

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 (α)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	<u>SKADVVIVGAGHGGAQ</u> (C) AIALQN
Internal	<u>LYIRPPTFWA</u>
Nested PCR primers	
A	5'-AARGCNGAYGTNGTAT-3'
B	5'-ATHGTNGGNGCNGGCA-3'
C	5'-GTNGGNGCKDATRTA-3'
PCR primers (probe)	5'-GGGCATGGCGGTGCACA-3' 5'-AGGCGGTCGAAGGTCTT-3'
Ferredoxin	
N-terminal	<u>PQITVVNQSGEESVSEGRITLMEVIRD</u>
Consensus	<u>RL(T/S/C)CQ(V/I/L)</u>
Nested PCR primers	
A	5'-ATHACNGTNGTNAAYCA-3'
B	5'-ATGGARGTNATHMGNGA-3'
C	5'-ANYTGRCAANSWNARNCG-3'
PCR primers (probe)	5'-ATGGAGGTTATTCCGCGACA-3' 5'-GCTGTCGAGCAGGTCGTTTC-3'
Oxygenase	
N-terminal	<u>TFVRNAYVVAALPEELSEKPLGRITLD</u>
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 *Synecococcus* 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 $9,110 \times g$ 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 $214,200 \times g$ 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 $5,000 \times g$ 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 μ l) typically contained 5 μ l of 10 times buffer (Invitrogen), 1.5 mM MgCl₂, 0.1% Triton X-100, 200 μ M 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* DH5 α 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 μ g) 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* DH5 α 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 $2 \times$ SSC with 0.1% SDS and then twice for 20 min at 65 °C in $0.1 \times$ 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 reductase_{DIC} protein. New sense and antisense primers based on the DNA sequence of the clone were designed (Table I) and a 148-bp probe was labeled in a PCR. The DIG-labeled probe consistently detected two

² D. Bryant, personal communication.

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 from *P. maltophilia* was resolved on a 0.7% agarose gel in 1× TAE buffer by pulsed field gel electrophoresis using a CHEF-DR III system (Bio-Rad). The apparatus was run at 6 V for 10 h with an initial switch

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 a 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 His₆ 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 A₆₀₀ 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 A₆₀₀ 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 µBondapak 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 GGGAAA 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 and internal amino acid sequence information previously obtained from purified reductase_{DIC}. The protein also had a flavin binding domain for FAD (consensus sequence TX₆AXGD) and

