Aromatic hydrocarbon dioxygenases in environmental biotechnology

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Aromatic hydrocarbon dioxygenases belong to a large family of Rieske non-heme iron oxygenases. The dioxygenases have a broad substrate specificity and catalyze enantiospecific reactions with a wide range of substrates. These characteristics make them attractive synthons for the production of industrially and medically important chiral chemicals and also provide essential information for the development of bioremediation technology.

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Abbreviations
NDO naphthalene 1,2-dioxygenase
PCB polychlorinated biphenyl

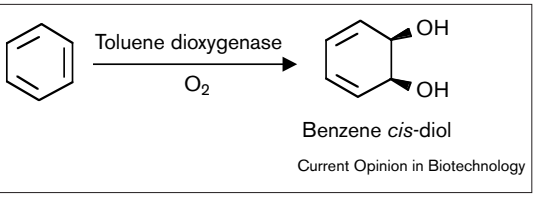
Introduction

Bacterial aromatic hydrocarbon dioxygenases are multicomponent enzyme systems that add dioxygen to the aromatic nucleus to form arene *cis*-diols, as shown in Figure 1 for the oxidation of benzene to *cis*-1,2-dihydroxycyclohexa-3,5-diene (benzene *cis*-diol) by toluene dioxygenase [1]. Today, more than 300 vicinal arene *cis*-diols have been identified as the initial reaction products in the bacterial oxidation of aromatic hydrocarbons ranging in size from toluene to benzo[a]pyrene. In almost all cases, the arene *cis*-diols produced are single enantiomers [2,3•,4••].

There are two principal reasons for the current interest in the structure and function of aromatic hydrocarbon dioxygenases. First, aromatic hydrocarbons are common contaminants of soils and groundwaters. The removal of these compounds from polluted environments by microorganisms represents a potential solution to the environmental problems posed by these pollutants. Dihydroxylation of the aromatic ring is a prerequisite for oxidation of the aromatic nucleus by bacterial ring-fission dioxygenases [5]. Aromatic hydrocarbons contain only the elements of carbon and hydrogen. Studies on the multicomponent dioxygenases that oxidize aromatic hydrocarbons to vicinal arene *cis*-diols are of paramount importance in providing the scientific foundations necessary for the development of bioremediation technology [6•,7].

The second reason for interest in aromatic hydrocarbon dioxygenases is related to industry's search for environmentally benign procedures for the synthesis of useful chemicals. The dioxygenases fulfill this 'green chemistry' requirement as they are a source of new enantiopure arene

Figure 1



Oxidation of benzene to benzene *cis*-diol by toluene dioxygenase.

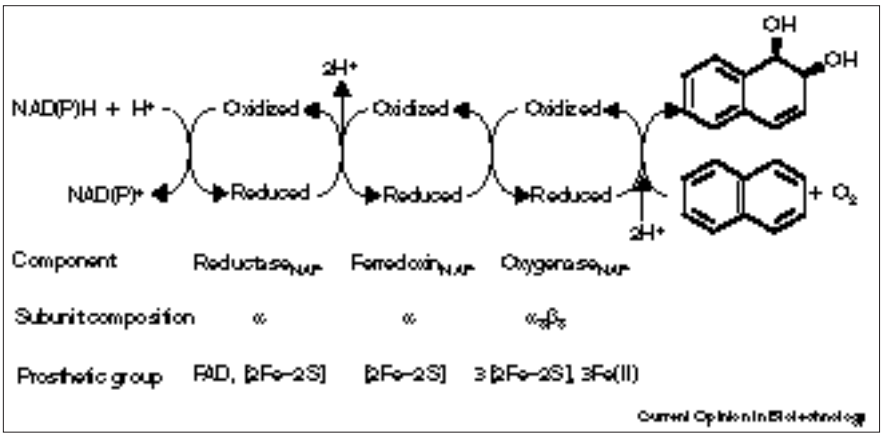
cis-diols that are not attainable by conventional chemical synthesis. The diols are used as synthons in the development of new compounds of industrial and medical importance. Two examples are polyphenylene and prostaglandin E2α. The extent of interest in arene *cis*-diols, including those formed from aromatic acids, in asymmetric synthesis can be seen in the publication of several extensive reviews in this field between 1990 and 1999 [3•,4••,8–11]. Here we focus on the classification of aromatic hydrocarbon dioxygenases and the role of these enzymes in biocatalysis and biodegradation.

