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2-Oxo-1,2-Dihydroquinoline 8-Monooxygenase: Phylogenetic Relationship to Other Multicomponent Nonheme Iron Oxygenases

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2-Oxo-1,2-dihydroquinoline 8-monooxygenase, an enzyme involved in quinoline degradation by *Pseudomonas putida* 86, had been identified as a class IB two-component nonheme iron oxygenase based on its biochemical and biophysical properties (B. Rosche, B. Tshisuaka, S. Fetzner, and F. Lingens, J. Biol. Chem. 270:17836–17842, 1995). The genes *oxoR* and *oxoO*, encoding the reductase and the oxygenase components of the enzyme, were sequenced and analyzed. *oxoR* was localized approximately 15 kb downstream of *oxoO*. Expression of both genes was detected in a recombinant *Pseudomonas* strain. In the deduced amino acid sequence of the NADH:(acceptor) reductase component (OxoR, 342 amino acids), putative binding sites for a chloroplast-type [2Fe-2S] center, for flavin adenine dinucleotide, and for NAD were identified. The arrangement of these cofactor binding sites is conserved in all known class IB reductases. A dendrogram of reductases confirmed the similarity of OxoR to other class IB reductases. The oxygenase component (OxoO, 446 amino acids) harbors the conserved amino acid motifs proposed to bind the Rieske-type [2Fe-2S] cluster and the mononuclear iron. In contrast to known class IB oxygenase components, which are composed of differing subunits, OxoO is a homomultimer, which is typical for class IA oxygenases. Sequence comparison of oxygenases indeed revealed that OxoO is more related to class IA than to class IB oxygenases. Thus, 2-oxo-1,2-dihydroquinoline 8-monooxygenase consists of a class IB-like reductase and a class IA-like oxygenase. These results support the hypothesis that multicomponent enzymes may be composed of modular elements having different phylogenetic origins.

Multicomponent oxygenases play an important role in the bacterial degradation of aromatic compounds. 2-Oxo-1,2-dihydroquinoline 8-monooxygenase catalyzes the second step of quinoline degradation by *Pseudomonas putida* 86: in a NADH-dependent oxygenation, 2-oxo-1,2-dihydroquinoline is converted to 8-hydroxy-2-oxo-1,2-dihydroquinoline (Fig. 1). As illustrated in Fig. 1, this enzyme system consists of two soluble protein components with four redox active centers, which constitute an electron transfer chain. Electrons are transferred from NADH via flavin adenine dinucleotide (FAD) and a chloroplast-type [2Fe-2S] cluster, which are located on the reductase component, to the substrate hydroxylating oxygenase component, which harbors Rieske-type [2Fe-2S] clusters and additional iron (26, 27).

Based on the number of its protein components and on its set of cofactors, the enzyme system belongs to the class IB multicomponent Rieske center nonheme iron oxygenases as defined by Batie et al. (1). However, it differs from known class IB enzymes, since the oxygenase component is a homomultimer and thus resembles class IA oxygenase components (27). This unusual property prompted us to investigate the phylogenetic relationship of multicomponent oxygenases.

Here we report the localization, expression, and comparative sequence analysis of the *oxoO* and *oxoR* genes, encoding the oxygenase and reductase components of 2-oxo-1,2-dihydroquinoline 8-monooxygenase from *P. putida* 86. Putative cofactor binding domains are located, and based on sequence alignments, the phylogenetic relationship of multicomponent oxygenases is discussed.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *P. putida* 86 had been isolated from soil by selective enrichment on quinoline as the carbon source (29). The recombinant strain *P. putida* mt-2 KT2440 (13/42) harbors the cosmid pCIB119 with a 30-kb insertion of genomic DNA of *P. putida* 86 and has been described previously (2). Fragments of this insert were cloned in the vector plasmid pUC18 (23, 37) with *Escherichia coli* TG1 (8) as the host strain. The plasmid pCIB119 is a double cosmid and was a kind gift of Stephen T. Lam (Ciba-Geigy, Research Triangle Park, N.C.).

