The nucleotide sequence of the Tn5271 3-chlorobenzoate 3,4-dioxygenase genes (*cbaAB*) unites the class IA oxygenases in a single lineage

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The nucleotide sequence of the 3-chlorobenzoate 3,4-dioxygenase genes, designated *cbaAB*, from the transposon Tn5271 was determined. The function of the two sequenced open reading frames was evaluated by mutagenesis and expression *in vivo* to show that the *cbaA* and *cbaB* genes code for dioxygenase and reductase proteins, respectively. Comparison of the deduced amino acid sequences of the *cbaAB* genes with sequences for other oxygenases revealed a clearly defined lineage among the class IA oxygenases that shows several unique features. This lineage includes phthalate 4,5-dioxygenase (*pht23*), and based on the available NH₃-terminal sequence of component A, also includes 4-sulphobenzoate 3,4-dioxygenase. Vanillate demethylase, encoded by the *vanAB* genes and formally a monooxygenase enzyme catalysing an oxidative demethylation, is also included in this lineage. The terminal chlorobenzoate dioxygenase (CbaA) component is characterized by a conserved Rieske-type [2Fe-2S]_R ligand centre. The reductase component (CbaB) contains a plant-type ferredoxin [2Fe-2S]_{Fd}, FMN-isoalloxazine and NAD-ribose-binding domains and the orientation of these domains is conserved in all known class IA reductases. These results support the hypothesis that alternative fusions of the electron transfer modules of the reductases arose early in the divergence of oxygenase systems. The over-riding evolutionary constraint acting on the divergence of the class IA oxygenases would appear to be the requirement for a carboxyl group *para* to the site of oxygen insertion into the aromatic ring.

Keywords: transposon, chlorobenzoate, dioxygenase, plasmid

INTRODUCTION

Complex mixtures of organic compounds found in contaminated soils and surface waters are a powerful selective force for the evolution of bacterial catabolic pathways. Many isolates have been recovered from these environments on the basis of their ability to utilize selected contaminants as their sole source of carbon and energy (Sayler *et al.*, 1990; van der Meer *et al.*, 1992). While the aromatic catabolic determinants of some genera

have been thoroughly studied, particularly the fluorescent pseudomonads and *Acinetobacter* (Gibson & Subramanian, 1984; Frantz & Chakrabarty, 1986; Reineke & Knackmuss, 1988; Harayama *et al.*, 1992), other genera are not well characterized. As new catabolic gene sequences from diverse contaminant-degrading species are determined, new insights into evolutionary lineages and origins of these elements are revealed (Neidle *et al.*, 1991).

The metabolism of chlorobenzoates illustrates the diversity of pathways for contaminant degradation that is likely to be encountered in natural communities or industrial consortia. There are now several known pathways for the metabolism of chlorobenzoates among the aerobic proteobacteria. The chlorobenzoate 1,2-dioxygenase pathway, wherein oxygen is introduced at the carboxyl and adjacent positions of the chlorobenzoate

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ring, forming chlorocatechols, has been thoroughly studied (Reineke & Knackmuss, 1988; Hickey & Focht, 1990; Harayama *et al.*, 1992). This pathway is initiated either by benzoate(toluato) 1,2-dioxygenases with broad substrate specificity (Fetzner *et al.*, 1992), or by *ortho*-halobenzoate 1,2-dioxygenases that are specific for *ortho*-substituted halobenzoates (Romanov & Hausinger, 1994). An operon representative of the former, *xyzXYZ* from the *Pseudomonas putida* plasmid pWW0, has been sequenced and the deduced amino acid sequences have been compared to the narrow-substrate-range benzoate 1,2-dioxygenase of *Acinetobacter calcoaceticus* (*benABC*) (Harayama *et al.*, 1991; Neidle *et al.*, 1991). Sequences of chlorocatechol *ortho*-ring-fission genes (*clcABD*) from *P. putida* (pAC27), required for complete metabolism of chlorobenzoates by this pathway, have also been determined. They reveal similarities to the corresponding catechol *ortho*-ring-fission genes found in *Pseudomonas* and *Acinetobacter*, and to other chloroaromatic *ortho*-ring-fission genes (Frantz & Chakrabarty, 1987; Neidle *et al.*, 1988; van der Meer *et al.*, 1991, 1992; Harayama *et al.*, 1992). Another known pathway involves the removal of a chlorine substituent by hydrolytic dehalogenation. This pathway functions in *Pseudomonas* sp. CBS-3 growing on 4-chlorobenzoate (Savard *et al.*, 1986; Scholten *et al.*, 1991). An early report on the metabolism of 3-chlorobenzoate in *Pseudomonas* attributed a similar transformation, to form 3-hydroxybenzoate, to a monooxygenase (Johnston *et al.*, 1972). Very little is known concerning the biochemistry and genetics of the latter pathway. Recently we described a new pathway in *Alcaligenes* sp. strain BR60 that expresses a 3-chlorobenzoate 3,4-dioxygenase activity (Nakatsu & Wyndham, 1993). This dioxygenase introduces oxygen at the carbons of benzoate distal to the carboxyl group, yielding protococatechuate and chloroprotocatechuate metabolites. The genes for this pathway are carried on a 3.7 kb *NotI*-*EcoRI* fragment within the catabolic transposon Tn5271, originally found on the 85 kb conjugative plasmid pBRC60 (Wyndham *et al.*, 1988, 1994; Nakatsu *et al.*, 1991; Nakatsu & Wyndham, 1993).

Oxygenases that are involved in the initial attack on aromatic substrates have been grouped according to the nature of the oxidation they carry out, and according to the properties of their electron transport proteins (Harayama *et al.*, 1992; Mason & Cammack, 1992). The aromatic-ring-hydroxylating dioxygenases have been classified into two-component systems and three-component systems, based on the number of proteins involved in electron transport and hydroxylation (Fetzner *et al.*, 1992). These broad classes have been further subdivided according to the detailed arrangements and cofactor requirements of their reductase and oxygenase components (Batie *et al.*, 1991; reviewed by Mason & Cammack, 1992). Briefly, these classes and their distinguishing features are: class I, two-component oxygenases in which a flavin and iron-sulphur cluster are combined in the same reductase (class IA: FMN cofactor; class IB: FAD cofactor); class II, three-component dioxygenases in which the flavin (reductases) and iron-

sulphur proteins (ferredoxins) are separate (class IIA: chloroplast-type ferredoxins; class IIB: Rieske-type ferredoxins); class III, three-component dioxygenase in which the reductase and ferredoxin are separate, but the reductase contains both a flavin and an iron-sulphur cluster. All of these classes have terminal oxygenase components with two common cofactors, a Rieske-type $[2Fe-2S]_R$ iron-sulphur centre and one mononuclear non-haem iron.

In order to place the 3-chlorobenzoate 3,4-dioxygenase of Tn5271 into the context of the evolution of bacterial oxygenases, we determined the activity expressed by the genes designated *cbaAB* from *Alcaligenes* sp. BR60, and their nucleotide sequence. From this sequence we derived putative amino acid sequences and compared these with known sequences for other oxygenases. This comparison revealed a clearly defined lineage among the biochemically defined class IA oxygenases that shows several unique features.