		<u>ADP Binding Domain [FAD]</u>																																																				
		G	G	G	A																																																	
DdmA2	-	MQR	ADV	VIV	GAG	HG	GAQ	CAI	ALR	QNG	FEG	IT	V	IG	RE	PE	Y	PY	ER	PP	LS	K	49																															
DdmA1	-	MSK	ADV	VIV	GAG	HG	GAQ	CAI	ALR	QNG	FEG	IT	V	IG	RE	PE	Y	PY	ER	PP	LS	K	49																															
RedA2	-	MR	S	ADV	VIV	GAG	HAG	AQ	CAI	ALR	QAG	YEG	S	I	AL	VG	RE	N	EV	PY	ER	PP	LS	49																														
ThcD	-	MS	I	V	I	I	GS	G	Q	AG	F	E	AA	V	SL	RS	H	GF	S	GT	IT	LV	GD	EP	GV	PY	QR	PP	LS	K	46																							
CamA	-	MN	AN	DN	V	V	I	V	GT	G	L	AG	V	E	V	A	F	G	L	R	A	S	G	W	E	G	N	I	R	L	V	G	D	A	T	V	I	P	H	H	L	P	P	LS	K	50								
DdmA2		EY	F	A	R	E	K	T	F	D	R	L	Y	I	R	P	P	T	F	W	A	E	K	N	I	E	F	K	L	G	T	E	V	T	K	V	D	P	K	A	H	E	L	T	L	S	N	G	E	S	99			
DdmA1		EY	F	A	R	E	K	T	F	D	R	L	Y	I	R	P	P	T	F	W	A	E	K	N	I	E	F	K	L	G	T	E	V	T	K	V	D	P	K	A	H	E	L	T	L	S	N	G	E	S	99			
RedA2		EY	F	S	R	E	K	S	F	E	R	L	Y	I	R	P	P	E	F	W	R	E	K	D	I	H	L	T	L	G	I	E	V	S	A	V	D	P	G	S	K	V	L	T	L	S	D	G	S	A	99			
ThcD		A	Y	L	H	S	D	P	D	R	E	S	L	A	L	R	P	A	Q	Y	F	D	D	H	R	I	T	L	T	C	G	K	P	V	V	R	I	D	R	D	A	Q	R	V	E	L	I	D	A	T	A	96		
CamA		A	Y	L	A	G	K	A	T	A	E	S	L	Y	L	R	T	P	D	A	Y	A	A	Q	N	I	Q	L	L	G	G	T	Q	V	T	A	I	N	R	D	R	Q	V	I	L	S	D	G	R	A	100			
DdmA2		Y	G	Y	G	K	L	V	W	A	T	G	G	D	P	R	R	L	S	C	.	Q	G	A	.	.	D	L	T	G	I	H	A	V	R	T	R	E	D	C	D	T	L	M	A	E	V	D	A	G	T	146		
DdmA1		Y	G	Y	G	K	L	V	W	A	T	G	G	D	P	R	R	L	S	C	.	Q	G	A	.	.	D	L	T	G	I	H	A	V	R	T	R	E	D	C	D	T	L	M	A	E	V	D	A	G	T	146		
RedA2		F	A	Y	G	Q	L	V	W	A	T	G	G	D	P	R	K	L	A	C	.	P	G	A	.	.	E	L	S	G	V	H	A	I	R	T	R	A	D	C	D	R	L	M	A	E	I	D	R	G	L	146		
ThcD		I	E	Y	D	H	L	L	A	T	G	A	R	N	R	L	L	P	V	.	P	G	A	.	.	N	L	P	G	V	H	Y	L	R	T	A	G	E	A	E	S	L	T	S	S	M	.	A	S	C	142			
CamA		L	D	Y	D	R	L	V	L	A	T	G	G	R	P	R	P	L	P	V	A	S	G	A	.	.	V	G	K	A	N	N	F	R	Y	L	R	T	L	E	D	A	E	C	I	R	R	Q	L	I	A	D	.	149