Media and growth conditions. For the preparation of plasmid DNA, overnight cultures in Luria-Bertani medium (28) with tetracycline, 50 μg/ml (for pCIB119), or ampicillin, 100 μg/ml (for pUC18), were used. Cometabolic conversion of 2-oxo-1,2-dihydroquinoline was investigated by using mineral salt medium (7) with 2-g/liter succinate and 40-mg/liter 2-oxo-1,2-dihydroquinoline. *Pseudomonas* strains were grown at 30°C, and *E. coli* strains were grown at 37°C.

DNA techniques. Plasmid DNA was prepared by alkaline lysis (28) or by using the Midi kit (Qiagen, Inc., Chatsworth, Calif.). DNA fragments were isolated from agarose gels according to the instruction manual for the Qiaex II gel extraction kit (Qiagen). Restriction digestions, dephosphorylation of DNA fragments, and DNA ligation were performed as described by the enzyme suppliers (Eurogentec, Seraing, Belgium; U.S. Biochemical Corp., Bad Homburg, Germany; and Pharmacia Biotech Inc., Freiburg, Germany). Transformation of *E. coli* TG1 with recombinant pUC18 DNA was carried out by using $CaCl_2$ (15). Cloned gene DNA was identified by DNA restriction of recombinant pCIB119 or pUC18, agarose gel electrophoresis, Southern blotting, and hybridization with digoxigenin-labeled DNA probes by standard methods (28). Mixed digoxigenin-labeled oligonucleotides were synthesized on the basis of the N-terminal amino acid sequences of the two components of 2-oxo-1,2-dihydroquinoline 8-monooxygenase as determined by Edman degradation. The 23-mer ATG AA(A/G) GA(C/T) CA(A/G) ATG CA(C/T) CA(A/G) GT, designated "Red," and the 17-mer GA(C/T) CA(A/G) CCT AT(C/T) AT(C/T) CG, designated "Ox," were used as probes for detection of the reductase and the oxygenase gene, respectively. The hybridization temperatures were 51°C for Red and 43°C for Ox. Stringent washes were performed at the same temperatures with 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS). For gene mapping of the recombinant cosmid, further DNA probes, f1,

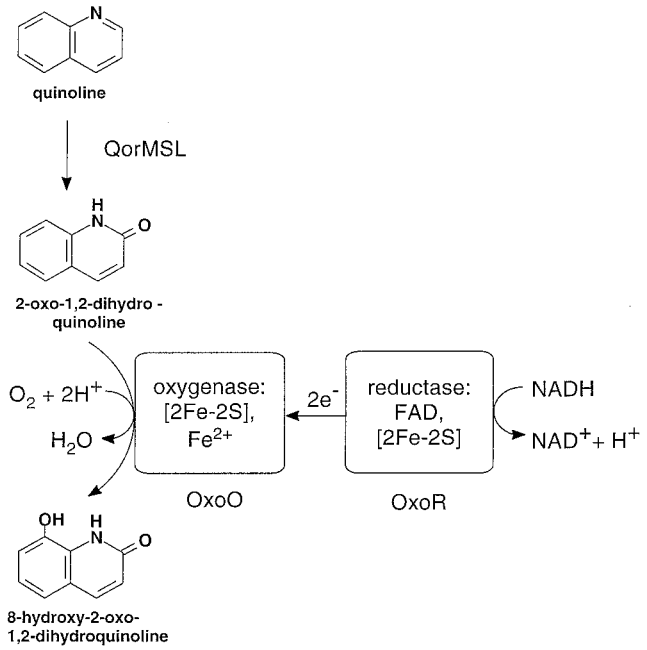

FIG. 1. Function of the gene products QorMSL (medium, small, and large subunits of quinoline 2-oxidoreductase [2]) and OxoO and OxoR (components of 2-oxo-1,2-dihydroquinoline 8-monooxygenase) in *P. putida* 86.

f2, and f3, were synthesized (see Fig. 2), using fragments of subcloned DNA as templates for digoxigenin random-primed DNA labeling (Boehringer Mannheim, Mannheim, Germany). Hybridizations were carried out at 70°C, and stringent washes were performed as follows: f1, 0.1× SSC, 80°C; f2, 1× SSC, 80°C; and f3, 2× SSC, 96°C. Immunological detection was performed with the DIG luminescence detection kit (Boehringer Mannheim).

