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The Alkene Monooxygenase from *Xanthobacter* Strain Py2 Is Closely Related to Aromatic Monooxygenases and Catalyzes Aromatic Monohydroxylation of Benzene, Toluene, and Phenol

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The genes encoding the six polypeptide components of the alkene monooxygenase from *Xanthobacter* strain Py2 (Xamo) have been located on a 4.9-kb fragment of chromosomal DNA previously cloned in cosmid pNY2. Sequencing and analysis of the predicted amino acid sequences indicate that the components of Xamo are homologous to those of the aromatic monooxygenases, toluene 2-, 3-, and 4-monooxygenase and benzene monooxygenase, and that the gene order is identical. The genes and predicted polypeptides are *aamA*, encoding the 497-residue oxygenase α-subunit (XamoA); *aamB*, encoding the 88-residue oxygenase γ-subunit (XamoB); *aamC*, encoding the 122-residue ferredoxin (XamoC); *aamD*, encoding the 101-residue coupling or effector protein (XamoD); *aamE*, encoding the 341-residue oxygenase β-subunit (XamoE); and *aamF*, encoding the 327-residue reductase (XamoF). A sequence with >60% concurrence with the consensus sequence of σ^{54} (RpoN)-dependent promoters was identified upstream of the *aamA* gene. Detailed comparison of XamoA with the oxygenase α-subunits from aromatic monooxygenases, phenol hydroxylases, methane monooxygenase, and the alkene monooxygenase from *Rhodococcus rhodochrous* B276 showed that, despite the overall similarity to the aromatic monooxygenases, XamoA has some distinctive characteristics of the oxygenases which oxidize aliphatic, and particularly alkene, substrates. On the basis of the similarity between Xamo and the aromatic monooxygenases, *Xanthobacter* strain Py2 was tested and shown to oxidize benzene, toluene, and phenol, while the alkene monooxygenase-negative mutants NZ1 and NZ2 did not. Benzene was oxidized to phenol, which accumulated transiently before being further oxidized. Toluene was oxidized to a mixture of *o*-, *m*-, and *p*-cresols (39.8, 18, and 41.7%, respectively) and a small amount (0.5%) of benzyl alcohol, none of which were further oxidized. In growth studies *Xanthobacter* strain Py2 was found to grow on phenol and catechol but not on benzene or toluene; growth on phenol required a functional alkene monooxygenase. However, there is no evidence of genes encoding steps in the metabolism of catechol in the vicinity of the *aam* gene cluster. This suggests that the inducer specificity of the alkene monooxygenase may have evolved to benefit from the naturally broad substrate specificity of this class of monooxygenase and the ability of the host strain to grow on catechol.

Xanthobacter strain Py2 is a gram-negative bacterial strain which was isolated on propene as a sole carbon and energy source (34). The metabolism of propene involves an alkene-specific monooxygenase which converts propene to epoxypropane. Further metabolism involves isomerization and carboxylation of the epoxide, ultimately yielding acetoacetate (Fig. 1), which feeds into the central metabolism (1, 6, 36).

The *Xanthobacter* strain Py2 alkene monooxygenase (Xamo) will catalyze the epoxidation of a range of alkenes, some with a high degree of stereospecificity, but will not hydroxylate the homologous alkanes (35). This contrasts with alkane monooxygenases such as those based on cytochrome P-450 (28) or nonheme iron (e.g., ω-hydroxylase [27] and methane monooxygenase [MMO] [11]), which appear to generate a highly reactive iron-oxygen intermediate which can attack both unactivated C-H bonds and carbon-carbon double bonds. This reaction specificity and stereospecificity make Xamo an enzyme of considerable interest as a biocatalyst for the production of chiral 1,2-epoxides. Additionally, Xamo has been shown to be responsible for catalyzing the initial step in the cometabolic degradation of a number of chlorinated alkenes of environmental concern, including vinyl chloride, trichloroethene, and 1,3-dichloropropene (9), and it can even be induced by the presence of these chlorinated alkenes, although there is no evidence for growth on these substrates (10). Xamo has been resolved into four components: an NADH-dependent reductase, a Rieske-type ferredoxin, an oxygenase, and a small protein which may be a coupling or effector protein (29). The oxygenase is an $\alpha_2\beta_2\gamma_2$ hexamer which was reported to contain approximately four atoms of nonheme iron per hexamer on the basis of colorimetric iron analysis and the lack of a significant UV- or visible-light chromophore. Sequence (25) and electron paramagnetic resonance evidence (12) has demonstrated that an alkene-specific monooxygenase derived from the gram-positive bacterium *Rhodococcus rhodochrous* (formerly *Nocardia corallina*) B276 has a binuclear nonheme iron center of the type found in soluble MMO. However, the *Xanthobacter* enzyme is more complex than that from *Rhodococcus*, having a two-component (reductase and ferredoxin) redox system typical of aromatic dioxygenases (3) and a more complex oxygenase structure.