		<u>ADP Binding Domain [NADH]</u>																																																				
		G	G	G	A																																																	
DdmA2		K	N	I	V	V	I	G	G	G	Y	I	G	L	E	A	A	V	L	S	K	M	G	L	K	V	T	L	L	E	A	L	P	R	V	L	A	R	V	A	G	E	D	L	S	T	F	Y	Q	K	196			
DdmA1		K	N	I	V	V	I	G	G	G	Y	I	G	L	E	A	A	V	L	S	K	M	G	L	K	V	T	L	L	E	A	L	P	R	V	L	A	R	V	A	G	E	D	L	S	T	F	Y	Q	K	196			
RedA2		T	Q	V	V	V	I	G	G	G	Y	I	G	L	E	A	A	V	L	T	K	L	N	C	H	V	T	L	L	E	A	M	P	R	V	L	A	R	V	A	G	T	E	L	S	T	F	Y	E	N	196			
ThcD		S	S	L	V	V	I	G	A	G	F	I	G	L	E	V	A	A	A	R	K	K	G	L	D	V	T	V	V	E	A	M	D	R	P	M	A	R	A	L	S	S	V	M	S	G	Y	F	S	T	192			
CamA		N	R	L	V	V	I	G	G	G	Y	I	G	L	E	V	A	A	T	A	I	K	A	N	M	H	V	T	L	L	D	T	A	A	R	V	L	E	R	V	T	A	P	P	V	S	A	F	Y	E	H	199		
DdmA2		E	H	V	D	H	G	V	D	L	R	T	E	V	M	V	D	.	.	S	L	V	G	E	N	G	K	V	T	G	V	Q	L	A	G	G	E	V	I	P	A	E	G	V	I	V	G	I	G	I	V	244		
DdmA1		E	H	V	D	H	G	V	D	L	R	T	E	V	M	V	D	.	.	S	L	V	G	E	N	G	K	V	T	G	V	Q	L	A	G	G	E	V	I	P	A	E	G	V	I	V	G	I	G	I	V	244		
RedA2		E	H	R	G	H	G	V	D	L	R	T	G	I	T	V	A	.	.	A	L	E	G	Q	E	.	S	V	T	G	V	R	L	G	D	G	S	V	L	P	A	Q	A	V	I	V	G	I	G	I	V	243		
ThcD		A	H	T	E	H	G	V	H	M	R	L	S	T	G	V	K	.	.	T	I	N	A	A	D	G	R	A	A	G	V	T	T	N	S	G	D	V	I	H	A	D	A	V	V	V	G	I	G	V	V	240		
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DdmA1		P	A	V	G	P	L	I	A	A	G	A	A	G	A	N	G	V	D	V	D	E	Y	C	R	T	S	L	P	D	I	Y	A	I	G	D	C	A	A	F	A	C	D	Y	A	G	G	N	V	M	R	294		
RedA2		P	A	V	A	P	L	I	E	A	G	A	A	G	D	G	V	T	V	D	E	Y	C	R	T	S	L	P	D	V	F	A	I	G	D	C	A	S	F	S	C	S	F	A	D	G	R	V	L	R	293			
ThcD		P	N	I	E	L	A	A	L	T	G	L	P	V	D	N	G	I	V	V	D	E	Y	L	R	T	P	D	E	N	I	S	A	I	G	D	C	A	A	P	I	P	G	K	A	G	.	L	V	R	289			
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ThcD		L	E	S	V	Q	N	A	V	D	Q	A	R	C	L	A	A	Q	L	T	G	T	S	T	H	Y	R	S	V	P	F	W	S	E	Q	Y	E	S	K	L	Q	M	A	G	L	T	A	G	A	D	339			
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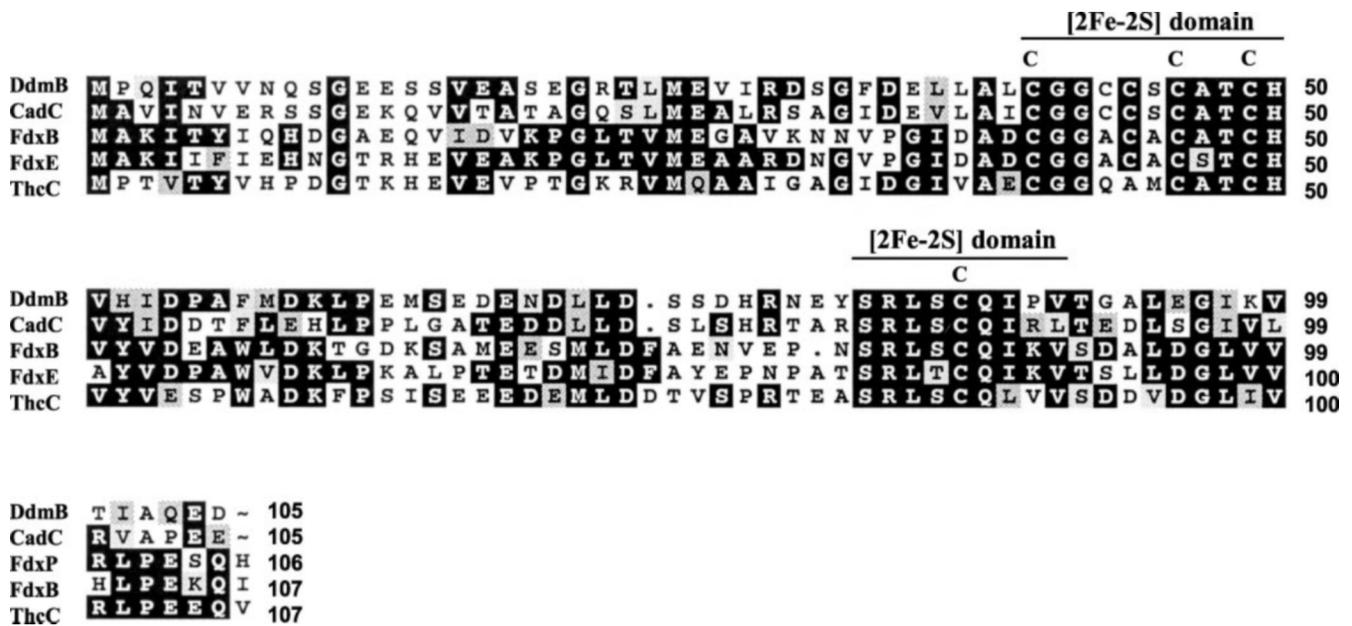