DNA sequencing and sequence analysis. Overlapping regions of DNA were sequenced in both directions by primer walking by using the DyeDeoxy terminator cycle sequencing method based on the protocol from Applied Biosystems Inc. Electrophoresis and detection were carried out with an ABI 373 Stretch DNA sequencer (Applied Biosystems Inc.). Computer analyses of the DNA sequences were performed with the GENMON program (Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany) and the HUSAR 4.0 program package (EMBL, Heidelberg, Germany), which includes the software, version 8.1, of the Genetics Computer Group of the University of Wisconsin. The programs FASTA, TFASTA, BLASTN, and TBLASTN were used to search for similar sequences, CLUSTAL was used to calculate multiple alignments, and TREE was used for the construction of dendrograms.

Expression of cloned genes. The recombinant clone *P. putida* mt-2 KT2440 (13/42), harboring the genes for 2-oxo-1,2-dihydroquinoline 8-monooxygenase on pCIB119, was tested for cometabolic conversion of 2-oxo-1,2-dihydroquinoline in mineral salt medium. Following substrate consumption, UV-visible spectra of culture supernatant in the range of 250 to 400 nm were recorded. Control experiments were performed with the wild-type *P. putida* strain 86 and the DNA recipient *P. putida* mt-2 KT2440. For the determination of enzyme activities in crude extracts, cells were harvested, washed, and disrupted by sonication as described previously (27). After removal of cell debris by centrifugation, the activities of both enzyme components were determined by spectrophotometrically measuring substrate-dependent NADH consumption at 365 nm. In order to determine the activity of the oxygenase component, the assay mixture was supplemented with an excess of reductase component, and vice versa (27).

Nucleotide sequence accession numbers. The DNA sequences presented in this report, encoding OxoO and OxoR, are deposited in the EMBL Nucleotide Sequence Library, Heidelberg, Germany, under accession numbers Y12654 (*oxoR*) and Y12655 (*oxoS, oxoO,* and *oxoH*).

RESULTS

Expression of recombinant 2-oxo-1,2-dihydroquinoline 8-monooxygenase. The recombinant clone *P. putida* mt-2 KT2440 (13/42), harboring the cosmid vector pCIB119 with a 30-kb insertion of genomic DNA of *P. putida* 86, was reported to carry and express the genes for quinoline 2-oxidoreductase,

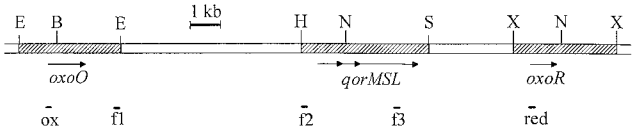

FIG. 2. Partial restriction map for the cloned *oxoO* and *oxoR* regions. *qorMSL* encodes the medium, small, and large subunits of quinoline 2-oxidoreductase (2). f1, f2, f3, ox, and red are the DNA probes used for the hybridization experiments (bars show locations but are not to scale). Restriction sites: B, *Bgl*II; E, *Eco*RI; H, *Hinc*II; N, *Nsi*I; S, *Sph*I; X, *Xma*I. The hatched areas represent the regions that have been sequenced. The arrows indicate the positions and the direction of transcription of the genes *oxoO, gorM, gorS, gorL,* and *oxoR.*