Organization, nomenclature and classification

Aromatic hydrocarbon dioxygenases belong to a large family known as aromatic-ring-hydroxylating dioxygenases (last reviewed in 1997) [12,13]. All members of this family have one or two electron transport proteins preceding their oxygenase components, as shown in Figure 2 for the naphthalene 1,2-dioxygenase (NDO) system. At present, the family also contains monooxygenases and enzymes that do not hydroxylate aromatic rings. The crystal structure of naphthalene dioxygenase has confirmed the long-suspected presence of a Rieske [2Fe–2S] cluster and mononuclear iron in each α subunit [14••]. We now suggest a change in the family name to Rieske non-heme iron oxygenases and reserve the designations dioxygenase and monooxygenase for systems that are unambiguous. Each oxygenase system should be identified by an EC number. For example, naphthalene 1,2-dioxygenase (EC 1.14.12.12) and 4-methoxybenzoate monooxygenase (EC 1.14.99.15). These assignments and the systematic names follow the 1991 suggestions of Locher *et al.* [15] and the 1992 recommendation of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology [16]. Members of the Rieske non-heme iron oxygenase family are currently classified according to the properties of their electron transport components [17]. The major problem with this scheme is the absence of any information on the oxygenases themselves and this can be confusing. For example, 2-oxo-1,2-dihydroquinoline monooxygenase and benzoate dioxygenase are

Figure 2

Organization of the components of the naphthalene dioxygenase system from *Pseudomonas* sp. strain NCIB 9816-4. We recommend elimination of the terms iron-sulfur protein and ISP_{NAP}, which were introduced by one of us (DT Gibson) in 1979 [62] to differentiate ferredoxins from iron-sulfur proteins that have enzymatic activity. The trivial name oxygenase with the appropriate subscript as shown above should be pertinant to all Rieske non-heme iron oxygenases.



assigned to class IB as both oxygenase systems have only an iron-sulfur flavoprotein reductase preceding their respective oxygenase components. This seems inappropriate because one enzyme is a monooxygenase. In addition, the quinoline oxygenase component has an $(\alpha)_6$ subunit composition in contrast to the $(\alpha\beta)_3$ subunit composition of benzoate dioxygenase. The Batie [17] classification system serves a useful purpose with regard to discussions on the evolution of different oxygenase systems. It has, however, outlived its usefulness as a classification scheme for multicomponent non-heme iron oxygenases. It is now clear that the catalytic components (α subunits) of all Rieske non-heme iron oxygenases are related to each other and several publications have shown this in sequence alignment or dendrogram form. In 1996, Werlen *et al.* [18] identified four dioxygenase families (Naphthalene, Toluene/Benzene, Biphenyl, and Benzoate/Toluate) based on amino acid sequence comparisons of the catalytic oxygenase α subunits. In addition, Nakatsu *et al.* [19] pointed out that oxygenases that only have α subunits are related to each other and form a distinct lineage separate from other members of the superfamily. Figure 3 shows that the oxygenase components can be considered to be a superfamily with clearly identifiable families, which we describe below.

In general, the clustering of oxygenases into families correlates with the native substrates oxidized by the members. The Toluene/Biphenyl Family (Figure 3) includes enzymes for the degradation of toluene, benzene, isopropylbenzene, chlorobenzenes, and biphenyl from both Gram-negative and Gram-positive organisms. The Naphthalene Family consists of enzymes for the degradation of naphthalene and phenanthrene. A branch from this group links it to a small group of enzymes for nitrobenzene and nitrotoluene degradation. All enzymes in the Naphthalene Family are from Gram-negative organisms. The Benzoate Family is a diverse group consisting of enzymes that oxidize aromatic acids (benzoate, toluate, anthranilate, 2-chlorobenzoate, trichlorophenoxyacetate, and isopropylbenzoate). Oxygenases of the Toluene/Biphenyl, Naphthalene, and Benzoate Families catalyze dioxygenation reactions on their native substrates and are heteromultimers consisting of α and β subunits.

