophoresis—A purified plasmid preparation time of 2 s and a final switch time of 2 s. The gel was stained with ethidium bromide (1 μg/ml water) to visualize the plasmid DNA and then blotted to a nylon filter. Sizes of megaplasmid DNAs were estimated relative to two sets of linear DNA markers.

Expression and Purification of Recombinant Proteins—A set of sense and antisense primers was designed for each of the cloned genes to introduce an NcoI restriction site at the 5′ end and an XhoI restriction site at the 3′ end of the coding sequence. Each primer pair was used in a PCR with *Pfu* polymerase and the appropriate genomic clone as template. The amplified products were digested with NcoI and XhoI and then ligated into a pET expression vector (Novagen) that had been digested with the same restriction enzymes. The ferredoxin (*ddmB*) and the reductase (*ddmA1*) genes were cloned into the pET 30b(+) vector and the oxygenase gene (*ddmC*) was cloned into the pET 32b(+) vector. The constructs were sequenced to verify that the coding sequence of each gene was in-frame with the vector sequence that encodes a N-terminal His_6 tag and then transformed into *E. coli* BL21(DE3) cells (Novagen). Recombinant proteins were expressed according to protocols in the pET system manual (Novagen). Cells transformed with the ferredoxin and reductase constructs were grown in 500 ml of LB medium supplemented with kanamycin (50 μg/ml) at 37 °C with shaking, induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside when the A_{600} reached 0.6, and then grown for an additional 3 h at 37 °C. Cells transformed with the oxygenase construct were grown in 500 ml of LB medium supplemented with ampicillin (75 μg/ml) at 37 °C with shaking, induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside when the A_{600} reached 0.6, and then grown at 15 °C for an additional 24 h. The recombinant proteins were purified on a nickel ion-charged affinity column (Novagen) according to the manufacturer's directions. Fractions from the column were analyzed by SDS-PAGE and Coomassie Blue staining.

Assay of Dicamba O-Demethylase Activity—Fractions containing the purified native or recombinant reductase, ferredoxin, and oxygenase proteins were combined in a standard reaction mixture (9) that was assayed for enzymatic activity by using high performance liquid chromatography (HPLC) to monitor the appearance of the DCSA reaction product (10). Reactions were performed at 30 °C for 10 min in a total volume of 500 μl. Assays were initiated by addition of dicamba after a 5-min preincubation at 30 °C. The reaction was terminated by the addition of 80 μl of 5% sulfuric acid. Samples were centrifuged, filtered, and 250 μl of each sample was then injected into a C-18 reverse phase μBondapack 4.6 × 150-mm column. The product mixture was separated using a linear gradient of 60 to 0% methanol in 40 mM Tris acetate (pH 7.2) using a Waters HPLC unit. DCSA retention time was determined to be 14.1 min (established using 250 μl of 500 mM DCSA as a standard). Set concentrations of DCSA were used as quantitation standards. For kinetic studies, DCSA in reaction samples was detected and quantified by fluorescence emission at 420 nm (excitation wavelength, 310 nm) after separation from other reaction products by reverse-phase HPLC. Enzymatic activity in fractions containing the particular component being purified was assayed in the presence of excess quantities of the other two components of dicamba *O*-demethylase, 25 mM potassium phosphate buffer (pH 7.2), 0.5 mM NADH, 10 mM magnesium chloride, 0.5 mM ferrous sulfate, and 0.5 mM dicamba.

RESULTS

Cloning of the Reductase Genes—A 148-bp reductase probe was generated by a two-step nested PCR approach described under "Experimental Procedures" and used to screen two *E. coli* size-fractionated genomic libraries of *P. maltophilia*, strain DI-6. Two genes (*ddmA1* and *ddmA2*), both of which encode the reductase component of dicamba *O*-demethylase, were identified. Sequence analysis showed that a 4.3-kb KpnI/EcoRI fragment that hybridized to the probe contained a 1224-bp open reading frame (ORF) preceded by Shine-Dalgarno (ribosome binding site) sequence GGGAAAA positioned 11 bases upstream from the initiation codon (data not shown). The ORF encoded a 43.7-kDa protein consisting of 408 amino acids (Fig. 1), a size that was consistent with the molecular mass of 45 kDa that was previously estimated for purified reductase$_{DIC}$ by SDS-PAGE (10). The amino acid sequence specified by the *ddmA1* gene matched the N-terminal