the first enzyme of the quinoline degradation pathway in *P. putida* 86 (2). In this study, the expression of 2-oxo-1,2-dihydroquinoline 8-monooxygenase, the second enzyme in this degradation pathway, was investigated, using the same recombinant strain. The rate of cometabolic transformation of 2-oxo-1,2-dihydroquinoline by clone 13/42 and the activities of both enzyme components of 2-oxo-1,2-dihydroquinoline 8-monooxygenase in crude extracts of clone 13/42 were comparable to those obtained with *P. putida* 86. Since neither substrate transformation nor reductase or oxygenase activity was detectable with the DNA recipient *P. putida* mt-2 KT2440, the recombinant cosmid DNA contained the genes *oxoO* and *oxoR*, encoding the functional oxygenase and reductase components of 2-oxo-1,2-dihydroquinoline 8-monooxygenase.

Localization and cloning of the genes *oxoO* and *oxoR*. Restriction fragments of the recombinant cosmid that hybridized with both of the oligonucleotide probes Ox and Red, which were deduced from the N termini of the oxygenase and the reductase of 2-oxo-1,2-dihydroquinoline 8-monooxygenase, were larger than approximately 20 kb. Thus, the 3.2-kb *Eco*RI fragment hybridizing with Ox and the 3.7-kb *Xma*I fragment hybridizing with Red were selected to subclone *oxoO* and *oxoR* separately in *E. coli* TG1 with pUC18 as a vector.

Restriction digestions of the recombinant cosmid with *Bgl*II and *Nsi*I followed by hybridizations with the digoxigenin-labeled DNA probes f1, f2, f3, and Red resulted in the gene map shown in Fig. 2. *oxoR* is localized approximately 15 kb downstream of *oxoO*. The genes for quinoline 2-oxidoreductase are situated in between.

Nucleotide sequences of *oxoO*, *oxoR*, and flanking regions. The nucleotide sequences of *oxoO* and *oxoR* and the deduced amino acid sequences are shown in Fig. 3. Both translational ATG start codons were preceded by a putative ribosome binding sequence, 5′-GGAG-3′. The N-terminal amino acid sequences of purified oxygenase and reductase as determined by Edman degradation totally matched the corresponding amino acid sequences derived from the nucleotide sequences. *oxoO* encodes a protein of 446 amino acids. Its calculated molecular mass is 51.2 kDa, which corresponds with the molecular mass of 55 kDa determined by SDS-polyacrylamide gel electrophoresis (PAGE) (27). The *oxoR* product is 342 amino acids in length, and its deduced molecular mass of 37 kDa agrees with the molecular mass of 38 kDa estimated by SDS-PAGE. The G+C contents of *oxoO* and *oxoR* are 61.6 and 63.2%, respectively, and match the G+C content of 62.5% reported for the genome of *P. putida* biovar A (24). Codon usage in the *oxoO* and *oxoR* genes showed preferential usage of G and C in the third position.

The nucleotide sequences of flanking regions of *oxoO* and *oxoR* were analyzed for further gene regions. The N-terminal-region-encoding part (coding for 272 amino acid residues) of a putative open reading frame was identified 53 nucleotides

