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

GENE ISOLATION, CHARACTERIZATION, AND HETEROLOGOUS EXPRESSION*

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Patricia L. Herman‡, Mark Behrens, Sarbani Chakraborty, Brenda M. Chrastil, Joseph Barycki, and Donald P. Weeks§

From the Department of Biochemistry, University of Nebraska-Lincoln, Lincoln, Nebraska 68588-0664

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. reductaseDIC, ferredoxinDIC, and oxygenaseDIC) and classified the oxygenase component of dicamba O-demethylase as a member of the Rieske non-heme iron family of oxygenses. 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 437- and 439-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 oxygenses 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 oxygenasePIC 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 reductaseDIC, ferredoxinDIC, and oxygenaseDIC components of dicamba O-demethylase (10). OxygenaseDIC is a homotrimer (α3) with a subunit molecular mass of ~40 kDa and contains a single Rieske [2Fe-2S] cluster. FerredoxinDIC 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. ReductasePIC is 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 oxygenasePIC can be classified as a member of the family of Rieske non-heme iron oxygenses (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...

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

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Dicamba O-Demethylase Genes

**Table I**

<table>
<thead>
<tr>
<th>Component</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reductase N-terminal</td>
<td>SKADVIVTGAGGCGAQ (C) AIALQN</td>
</tr>
<tr>
<td>Reductase Internal</td>
<td>LIYRPFPFPA</td>
</tr>
</tbody>
</table>

**Nested PCR primers**

- A | 5'-AARGCNGAYGTGNTNAT-3' |
- B | 5'-ATGHTNGGNCGCGONCA-3' |
- C | 5'-GTNGNENGCRCQAPFTA-3' |

**PCR primers (probe)**

- 5'-GGGCGATGCGCCTGACA-3' |
- 5'-AGGCGCTTGGAAGGTCTT-3' |

**Ferredoxin**

- N-terminal | POITTVNQGEESSVEASEGTMEIVRD |
- Consensus | RL(T/S/C)C O(I/V/L) |

**Nested PCR primers**

- A | 5'-AHTACNGTNGTNAAYCA-3' |
- B | 5'-ATGAGGNTNATMONGA-3' |
- C | 5'-ANYTCRGCANSWNRCG-3' |

**PCR primers (probe)**

- 5'-ATGAGGTTATATCGCGACA-3' |
- 5'-GCTGGCAGCATGGCTGC-3' |

**Oxygenase**

- N-terminal | TFRVNNYYVALFPELESKPLRGLLDD |
- Oligonucleotide (probe) | 5'-AAYNGTGGTAYGTGSC-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 *Synchococcus* 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 A600 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 lysosome (5 mg/ml) for 30 min at 37 °C, and then lysed in 1% Sarkosyl. The lysate was centrifuged to equilibrium in a CaCl2-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*sub*, ferre

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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 149-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 27 residues of the N-terminal amino acid sequence was determined for the purified ferredoxin, and the oxygenase gene, a degenerate oligonucleotide (17-mer, 32 variants) was hybridized to DNA on the blot at temperatures ranging from 35 to 60 °C. At 45 °C, the probe specified by the sequence NAWYVA (Table I) was designed and synthesized to the GenBank data base showed that it was 35% matched to the 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 A660 reached 0.6, and then grown for an additional 3 h at 37 °C. Cells were harvested, washed with the over the top of 60% ethidium bromide (1 μg/ml) in 37 °C with shaking, induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside when the A660 reached 0.6, and then grown 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 for Western analysis.