METHODS

Strains, plasmids and growth conditions. The growth and maintenance of *Alcaligenes* sp. strain BR60 (pBRC60), strain BR6024 (chloramphenicol resistant, tryptophan auxotroph) and *Escherichia coli* strains have been described previously (Wyndham *et al.*, 1988; Wyndham & Straus, 1988; Nakatsu *et al.*, 1991; Nakatsu & Wyndham, 1993). *E. coli* strain DH5 α was used as the host for the broad-host-range pBW13 constructs pBRCN5, pBRCN7, pBRCN11, pBRCN12, pBRCN15 and pBRCN16, containing the dioxygenase coding region of Tn5271 and deletion and point mutations of this region (Nakatsu & Wyndham, 1993; this study). *E. coli* strain JM109 was the host for the pUC18 plasmid construct pBRH2, containing the cloned *HindIII* restriction fragment H2 of Tn5271 (Wyndham *et al.*, 1988). *E. coli* strain HB101 was used as the host for single-stranded DNA synthesis from clones in M13mp18 and M13mp19 (Yanisch-Perron *et al.*, 1985).

DNA cloning, sequencing and mutation. DNA extraction and cloning was performed according to previously described methods (Nakatsu *et al.*, 1991). The *HindIII* fragment H2 of Tn5271, originally cloned into the *HindIII* site of pUC18 to give pBRH2 (Wyndham *et al.*, 1988), was the source of the DNA sequenced in this study. Plasmid pBRH2 was digested with *EcoRI* and the fragments E11 (2.9 kb), E13 (1.6 kb) and E14 (1.5 kb) were ligated into the replicative forms of phage M13mp18 and M13mp19 in both orientations. These constructs were transformed into *E. coli* HB101 to produce single-stranded DNA for sequencing in both directions using the commercial dideoxy chain-termination Sequenase kit (United States Biochemical). Oligonucleotides for sequencing were prepared as described by Nakatsu *et al.* (1991).

The *cba* gene region was mutagenized to determine the requirements for chlorobenzoate dioxygenase activity by creating a deletion and two site mutations, followed by the evaluation of expression *in vivo*. Unique *ScaI* restriction sites within *cbaA* were used to create a deletion of the internal fragment of pBRCN5 *in vitro*, by restriction digestion and ligation, followed by transformation into *E. coli* DH5 α and triparental mating into *Alcaligenes* sp. BR6024 (Nakatsu & Wyndham, 1993). The only known unique site within the second ORF (*cbaB*) was for *EcoRV*, but this site also existed within the vector pBW13. Therefore, the *PstI*-*HindIII* frag-

ment of pBRH2 was cloned into pUC18, then the *EcoRV* site was mutated by restriction digestion and treatment with exonuclease III for 1 s according to the manufacturer's directions (Boehringer Mannheim). The site was then filled, religated and the plasmid was transformed into *E. coli* DH5 α as described (Sambrook *et al.*, 1989). The modified fragment was then excised from pUC18, cloned into the broad-host-range vector pBW13 and conjugated into *Alcaligenes* sp. strain BR6024 as described previously (Nakatsu & Wyndham, 1993). A third mutation was created 3' to the *cbaB* ORF by digesting pBRCN5 with *Bst*EII and treating with exonuclease III as described above. The presence of the specified modifications to all plasmids was confirmed by plasmid isolation and restriction mapping.

Sequence analyses. The National Center for Biotechnology Information (NCBI) BLAST and BLASTX programs were used to search for similarities to the *cbaAB* nucleotide sequences and deduced amino acid sequences (Altschul *et al.*, 1990). The non-redundant components of peptide sequence databases, including SwissProt, PIR, GenPept (translations from GenBank) and GUPdate (cumulative daily updates to the major release), were searched. The GCG sequence analysis programs of the University of Wisconsin (Devereux *et al.*, 1984), provided through the Molecular Biology Database System of the National Research Council of Canada, were used for all analyses. Amino acid sequence alignments were carried out using the GAP, BESTFIT and PILEUP programs of the GCG7 package. For these alignments a gap weight of 3.0 and a gap length weight of 0.1 were used. Complete sequences were used for alignments within the class IA oxygenase group containing Cba, Van and Pht protein sequences. Complete sequences and partial sequences were used for alignments between amino acid sequences of class IA and the other classes of oxygenases as defined by Batie *et al.* (1991). The sequences of the 200 amino acids spanning the Rieske-type [2Fe-2S]_R domains of the terminal oxygenase components were aligned to show similarities across dioxygenase classes. Similarly, 200 amino acids containing the contiguous FMN- (FAD-) isalloxazine binding and NAD-ribose-binding domains of the reductase components, and 110 amino acids spanning the plant ferredoxin-like [2Fe-2S]_{FD} domains of these same components, were aligned individually. The PILEUP program uses a progressive alignment method the order of which is based on pairwise similarity scores for each pair of sequences. The pairwise similarity scores were used to cluster the sequences by similarity. Relative evolutionary distances were presented graphically as dendrograms using these pairwise similarity scores.

RESULTS

Localization and nucleotide sequence of the *cbaAB* genes

A restriction digest map of Tn5271, showing the flanking IS1071 elements and the non-repeated region carrying the *cbaAB* (3-chlorobenzoate 3,4-dioxygenase) genes is presented in Fig. 1. This figure illustrates the subclones constructed to determine the function of the *cbaAB* genes.

The nucleotide sequence from position 4600 to 6938 is presented in Fig. 2. The nucleotide numbering system begins with nucleotide 1 as the first G in the left inverted repeat of the IS1071 L element of Tn5271 (Fig. 1; GenBank accession number M65135; Nakatsu *et al.*, 1991). Previous studies (Nakatsu & Wyndham, 1993)

demonstrated that unique protein products were produced only in the left to right (*Pst*I to *Hind*III) orientation, the upper strand in this study, therefore only the deduced amino acid sequences of the ORFs of the upper strand are included in Fig. 2. The ORFs were designated *cbaA* and *cbaB*.

Conjugation of the pBRCN5 construct containing these ORFs plus flanking DNA into *Alcaligenes* sp. BR6024 restored the ability of this mutant to grow on 3-chlorobenzoate. Elimination of the *Sca*I fragment within the *cbaA* ORF to give the pBRCN7 plasmid resulted in a failure to restore the degradative phenotype.

The clone pBRCN15 was created by eliminating the *EcoRV* restriction enzyme site within *cbaB* of pBRCN5 by exonuclease digestion. The introduction of this plasmid into *Alcaligenes* sp. strain BR6024 did not initially produce 3-chlorobenzoate-degrading transconjugants. After prolonged incubation (3 weeks), colonies did appear on the 3-chlorobenzoate selection plates. When these putative transconjugants were patched to fresh 3-chlorobenzoate selection plates, growth of 10% (5/50) of the patched colonies occurred. If the tetracycline resistance marker of pBRCN15 was used for selection of transconjugants on succinate plates, followed by patching of transconjugants onto 3-chlorobenzoate, no growth occurred even after prolonged incubation. These observations suggest CbaB is required for 3-chlorobenzoate 3,4-dioxygenase activity, and that we observed mutational recovery of this activity due to repair of the deletion around the *EcoRV* site *in vivo*.