We have recently reported the sequencing of the first open reading frame (ORF) in the Xamo gene cluster (42). The predicted polypeptide sequence showed strong homology to

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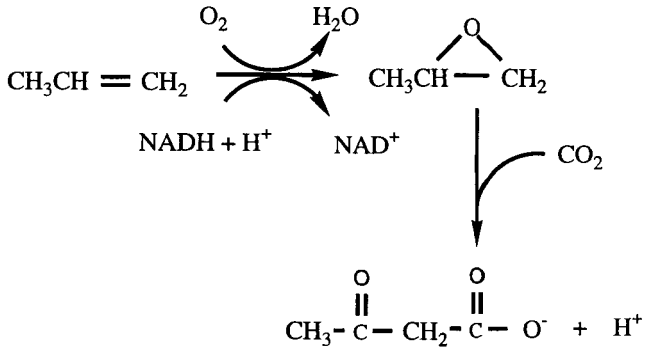

FIG. 1. Initial steps in the metabolism of propene in *Xanthobacter* strain Py2.

the nonheme iron binding subunit in aromatic monooxygenases and MMO, particularly around the iron binding domain. Modelling of the predicted *Xanthobacter* polypeptide on the coordinates of the α-subunit in the MMO crystal structure (24) allowed us to conclude that Xamo is also a nonheme iron monooxygenase. In this paper, we report the entire sequence of the six ORFs which encode Xamo. The predicted amino acid sequences of all six polypeptides encoded by these ORFs show strong homology with those of aromatic monooxygenases, including benzene monooxygenase and toluene 4-monooxygenase. This led us to investigate whether Xamo could catalyze the monohydroxylation of aromatic hydrocarbons or grow on them as sole sources of carbon and energy.

MATERIALS AND METHODS

Bacterial strains. The following strains were used: *Escherichia coli* DH 10B F$^-$ *mcrA* Δ(*mrr hsdRMS mcrBC*) ϕ80d *lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL nupG* (GIBCO BRL) and *Xanthobacter* strains Py2 (34), NZ1, and NZ2. The latter two are independently isolated alkene monooxygenase-negative (Amo$^-$) mutants of *Xanthobacter* strain Py2 (41).

Materials. Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were obtained from Promega. Restriction enzymes were supplied by Boehringer and used as recommended by the manufacturer. DNA ligase was purchased from New England Biolabs.

Bacterial growth. *E. coli* cells were routinely grown in liquid Luria-Bertani medium (10 g of casein peptone/liter, 5 g of yeast extract/liter, 5 g of NaCl/liter [pH 7.2]) or on Luria-Bertani plates at 37°C. For biotransformation assays, *Xanthobacter* strain Py2 was grown on an ammonia mineral salts medium (AMS) supplemented with 10% (vol/vol) propene or 0.5% fructose, and the mutants NZ1 and NZ2 were grown on AMS supplemented with 10% (vol/vol) propene and 0.01% (vol/vol) propene oxide, as previously described (41).

For testing growth on aromatic substrates, *Xanthobacter* strain Py2 and its mutants were grown on AMS supplemented with 2 and 4 mM benzene, toluene, or phenol. Where growth was not seen, the toxicity of aromatic substrates was tested by comparing the growth of *Xanthobacter* strain Py2 on 0.5% fructose in AMS with and without aromatic substrate supplementation.

Sequencing strategy and sequence analysis. An 11.2-kb *Eco*RI fragment from the cosmid clone pNY2, previously subcloned as pNY2C and shown to complement Amo$^-$ mutants of *Xanthobacter* strain Py2 (41), was used to prepare nested deletions by using the Deletion Factory system, version 2 (GIBCO BRL), as described previously (42). These were sequenced in both directions by using BigDye Terminator Cycle Sequencing (Perkin-Elmer) with Ampli*Taq* FS DNA polymerase and were analyzed on an ABI 377 automated sequencer (Applied Biosystems). DNA and deduced amino acid sequences were analyzed by using the MacVector (version 4.5.3) and AssemblyLIGN (version 1.07) software packages (Eastman Kodak Co.). Homologous protein searches were carried out with FASTA 3 (22) and PSI-BLAST (2). Pairwise alignments were made with the GAP program in the Wisconsin package (7). Multiple alignments were run under Clustal W (32). The search for σ^{54}-dependent promoter sequences was done remotely with the SEQSCAN program (26a).