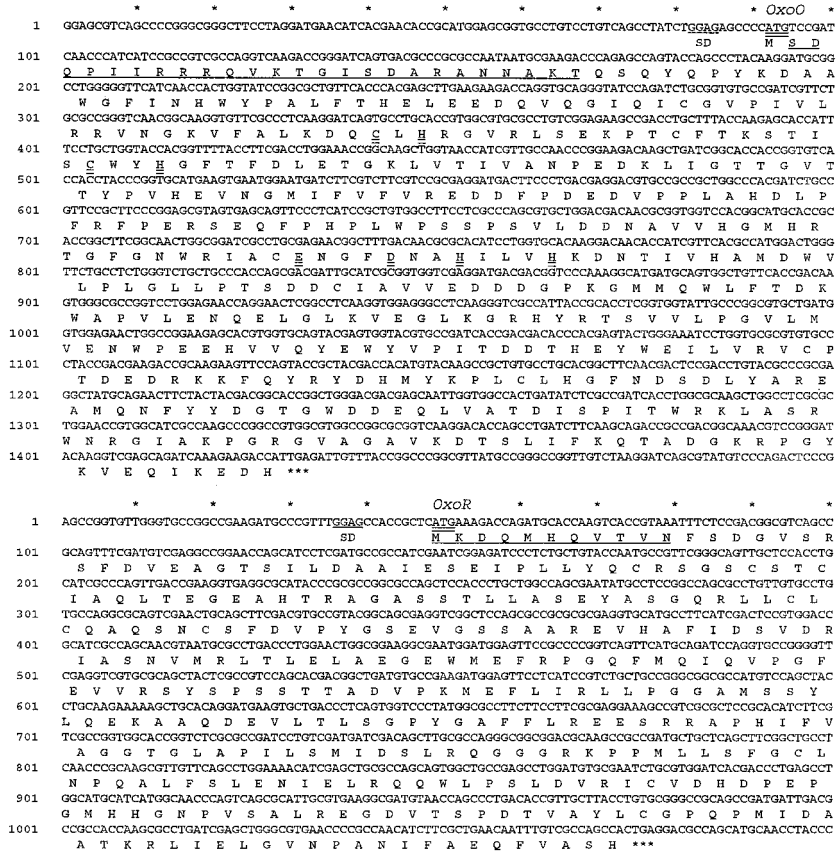

FIG. 3. Nucleotide sequences of the *oxoO* and *oxoR* structural genes. Potential ribosome binding sites (SD; underlined), start codons (double underlined), and stop codons (three asterisks) are indicated. The deduced amino acid sequences of the oxygenase (OxoO) and reductase (OxoR) components are presented below the corresponding DNA sequences. Underlined amino acid sequences are identical to those determined by Edman degradation. Conserved amino acids in OxoO which are supposed to coordinate the Rieske-type iron sulfur cluster ($CXHX_{18}CXXH$) (1, 13, 16) and the mononuclear iron center (EXXXDXXHXXXH) (14) are double underlined.

downstream of the stop codon of *oxoO*. Comparison with DNA and protein databases revealed homologies between the encoded protein (amino acid identity of about 30%) and a number of hydrolases, which are involved in the microbial degradation of aromatic compounds, catalyzing the hydrolysis of *meta* cleavage products. 2,6-Dioxo-6-phenylhexa-3-enoate hydrolase (encoded by *bphD* [12]) and 2-hydroxymuconic semialdehyde hydrolase (encoded by *dmpD* [22]), both from *P. putida*, were among the top matches.

Upstream of *oxoO*, part of another potential open reading frame, which ended 242 nucleotides in front of the *oxoO* start codon, was identified. The deduced amino acid sequence (244 residues) showed similarity to transcription-regulatory (DNA-binding) proteins encoded by the *araC-xylS* family (25). For instance, an amino acid identity of 32% was calculated for the protein ThcR, which is assumed to regulate the degradation of thiocarbamates in a *Rhodococcus* sp. (17).

The flanking DNA sequences 0.5 kb upstream and 2 kb downstream of *oxoR* showed no relevant similarity to any sequences available in DNA databases.

DISCUSSION

The structural genes for 2-oxo-1,2-dihydroquinoline 8-monooxygenase, a two-component nonheme iron oxygenase system involved in quinoline degradation by *P. putida* 86, were sequenced and analyzed. From the following observations we concluded that *oxoO* and *oxoR* code for its oxygenase and reductase components: (i) the N-terminal amino acid sequences deduced from the nucleotide sequences of *oxoO* and *oxoR* were identical to the N-terminal sequences of the enzyme components as determined by Edman degradation; (ii) the predicted molecular masses of OxoO and OxoR corresponded to the molecular masses of the enzyme components estimated by SDS-PAGE (27); and (iii) functional expression of both enzyme components was detected as in vivo and in vitro activity in a recombinant clone of *P. putida* mt-2 KT2440 that harbors both *oxoO* and *oxoR* from *P. putida* 86.