FIG. 2. Alignment of the predicted amino acid sequence encoding the ferredoxin component of dicamba O-demethylase with the sequences of other members of the adrenodoxin family. Proteins in this family contain a [2Fe-2S] domain with the consensus sequence CX₅CX₂CX_{36/37}C. DdmB (AY786442), ferredoxin 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*; TheC (P43493), rhodocoxin from *R. erythropolis* (GenBank accession numbers in parentheses).

two ADP binding domains (for FAD and NADH, respectively) with the consensus sequence GXGX₂GX₃A. 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 (TheD) 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 molecular mass of 14 kDa that was previously estimated for purified ferredoxin_{DIC} by SDS-PAGE (10). The amino acid sequence predicted by the *ddmB* gene matched the N-terminal

and internal amino acid sequence information previously obtained from purified ferredoxin_{DIC}. The protein also had a [2Fe-2S] domain with the consensus sequence CX₅CX₂CX_{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 CXHX₁₆CX₂H and a non-heme Fe(II) domain with the consensus sequence (D/E)₃DX₂HX₄H. 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 from 36% with the oxygenase component of toluenesulfonate methyl-monooxygenase (TsaM) from *Comamonas testosteroni* T-2 to 34% with the oxygenase component of vanillate

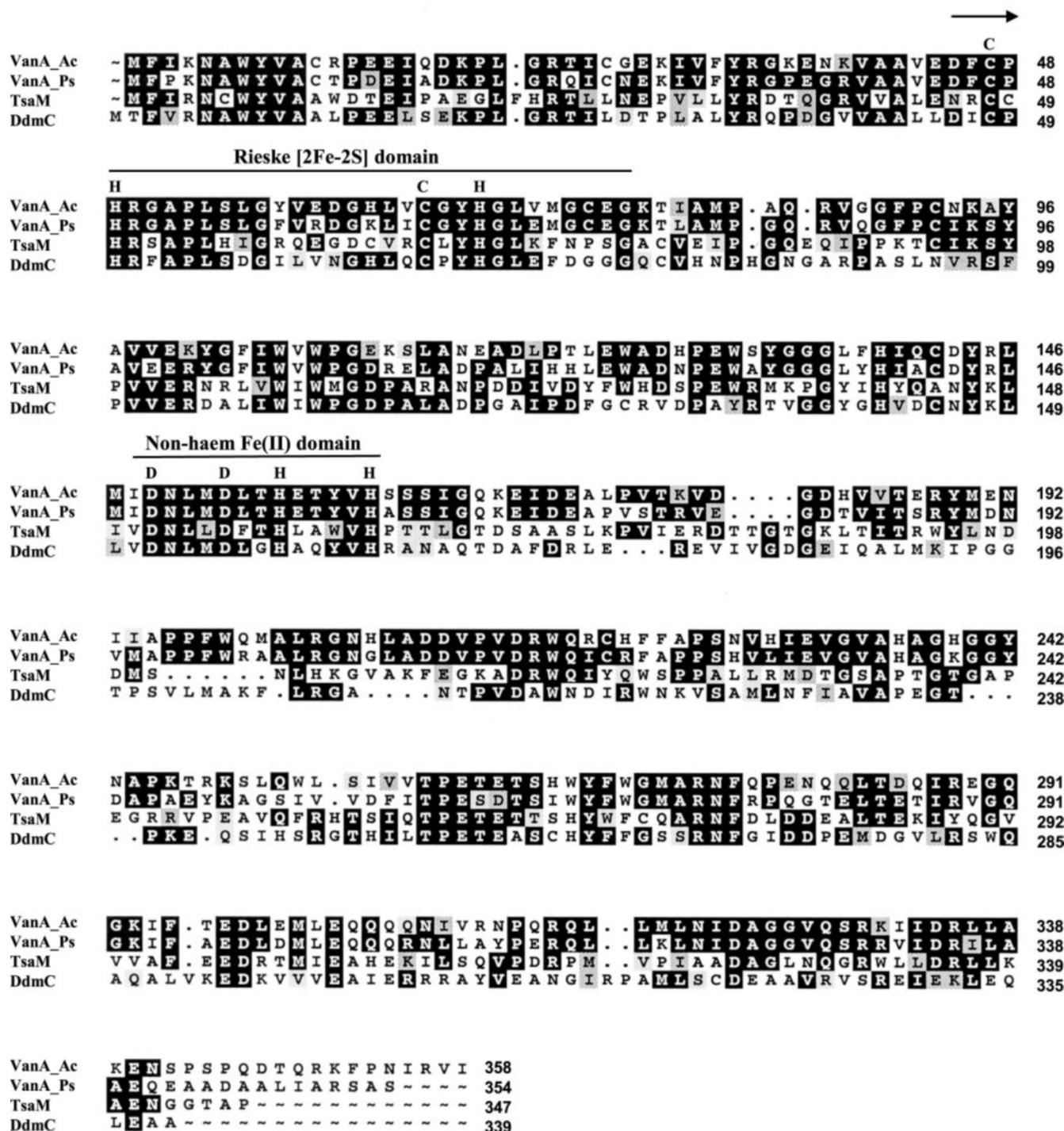


FIG. 3. Alignment of the predicted amino acid sequence encoding the oxygenase component of dicamba O-demethylase with the sequences of other monoxygenases that are members of the Plthalate family of Rieske non-heme iron oxygenases. Proteins in this family are homomultimers containing a Rieske [2Fe-2S] domain with the consensus sequence CXHX₁₆CX₂H and a non-heme Fe(II) domain with the consensus sequence (D/E)X₃DX₂HX₄H. *VanA_Ac* (AAC27107), oxygenase component of vanillate demethylase from *Acinetobacter* sp. ADP1; *VanA_Ps* (O05616), oxygenase component of vanillate demethylase from *Pseudomonas* sp. HR199; *TsaM* (AAC44804), oxygenase component of toluenesulfonate methyl-monoxygenase from *C. testosteroni* T-2; *DdmC* (AY786443) oxygenase component of dicamba O-demethylase from *P. maltophilia*, strain DI-6 (GenBank accession numbers in parentheses).

demethylase (*VanA*) from *Acinetobacter* sp. ADP1 (Fig. 3).