Biotransformation assays with alkene monooxygenase (AMO). Assays were carried out on resting cell suspensions of *Xanthobacter* strain Py2 and the mutants NZ1 and NZ2. Cells were harvested in late-exponential phase, washed once, and resuspended in 25 mM phosphate buffer, pH 7.5, to an optical density at 600 nm of 10. Cell suspensions (1 ml) were preincubated for 2 min at 30°C in 7-ml Suba-sealed (W. H. Freeman, Barnsley, United Kingdom) flasks before addition of either benzene, toluene, or phenol to a final concentration of 2 mM. The rate of product formation was assayed by gas chromatography (Philips PU4500) by taking 5-μl liquid samples at suitable time intervals, separating them on a Tenax TA (60/80 mesh) column (Phase Separations, Deeside, United Kingdom) at 210°C with nitrogen as the carrier gas at 40 ml · min^{-1}, and carrying out flame ionization detection. Product concentrations were determined by reference to external standards, by using a Shimadzu CR3A recording integrator. The identities of the reaction products were confirmed by coretention with authentic standards on both the Tenax column and (for phenol) 5% (wt/vol) OV-17 on a Chromosorb W.HP (80/100 mesh) column at 130°C with nitrogen as the carrier gas at 40 ml · min^{-1}. To separate and identify *o*-, *m*-, and *p*-cresol and benzyl alcohol, the samples were analyzed by capillary gas chromatography (model 436; United Technologies Packard) on a 50-m by 0.25-mm Lipodex C column (Macharey-Nagel) at 120°C with helium as the carrier gas at 0.77 ml · min^{-1}. The split ratio was 1:100. For analysis on the latter two columns, the reaction mixtures were extracted with 0.5 ml of ethyl acetate and the extract was dried over sodium sulfate.

Propene oxide formation and degradation were measured as previously described (41).

Nucleotide sequence accession number. The sequences described in this report have been deposited with the EMBL data bank and are available under accession no. AJ012090.

RESULTS AND DISCUSSION

Preliminary sequence analysis of *Xanthobacter aam* genes. The genes encoding Xamo have previously been cloned as a 25.7-kb insert in the broad-host-range cosmid vector pLAFR5 and shown to express propene-inducible AMO when transferred to *Xanthobacter autotrophicus* JW33, although it was not expressed in *E. coli* (41). Mapping of the cosmid showed that it overlapped with a region sequenced by Swaving et al. (31) which contained the genes encoding components of the epoxide isomerase-carboxylase complex. Additionally, deletions from one end of the cosmid resulted in loss of expression of the AMO and complementation of Amo$^-$ mutants, suggesting that the AMO was encoded in a fragment of 11.2 kb flanked on one side by the cosmid junction and on the other by the isomerase-carboxylase genes. By using a nested deletion strategy, a large part of this fragment has now been sequenced, revealing the presence of six closely spaced ORFs in a 4.9-kb DNA fragment, consistent in size and predicted amino acid sequence with the reported components of Xamo (29). These ORFs have been designated *aamA* through *aamF* (Table 1); the designations refer to "alkene and aromatic monooxygenase," for reasons that will become clear in this manuscript. We have already reported (42) that the predicted amino acid sequence of the *aamA* gene product (497 residues; predicted mass, 58,037 Da; gene previously referred to as *xamoA* [42]) has a high degree of sequence similarity to the α-subunit of nonheme iron monooxygenases, particularly the aromatic monooxygenases, allowing us to model part of the sequence of this polypeptide on the coordinates of the MMO α-subunit, obtained from the crystal structure.

aamB encodes a polypeptide of 88 amino acids (9,740 Da) with a high degree of overall similarity to the γ-subunit of the $\alpha_2\beta_2\gamma_2$ hexameric nonheme iron monooxygenases; *aamC* encodes a polypeptide of 122 amino acids (13,359 Da) with a high degree of similarity to the ferredoxins of four-component nonheme iron monooxygenases and aromatic ring-hydroxylating bacterial dioxygenases; *aamD* encodes a polypeptide of 101 amino acids (11,193 Da) which is most similar to the small "coupling" proteins of four-component nonheme iron monooxygenases and also the *dmpM* gene product (P2) of the three-component phenol hydroxylase from *Pseudomonas* sp. strain CF600 (19); *aamE* encodes a polypeptide of 341 amino acids (38,188 Da) which is homologous to the oxygenase β-subunits of $\alpha_2\beta_2\gamma_2$ hexameric monooxygenases and also the β-subunit of the *R. rhodochrous* B276 AMO, which is probably tetrameric

TABLE 1. Genes with predicted products which are most homologous to those of *aamA* through *aamF*