The clone pBRCN16 was created by eliminating the *Bst*EII restriction enzyme site that lies approximately 0.3 kb beyond the stop codon of *cbaB*. The clones pBRCN11 and pBRCN12 were created by nested deletions in the same region of DNA, 3' to the *cbaB* gene (Nakatsu & Wyndham, 1993). The introduction of these plasmids into *Alcaligenes* sp. strain BR6024 did not produce 3-chlorobenzoate-degrading transconjugants. However, the pBRCN16 and pBRCN11 transconjugants metabolized 3-chlorobenzoate to dihydrodiol intermediates that were unstable (Nakatsu & Wyndham, 1993). The major product accumulating in the medium of these transconjugants was a chlorohydroxybenzoate, presumed to be the product of spontaneous dehydration of a chlorodihydrodiol intermediate. The pBRCN12 transconjugants failed to metabolize 3-chlorobenzoate, indicating that the nested deletion had eliminated an essential sequence required for dioxygenase expression. Restriction mapping revealed that this deletion extended into the *cbaB* gene.

Sequence characterization

The first ORF, *cbaA*, has potential initiation codons at nucleotides 4631, 4685 and 4697 of Tn5271 (Fig. 2). The potential ribosome-binding site preceding nucleotide 4631 is AGGAG, very close to the ribosomal-binding site consensus sequence for *E. coli*, therefore this was chosen as a tentative initiation codon. The *cbaA* gene potentially codes for a protein of 432 amino acids with a deduced molecular mass of 48932 Da. The second ORF, *cbaB*, has

Fig. 1. Restriction enzyme digest map of Tn5271 showing the location of the *cbaA* and *cbaB* genes. (a) Map of the entire Tn5271 element. Restriction enzymes used were: Bs, *Bst*Ell; E, *Eco*RI; Ev, *Eco*RV; H, *Hind*III; N, *Not*I; P, *Pst*I; Sc, *Scal*. (b) The region of Tn5271 cloned into the IncP β broad-host-range vector pBW13 to form pBRCN5. The *cbaA* and *cbaB* genes are designated by arrows (encoded on the upper strand as drawn, with the 5' end to the left). Downstream of *cbaB* there is another ORF (ORF3) not fully characterized. Distances are marked in kb relative to the numbering system for Tn5271, with nucleotide 1 being the first G in the left inverted repeat of IS1071 L (Nakatsu *et al.*, 1991). The mutations of pBRCN5 used to determine functions of the ORFs are shown below. The construction of pBRCN7, pBRCN11 and pBRCN12 was previously described (Nakatsu & Wyndham, 1993). The pBRCN15 and 16 mutations were created by deletion of nucleotides at restriction enzyme sites for *Eco*RV and *Bst*Ell, respectively.

an initiation codon at nucleotide 5983 with a potential ribosome-binding site with the sequence GGAGG. The *cbaB* gene potentially codes for a 288 amino acid protein with a deduced molecular mass of 31 704 Da.

The G + C composition of *cbaA* is 58.7% and of *cbaB* is 59.2%, in the expected range for the *Alcaligenes* sp. BR60 family *Comamonadaceae* (57–70% G + C) (Willems *et al.*, 1991). This G + C composition is similar to the 58.8% G + C content for the IS1071 transposase sequence flanking *cbaAB* within Tn5271 (Nakatsu *et al.*, 1991). There is very little information on codon usage by species within the *Comamonadaceae*, however we can comment on the differences in codon usage when compared to codons used by fluorescent *Pseudomonas* species (West & Iglewski, 1988). For example, arginine AGA and AGG are used nine times in the *cbaAB* genes, which is unlike codon usage in the fluorescent pseudomonads. Harayama *et al.* (1991) found that there was an exceptional use of these same codons in the *xytX* gene of *P. putida*, prompting them to speculate on alternative origins for this gene. Codons considered rare in *Pseudomonas aeruginosa*, but used frequently in *cbaAB* are: arginine-CGA, alanine-GCA, isoleucine-ATT, leucine-TTG, glycine-GGG and GGA, and valine-GTT. In *cbaA* alanine-GCT and arginine-CGT and AGG, and in *cbaB* asparagine-AAT, cysteine-TGT, isoleucine-ATA and serine-AGT, are all rare codons for *Pseudomonas* that are used in the *cbaAB* genes.

These codon usage patterns suggest the *cbaAB* genes do not derive from fluorescent *Pseudomonas* species.

Alignments of oxygenase amino acid sequences

The BLASTX search of peptide sequence databases for similarities to the *cbaA* gene product listed the products of *phb3* and *vanA* as the closest matching full sequences (Fig. 3). The *phb12345* genes were described by Nomura *et al.* (1992). They are found on a 7 kb *Eco*RI fragment of the PHT plasmid of *P. putida* strain NMH102-2 and their expression allows this strain to grow with phthalate as sole source of carbon. The chromosomal *vanAB* genes encode vanillate demethylase activity in a *Pseudomonas* species (Brunel & Davison, 1988). The latter activity has been described as a monooxygenase-catalysed oxidative demethylation. In addition to the above matches, similarity was also detected to a partial amino acid sequence of the NH₃-terminal 35 residues sequenced from the purified component A of the 4-sulphobenzoate 3,4-dioxygenase of *Comamonas testosteroni* strain T2 (Locher *et al.*, 1991). The NH₃-terminal sequence of the 4-sulphobenzoate 3,4-dioxygenase showed 48% identity to CbaA over the available 35 amino acid sequence. This was a much greater similarity than for the CbaA/Phb3 comparison in the same region of the sequence (37%). This suggests that when the complete 4-sulphobenzoate 3,4-dioxygenase sequence