Localization of the Oxygenase Gene (*ddmC*) to Megaplasmid DNA—Plasmid DNA was isolated from cells of *P. maltophilia* that had been grown with dicamba as the sole carbon source and resolved by pulsed field gel electrophoresis. A DIG-labeled DNA probe that included most of the coding sequence of the oxygenase gene (856 bp) hybridized most strongly under stringent conditions to at least two megaplasmids, the smallest of which migrated, as isolated, near a linear 48.5-kb DNA marker

(Fig. 4). The smaller plasmid displayed a significantly stronger hybridization signal suggesting the possibility that this plasmid contains multiple copies of the *ddmC* gene. The blot was stripped and sequentially hybridized under stringent conditions to DIG-labeled DNA probes that contained the entire coding regions of the ferredoxin (310 bp) and reductase (1227 bp) genes. No signal was detected with either of these probes, even when the blot was exposed to film for 24 h (data not shown). However, both probes produced a strong signal when

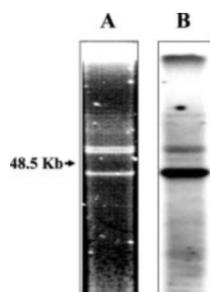


FIG. 4. Localization of the oxygenase gene (*ddmC*) to megaplasmid DNA from *P. maltophilia*, strain DI-6. Plasmid DNA was isolated from cells of *P. maltophilia* using a standard protocol (Qiagen) for very low copy number plasmids. The plasmid preparation was resolved on a 0.7% agarose gel in 1× TAE buffer by pulsed field gel electrophoresis using a CHEF apparatus set at 6 V for 10 h with an initial switch time of 2 s and a final switch time of 2 s, and then blotted to a nylon filter. The blot was hybridized to a DIG-labeled DNA probe that included most of the coding sequence (856 bp) of the oxygenase gene under conditions of high stringency. A, CHEF gel of megaplasmid DNA stained with ethidium bromide. B, blot from gel in panel A hybridized to the oxygenase gene probe.

they were hybridized under the same stringent conditions to total DNA from cells of *P. maltophilia* that had been digested with various restriction enzymes and blotted to a nylon membrane (data not shown).

Heterologous Expression and Enzymatic Activity of the Recombinant Components of Dicamba *O*-Demethylase—Analysis by SDS-PAGE showed that the ferredoxin and reductase (DdmA1) proteins were expressed at high levels in the soluble fraction of lysates from transformed *E. coli* BL21 cells that had been grown at 37 °C. However, the oxygenase protein was clearly sequestered into the inclusion body fraction when *E. coli* cells transformed with the oxygenase construct were grown at this temperature. Subsequent experiments at different culture temperatures ranging from 15 to 30 °C showed that high levels of the expressed oxygenase protein could be detected in the soluble fraction when the cells were grown at 15 °C for ~24 h. The three recombinant proteins were purified to homogeneity by passing each cell lysate through a nickel-charged affinity column (Fig. 5). The individual components displayed no enzymatic activity when they were tested singly or in pairs in the presence of excess Fe(II). (Earlier studies (10) demonstrated a marked, 6-fold stimulation of enzymatic activity by addition of exogenous iron(II).) However, enzymatic activity was obtained when the three components were mixed. With oxygenase_{DIC} as the limiting component, the specific activity of purified recombinant dicamba *O*-demethylase was 134 nmol/min/mg. Purified native dicamba *O*-demethylase, again with oxygenase_{DIC} as the limiting component, had a specific activity of 110 nmol/min/mg. In experiments measuring the kinetics of the oxygenase reaction (*i.e.* in reactions with limiting oxygenase and excess reductase and ferredoxin), approximate molar ratios of oxygenase:reductase:ferredoxin in the reaction were 1:10.5:36.6.

DISCUSSION

Previous studies have suggested that the initial step in the degradation of dicamba by *P. maltophilia*, strain DI-6, is a demethylation reaction that results in the formation of DCSA (7, 8). We recently described the purification of the enzyme that catalyzes this reaction, dicamba *O*-demethylase, and designated the three separate components that were required for enzymatic activity as reductase_{DIC}, ferredoxin_{DIC}, and oxygenase_{DIC} (9, 10). In the current investigation, the genes that encode the three components of dicamba *O*-demethylase were cloned and identified. The *ddmA1* and *ddmA2* genes encode nearly identical reductase proteins with homology to the FAD-

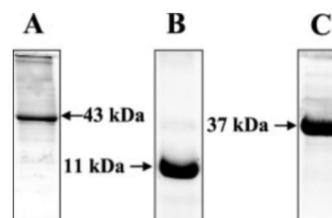


FIG. 5. Expression and purification of recombinant proteins encoding the three components of dicamba *O*-demethylase from *E. coli*. Each gene was cloned into a pET expression vector and the recombinant protein was purified from an *E. coli* lysate by passage through a His tag affinity column as described under "Experimental Procedures." Samples of the purified fractions were separated by SDS-PAGE and visualized by staining with Coomassie Brilliant Blue R-250. A, reductase (10 μg); B, ferredoxin (20 μg); C, oxygenase (40 μg).

dependent pyridine nucleotide reductase family. The *ddmB* gene specifies a ferredoxin that belongs to the adrenodoxin family of [2Fe-2S] bacterial ferredoxins. The *ddmC* gene encodes an oxygenase with homology to those members of the Phthalate family of Rieske non-heme iron oxygenases that function as monooxygenases (11).