Gene	Size (aa)[a]	% Identity	% Similarity	Organism	Accession no.[b]	Reference
aamA	497					
bmoA	500	47.69	67.0	*Pseudomonas aeruginosa* JI104	D83068	16
tbhA	501	46.23	64.39	*Burkholderia cepacia* AA1	AF001356	
tmoA	500	45.07	63.98	*Pseudomonas mendocina* KR1	M65106	39
tbuA1	501	44.06	64.39	*Ralstonia pickettii* PKO1	U04052	4
phlK	501	43.86	64.59	*Ralstonia eutropha* JMP134	AF06589	
phhN	516	27.16	46.91	*Pseudomonas putida* P35X	X79063	19
memA	527	22.86	45.16	*Methylococcus capsulatus* (Bath)	M90050	30
memA	525	22.82	46.47	*Methylosinus trichosporium* OB3b	X55394	5
amoC	501	22.08	48.33	*Rhodococcus rhodochrous* B-276	D37875	25
aamB	88					
tbuU	86	36.47	60.00	*Ralstonia pickettii* PKO1	U04052	4
phlL	86	35.29	60.00	*Ralstonia eutropha JMP134*	AF06589	
tmoB	84	34.94	53.00	*Pseudomonas mendocina* KR1	M65106	39
bmoB	88	31.76	60.00	*Pseudomonas aeruginosa* JI104	D83068	16
aamC	122					
bmoC	111	44.95	71.56	*Pseudomonas aeruginosa* JI104	D83068	16
tbhC	99	42.86	68.37	*Burkholderia cepacia* AA1	AF001356	
tmoC	112	39.45	66.05	*Pseudomonas mendocina* KR1	M65106	39
tbuB	111	38.53	68.81	*Ralstonia pickettii* PKO1	U04052	4
phlM	111	37.61	68.81	*Ralstonia eutropha* JMP134	AF06589	
bphA3	107	34.91	51.89	*Rhodococcus* sp. strain RHA1	D32142	17
hcaA3	106	30.48	54.29	*Escherichia coli*	Y11070	
msmC	123	23.64	48.18	α-Proteobacterium M2	U59152	13
aamD	101					
bmoD1	147	45.00	66.00	*Pseudomonas aeruginosa* JI104	D83068	16
tbuV	104	43.00	66.00	*Ralstonia pickettii* PKO1	U04052	4
phlN	105	43.00	65.00	*Ralstonia eutropha* JMP134	AF065891	
tbhD	104	43.00	63.00	*Burkholderia cepacia* AA1	AF001356	
tmoD	103	39.39	62.63	*Pseudomonas mendocina* KR1	M65106	39
dmpM	90	23.60	48.31	*Pseudomonas* sp. strain CF600	M60276	20
aamE	341					
tbuA2	329	38.34	56.44	*Ralstonia pickettii* PKO1	U04052	4
phlO	332	37.08	56.53	*Ralstonia eutropha* JMP134	AF065891	
tbhE	332	34.36	53.37	*Burkholderia cepacia* AA1	AF001356	
tmoE	327	34.26	55.56	*Pseudomonas mendocina* KR1	M65106	39
dsoB	333	24.84	50.97	*Acinetobacter* sp. strain 2OB	D85083	15
amoA	343	24.77	43.50	*Rhodococcus rhodochrous* B-276	D37875	25
	333	22.96	49.06	*Acinetobacter calcoaceticus*	Z36090	8
dmpL	331	22.93	46.18	*Pseudomonas* sp. strain CF600	M60276	20
phhL	331	22.29	45.22	*Pseudomonas putida* P35X	X79063	19
aamF	327					
tbuC	334	41.92	63.04	*Ralstonia pickettii* PKO1	U04052	4
phlP	332	41.87	64.69	*Ralstonia eutropha* JMP134	AF065891	
xylA	346	37.3	57.68	*Sphingomonas aromaticivorans* F199	AF079317	
caraD	329	37.11	59.43	*Pseudomonas* sp. strain CA10	D89064	26
	329	37.1	59.75	*Pseudomonas stutzeri* OM1	AB001723	21
tmoF	326	33.02	57.00	*Pseudomonas mendocina* KR1	M65106	40
amoD	342	32.6	57.99	*Rhodococcus rhodochrous* B-276	D37875	25

[a] aa, amino acids.
[b] Refers to NCBI nucleotide accession no.

(18, 25). As is the case with most other multicomponent mono- and dioxygenases, the final ORF (*aamF*) encodes a reductase homolog (327 amino acids [34,171 Da]).

Gene order. On the basis of the comparisons above, it is reasonable to conclude that *aamA* through *aamF* encode the oxygenase α-subunit, oxygenase γ-subunit, ferredoxin, coupling or effector protein, oxygenase β-subunit, and reductase, respectively (Fig. 2). This gene order is identical to that of the four-component benzene and toluene monooxygenases (4, 39, 40) and the *Ralstonia eutropha* JMP134 phenol hydroxylase, to which the individual subunits show the highest homology. The extent of sequence identity or similarity between the component polypeptides of the hexameric monooxygenase subunits of these enzymes also shows a clear grouping, separating them from MMO (5, 30) and the almost identical pair of phenol hydroxylases from *Pseudomonas* sp. strain CF600 (20) and *Pseudomonas putida* P35X (19), all of which have hexameric oxygenases, and the tetrameric *R. rhodochrous* B276 AMO