cbaAB

4620		4631		4600	
AGGAGCAAGCT		ATG GCA TTG ACC AAG GAC AAC GAA GAT TTG GTG CGA GTG GGC CGT		AACAACATCCGCAAAATTTTC	
[CbaA]		Met Ala Leu Thr Lys Asp Asn Glu Asp Leu Val Arg Val Gly Arg			
4676		GGA ACC CCG ATG GGC GGC CTC ATG CGC GAG TAC TGG ATC CCC GCA TTG AAA TCG			
Gly Thr Pro Met Gly Gly Leu Met Arg Glu Tyr Trp Ile Pro Ala Leu Lys Ser					
4730		ACC GAG CTC GAG GCT GGC GGA AGT CCT GTG AGG CTG CTG CTC TTG GGC GAA AAG			
Thr Glu Leu Glu Ala Gly Gly Ser Pro Val Arg Leu Leu Leu Gly Glu Lys					
4784		CTC GTC GCC TTC AGG GAG CCG TCG GGT GCG GTC GGC GTG ATG GAC AGC CGA TGC			
Leu Val Ala Phe Arg Glu Pro Ser Gly Ala Val Gly Val Met Asp Ser Arg Cys					
4838		CCC CAC CGT GGC GTT TCC CTG TTC ATG GGG CGC GTT GAA GAG GGC GGC CTG CGC			
Pro His Arg Gly Val Ser Leu Met Gly Arg Val Glu Glu Gly Gly Leu Arg					
4892		TGC GTC TAC CAC CCG TGG AAA TTC AGC GCT GAG GGC AAA TGC GTT GAC ATG CCC			
Cys Val Tyr His Gly Trp Lys Phe Ser Ala Glu Gly Lys Cys Val Asp Met Pro					
4946		AGT GTC CGT CCT GAA GAC GAA TTC AAA AAC AGC GTG CGC GTG GCG CGA TAC CCG			
Ser Val Arg Pro Glu Asp Glu Phe Lys Asn Ser Val Arg Val Ala Arg Tyr Pro					
5000		GTG AAA GAA ATG GCG GGC GTT GTC TGG GTG TAC ATG GGT ACT CGA AAA GTT TTG			
Val Lys Glu Met Ala Gly Val Val Trp Val Tyr Met Gly Thr Arg Lys Val Leu					
5054		CCC GAG CTC CCG CCG CTG GAA GTG CTG AAT CTT CCG GAA AAC GAA GTG GAT GTC			
Pro Glu Leu Pro Arg Leu Glu Val Leu Asn Leu Pro Glu Asn Glu Val Asp Val					
5108		ATT TGC CTC CAG CGA AAG TCG AAC TGG TTG CAA AAC CTG GAA GGT GAA ATT GAT			
Ile Cys Leu Gln Arg Lys Ser Asn Trp Leu Gln Asn Leu Glu Gly Glu Ile Asp					
5162		ACC TCG CAT TTC AAC TTC CTT CAC GTG GGC GGA CTC CAC GCG GAC GAA GTG CCG			
Thr Ser His Phe Asn Phe Leu His Val Gly Gly Leu His Ala Asp Glu Val Pro					
5216		GAC GAT CAC CCA TTG AAA TAC ACC GCC CAA GTC GCT CCG CAG TAC CTG GTC AAG			
Asp Asp His Pro Leu Lys Tyr Thr Ala Gln Val Ala Pro Gln Tyr Leu Val Lys					
5270		GAA ACG GCA TTG GGC ACC TGC TAC GCG GCT CAA GTG CCG GCG GAA GAG GAT CAC			
Glu Thr Ala Leu Gly Thr Cys Tyr Ala Ala Gln Val Pro Ala Glu Glu Asp His					
5324		ACC TAC ACG CCG TTT GCG CAT TTC CTT TTC CCG TTC TGG GCG CTC ATT CCG CAA			
Thr Tyr Thr Arg Phe Ala His Phe Leu Phe Pro Phe Trp Ala Leu Ile Pro Gln					
5378		GCC GAT ATT GCG CAG AAC ATT TTG GCC AGA GCC TGG GTG CCC ATG GAC GAT GAG			
Ala Asp Ile Ala Gln Asn Ile Leu Ala Arg Ala Trp Val Pro Met Asp Asp Glu					
5432		CAC ACC ATG ATG TTC TTC CCG TGG ACG GGA TCC AAG GCC AAA GCG CTC GAT			
His Thr Met Met Phe Phe Phe Arg Trp Thr Gly Ser Lys Ala Lys Arg Leu Asp					
5486		ACG CCA TTG AAG AGC GGT TCG CCC ATG CCC GGG GTC ACG CTG ACG GAC ATG AAG			
Thr Pro Leu Lys Ser Gly Ser Pro Met Pro Gly Val Thr Leu Thr Asp Met Lys					
5540		TAC AAG GAA AAC ACG ACG GAT TGG TAC GGC CGC TGG CAG CCG CTC GGC GAT GAG			
Tyr Lys Glu Asn Thr Thr Asp Trp Tyr Gly Arg Glu Thr Leu Thr Asp Met Gly Pro					
5594		AGC AAC GAT TGG CTC ATC GAT CGA GAT TTG CAA AAA GTA GGC CCG GTG TTC TCC			
Ser Asn Asp Trp Leu Ile Asp Arg Asp Leu Gln Lys Val Gly Arg Val Phe Ser					
5648		GGT ATT TAC GGA ATC CAC GCA CAG GAT CAG GCC ATG ACC GAC AGC ATG GGG CCG			
Gly Ile Tyr Gly Ile His Ala Gln Asp Gln Ala Met Thr Asp Ser Met Gly Pro					
5702		ATC ATT GAC CAC GGC CTG GAG CAA TTG GCA CCG ACC GAT CTC ATG ATC GTC AGA			
Ile Ile Asp His Gly Leu Glu Gln Leu Ala Pro Thr Asp Leu Met Ile Val Arg					
				5756	
				ACC CGT CGC CGG ATT CTC AAG GCG CTC CGT GCG CAC GAA GCG AAT GGC ACC CTG	
				Thr Arg Arg Arg Ile Leu Lys Ala Leu Arg Ala His Glu Ala Asn Gly Thr Leu	
				5810	
				CCC CCG GGT GTT GAC GAG GCG GAT CAG TAC TTC ACG CCC AGG AGC GGC TAT TAC	
				Pro Pro Gly Val Asp Glu Ala Asp Gln Tyr Phe Thr Pro Arg Ser Gly Tyr Tyr	
				5864	
				CTC ACG CCG AAA AGC GTC GAC TGG GAG ACC GCT TAC GAA CAA AGG ATC GAG GGC	
				Leu Thr Pro Lys Ser Val Asp Trp Glu Thr Ala Tyr Glu Gln Arg Ile Glu Gly	
				5918	
				CTC GTG CCG TGA	
				Leu Val Arg *	
				5968	
				CGCGGAGGTGATGCC	
				[CbaB] Met Val Ala Ile Asp Gln His Asp Thr Tyr Ser Val Arg Val	
				6025	
				ATA TCC CGA TCG CAC CTG AGC AAG GAT ATT GTT CAG GTG GAA CTC GAG GAA TCG	
				Ile Ser Arg Ser His Leu Ser Lys Asp Ile Val Gln Val Glu Leu Glu Glu Ser	
				6079	
				AGC CAG CGA CCA TTG CCG GAC TAT GAG CCG GGA TCG CAC GTA GAC ATC TAT GTC	
				Ser Gln Arg Pro Leu Pro Asp Tyr Glu Pro Gly Ser His Val Asp Ile Tyr Val	
				6133	
				CAG GAT GAC CTT GTC CCG CAA TAC TCG CTG GTC AAG GCG TCC GAC GCG CAG GCC	
				Gln Asp Asp Leu Val Arg Gln Tyr Ser Leu Val Lys Ala Ser Asp Ala Glu Ala	
				6187	
				TCG TAC CAG ATC GCC TTC AAG GTC AAG CCG GAT CAG GGC AGT GCG ACG GAG CTG	
				Ser Tyr Gln Ile Ala Phe Lys Val Lys Arg Asp Gln Gly Ser Ala Thr Glu Leu	
				6241	
				ATG TGC GAG TTG CTC CGT GTC GGT GCA ACA ACC CGA ATC TCT GCG CCG AGG AAC	
				Met Cys Glu Leu Leu Arg Val Gly Ala Thr Thr Arg Ile Ser Ala Pro Arg Asn	
				6295	
				GCT TTT GCC CTG GAT CCG CAG GCG GCG GAG ACC GTC CTG ATT TGT GGC GGA ATA	
				Ala Phe Ala Leu Leu Asp Pro Gln Ala Arg Glu Thr Val Leu Ile Cys Gly Ile	
				6349	
				GGA ATA ACG CCC ATG GTT CAT ATG GCC ATG ACC CTG GTC AAA GCG AAG CCG CCA	
				Gly Ile Thr Pro Met Val His Met Ala Met Thr Leu Val Lys Ala Lys Arg Pro	
				6403	
				TGG AGC ATG CAC ATT GCC TCC CCG GAT GGC GAT GAG CTC GAT TTG CTG GGG CCG	
				Trp Ser Met His Ile Ala Ser Arg Asp Gly Leu Leu Asp Leu Leu Gly Pro	
				6457	
				CTG GGA AGT TGC TCC GAG ATC AGC CCG TAC ATC TCG TCC CAG GGG GAC AGA TTG	
				Leu Gly Ser Cys Ser Glu Ile Ser Arg Tyr Ile Ser Ser Gln Gly Asp Arg Leu	
				6511	
				CCG ATT CCG GAC CTG GCC GAG CCG GCA CCT GCG AAT GCG CAT CTG TAC TTC TGC	
				Pro Ile Arg Asp Leu Ala Glu Arg Ala Pro Ala Asn Ala His Leu Tyr Phe Cys	
				6565	
				GGG CCT GAG GGC ATG TTG CAG GAA TTC ACG GTG AAT CTT GCC CCG AGT GGG GCG	
				Gly Pro Glu Gly Met Leu Gln Glu Phe Thr Val Asn Leu Ala Arg Ser Gly Ala	
				6619	
				CAG TAT GTC GTG CCG GAG GGC GAG ACG ATA TTG GAT GTC CTG CCG AAT GCC GGC	
				Gln Tyr Val Val Arg Glu Gly Glu Thr Ile Leu Asp Val Leu Arg Asn Ala Gly	
				6673	
				CAC CAT GTC ACG TCC TCC TGT CCG CAG GGG ATT TGT GGC ATG TGT GAA ACC ACT	
				His His Val Thr Ser Ser Cys Arg Gln Gly Ile Cys Gly Met Cys Glu Thr Thr	
				6727	
				TTG ATT TCC GGC GTG CCG GAC CAC AGG GAC CCG TTG CTG ACC GAT AGC GAA AAG	
				Leu Ile Ser Gly Val Pro Asp His Arg Glu Thr Thr Thr Thr Thr Thr Thr Thr	
				6781	
				GCC TCG GGT CGA ACC ATG TTG ATT TGC TGT TCA CCG GCG CTC AGC CCG GAA CTC	
				Ala Ser Gly Arg Thr Met Leu Ile Cys Cys Ser Arg Ala Leu Ser Pro Glu Leu	
				6835	
				ACC CTG GAT CTG TGA AT	
				Thr Leu Asp Leu * [ORF3] Met Arg Ile Gly Val Val Gly Leu Gly Lys Ala	
				6885	
				TTT ATG CTG ATG CTG CCG ACG TTT CTC ATG GAT CCG CCG GTT CAA CTG GTC GCT	
				Phe Met Leu Met Leu Pro Thr Phe Leu Met Asp Arg Arg Val Gln Leu Val Ala	