The characteristics of the isolated genes support our previous classification of dicamba *O*-demethylase as a three-component enzyme system containing a terminal Rieske non-heme iron oxygenase (10). Such enzyme systems typically consist of a flavin-containing reductase that accepts reducing equivalents from NADH, a [2Fe-2S] cluster that is either part of the reductase molecule or on a separate ferredoxin, and a terminal oxygenase with a Rieske [2Fe-2S] cluster and a non-heme mononuclear iron center (15, 16). The Rieske non-heme iron oxygenases have traditionally been classified according to the properties of their electron transport components (17). However, a number of recent studies have presented dendrograms and sequence alignments to show that the catalytic components (α subunits) of all Rieske non-heme iron oxygenases are evolutionarily related to each other (18–21). There is also considerable evidence to suggest that Rieske oxygenases have the same reaction mechanism because the catalytic α subunits of these enzymes share two conserved motifs, a Rieske [2Fe-2S] domain and a mononuclear iron domain that is probably the site of oxygen activation (16, 22). Using a rootless tree analysis, Gibson and Parales (11) showed that the non-heme Rieske oxygenases can be clustered into four major families according to the native substrate oxidized by each enzyme system. The oxygenases in the Phthalate family are homomultimers with an (α)_n subunit configuration, whereas those in the Toluene, Naphthalene, and Benzoate families are heteromultimers with ($\alpha\beta$)_n subunit configurations. When an evolutionary tree of the known Rieske oxygenases was constructed, it became evident that oxygenases with (α)_n subunits divided into two distinct evolutionary lines, that is, the monooxygenases and dioxygenases (20). Because both of these groups share a conserved Rieske [2Fe-2S] center and a mononuclear iron binding site, this subdivision was attributed to differences in the binding site for the aromatic substrate and oxygen (20).

Biochemical and physical analyses of the purified oxygenase component of dicamba *O*-demethylase showed that it is a homotrimer composed of three identical ~40-kDa α subunits (10). In the classification scheme of Gibson and Parales (11), dicamba *O*-demethylase most closely aligns with members of the Phthalate family that function as monooxygenases. Identification of the reaction products of dicamba *O*-demethylase as DCSA and formaldehyde (10) confirm the identity of this enzyme as a monooxygenase. However, dicamba *O*-demethylase is unique in comparison to other monooxygenases in the Phthalate family. The derived amino acid sequence encoded by

the *ddmC* gene is 36% identical over its entire length to the oxygenase component of toluenesulfonate monooxygenase (TsaM) from *C. testosteroni* T-2 and 34% identical to the oxygenase component of vanillate demethylase (VanA) from *Acinetobacter* sp. ADP1. Both of these homologous enzymes are two-component monooxygenase systems in which electrons are shuttled from the low-potential donor NADH to oxygen via a reductase component that contains flavin (FAD or FMN) and NAD binding sites and a [2Fe-2S] cluster (21). In contrast, dicamba O-demethylase is a three-component enzyme system in which the [2Fe-2S] cluster that is involved in the transfer of electrons from NADH to oxygen is not contained within the reductase_{DIC} component, but is associated with a separate ferredoxin_{DIC} molecule.

More than 50 different Rieske oxygenase systems have been isolated from bacteria and characterized (16). Of the enzyme systems whose genes have been cloned and identified, the electron transport components of dicamba O-demethylase (*i.e.* reductase_{DIC} and ferredoxin_{DIC}) are most similar to those of dioxin dioxygenase, a three-component enzyme system from *Sphingomonas* sp. RW1. The reductase component of dioxin dioxygenase, RedA2, is 69% identical to the DdmA1 component of dicamba O-demethylase with NADH and FAD binding sites in identical positions at the N-terminal end of the polypeptides. Furthermore, a second isofunctional reductase, RedA1, has been isolated from *Sphingomonas* sp. RW1 (23). Unlike the two reductase components of dicamba O-demethylase that are 99.3% identical to each other, the N termini of the reductase components of dioxin dioxygenase are very different (23). However, it was shown that both monomeric flavoproteins could function in the channeling of electrons from NADH to the [2Fe-2s] ferredoxin component, Fdx1 (24). Fdx1 is a member of the adrenodoxin family of ferredoxin proteins and has 30% identity with the ferredoxin component of dicamba O-demethylase (DdmB). The similarities between the electron transfer components of dioxin dioxygenase and dicamba O-demethylase does not extend to their oxygenase components. The oxygenase component of dicamba O-demethylase is a homotrimer (α)₃, whereas the oxygenase component of dioxin dioxygenase is a heterodimer ($\alpha\beta$)₂ composed of a 48.3-kDa catalytic α subunit and a smaller 20.9-kDa β subunit (25).