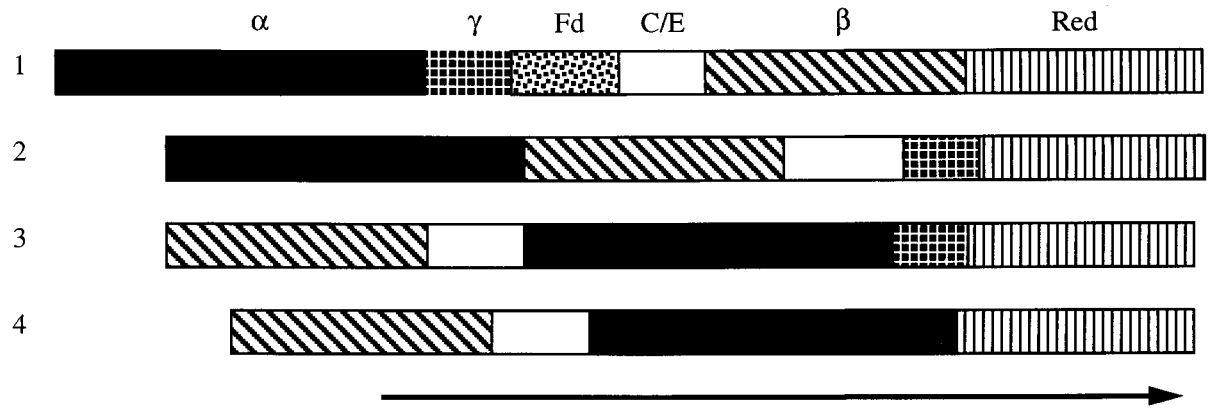

FIG. 2. Gene orders of the Xamo and related gene clusters. The monooxygenase components encoded are indicated at the top, as follows: α, β, and γ, oxygenase α-, β-, and γ-subunits; Fd, ferredoxin; C/E, coupling or effector protein; Red, reductase. The arrow indicates the direction of transcription. Diagram 1, Xamo and the benzene (*bmo*), toluene 3- (*tbh*), toluene 4- (*tmo*), toluene/benzene (*tbu*), and phenol (*phl*) monooxygenases; diagram 2, MMO (an additional ORF [not shown] lies between the genes encoding the γ-subunit and the reductase); diagram 3, phenol hydroxylase (*dmp* and *phh*); diagram 4, *R. rhodochrous* B276 AMO.

(25). All of the latter appear to be three-component systems, lacking the separate ferredoxin component, and have a gene order different from that of the Xamo–aromatic ring monooxygenase family. In MMO the genes encoding the β- and γ-subunits are in reverse order with respect to that for the "coupling" protein, while in the *R. rhodochrous* B276 AMO and the pseudomonad phenol hydroxylases, the first structural gene encodes the oxygenase β-subunit, and the α-subunit is actually the third structural gene in the sequence.

Regulatory features and codon usage. Potential ribosome binding sites precede the ATG or GTG translational initiation sites by 4 to 13 bp in all six ORFs, namely AGGA for *aamA*, *aamB*, *aamE*, and *aamF* and GAGG for *aamC* and *aamD* (Fig. 3). The translational stop codon is TAA for *aamA* and *aamD* and TGA for the other genes. No homolog of the −35 and −10 consensus sequence of σ^{70}-dependent promoters was found immediately upstream of the *aamA* gene, which might explain the lack of expression of the cosmid pNY2 in *E. coli*. However, a possible σ^{54} (RpoN)-dependent promoter sequence (GGG CACGCCATGCGCT) is present approximately 300 bp upstream of the translational start site of *aamA*.

Xanthobacter spp. have genomes which are typically composed of 65 to 70% G+C (38). While this feature is evident when the combined frequencies of all six ORFs (67% GC) are considered, there is significant variation, from 62.4% (*aamC*) to 71.9% (*aamF*, among the different coding regions. A high GC content implies that codons in which the final base in the triplet is G or C should be more frequently used, and this is also evident (90.5% G/C). The codon usage is similar to that described for other genes in *Xanthobacter* spp. (31, 33).

Intriguingly, the codons UUA (Leu), CUA (Leu), GUA (Val), UCA (Ser), and AGA (Arg) are not used once either in the 1,476 codons characterized here or in the 1,469 codons described by Swaving et al. (31) from the same organism.

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GACCGGTCGGGCGATCCGCCGCCTCATGGCGGgggcacgccatgcgctCCGGCCCGCTCACGGGAACGGCCCCGCCGAT  :  79
CGAACCGGAGGGCGTCGCCGCGGCGGCGTCACGGTCGAGGTGTGGGTGTTGCCCCTTGCTCAATGTCCGTCAGATCGCC  : 158
CTCGCCCCAAGCGAAAGCTGCTTTCGTATTGCACGACGGCTCAATTGAGCGGCGCGGCGATCGGATCGCGCGCGCTGTC  : 237
TTGTGAGATCACGTTGGGCGCACCTTGCCGCTCATCGCAATAAGAGCCGTTGTTATTGGCCAACAACAGTAGATCTGTG  : 316

AGGGAGGACATCCATGGCGCTCTTGAATCGG--1455 n--AACCGGATCGCCGCCGAGTAAACGGCGACAACAACAAG  :1840
        aamA  M  A  L  L  N  R - 485 aa - N  R  I  A  A  E  *

AGCATTCTGGAGGAAACCCATGTCTTTGTTCCCCATCGTG--222 n --TTCGACGTGAGGTTCAAGAAGTGAACCTT  :2131
              aamB  M  S  L  F  P  I  V --74 aa -- F  D  V  R  F  K  K  *
                                                          aamC  M  N  L