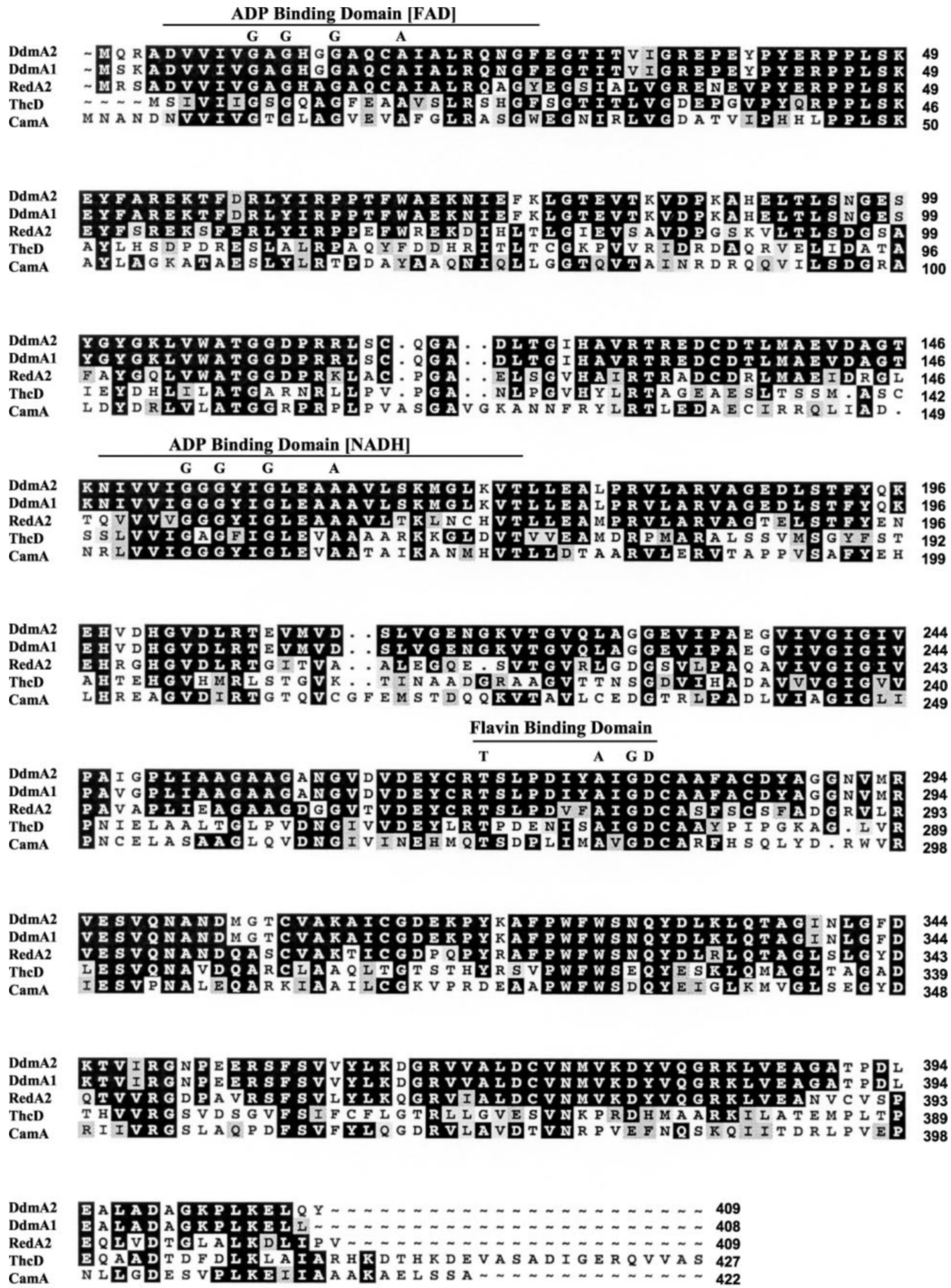

FIG. 1. **Alignment of the two predicted amino acid sequences encoding the reductase component of dicamba *O*-demethylase with the sequences of other members of the FAD-dependent pyridine nucleotide reductase family.** Proteins in this family have two ADP binding domains (for FAD and NADH, respectively) with the consensus sequence GXGX$_2$GX$_3$A and a flavin binding domain (for FAD) with the