Multicomponent Rieske center nonheme iron mono- and dioxygenases are known as enzymes that participate in bacterial degradation of aromatic compounds. They consist of two or three soluble proteins that constitute an electron transport chain, transferring electrons from NAD(P)H via flavin and [2Fe-2S] centers to a non-heme-bound mononuclear iron as the site of dioxygen activation (1, 16). The substrate-hydroxylating terminal oxygenases always harbor Rieske-type [2Fe-2S] centers and mononuclear iron. Conserved amino acid motifs that are supposed to coordinate these cofactors (1, 13, 14, 16) were identified in the oxygenase component of 2-oxo-1,2-dihydroquinoline 8-monooxygenase and are indicated in Fig. 3.

Batie et al. (1) grouped the multicomponent Rieske center nonheme iron oxygenases into classes I through III based on the different proteins involved in electron transport from the cosubstrate NAD(P)H to the terminal oxygenase component.

Class I reductase components contain flavin (flavin mononucleotide in class IA and FAD in class IB) and a chloroplast-type [2Fe-2S] center. In class II enzymes, the flavin (always FAD) and the [2Fe-2S] center (IIA, chloroplast type; IIB, Rieske type) are located on separate components. Class III systems harbor FAD and chloroplast-type [2Fe-2S] in the reductase as well as Rieske-type center in an additional component. This biochemical classification is supposed to have a strong evolutionary basis (10, 18, 19).

In order to investigate the evolutionary relationship between reductase and oxygenase components from different classes, dendrograms of amino acid sequences had been established (18, 38). As shown in Fig. 4A, the oxygenase components of each distinct class form a separate branch, thus confirming an evolutionary basis for their biochemical grouping. However, although 2-oxo-1,2-dihydroquinoline 8-monooxygenase was classified as a class IB system (27), OxoO shows only distant relatedness to class IB oxygenase sequences. Surprisingly, it turned out to be most related to class IA oxygenases, which form a distinct branch. The assignment to class IA is supported by the previous observation that the native oxygenase component of 2-oxo-1,2-dihydroquinoline 8-monooxygenase, like all known class IA oxygenases (26, 27), is a homomultimer, whereas class IB oxygenases are composed of differing subunits.

On the other hand the dendrogram of reductases (Fig. 4B) revealed that the amino acid sequence of OxoR is most similar to the sequences of a branch of class IB reductases and there is a far distance to IA reductases, which again belong to a distinct group. The dendrogram supports the evolutionary divergence between group IA and IB reductases. In order to develop a more comprehensive understanding of the phylogenetic relationship of multicomponent oxygenases, the dendrograms of the sequences of DmpP, MmoC, and XylA are also shown in Fig. 4. Obviously, these reductases are related to class IB and class III reductases. In spite of this sequence similarity, these multicomponent oxygenase systems do not belong to any of the classes as defined by Batie et al. (1), because their oxygenase components do not contain a Rieske center.

The evolutionary divergence between group IA and IB reductases is also illustrated by the different arrangements of their cofactor binding sites (Fig. 5): the amino acid sequence for OxoO was aligned with the class IB reductase sequences of BenC (19), XylZ (11), and CbdC (9). The overall amino acid identity of the sequences was 19.5%. OxoO and XylZ appeared most similar, with 32.6% amino acid identity. In the N-terminal regions of the reductases, the conserved amino acid motif $CX_4CXXCX_{24-34}C$ indicates the presence of a chloroplast-type [2Fe-2S] cluster (16, 19). Downstream, sequence motifs homologous to known FAD- and NAD-binding domains (5, 18, 19) were found. The arrangement of the chloroplast-type [2Fe-2S]-, flavin-, and NAD-binding sites (Fig. 5B) is conserved in class IB and class III (5) and is even found in the reductase component MmoC of methane monooxygenase from *Methylococcus capsulatus* (31), which shows 32.5% amino acid identity with OxoR. As indicated in Fig. 5B, the arrangement of the cofactor binding sites is changed in class IA reductases. This fact has been attributed to an evolutionary divergence by alternative fusions of the distinct modular domains (5, 18).