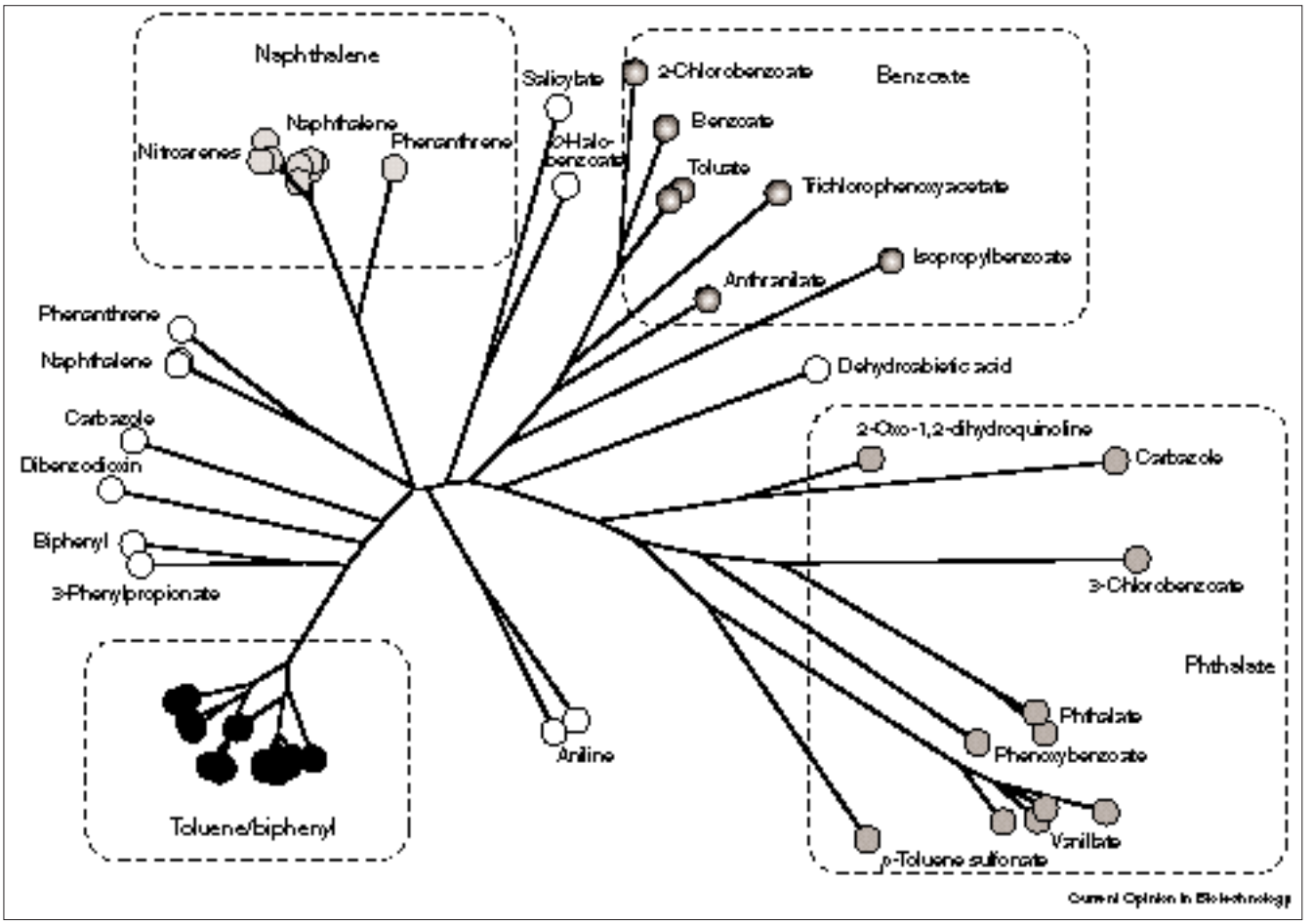
The Phthalate Family is a large and diverse group of enzymes that have an α_n subunit configuration. This family contains enzymes that oxidize aromatic acids (vanillate, phthalate, 3-chlorobenzoate, phenoxybenzoate, and *p*-toluene sulfonate). An offshoot branch of this group includes enzymes that oxidize carbazole and 2-oxo-1,2-dihydroquinoline. The Phthalate Family contains both dioxygenases and monooxygenases and represents the most diverse group in terms of amino acid sequence and primary substrate.

Several oxygenases do not cluster with any of these families. Enzymes for aniline, dibenzodioxin, 3-phenylpropionate, salicylate, *o*-halobenzoate, and dehydroabeitate oxygenation, as well as a second carbazole dioxygenase, a biphenyl dioxygenase, two naphthalene dioxygenases from *Rhodococcus* sp. and a phenanthrene dioxygenase from *Nocardioides* sp. are related but do not fall within any of the clusters on the phylogenetic tree (Figure 3).