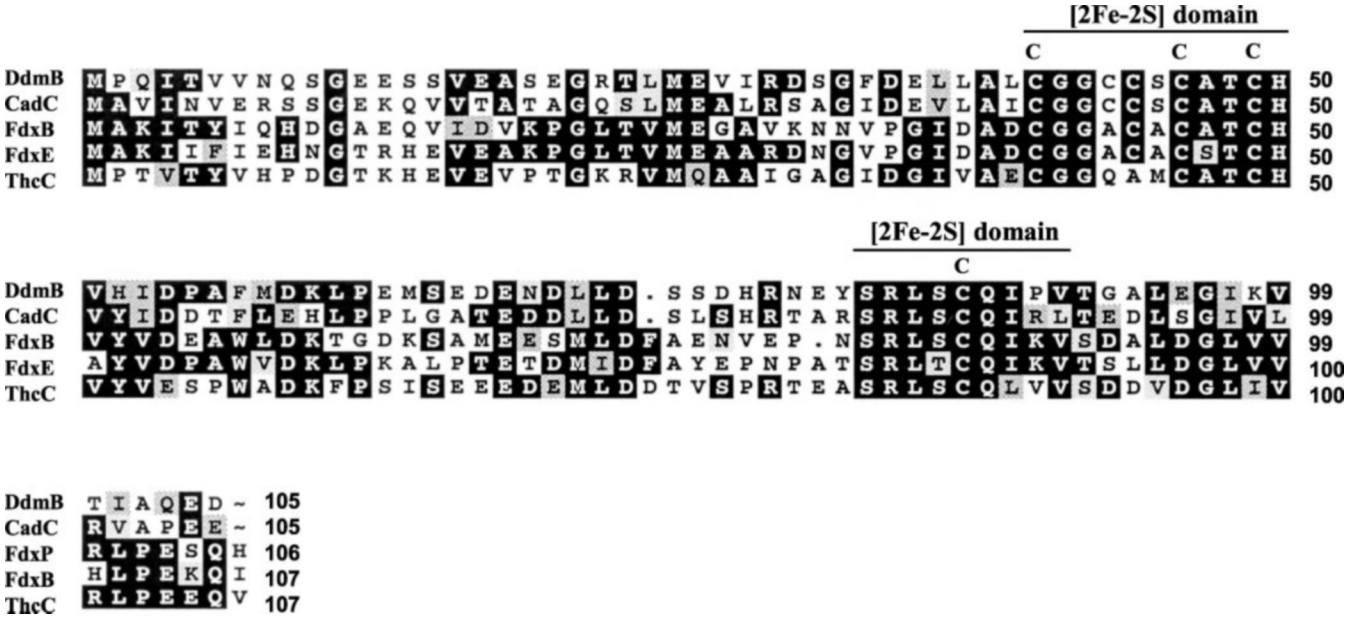

FIG. 2. **Alignment of the predicted amino acid sequence encoding the ferredoxin component of dicamba *O*-demethylase with the sequences of other members of the adrenodoxin family.** Proteins in this family contain a [2Fe-2S] domain with the consensus sequence $CX_5CX_2CX_{36/37}C$. *DdmB* (AY786442), ferredoxin component of dicamba *O*-demethylase from *P. maltophilia*, strain DI-6; *CadC* (BAB78524), ferredoxin component of 2,4-D oxygenase from *Bradyrhizobium* sp. HW13; *FdxP* (P37098), ferredoxin from *C. crescentus*; *FdxE* (CAA72162), ferredoxin from *R. capsulatus*; *ThcC* (P43493), rhodocoxin from *R. erythropolis* (GenBank accession numbers in parentheses).

two ADP binding domains (for FAD and NADH, respectively) with the consensus sequence $GXGX_2GX_3A$. These conserved features were consistent with the yellow color and UV fluorescence previously observed for reductase$_{DIC}$ (10). The derived amino acid sequence was homologous over its entire length to other members of the FAD-dependent pyridine nucleotide reductase family. The identities ranged from 69% with the cytochrome P450-type reductase component of dioxin dioxygenase (RedA2) from *Sphingomonas* sp. RW1 to 38% with rhodocoxin reductase (ThcD) from *Rhodococcus erythropolis* and putidaredoxin reductase (CamA) from *Pseudomonas putida* (Fig. 1).