CACGCCCCC--330 n --CCCAAAGAGGGAGAGGCCTGAAAATGAGCAACGCCACCGTC--267 n --CGCTGGTAC  :2787
                                    aamD  M  S  N  A  T  V -- 89 aa-- R  W  Y
 H  A  P --110 aa-- P  K  E  G  E  A  *

TACAAGTCCTAAGCCGCAAGGCCATAAGATCTGGGAGGAACACGTGACACAGCAGCGCCCC--987 n--GGCCTCGGG  :3844
 Y  K  S  *                          aamE  M  T  Q  Q  R  P --329 aa- G  L  G

CACGATGCCTGAGGCGCGTGACGCGGCCGCCACGGCCGAGCGTCCGGGGCAGGACGGGGCATTTCACGTGCGGCTGAAC  :3923
 H  D  A  *                                                    aamF  M  R  L  N

GACGGC--945 n--TTTTTCGACCGTTTCGTGTGACGCGCAAAGAAGAACA                              :4911
 D  G --315 aa- F  F  D  R  F  V  *
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FIG. 3. Regulatory features of the *aam* gene cluster, showing the putative σ^{54} (RpoN)-dependent promoter sequence (lowercase letters in the top line) and ribosome binding sites (underlined).

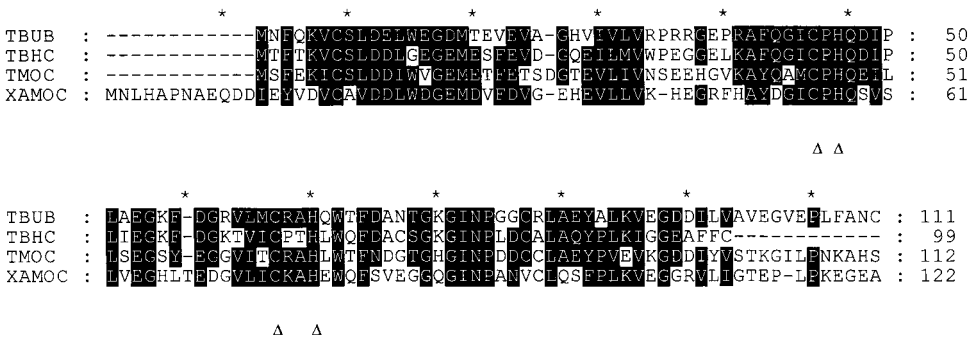

FIG. 4. Homology between the putative ferredoxin of Xamo (XAMOC), encoded by *aamC*, and ferredoxins from four-component aromatic ring monooxygenases. Dark highlighting indicates residues which are identical or functionally conserved in at least three sequences. The two cysteines and two histidines which form the metal binding sites are shown. TBUB, TBHC, and TMOC, ferredoxins from the *Ralstonia pickettii* PKO1 toluene 3-monooxygenase (4), the *Burkholderia cepacia* AA1 toluene 3-monooxygenase, and the *Pseudomonas mendocina* KR1 toluene 4-monooxygenase (39), respectively.

Further analysis of *aamC* and *aamD*. (i) *aamC*. Alignment of the amino acid sequence of the *aamC* gene product with that of the homologous monooxygenase and dioxygenase ferredoxins (Fig. 4) clearly shows that a number of key residues are absolutely conserved, including the pairs of cysteine and histidine residues which coordinate the 2Fe–2S cluster. The latter are diagnostic of Rieske-type iron-sulfur proteins and confirm the spectroscopic assignments (14, 29). Although it is clear that the *aamC* gene product is most similar to the ferredoxins present in the four-component aromatic ring monooxygenases, XamoC contains an additional 12 amino acids at the N terminus which are not seen in any of the near homologs. This enzyme contains a high proportion of acidic residues and could well be important in protein-protein interactions.

(ii) *aamD*. Alignments of the predicted amino acid sequence of the *aamD* gene product (Fig. 5) show that it clearly falls into the aromatic ring monooxygenase family and is more distantly related to the coupling protein in MMO (not shown). The role of this small protein, which does not appear to contain any cofactors, in the catalytic cycle of monooxygenases is still unclear. Small and Ensign (29) reported that this small protein was obligately required for steady-state alkene epoxidation. In MMO the coupling protein is necessary to couple electron transfer to substrate oxidation, and it is also known to affect the regioselectivity of substrate oxidation and the redox potential of the oxygenase binuclear nonheme iron center. *dmpM* encodes the homologous coupling protein (P2) in the phenol hydroxylase from *Pseudomonas* sp. strain CF600. A solution structure for this protein has recently been determined by nuclear magnetic resonance (23), and although the structure was poorly defined in places, it is evident that this small protein has a hydrophobic cavity which could well act to bind substrate, either to deliver it to the oxygenase, or to act as a substrate-dependent regulator of electron transport. The conserved residues indicated in Fig. 5 are all either within, or at the ends of, regions of secondary structure evident from the P2 structure (23).