**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 20 μl containing 1 mM DCSA, 1 mM NADPH, 0.5 mM ferrous sulfate, 5 μM ferredoxin, 5 μM ferredoxin, and 5 μM ferredoxin. The details of the reaction were as described previously (9) except that the purified reductase was added in a 10 mM magnesium phosphate buffer (pH 7.2), 0.5 mM NADPH, 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 1274-bp inserts (ORF) encoded a Shine-Dalgarno (ribosome binding site) sequence GGAAAA 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 reductaseDIC 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 reductaseDIC. The protein also had a flavin binding domain for FAD (consensus sequence TXAAXGD) and
FIG. 1. Alignment of the two predicted amino acid sequences encoding the reductase component of dicamba \( \text{O} \)-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 \( \text{GXXGXXGXX} \) and a flavin binding domain (for FAD) with the consensus sequence \( \text{TXXGD} \). DdamA1 (AY786444) and DdamA2 (AY786445), reductase components of dicamba \( \text{O} \)-demethylase from \( \text{P. maltophilia} \); strain DI-6; RedA2 (CAA05635), reductase component of dioxin dioxygenase from \( \text{Sphingomonas} \) sp. RW1; ThcD (P43494), rhodocoxin reductase from \( \text{R. erythropolis} \); CamA (P16640), putidaredoxin reductase from \( \text{P. putida} \) (GenBank accession numbers in parentheses).
two ADP binding domains (for FAD and NADH, respectively) with the consensus sequence GXGXXGXXA. These conserved features were consistent with the yellow color and UV fluorescence previously observed for reductaseDIC (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 315 bp preceded by a ribosome binding site with the sequence GGAG situated 9 bases upstream from the initiation codon (data not shown). The amino acid sequence predicted by the second reductase gene (ddmA2) was 99.3% identical to the sequence predicted by the first reductase gene (ddmB). 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 Oxygenase 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 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 amino acid sequence specified a 37.3-kDa protein composed of 339 amino acid residues (Fig. 2), a size that was consistent with the molecular mass of 14 kDa that was previously estimated for purified ferredoxin subunit obtained from purified ferredoxin subunit (10). The amino acid sequence predicted by the ddmC gene matched the N-terminal amino acid sequence previously obtained from purified ferredoxin subunit. The protein also had a [2Fe-2S] domain with the consensus sequence [2Fe-2S] domain with the consensus sequence C[36-37C], a conserved feature that was consistent with the previous biochemical characterization of ferredoxin subunit (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 (FdxE) 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 oxidase subunit 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 oxidase subunit by SDS-PAGE (10). The amino acid sequence predicted by the ddmC gene matched the N-terminal sequence information previously obtained from purified oxidase subunit. In addition, the protein had a Rieske [2Fe-2S] domain with the consensus sequence CXXHXXCXXH and a non-heme Fe(II) domain with the consensus sequence D/EHXDXHXH. Both of these conserved features were consistent with the previous biochemical characterization of oxidase subunit (10). The derived amino acid sequence was homologous over its entire length to those members of the diverse Pththalate family of Rieske non-heme iron oxidases that function as a monooxygenase (11). The identities ranged from 36% with the oxidase component of toluenesulfonate methyl-monoxygenase (TsaM) from Comamonas testosteroni T-2 to 34% with the oxidase component of vanillate...
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
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 mem-
brane (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 differ-
cent 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 activ-
ity in pairs in the presence of excess Fe(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 measur-
ing 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 design-
ated the three separate components that were required for
enzymatic activity as reductase_{DIC}, ferredoxin_{DIC}, and oxygen-
ase_{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-
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 en-
codes 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 previ-
ous classification of dicamba O-demethylase as a three-compo-
ent 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 reduc-
tase 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). How-
ever, a number of recent studies have presented dendrograms
and sequence alignments to show that the catalytic compo-
nents (α subunits) of all Rieske non-heme iron oxygenases are
evolutionarily related to each other (18–21). There is also con-
siderable 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), subunit configuration, whereas those in the Toluene,
Naphthalene, and Benzoate families are heteromultimers with
(αβγ), subunit configurations. When an evolutionary tree of the
known Rieske oxygenases was constructed, it became evident
that oxygenases with (αγ), subunits divided into two distinct
evolutionary lines, that is, the monooxygenases and dioxygen-
ases (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 ho-
motrimer composed of three identical ~40-kDa α subunits (10).
In the classification scheme of Gibson and Parales (11), dic-
amba O-demethylase most closely aligns with those members
of the Phthalate family that function as monooxygenases. Iden-
tification of the reaction products of dicamba O-demethylase
as DCSA and formaldehyde (10) confirm the identity of this en-
zyme 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

**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 hy-
bridized to the oxygenase gene probe.

**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).
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 do not extend to their oxygenase components. The oxygenase component of dicamba *O*-demethylase is a homotrimer (α)₂β, whereas the oxygenase component of dioxin dioxygenase is a heterodimer (αβ)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 (αβ)₂ subunit structure and whose α subunit contains the catalytic domain of the enzyme. The amino acid sequence of the oxygenase 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 constructed 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 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 _ddmA_, _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<sub>ddmC</sub> 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<sub>ddmC</sub>, ferredoxin<sub>ddmC</sub>, and oxygenase<sub>ddmC</sub> 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<sub>ddmC</sub> (_ddmC_) gene for high-level expression in plants and, in a report to be published elsewhere, 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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REFERENCES


3 M. Behrens, N. Mutlu, S. Chakraborty, P. L. Herman, T. Clemente, and D. P. Weeks, unpublished data.