Sequence analysis showed that a 3.0-kb ApaI fragment that hybridized to the same 148-bp reductase probe contained an ORF of 1227 bp preceded by a ribosome binding site with the sequence GGAG situated 9 bases upstream from the initiation codon (data not shown). The coding sequence specified a 43.9-kDa protein consisting of 409 amino acids (Fig. 1). The amino acid sequence predicted by the second reductase gene (*ddmA2*) was 99.3% identical to the sequence predicted by the first reductase gene (*ddmA1*). As expected, *in vitro* dicamba *O*-demethylase assays in which DdmA2 was substituted for DdmA1 demonstrated that the two enzymes possessed identical or nearly identical activities (data not shown).

Cloning of the Ferredoxin Gene—A 149-bp ferredoxin probe was generated by a two-step nested PCR approach described under "Experimental Procedures" and used to screen an *E. coli* size-fractionated genomic library of *P. maltophilia*, strain DI-6. A single gene (*ddmB*) that encodes the ferredoxin component of dicamba *O*-demethylase was identified. Sequence analysis showed that a 900-bp XhoI/PstI fragment that hybridized to the probe contained an ORF of 315 bp preceded by a ribosome binding site with the sequence AGGGGA situated 10 bases upstream from the initiation codon (data not shown). The coding sequence specified an 11.4-kDa protein composed of 105 amino acid residues (Fig. 2), a size that was consistent with the and internal amino acid sequence information previously obtained from purified ferredoxin$_{DIC}$. The protein also had a [2Fe-2S] domain with the consensus sequence $CX_5CX_2CX_{36/37}C$, a conserved feature that was consistent with the previous EPR spectroscopic analysis of ferredoxin$_{DIC}$ (10). The derived amino acid sequence was homologous over its entire length to other members of the adrenodoxin family of [2Fe-2S] bacterial ferredoxins. The identities ranged from 53% with the ferredoxin component of 2,4-D oxygenase (CadC) from *Bradyrhizobium* sp. strain HW13 to 38% with a ferredoxin (FdxP) from *Caulobacter crescentus* (Fig. 2).

Cloning of the Oxygenase Gene—A 17-mer degenerate oligonucleotide probe based on the N-terminal amino acid sequence of purified oxygenase$_{DIC}$ was used to screen an *E. coli* size-fractionated genomic library of *P. maltophilia*, strain DI-6. A gene, designated *ddmC*, which encodes the oxygenase component of dicamba *O*-demethylase was identified. Sequence analysis showed that a 3.5-kb XhoI/SstII fragment that hybridized to the probe contained an ORF of 1017 bp preceded by a ribosome binding site with the sequence AAGGAG located 7 bases upstream from the initiation codon (data not shown). The coding sequence specified a 37.3-kDa protein composed of 339 amino acid residues (Fig. 3), a size that was consistent with the molecular mass of 40 kDa that was previously estimated for purified oxygenase$_{DIC}$ by SDS-PAGE (10). The amino acid sequence predicted by the *ddmC* gene matched the N-terminal sequence information previously obtained from purified oxygenase$_{DIC}$. In addition, the protein had a Rieske [2Fe-2S] domain with the consensus sequence $CXHX_{16}CX_2H$ and a non-heme Fe(II) domain with the consensus sequence $(D/E)X_3DX_2HX_4H$. Both of these conserved features were consistent with the previous biochemical characterization of oxygenase$_{DIC}$ (10). The derived amino acid sequence was homologous over its entire length to those members of the diverse Phthalate family of Rieske non-heme iron oxygenases that function as a monooxygenase (11). The identities ranged

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