Fig. 2. Nucleotide and deduced amino acid sequence of the *cbaA* and *cbaB* genes. The nucleotide sequence of the upper strand of Tn5271 between nucleotide positions 4600 and 6938 (nucleotide 1 defined as the first G in the sequence of the left IS1071 element of Tn5271; Nakatsu *et al.*, 1991) is shown. Potential ribosomal-binding sites are underlined and initiation codons are in bold letters. Stop codons are indicated by an asterisk. The first 87 nucleotides of an ORF downstream of *cbaB* that has been partly characterized are also shown.

is eventually determined, the alignment with CbaA will likely be very strong.

Fig. 4(a) shows an alignment of amino acids 50–108 of CbaA with similar regions of the terminal oxygenase components representing several other oxygenase classes. Within this region there is 58% identity between CbaA and Pht3, and 35% identity between CbaA and VanA. Alignments in this region are similar to previously determined conserved regions containing the proposed cysteine and histidine ligands of Rieske-type $[2\text{Fe}-2\text{S}]_{\text{R}}$ centres of several dioxygenases (Neidle *et al.*, 1991; Mason & Cammack, 1992). In addition to the Rieske-type $[2\text{Fe}-2\text{S}]_{\text{R}}$ cofactor, class IB terminal oxygenase components have been shown to bind mononuclear, non-

haem Fe(II) (Yamaguchi & Fujisawa, 1982). This cofactor is thought to be involved in oxygen binding (Mason & Cammack, 1992). Histidine and tyrosine residues conserved in the class IB, IIB and III oxygenases have been proposed to coordinate this Fe(II) (Neidle *et al.*, 1991). In the class IA alignment of Fig. 3, an aspartate and two histidine residues are conserved in a D-x-x-H-x-x-x-H motif in about the same position relative to the Rieske domain (separated by 90–100 amino acids) as in the other oxygenases. No tyrosine residues are conserved in this region.

The relative evolutionary distances of the terminal oxygenase components from different classes were determined by aligning the NH_3 -terminal 250 amino acids of

(a)

Class IA:	
CbaA (59-93)	SGAVGVMSR C P H RGVSLFMG-RVEEGLR C VY H GW
Pht3 (60-94)	DGRVGVMEY C P H RRVSIIYG-RNENSLR C LY H GW
VanA (14-48)	GQRVAALDF C P H RGAPLSLG-SIQDGKLV C GY H GL
	* * * * *
Class IB:	
BenA (85-120)	NGELNAMINA C S H RGAQLLGHKRGNTTYYT C PF H GW
XylX (80-115)	DGELNAFVNA C S H RGATLCRRFSGNKATHT C SF H GW
	* * * *
Class IIB:	
BphA (90-125)	DKSIKVFLNQ C R H RGMRICRSAGNAKAFT C SY H GW
TodC1 (86-121)	DASIAVFLNQ C R H RGMRICRADAGNAKAFT C SY H GW
BnzA (86-121)	DASIAVFLNQ C S H RGMRICRADAGNAKAFT C SY H GW
	* * *
Class III:	
NdoB (71-106)	DGSIRAFLNV C R H RGKTLSVEAGNAKFV C SY H GW
	* * *

(b)