Structure–function relationships

The structure of NDO was reported in 1998 and now serves as the prototype for this class of enzymes [14••]. NDO is an $\alpha_3\beta_3$ hexamer and each α subunit contains a Rieske [2Fe–2S] cluster and non-heme iron(II) coordinated by His208, His213, Asp362 (bidentate) and a water molecule. This is in contrast to the proposed consensus sequence ($Glu–X_{3–4}–Asp–X_2–His–X_{4–5}–His$) based on site-directed mutagenesis of the toluene dioxygenase α subunit [20]. Asp205 in NDO plays a role in the transfer of electrons between the Rieske [2Fe–2S] cluster in one α subunit and the non-heme iron (II) center at the active site in an adjacent subunit [21]. The conserved glutamate residue might play a role in α–α subunit interactions [14••].

Figure 3



Phylogenetic tree of the α subunits of Rieske non-heme iron oxygenases. Proteins were chosen based on the availability of complete deduced amino acid sequences in the Genbank database and published identification of their native substrates. For clarity, bacterial names, protein designations and accession numbers have been omitted. The tree was constructed using the AllAll program of the Computational Biochemistry Research Group [63].

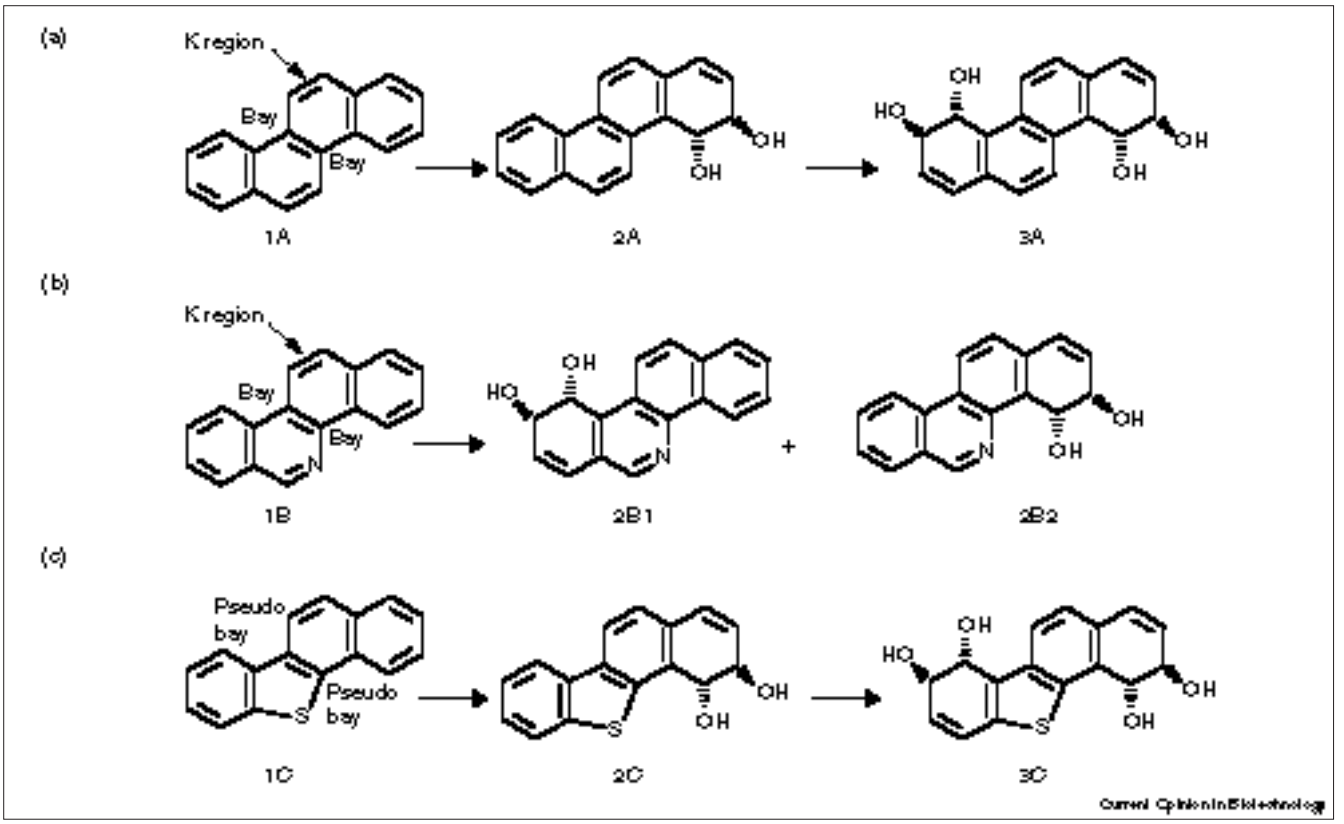
The coordinating ligands to the non-heme iron in NDO form a 2-His-1-carboxylate facial triad [22], which is present in all Rieske non-heme iron oxygenases and several other oxidoreductases. NDO resembles cytochrome P450 in its ability to catalyze monohydroxylation, sulfoxidation, desaturation, dehydrogenation, and *O*- and *N*-dealkylation reactions [2]. P450 does not, however, catalyze the enantiospecific *cis*-hydroxylation of arenes and NDO does not oxidize alkenes to epoxides. Recent model studies with synthetic non-heme iron complexes and hydrogen peroxide suggest the formation of an Fe(III) peroxo species that can oxidize olefins to *cis*-diols [23•]. It is not known if this system can add dioxygen stereospecifically to aromatic hydrocarbons. It seems probable that all Rieske non-heme iron oxygenases will, as with cytochrome P450, have the same reaction mechanism with substrate specificity being determined by amino acids at the active site.