Thus, 2-oxo-1,2-dihydroquinoline 8-monooxygenase consists of a class IA-like oxygenase and a class IB-like reductase. Consequently, the biochemical classification of the enzyme system as a whole (class IB) does not correspond to the genetic relationships of the individual components. The unusually far distance (15 kb) between the genes *oxoO* and *oxoR* also may hint at independent origins of the genes. An example of independently organized component genes was reported by Chang and Zylstra (4), who found that the reductase and oxygenase

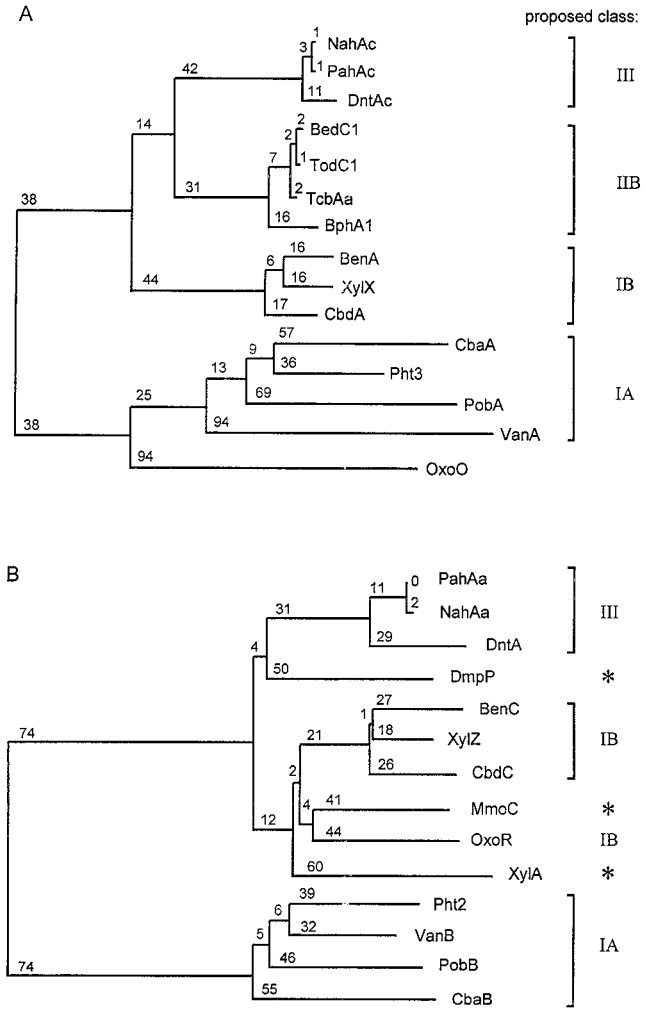

FIG. 4. Dendrograms of oxygenase components (alpha subunits in the case of heteromultimeric oxygenases) (A) and reductase components (B). The dendrograms were calculated by the program TREE of the Genetic Computer Group software package. The lengths of the branches and the numbers indicate the relative phylogenetic distances among the amino acid sequences. The class designation is based on the system of Batie et al. (1). Only components with similar cofactor compositions have been included: class II reductases are not considered since the [2Fe-2S] center is lacking. Asterisks indicate reductases of nonclassified multicomponent oxygenase systems. Bed, benzene dioxygenase from *P. putida* ML2 (36); Ben, benzoate 1,2-dioxygenase from *Acinetobacter calcoaceticus* (19); Bph, biphenyl dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 (34); Cba, 3-chlorobenzoate 3,4-dioxygenase from Tn*5271* of *Alcaligenes* sp. strain BR60 (18); Cbd, 2-halobenzoate 1,2-dioxygenase from *P. cepacia* 2CBS (9); Dmp, phenol hydroxylase from *Pseudomonas* sp. strain CF600 (21); Dnt, 2,4-dinitrotoluene dioxygenase from *Burkholderia* sp. strain DNT (32); Mmo, methane monooxygenase from *M. capsulatus* (Bath) (31); Nah, naphthalene dioxygenase from *P. putida* G7 (30); Pah, polycyclic aromatic hydrocarbon dioxygenase from *P. putida* OUS82 (35); Pht, phthalate dioxygenase from *P. putida* (20); Pob, phenoxybenzoate dioxygenase from *P. pseudoalcaligenes* POB310 (6); Tcb, chlorobenzene dioxygenase from *Pseudomonas* sp. strain P51 (38); Tod, toluene 1,2-dioxygenase from *P. putida* F1 (39); Van, vanillate demethylase from a *Pseudomonas* sp. (3); XylA, xylene monooxygenase (33), and XylXYZ, benzoate 1,2-dioxygenase (11), both from *P. putida* and encoded by the TOL plasmid pWW0.