[2Fe-2S]_R-Peptide Class

```
graph LR; Root --- Node1; Node1 --- Node2; Node2 --- CbaA; Node2 --- Node3; Node3 --- Pht3; Node3 --- VanA; Root --- Node4; Node4 --- Node5; Node5 --- BenA; Node5 --- XylX; Node4 --- Node6; Node6 --- BphA; Node6 --- Node7; Node7 --- TodC1; Node7 --- BnzA; Node4 --- NdoB;
```

The phylogenetic tree shows the following relationships:

- Class IA: CbaA, Pht3, VanA
- Class IB: BenA, XylX
- Class IIB: BphA, TodC1, BnzA
- Class III: NdoB

Fig. 4. (a) Alignment of the conserved Rieske $[2Fe-2S]_R$ centres of the oxygenase components of *cis*-diol-forming oxygenases. The class designation for oxygenases is based on the system of Batie *et al.* (1991). The two cysteine and two histidine residues are known to be the ligands of the $[2Fe-2S]_R$ centre. *, Amino acids above this position are conserved in all of the aligned sequences; -, gap introduced into the alignment. Spaces flanking conserved residues are used for emphasis only. CbaA, chlorobenzoate dioxygenase (this study); Pht3, phthalate 4,5-dioxygenase (Nomura *et al.*, 1992); VanA, vanillate demethylase (Brunel & Davison, 1988); BphA, biphenyl dioxygenase (Erickson & Mondello, 1992); TodC1, toluene dioxygenase (Zylstra & Gibson, 1989); NdoB, naphthalene dioxygenase (Kurkela *et al.*, 1988); BenA, benzoate dioxygenase (Neidle *et al.*, 1991); XylX, toluate dioxygenase (Harayama *et al.*, 1991); BnzA, benzene dioxygenase (Irie *et al.*, 1987). (b) Relative evolutionary distances of terminal oxygenase components determined using the NH_3 -terminal 250 amino acids of all sequences and drawn as a dendrogram based on pairwise similarity scores.

are short regions containing consensus amino acid sequences for the binding of flavin and nicotinamide cofactors (Neidle *et al.*, 1991). These regions of similarity are shown in Fig. 5(a, b), aligned with sequences of class IB reductase components showing similar cofactor binding regions. Also shown are the corresponding sequences for the xylene monooxygenase reductase (XylA). A third region of conserved sequence includes four cysteine

The second component, CbaB, had the highest similarity scores to the reductase components of phthalate 4,5-dioxygenase (Nomura *et al.*, 1992) and vanillate demethylase (Brunel & Davison, 1988). There are three regions of conserved sequence in these components. The first two

(a)
FMN(FAD)-Isoalloxazine ring binding domain.

Class IA (FMN):	
CbaB (56-62)	LVRQYSLVK
Pht2 (57-65)	SIRNYLSN
PDR (53-61)	SRRTYSLCN
VanB (48-54)	LVRPYSLCN
	* **
Class IB (FAD):	
BenC (166-172)	ETRSYFSS
XylZ (153-159)	QTRYSFSS
	* **
Xylene monooxygenase:	
XylA (158-164)	AVRSYFSA
	* **

(b)
NAD-ribose binding domain.

Class IA:	
CbaB (112-134)	ARETVLICGGIGITPMVHMAMTLVKAKRP
Pht2 (114-136)	ARQFIFVAGGIGITPILSMRHLKASTDL
PDR (111-139)	AKSFILVAGGIGITPMLSMARQLRAEGLR
VanB (106-126)	SSRSLLFAGGIGITPILAMAQVLAARGDT
	***** *
2° Structure:	[aaab88888tttaaaanooooooooooooottt]
Class IB:	
BenC (219-242)	KRPVLMAGGTGIAPFLSMLQVLEQKGSE
XylZ (206-229)	KRPLLLLAGGTGLAPFTAMLEKIAEQGGE
	** * * *
Xylene monooxygenase:	
XylA (216-238)	NATHMVCVAGGTGLAPIKCVLQSMTQAQRE
	** * *

(c)
[2Fe-2S]_{Fd}-binding domain.

Class IA:	
CbaB	TSS C RQGI C GM C ETTLSIGVDPH---RDRL-LTDSKASGRMTLI C C
Pht2	PSS C ESGT C GS C RTRLIEGDVEH---RDMV-LREDE--QHDQIHI C V
PDR	PSS C ESGT C GS C KTGLCGGAADH---RDDV-LAAAA--KGTQIIV C V
VanB	PLA C EQGI C GT C LTRVLGDGEPEH---RDSF-LTDAERARNDQIFP C C
	* * * * * * * * * * * *
Class IB:	
BenC	PMD C REGE C GT C RAFCESGNYDMPEDNYIEKALTPEEAQOG-YVLA C Q
XylZ	PLD C RDGA C GA C KCRAESGYSYSL-GEEYIEDALSEAEQOG-YVLT C Q
	* * * * * * * * * * *
Xylene monooxygenase:	
XylA	PHD C KVG C GT C KYKLISGRVNE--LTSSAMGLSGDLYQSG-YRLG C Q
	* * * * * * * * * *
Plant Fd:	
Fer1	PYS C RAGA C ST C AGKLISGTVDO---SDQSFLDDDDQIEAG-YVLT C V
Fer2	PFS C RAGA C ST C AGKLVSIGTVDO---SDQSFLDDDDQIEAG-YVLT C V
	* * * * * * * * * *

Fig. 5. Conserved regions in the reductase component of dioxygenases. *, Amino acids above this position are conserved in all of the aligned sequences above; -, gap introduced into the alignment. Spaces flanking conserved residues are used for emphasis only. CbaB, chlorobenzoate dioxygenase reductase (this study); Pht2, phthalate 4,5-dioxygenase reductase (Nomura *et al.*, 1992); PDR, phthalate dioxygenase reductase (Correll *et al.*, 1992) (note that the sequences reported for the PDR protein were determined by peptide sequencing or were inferred from electron density maps); VanB, vanillate demethylase reductase (Brunel & Davison, 1988); BenC, benzoate dioxygenase reductase (Neidle *et al.*, 1991); XylZ, toluate dioxygenase reductase (Harayama *et al.*, 1991); XylA, xylene monooxygenase reductase (Suzuki *et al.*, 1991); Fer1, ferredoxin, *Fischerella* sp. (Hase *et al.*, 1978; GenBank accession number A00252); Fer2, ferredoxin, *Anabaena variabilis* (Chan *et al.*, 1983). (a) The FMN(FAD)-isoalloxazine ring-binding region. The numbers in brackets indicate the amino acid number. (b) The NAD-ribose-binding region. The numbers in brackets indicate the amino acid number. (c) Conserved cysteine residues involved in binding the [2Fe-2S]_{Fd} centre characteristic of plant-type ferredoxins. Amino acid residues used in these alignments: CbaB (234-276); Pht2 (272-312); PDR (269-309); VanB (260-302); BenC (48-94); XylZ (37-82); XylA (49-88); Fer1 (38-80); Fer2 (38-80).

residues characteristic of the chloroplast-type ferredoxin [2Fe-2S]_{Fd}-binding domain (Otaka & Ooi, 1989). Fig. 5(c) shows an alignment of the class IA and IB sequences in this region with the xylene monooxygenase reductase and plant ferredoxin sequences.