Does Xamo function as an aromatic hydrocarbon monooxygenase? Despite the similarity of the primary sequence of the Xamo oxygenase α-subunit to that of the aromatic monooxygenases, there are certain features which clearly link it to the *R. rhodochrous* B276 AMO and also to MMO. In particular, it was noted previously that at the position equivalent to Cys151 in the *memA* gene product, the AMO enzymes have an acid residue, whereas the aromatic hydroxylases have a Gln residue (Fig. 6). Additionally, residues 206 and 208 in the *memA* protein sequence are small aliphatic amino acids, whereas in all of the aromatic ring monooxygenases they are Phe's. This is particularly significant because Glu209 in the *memA*-encoded gene product is one of the acidic residues which coordinate the binuclear nonheme iron center, and it is probable that the residue at position 208 (and its equivalent in homologous

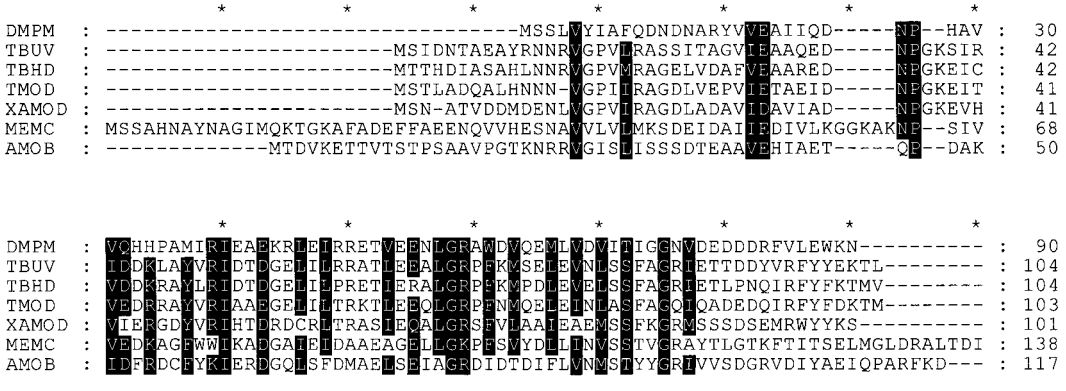

FIG. 5. Homology between the putative small coupling or effector protein of Xamo (XAMOD), encoded by *aamD*, and the coupling or effector proteins of aromatic ring monooxygenases, MMO, and the *R. rhodochrous* B276 AMO. Dark highlighting indicates residues which are identical or functionally conserved in at least six sequences. DMPM, TBUV, TBHD, TMOD, MEMC, and AMOB, small coupling or effector proteins from the *Pseudomonas* sp. strain CF600 phenol hydroxylase (20), the *R. pickettii* PKO1 toluene 3-monooxygenase (4), the *B. cepacia* AA1 toluene 3-monooxygenase, the *P. mendocina* KR1 toluene 4-monooxygenase (39), the *Methylosinus trichosporium* OB3b MMO (5), and the *R. rhodochrous* B276 AMO (25), respectively.