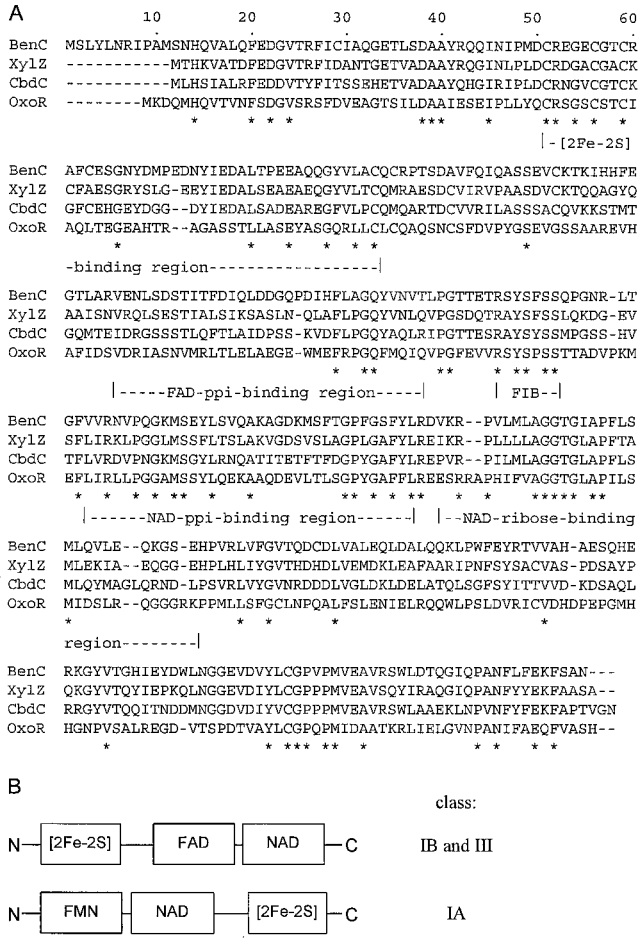

FIG. 5. Putative cofactor binding domains in the reductase components of multicomponent oxygenases. (A) Alignment of the deduced amino acid sequence of OxoR with the published class IB reductase sequences of BenC, XylZ, and CbdC (abbreviations are defined in the legend to Fig. 4). Conserved amino acid residues (asterisks) and potential cofactor binding domains are indicated (5, 16, 18, 19). FIB, FAD-isoalloxazine-ring-binding site. (B) Arrangement of NAD-, flavin-, and ferredoxin-binding domains in reductase components as deduced from sequence alignments (5). The class designation is based on the system of Batie et al. (1).

genes of phthalate dioxygenase from *Pseudomonas cepacia* DBO1 are located on separate operons.

All these findings indicate that modular protein elements have been combined during evolution. Whereas many mutations may be necessary for the development of a novel catalytic specificity, the genetic rearrangement and functional combination of catalytically active protein modules may open up novel metabolic pathways in a few steps.

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