Substrate specificity of Rieske non-heme iron dioxygenases

Biocatalysis

Mutants that do not express arene *cis*-diol dehydrogenase have played a major role in determining the regioselectivity and enantioselectivity of different dioxygenases. A comprehensive 1998 review lists the structure, enantiopurity and absolute configuration of 163 vicinal *cis*-diols formed collectively by toluene, naphthalene, biphenyl and benzoate dioxygenases [3•]. New structures appear regularly in the literature and it is estimated that the total number of arene *cis*-diols formed by a small number of dioxygenases probably exceeds 300 [3•,4••]. In the current review period, a new class of metabolites produced by *Sphingomonas yanoikuyae* B8/36 (biphenyl dioxygenase) are the *bis-cis*-diols (*cis*-tetraols) formed from polycyclic aromatic hydrocarbons and related heterocyclic compounds [24•]. In all cases, dihydroxylation occurs at the regions of lower electron density (bay

Figure 4



Oxidation of **(a)** chrysene (1A), **(b)** benzo[c]phenanthridine (1B) and **(c)** benzo[b]naphtho[2,1-d]thiophene (1C) to *mono-cis*-dihydrodiols (2A, 2B1, 2B2 and 2C) and *bis-cis*-dihydrodiols (3A and 3C) by *Sphingomonas yanoikuyae* B8/36.

or pseudobay regions) shown in Figure 4. *S. yanoikuyae* B8/36 also oxidizes the tricyclic azaarenes acridine and phenazine to *cis*-tetraols [25]. These products, together with those shown in Figure 4, represent a unique source of chiral synthons for asymmetric synthesis of industrially and medically useful chemicals.

Although many arene *cis*-diols can be produced in significant quantities, only a few have been used in organic synthesis. 1-chloro-(2*S*,3*S*)-dihydroxycyclohexa-4,6-diene (chlorobenzene *cis*-2,3-diol) is a versatile synthon produced by *Pseudomonas putida* F39/D (toluene dioxygenase). Explicit details of the synthesis of this compound have been published [26•]. In addition, several arene *cis*-diols and primary synthons derived from them are listed by Aldrich. A recent review by Hudlicky *et al.* [4••] provides his rationale for synthesis based on the types of bond-forming reactions that are available in arene *cis*-diols and how these reactions can be utilized to approach the desired target chiral molecules. Examples given include the synthesis of conduritols, conduramines, inositols, pinitols, the enantiodivergent synthesis of erythroses and several natural products including pancristatin, lycoricidine, narciclasine and morphine.