One of the few Rieske non-heme iron oxygenases for which a detailed structural analysis exists is naphthalene 1,2-dioxygenase (Protein Data Bank code 1NDO) (26, 27), a three-component enzyme whose oxygenase component has an ($\alpha\beta$)₃ subunit structure and whose α subunit contains the catalytic domain of the enzyme. The amino acid sequence of the dioxygenase α subunit shares only 15% identity with oxygenase_{DIC}. Despite this low apparent homology, a suitable model for a single oxygenase_{DIC} subunit was generated using 3D-PSSM (28) (Fig. 6B). Gel filtration data suggest that the native oligomeric state of oxygenase_{DIC} is a homotrimer (10), and as such, a trimeric model of the enzyme (Fig. 6C) was built by superimposing the oxygenase_{DIC} monomer onto the three core α subunits of naphthalene 1,2-dioxygenase. Ribbon renderings of the models were made with Chimera (29). The potential similarity in the native structures of the trimeric α subunits of the dioxygenase and oxygenase_{DIC} suggest, in analogy to the situation in the dioxygenase, the possibility that there is intersubunit transfer of electrons from the Rieske iron-sulfur cluster of one subunit of oxygenase_{DIC} to the free iron atom at the catalytic site of a closely adjacent oxygenase_{DIC} subunit. The conservation of the domain order within the Rieske non-heme iron oxygenase family (*e.g.* the alignment depicted in Fig. 3) and our current modeling raise the possibility that members of this family (be they monooxygenases, like oxygenase_{DIC}, or

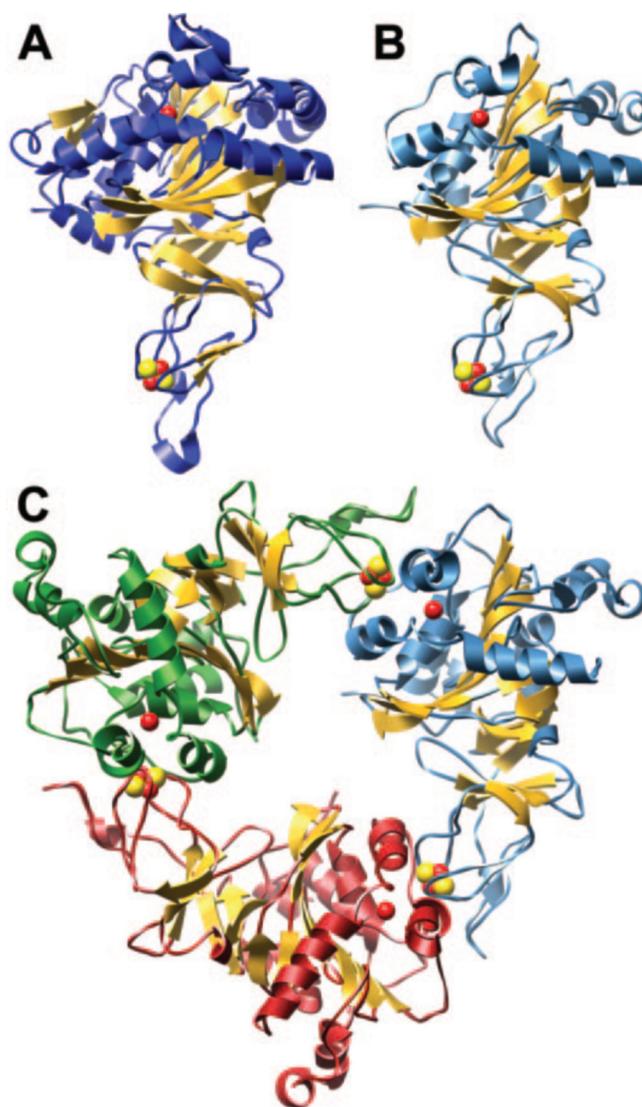


FIG. 6. Comparison of the known structure of the α subunit of naphthalene dioxygenase with the predicted oxygenase_{DIC} subunit structure. Comparison of single subunits of the α subunit of naphthalene dioxygenase (A) and the subunit of oxygenase_{DIC} (B) and the predicted native, homotrimeric form of oxygenase_{DIC} (C) based on the known native trimeric configuration of the α subunits of naphthalene dioxygenase.

dioxygenases, like naphthalene 1,2-dioxygenase) may not only be evolutionarily related, but perhaps also structurally conserved. Complete structural analyses of oxygenase_{DIC} will be required to confirm or deny the predicted resemblance of oxygenase_{DIC} with the α subunit configuration of naphthalene 1,2-dioxygenase.