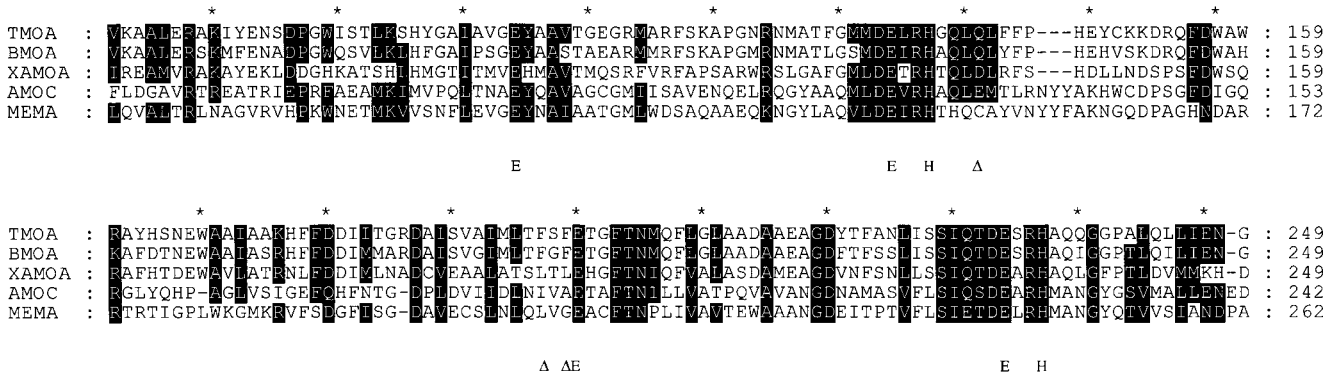

FIG. 6. Homology between the putative oxygenase iron binding subunit of Xamo (XAMOA), encoded by *aamA*, and the iron binding subunits of aromatic ring monooxygenases, MMO, and the *R. rhodochrous* B276 AMO. Only the sequence around the iron binding sites is shown. Dark highlighting indicates residues which are identical or functionally conserved in at least four sequences. The glutamate and histidine ligands that coordinate the binuclear iron center (24) in MMO (and are completely conserved in the other homologs) are shown as subscripts in capital letters, and the residues referred to in the text are indicated by triangles. TMOA, BMOA, AMOB, and MEMA, iron binding subunits from the *P. mendocina* KR1 toluene 4-monooxygenase (39), the *Pseudomonas aeruginosa* JI 104 benzene monooxygenase (16), the *R. rhodochrous* B276 AMO (25), and the *M. trichosporium* OB3b MMO (5), respectively.

polypeptides) has a significant effect on the approach and orientation of the substrate. Aromatic residues at positions equivalent to positions 206 and 208 in the *memA*-encoded protein sequence should therefore facilitate the approach of aromatic substrates.

Despite these differences, the evident homology between all of the Xamo components and those of aromatic ring monooxygenases suggested that Xamo might hydroxylate aromatic substrates. By using propene-grown resting cells, *Xanthobacter* strain Py2 was shown to convert benzene to phenol and to convert toluene to a mixture of cresols and a trace of benzyl alcohol (Table 2). It should be noted that, although the rates of aromatic hydrocarbon oxidation are similar to those cited for propene oxidation, the latter was carried out at pH 9 to limit the further metabolism of epoxypropane. Propene oxidation assays measured by substrate disappearance at pH 7.2 give rates at least 5 times higher (10) than those observed here for aromatic hydrocarbon oxidation at a similar pH. Confirmation that oxidation was due to the AMO was obtained by using the Amo$^-$ mutants NZ1 and NZ2, which cannot oxidize propene but retain the ability to grow on propene oxide. Additionally, resting cells of noninduced control cultures of the wild type grown on AMS fructose were also unable to oxidize propene or the aromatic substrates. In the biotransformation of benzene, it was noticed that phenol accumulation was transient and that phenol was metabolized as soon as the benzene had been converted. By repetition of the same assays with phenol as a substrate, it was evident that phenol was also oxidized by the AMO, and thus, benzene was being oxidized consecutively by the same enzyme. However, tests with pure cresols indicated that these were not good substrates for Xamo. Therefore, in the biotransformation of toluene, the resulting cresols remained at their final concentrations once the toluene had been completely oxidized.

TABLE 2. Whole cell propene, benzene, and toluene monooxygenase activities, and phenol hydroxylase and propene oxide degradation activities, of *Xanthobacter* strain Py2 and the Amo$^-$ mutants NZ1 and NZ2[a]

Strain	Activity[b] on:				
	Benzene	Toluene	Phenol	Propene	Propene oxide
Xanthobacter strain Py2	13.8	19.5	8.4	20.1	50.5
NZ1	0	0	0	0	51.2
NZ2	0	0	0	0	49.8

[a] No activity was detected with any of the substrates with *Xanthobacter* strain Py2 cells grown with 0.5% (wt/vol) fructose as the sole carbon source.

[b] Activities, in nanomoles per minute per milligram of protein, refer to the rate of product formation, except for the activities on phenol and propene oxide, which were measured as rates of substrate disappearance.

Biotransformation of a range of substrates is a common feature of iron-containing monooxygenases, but it is generally observed that the range of substrates used for growth is much more restricted, usually because of the greater inducer specificity or inability to further metabolize the hydroxylated product. Given the evidence for metabolism of propene oxide via isomerization and carboxylation, it seemed unlikely that aromatic hydrocarbons would act as growth substrates. Thus we were surprised to find that although benzene and toluene were not growth substrates (even at nontoxic concentrations), growth of Py2 was observed on 2 and 4 mM phenol, and this required a functional Xamo, as seen by the fact that the Amo$^-$ mutants failed to grow at any concentration. Cells grown on phenol were able to oxidize propene and metabolize propene oxide in resting cell assays, but at rates lower than those obtained with propene-grown cells, confirming that phenol induces both Xamo and the epoxide carboxylase. Growth on phenol presumably occurred as a result of oxidation to catechol and subsequent ring cleavage reactions; certainly *Xanthobacter* strain Py2 (and the Amo$^-$ mutants) can grow on catechol as a sole carbon and energy source. However, although the sequencing is not complete, we have found no evidence for genes encoding ring cleavage enzymes in the vicinity of the *aamA*-through-*aamF* cluster. At this stage we can only speculate that the broad range of inducers previously described for Xamo also includes phenol and that the resulting catechol can induce the necessary ring cleavage pathway. The ability to grow on phenol via the hybrid route may well have provided selective pressure for the evolution of inducer specificity to include phenol. Interestingly, we have been unable to detect induction with the nongrowth substrates, benzene and toluene (2 mM), in cells grown on either glucose or isopropanol, although at this stage we cannot exclude the possibility that they are weak inducers.

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