Commercial applications

1999 was a quiet year in terms of the use of Rieske non-heme iron oxygenases or their reaction products in commercial processes. Crixivan (Indinavir) is an orally-active HIV protease inhibitor. It has five chiral centers and a key intermediate in its synthesis is (-)-*cis*-(1*S*,2*R*)-1-aminoindan-2-ol. This compound can be synthesized directly from enantiopure *cis*-(1*S*,2*R*)-dihydroxyindan. Toluene dioxygenase oxidizes indene to produce the desired enantiomer [27]; however the enantiopurity is low (30% enantiomeric excess [ee]). Studies with a recombinant *Escherichia coli* strain expressing toluene dioxygenase and dihydrodiol dehydrogenase led to the production of enantiopure *cis*-(1*S*,2*R*)-dihydroxyindan (ee > 99%) as a result of selective dehydrogenation of the *cis*-(1*R*,2*S*)-enantiomer. The yields of *cis*-(1*S*,2*R*)-dihydroxyindan under optimal transformation conditions were said to be 'encouraging' [28]. Transformation of *Rhodococcus erythropolis* strain SQ1 with a cosmid clone from *Rhodococcus* strain I-24 led to a transformant that produces *cis*-(1*R*,2*S*)-dihydroxyindan. The genes catalyzing the formation of the *cis*-(1*R*,2*S*)-enantiomer are thought to be the naphthalene-inducible naphthalene dioxygenase genes first detected in *Rhodococcus* sp. strain I-25 [29].

The ability of naphthalene dioxygenase to oxidize indole through a putative *cis*-diol to indoxyl which autooxidizes to indigo was reported in 1983 [30]. Since that time, scientists at Genencor International (Palo Alto, CA) have constructed a recombinant strain of *E. coli* that efficiently converts glucose to indole. This strain expresses the *nahAaAbAcAd* genes from *P. putida* G7 that encode the naphthalene dioxygenase system. The current status of this research and development program is unknown.

Perhaps the most exciting biocatalysis breakthrough in 1999 was Banwell's synthesis of L-ascorbic acid (vitamin C) from chlorobenzene *cis*-dihydrodiol. This is the first synthesis of vitamin C from a non-carbohydrate precursor [31].

Biodegradation

Requirements for the design of bacteria for use in bioremediation have been described in recent review articles [6•,7]. Because many xenobiotic compounds have been present in the environment for a very short time, bacteria have not evolved efficient pathways for their degradation, and the construction of pathways may be required. A detailed understanding the enzymology and molecular biology of known pathways is an essential starting point for these studies.

Construction of hybrid dioxygenases and analysis of their substrate specificities has resulted in two sets of apparently conflicting results regarding the function of dioxygenase β subunits. One set of data indicates that the α subunit alone controls substrate specificity in dioxygenases of the Naphthalene Family [32,33] and Toluene/Biphenyl Family [34,35]. Other studies indicate that the β subunit contributes significantly to substrate specificity in biphenyl and toluene dioxygenases [36–38]. The crystal structure of naphthalene dioxygenase indicates that there are no β subunit residues near the active site of the enzyme [14••]. It will require the crystal structure of a Toluene/Biphenyl Family member to determine whether amino acids in the β subunit of these enzymes interact directly with substrates or influence substrate specificity at a distance.

Results from a large number of recent studies using *in vivo* and *in vitro* approaches for the generation of hybrid and mutant dioxygenases have confirmed and reconfirmed the conclusion that a very small number of amino acid differences in biphenyl dioxygenases are responsible for large differences in polychlorinated biphenyl (PCB) congener specificity [39•,40–44]. In most cases, the best enzymes generated in these studies have the combined degradation abilities of the two very closely related parental enzymes; however, a gene shuffling approach resulted in variants that acquired novel PCB-oxidizing activities [39•]. We predict that gene shuffling with less-closely related enzymes with demonstrated PCB-oxidizing capacities (such as naphthalene dioxygenase [45]) will result in a new series of enzymes with unique PCB-oxidizing capacities.

Brühlmann and Chen [46•] demonstrated that many of the potentially toxic PCB metabolites generated by hybrid or mutant dioxygenases are not completely metabolized and pointed out that a whole-pathway approach will be necessary to generate useful strains for the mineralization of PCBs. An *in vivo* approach to whole-pathway engineering primarily for the degradation of single-ring chlorinated aromatics has been explored extensively by Reineke [47••] and a recent contribution by Hrywna *et al.* [48•] describes the *in vitro* construction of strains that efficiently degrade 2-chlorobiphenyl and 4-chlorobiphenyl to metabolites that can enter the TCA cycle. Studies of the specificity of diol dehydrogenases and *meta*-cleavage pathway enzymes from various sources [45,49,50] have identified some broad-specificity enzymes that might be useful for the construction of complete pathways for the degradation of PCBs by either *in vivo* or *in vitro* methods.