The genes that specify the three components of dioxin dioxygenase have an atypical location in the genome of *Sphingomonas* RW1 (25). Usually, the genes encoding the components of the known Rieske oxygenases are found close together in tightly regulated transcriptional units (30). However, the dioxin dioxygenase genes have been localized to three different loci in the bacterial genome. Likewise, our current evidence suggests that the dicamba O-demethylase genes are monocistronic and are not clustered in a single transcriptional unit in the genome of *P. maltophilia*, strain DI-6. Hybridization experiments showed that the *ddmC* gene is present on a megaplasmid in cells of *P. maltophilia* (Fig. 4). The plasmid location of the gene that encodes oxygenase_{DIC} is not surprising because it has been well established that the catabolic genes used by

microorganisms to degrade halogenated organic compounds in their environment are often located on large plasmids (31). Furthermore, Cork and Khalil (32, 33) isolated a large 250-kb plasmid designated as pDK1 from cells of *P. maltophilia*, strain DI-6, and demonstrated by curing experiments that its presence was correlated with the ability of the cells to grow on dicamba. It was surprising that the full-length reductase and ferredoxin probes failed to hybridize to plasmid DNA, although the same probes hybridized strongly to a preparation of genomic DNA from *P. maltophilia*. Two possible explanations for this result are that the *ddmA* and *ddmB* genes are located on the *P. maltophilia* chromosome or that the megaplasmid containing the reductase and ferredoxin genes was not successfully isolated by the Qiagen method employed in this study. In the latter scenario, the megaplasmid DNA was sheared during the isolation procedure and excluded from the plasmid preparation along with the chromosomal DNA. The region surrounding the oxygenase gene (*ddmC*) extending ~2 kb in both directions has been sequenced and analyzed (data not shown). A search for possible open reading frames in this sequence revealed no ORFs with homology to a reductase or ferredoxin gene. Thus, our current evidence suggests that the *ddmA* and *ddmB* genes are not directly adjacent to the *ddmC* gene and more experiments will be required to ascertain their exact locations in the genome of *P. maltophilia*. One approach will be to construct a genomic cosmid library that will then be hybridized sequentially with probes for the dicamba O-demethylase genes to establish if there is cross-hybridization to any single cosmid clone in the library. Another approach will be to cure the megaplasmids from strain DI-6 and determine whether the *ddmA* and *ddmB* genes are associated exclusively with the bacterial chromosome.

The proteins specified by the *ddmA1*, *ddmB*, and *ddmC* genes were successfully overexpressed in *E. coli* and purified to homogeneity. The specific activity of a reconstituted mixture of the three purified recombinant proteins (with oxygenase_{DIC} as the limiting component) was 134 nmol/min/mg, a result that is similar to the specific activity of 110 nmol/min/mg that was measured for a mixture of the three purified native proteins. Therefore, it is reasonable to conclude that all of the components of dicamba O-demethylase that are required for optimal enzymatic activity have been identified. The slightly higher activity of the recombinant protein mixture can be explained by the greater purity of the recombinant protein fractions (Fig. 5) relative to that of the native protein fractions (see Fig. 1 in Ref. 10).

Future investigations will concentrate on the organization and regulation of the genes that encode dicamba O-demethylase. Likewise, it will be important to better understand how the reductase_{DIC}, ferredoxin_{DIC}, and oxygenase_{DIC} components physically interact with each other in a sequential manner to efficiently transfer electrons and catalyze the demethylation of the substrate dicamba. From the point of view of potential applications, it is possible that the catabolic genes in *P. maltophilia*, strain DI-6, have novel properties that can be used in the genetic engineering of microorganisms that can deal effectively with accidental spills of dicamba and perhaps other environmental pollutants (34, 35). Finally, we should note that

in converting dicamba to DCSA, dicamba O-demethylase eliminates all herbicidal activity. Based on this knowledge, we have genetically engineered the oxygenase_{DIC} (*ddmC*) gene for high-level expression in plants and, in a report to be published elsewhere,³ have demonstrated that transgenic *Arabidopsis*, tobacco, tomato, and soybean plants bearing this modified gene are resistant to treatments with high levels of dicamba. This new trait should allow use of dicamba as an inexpensive, environmentally friendly herbicide to effectively eliminate the costly competition between broadleaf crop plants and broadleaf weeds for sunlight, water, and essential nutrients.

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