In order to evaluate relative evolutionary distances of the reductase components, the protein sequences were split into two separate parts. This was necessary because the electron transfer components of different reductases have been found to be organized in modular structures that are fused in different ways to form the primary structure (Correll *et al.*, 1992). The first part examined included the FMN/FAD-isoalloxazine- and NAD-ribose-binding domains together in sequences averaging 200 amino acids in length. The second part contained the [2Fe-2S]_{Fd}-binding domains of the reductases and two plant ferredoxins and averaged 110 amino acids in length. Fig. 6(a) presents a scheme of alignments between two class IA reductases (VanB and CbaB) and a class IB reductase (BenC) to illustrate the alternative arrangements of the conserved regions containing FMN/FAD-isoalloxazine-, NAD-ribose-, and ferredoxin-like ([2Fe-2S]_{Fd}-binding domains (Neidle *et al.*, 1991; Correll *et al.*, 1992). Fig. 6(b) illustrates the relative evolutionary distances of these and other class IA and IB dioxygenase reductases as dendrograms based on pairwise similarity scores for the FMN/FAD-isoalloxazine- and NAD-ribose domain (left), and ferredoxin-like ([2Fe-2S]_{Fd}) domain (right). Xylene monooxygenase reductase (XylA) was included in these alignments as an outlier as it has previously been shown to contain the three conserved domains shown in Fig. 5, in the same orientation as the class IB reductases (Neidle *et al.*, 1991).

Class IIB reductase components from the biphenyl and toluene catabolic pathways (BphG and TodA, respectively) and their associated ferredoxin sequences (BphF and TodB) (Furukawa *et al.*, 1989; Zylstra & Gibson, 1989; Erickson & Mondello, 1992) showed very little similarity to the reductase components of the class IA oxygenases (data not shown). No similarity was found in the alignments in the region expected for the FMN/FAD-isoalloxazine consensus. Some similarity was detected between the potential NAD-ribose-binding domain shown in Fig. 5(b), and the second dinucleotide-binding $\beta\alpha\beta$ -fold described for the TodA sequence (Neidle *et al.*, 1991; Mason & Cammack, 1992). The ferredoxin domains were not similar. This was expected because the class IIB ferredoxins have spectroscopic properties and consensus sequences characteristic of Rieske-type [2Fe-2S]_R proteins as opposed to chloroplast-type ferredoxin [2Fe-2S]_{Fd} proteins (Morrice *et al.*, 1988; Mason & Cammack, 1992).

Another ORF, tentatively designated *cbaC*, has been identified from DNA sequence data extending beyond the *cbaB* gene (data not shown). Mutation of this region of the cloned chlorobenzoate dioxygenase genes of pBRCN5 resulted in metabolism of 3-chlorobenzoate to dihydrodiol intermediates (results presented above). Expression studies using the thermally regulated T7 polymerase-promoter of pGEM3Zf indicated that the region down-

Fig. 6. (a) Scheme of the complete alignment of the FMN/FAD-isoalloxazine, NAD-ribose- and [2Fe-2S]_{Fd}-binding domains of reductase components of class IA (VanB, CbaB) and class IB (BenC) oxygenases. The amino acid sequences are represented by bars with amino acid numbers indicated. Vertical lines represent conserved amino acid pairs. The VanB and CbaB sequences begin with N-terminal FMN/FAD-isoalloxazine- and NAD-ribose-binding domains, followed by C-terminal ferredoxin [2Fe-2S]_{Fd}-binding domains. The BenC sequence begins with an N-terminal ferredoxin [2Fe-2S]_{Fd}-binding domain and ends with a C-terminal FMN/FAD-isoalloxazine- and NAD-ribose-binding domain. (b) Cluster diagrams showing potential evolutionary relationships between amino acid sequences representing the two parts of the reductase proteins of oxygenases. The left diagram is based on pairwise similarity scores between the approximately 200 amino acids spanning the FMN/FAD-isoalloxazine- and NAD-ribose-binding domains. The right diagram is based on similarities between the approximately 110 amino acids spanning the ferredoxin [2Fe-2S]_{Fd}-binding domain. Mono signifies a monooxygenase class.

stream of *chaB* encodes a 42 kDa protein (Nakatsu & Wyndham, 1993). These preliminary results suggest this region of Tn5271 encodes a 3-chlorobenzoate 3,4-dihydrodiol dehydrogenase. Sequencing, database comparisons, and expression studies are continuing on this ORF in an effort to identify the product and determine its similarity to known dehydrogenases.

DISCUSSION

Earlier expression studies (Nakatsu & Wyndham, 1993), using constructs with various fragments of Tn5271 placed under the control of either the hybrid *tac* promoter of the broad-host-range plasmid pMMB66HE or the T7 promoter of pGEM3Zf, showed that two protein products (51 and 42 kDa) were formed from clones capable of restoring 3-chlorobenzoate degradation. The pattern of metabolites formed by clones that did not express the 42 kDa protein was consistent with its assignment as a dihydrodiol dehydrogenase. This protein was expressed from a cloned *ScaI*-*HindIII* fragment extending from position 5.7 to 9.8 kb (Fig. 1), which includes the *chaB* gene and the ORF downstream of *chaB*. However, based on the size of the expressed protein (42 kDa) we assume that it was the product of the downstream ORF and not of *chaB*. No evidence for expression of a 32 kDa protein corresponding in size to CbaB was found, using two different expression vectors. However, the evidence presented here for the elimination of 3-chlorobenzoate metabolism *in vivo* in cells carrying the mutated *chaB* gene on pBRCN15 and pBRCN12 indicates that the *chaB* gene

must be expressed for dioxygenase activity. Therefore, we contend that 3-chlorobenzoate 3,4-dioxygenase is a two-component enzyme made up of a terminal dioxygenase (CbaA) plus a reductase (CbaB) and that the CbaB reductase is unstable and is degraded during the extraction procedure used to investigate expression *in vivo*.

Characterization of the presumptive CbaC dehydrogenase encoded downstream of *chaB* remains to be completed. Expression of the entire operon in natural hosts of Tn5271 probably depends on the activity of an upstream promoter contained within an A + T-rich region of 142 bp (60% A + T), located between the *NotI* site (Fig. 1) and the putative ribosome-binding site of *chaA* (complete sequence not shown). There are no obvious similarities between this sequence and the known promoter sequences of other multi-component oxygenases, including the P_m or P_u promoters of the TOL plasmid and the putative promoter regions of the *pht12345* and *vanAB* operons. Characterization of the *chaAB* promoter is in progress.