In the past decade, sphingomonads have emerged as versatile organisms capable of degrading a wide range of environmental pollutants. In most other organisms, the genes encoding Rieske non-heme iron oxygenases are usually found in tightly regulated single transcriptional units, which are often part of a larger regulon. In contrast, the genes encoding the dibenzo-*p*-dioxin dioxygenase system in *Sphingomonas* sp. strain RW1 are located on three different segments of the chromosome. Sequence analysis showed the presence of three additional oxygenase (α) subunits of unknown function [51]. A similar situation exists in *Sphingomonas yanoikuyae* B1. This strain, once thought to contain only the *bphA1A2A3A4* genes encoding a biphenyl dioxygenase system [52], contains no less than five oxygenases (*bphA1*[a–e], *bphA2*[a–e]) scattered on a 40 kb DNA fragment [53,54]. Insertional inactivation of each *bphA1* gene did not yield a bph$^-$ phenotype (GJ Zylstra, personal communication). The complete nucleotide sequence of the PNL1 plasmid of *S. aromaticivorans* F199 shows the presence of six *bphA1*[a–f], *bphA2*[a–f] dioxygenase homologs scattered throughout the plasmid [55•]. Only single copies of *bphA3* and *bphA4* have been detected in *S. yanoikuyae* B1 and *S. aromaticivorans* F199. This suggests that the reductase and ferredoxin components can serve as electron transport systems for multiple dioxygenases [54,55•,56].

There is little doubt that ongoing biodegradation and genetic studies provide essential information for the construction of robust organisms that may be of use in the bioremediation of polluted environments. This theme is echoed throughout several papers in the November 1999 issue of the *Journal of Industrial Microbiology and Biotechnology* [57], an issue devoted entirely to 'The Genus *Sphingomonas*'.

Two critical steps in the biodegradation of aromatic compounds precede the dioxygenation of the aromatic ring: chemotaxis to the aromatic compound, and uptake of the compound. Although it has been generally accepted that

aromatic hydrocarbons can diffuse across bacterial membranes, a surprising number of open reading frames that appear to encode membrane proteins similar to porins or transporters have been identified in gene clusters for aromatic compound degradation. The function of these proteins remains to be determined. In addition, solvent efflux systems have also been identified and characterized [58,59]. A recent study demonstrated chemotaxis to naphthalene and biphenyl by *Pseudomonas putida* G7 [60], and a gene encoding a membrane-bound transducer protein required for chemotaxis to naphthalene is located on the NAH7 plasmid with genes for naphthalene degradation [61•]. These aspects of biodegradation may be important to consider when constructing strains for bioremediation purposes.

Conclusions

Multicomponent aromatic hydrocarbon dioxygenases add dioxygen to the aromatic ring to form arene *cis*-diols. They are currently members of the aromatic-ring-hydroxylating dioxygenase superfamily and are classified by the number and properties of the electron transport proteins that precede the catalytic oxygenase component. For reasons presented here, it now seems logical to use the designation Rieske non-heme iron oxygenases for the superfamily and to classify oxygenases into families based on their native substrates and the amino acid sequences of their respective α subunits. Outliers that do not fall into a family category are easily identified by this system.

The similarity of the reactions catalyzed by naphthalene dioxygenase and cytochrome P450 are noted, as is a biomimetic model for the oxidation of olefins to *cis*-diols. The remarkable number of substrates oxidized to single enantiomers by toluene and naphthalene dioxygenases has led to their use in asymmetric synthesis of many biologically active products. Only a few of these have been targeted for commercial use. The aromatic hydrocarbon dioxygenases, together with many other Rieske non-heme iron oxygenases, initiate the biodegradation of a wide range of environmental pollutants. The pros and cons of strategies used to develop hybrid dioxygenases for improved polychlorinated biphenyl degradation are being evaluated.

Finally, the recent emergence of sphingomonads as a source of new Rieske non-heme iron oxygenases with novel organizational and regulatory properties complements the list of organisms that have potential use in the development of bioremediation technology.

Update

Recent site-directed mutagenesis studies with naphthalene dioxygenase show the importance of Phe352 in determining the regioselectivity of the enzyme with naphthalene, biphenyl, and phenanthrene as substrates [64]. Further structural studies show the presence of an indole-oxygen adduct at the active site of naphthalene dioxygenase [65].

Acknowledgements

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