The biochemical classification scheme for bacterial oxygenases described by Batie *et al.* (1991) is continually being reinforced as new aromatic ring oxygenases are characterized (Bünz & Cook, 1993; Romanov & Hausinger, 1994). As the amino acid sequences of these oxygenases become known, evidence is accumulating that the biochemical classification has a strong evolutionary basis (Neidle *et al.*, 1991; Harayama *et al.*, 1992). In this study the class IA oxygenases have been united in a single lineage on the basis of similarities between their component amino acid sequences. Relative evolutionary

Fig. 7. Reactions carried out by the class IA oxygenases. The requirement for a carboxyl group on the ring is common to all the sequenced class IA oxygenases. Substrate R-groups vary as indicated for the phthalate dioxygenase (Pht23), chlorobenzoate dioxygenase (CbaAB), sulphobenzoate dioxygenase (SbaAB) and vanillate demethylase (VanAB) reactions. Chlorobenzoate dioxygenase (CbaAB) is active with 3-chloro- and 3,4-dichloro-benzoates (Nakatsu & Wyndham, 1993). The dihydrodiol products of Pht23 and SbaAB are converted to 4,5-dihydroxyphthalate and protocatechuate, respectively (Nomura *et al.*, 1992; Locher *et al.*, 1991). The dihydrodiol products of CbaAB are converted to protocatechuate and 4-chloroprotocatechuate (Nakatsu & Wyndham, 1993). VanAB is reported to catalyse a reaction mechanism involving introduction of one atom of oxygen at the methyl-carbon at position R1 to generate a hemiacetal intermediate that spontaneously decomposes to protocatechuate and formaldehyde (Brunel & Davison, 1988).

distances determined from pairwise amino acid sequence comparisons correspond well with the biochemical classification system of Batie *et al.* (1991). The terminal oxygenase components of class IA, in the region containing the $[2\text{Fe-2S}]_{\text{R}}$ -binding domains, are more closely related to each other than to the terminal oxygenases of the other classes (Figs 3 and 4). The reductase components of the class IA oxygenases (Fig. 5) show similar cofactor- and iron-binding domains to the class IB reductases and xylene monooxygenase reductase. However, the orientation of the chloroplast-type $[2\text{Fe-2S}]_{\text{Fd}}$ -binding domain and the FMN/FAD-isoalloxazine- and NAD-ribose-binding domains are reversed in all of the known class IA reductases, including the 3-chlorobenzoate dioxygenase reductase described here, compared to other dioxygenase reductases (Fig. 6). This reversal has been attributed to alternative fusions of the three distinct domains of these reductases (Correll *et al.*, 1992). Determination of the crystal structure of phthalate dioxygenase reductase has shown that alternative fusions of the cofactor- and iron-

binding domains of the reductases can be achieved by short peptide linkers that cause minimal disturbance to the structure of the reductase (Correll *et al.*, 1992). This would suggest that alternative fusions may arise frequently during the evolution of these electron transport systems. However, the sequence comparisons and relative evolutionary distance diagrams presented in Figs 4 and 6 indicate that the class IA oxygenases form a unified lineage that has conserved the unique fusion arrangement of the reductase domains despite considerable divergence in sequence and function. This suggests that the observed fusion of the cofactor- and iron-binding domains of the class IA dioxygenase reductases probably occurred early in the evolution of the electron transport systems of bacterial oxygenases.

There is good evidence that operons coding for pollutant biodegradation are assembled in a stepwise manner from existing catabolic genes (van der Meer *et al.*, 1992). It is possible that reductase genes for one class of oxygenase may be recruited into another class. Recently a reductase gene (*pcpD*) has been described that encodes an amino acid sequence that is > 55 % similar to the VanB and Pht2 class IA reductases (Lange *et al.*, 1994). This gene is located 13 nucleotides downstream from the pentachlorophenol 4-monooxygenase gene *pcpB* of *Flavobacterium* sp. strain ATCC 39723, suggesting it is co-transcribed. Unlike the class IA oxygenases, however, pentachlorophenol 4-monooxygenase (PcpB) is a NADPH-dependent flavoprotein monooxygenase (Orser *et al.*, 1993). This observation suggests that the class IA reductases may have been recruited for a variety of aromatic ring oxidation reactions.

The unique features of the class IA dioxygenases may be used in the future to recruit new oxygenases to this class. For example, the 4-sulphobenzoate 3,4-dioxygenase of *C. testosteroni* T2 belongs to this class on the basis of biochemical studies (Locher *et al.*, 1991). Based on the N-terminal 35 amino acids sequenced from the terminal oxygenase subunit, compared to the corresponding sequences of the CbaA and Pht3 proteins, this dioxygenase belongs to the class IA lineage defined here. It remains for complete sequencing of this dioxygenase and the associated reductase to establish its evolutionary similarity. Isolation and sequencing of dehalogenating class IA dioxygenases other than CbaAB, for example the 4-chlorophenylacetate 3,4-dioxygenase of *Pseudomonas* sp. CBS3 (Markus *et al.*, 1986; Schweizer *et al.*, 1987) is also expected to strengthen the conclusions presented here.

Both monooxygenase (vanillate demethylase) and dioxygenase enzymes belong to the class IA oxygenases. The terminal oxygenase components of these enzymes are more closely related to one another than to dioxygenases from different classes, suggesting that biochemical variation may arise rapidly within a single lineage of oxygenase genes. Sequence comparisons have not shed light on the important amino acid domain(s) determining substrate binding and monooxygenase versus dioxygenase activity. Biochemical studies have shown that the aromatic hydrocarbon dioxygenases may catalyse

monooxygenase reactions (Wackett *et al.*, 1988), and the 4-methoxybenzoate monooxygenase of *P. putida* can dihydroxylate the alkyl side chain of 4-methoxystyrene (Wende *et al.*, 1989). Therefore, the fate of the oxygen atoms at the active site of these enzymes may not be as important an evolutionary character as the orientation of attack on the substituted aromatic ring.

We speculated earlier that there may be an evolutionary relationship between the 4-sulphobenzoate 3,4-dioxygenase and the 3-chlorobenzoate 3,4-dioxygenase because both host organisms belong to the *Comamonadaceae*, and because the relative stereospecificity of the reactions was similar (Nakatsu & Wyndham, 1993). An examination of Fig. 7, showing the orientations of oxygen insertion into the four different substrates of the class IA oxygenases, supports this conclusion. The over-riding evolutionary constraint acting on the divergence of the class IA oxygenases would appear to be the requirement for a carboxyl group *para* to the site of oxygen insertion. The amino acid(s) of the dioxygenase component that orient the substrate within the active site and that presumably recognize the carboxyl group are not known, however they should be conserved in all class IA dioxygenase sequences. Conserved amino acids that may serve this function in the class IA dioxygenases are the three arginines at positions 329, 377 and 378 (Fig. 3, CbaA numbering). Arginines also make up three of the nine conserved residues in an alignment of the β -subunits of the class IB, IIB and III oxygenases that are required for substrate binding (Neidle *et al.*, 1991). Further sequencing, mutagenesis and expression studies are required to establish the nature of active sites and the mechanistic and evolutionary relationships between dioxygenase and monooxygenase reactions in these enzymes.

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