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MINIREVIEW

Biodegradation of Dibenzo-*p*-dioxin and Dibenzofuran by Bacteria

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Polychlorodibenzofurans and polychlorodibenzo-*p*-dioxins are among the most toxic xenobiotics released into the biosphere and the cause of significant public concern because of their apparent ubiquity - albeit at low levels- in food and environment. Several bacteria able to degrade nonchlorinated dioxin and dibenzofuran and in some cases to attack chlorinated analogues have recently been isolated. This opens up the possibility that bioremediation processes may ultimately be developed to eliminate these toxic compounds from contaminated sites. In this review we summarize current knowledge on the genetics and biochemistry of dioxin and dibenzofuran degradation by *Sphingomonas* sp. RW1, a gram-negative bacterium, and highlight the unusual nature of the genetic organization of these pathways.

Biodegradation of dibenzofuran and dibenzo-*p*-dioxin

Aromatic and aliphatic hydrocarbons, and particularly synthetic derivatives thereof, constitute major environmental pollutants. Microorganisms are major agents of carbon mineralization and are largely responsible for eliminating such compounds from the biosphere. They are, however, unable or poorly able to catabolize effectively the more toxic industrial pollutants that have entered the environment. The effective exploitation of microbial catabolic activities in biotechnological processes to detoxify and destroy the more recalcitrant environmental pollutants requires that we build a knowledge base on relevant catabolic processes in the environment, and the ecological factors which influence their efficiency, so that rational strategies to optimize required activities can be developed from an understanding of the critical metabolic processes.

Microbial degradation of monocyclic aromatics such as benzene, toluene and their chloroderivatives (Table 1) has been intensively studied (73), and the various metabolic strategies and mechanisms exploited by microorganisms in the utilization of these compounds as carbon and energy sources have been the subject of numerous reviews (30, 55). More recently, microbial metabolism of bicyclic and polycyclic aromatics, such as polychlorinated biphenyls (PCB), dibenzo-*p*-dioxins (DBD), dibenzofurans (DBF) and polyaromatic hydrocarbons (PAH), have received increasing attention (1, 67). Whereas microbial transformations of PCBs and PAHs have been characterized extensively (5, 24, 27, 32, 33, 37, 38), and research currently focusses on improvement of the catabolic properties of the enzymes and direct applications (24, 41, 63), progress on microbial transformation of DBF and DBD has been slow due to the difficulty of isolating microorganisms able to attack these compounds, the lack of commercially available, environmentally-relevant chlorinated congeners, and the problems of studying these highly toxic compounds. It is well known that some DBD congeners, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), are extremely toxic: TCDD is 500 times higher than cyanide and the ingestion of 1 mg/kg is lethal for adult humans. TCDD is also cancerogenic and teratogenic. Its high chemical stability and hydrophobicity make it very persistent and difficult to remove from contaminated sites (25).

Chlorinated DBDs and DBFs are created as byproducts or trace contaminants in a variety of chemical processes (manufacture of herbicides, insecticides and fungicides, paper pulp bleaching and metal smelting), as well as in combustion processes (burning of solid wastes in incinerators, motor vehicle exhausts and accidental fires). The 1976 Séveso example in which an accident in a chemical plant manufacturing the herbicide 2,4,5-trichlorophenol

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Table 1. Properties of the different polypeptides involved in the dibenzo-*p*-dioxin and dibenzofuran pathways

Protein	Function	Molecular mass (kDa)	Gene	Similarities			Reference
				% indentity[a]	Netserv citation	Species	
DxnA1	Dioxin dioxygenase (α subunit)	47.7	*dxnA1*[b]	Nter sequence : no significant similarity			
DxnA2	Dioxin dioxygenase (β subunit)	19.7	*dxnA2*[b]	No sequence yet available			
Fdx1	Ferredoxin (electron transport)	12	*fdx1*	44%	FER6-RHOCA	*Rhodobacter capsulatus*	50
				42%	FER-CAUCR	*Caulobacter crescentus*	69
				39%	PUTX-PSEPU	*Pseudomonas putida*	54
				39%	TERP-PSESP	*Pseudomonas* sp.	53
				38%	THCC-RHOSO	*Rhodococcus* sp. NI86/21	48
RedA1	Reductase (electron supply)	44	*redA1*	Nter sequence : no significant similarity			
RedA2	Reductase (electron supply)	44	not described	Nter sequence : no significant similarity			
DbfB	2,2'3-trihydroxybiphenyl-1,2-dioxygenase	32	*dbfB*	34%	BPHC-PSEPS	*Pseudomonas pseudoalcaligenes*	29
				31%	BPHC-PSES1	*Pseudomonas* sp. KKS02	40
				29%	BHC1-RHOGO	Rhodococcus globerulus P6	7
				28%	BPHC-BURCE	*Burkholderia cepacia* LB400	37
				27%	TODE-PSEPU	*Pseudomonas putida*	72
H1	Hydrolase	31	Not described	Nter sequence : no significant similarity			
H2	Hydrollase	29	Not described	Nter sequence : no significant similarity			

[a] Percentage of amino-acids that are identical when sequences are aligned with sequences listed in the EMBL / GENBANK / DDBJ data banks by using the algorithm of Needleman and Wunsch via Blitz program of the European BioInformatic Institute facilities.

[b] Preliminary results.

resulted in the formation and discharge over the italian town of large quantities of TCDD, is well known (36). More recently, a fire accident in a main building of Dusseldorf Airport in Germany in December 1995, resulted in the production of smoke containing TCDD.

Destruction of DBFs and DBDs can be accomplished non-selectively by different chemical processes such as photolysis (18) or oxidation by strong oxidants such as ruthenium tetroxide (8), or by microbial reductive dehalogenation (11, 46, 66). However, in most cases, DBDs and DBFs are widely dispersed in the environment at low absolute but toxicologically high concentrations. Chemical or thermal treatment of such contamination is unaffordable and environmentally unacceptable. In principle, therefore, the highly specific, and hence for non-target compounds and the biota non destructive approach of biodegradation, is an attractive prospect. Until now, however, no microorganisms able to mineralize highly chlorinated dibenzofurans and dibenzo-p-dioxins have been isolated. Both the ether bridges between the two aromatic rings and the chloro-substituents determine the resistance of such compounds to biochemical attack. On the other hand, bacteria able to aerobically degrade unchlorinated and monochlorinated congeners have recently been isolated, and it is known that anaerobic microbial consortia can reductively dehalogenate a wide range of polychlorinated organic compounds. There is therefore a distinct possibility that a combination of an initial reductive dehalogenation step followed by aerobic degradation of the carbon skeleton may prove to be successful.

The extensive arsenal of catabolic enzymes of the bacterium *Sphingomonas* sp. RW1

Several bacteria able to attack dibenzofuran and dibenzo-*p*-dioxin under aerobic conditions have been isolated during the last few years, some of which are also able to mineralize these molecules. *Pseudomonas* sp. strain HH69 isolated from the Elbe River (26, 35), *Terrabacter* sp. strain DPO1361 isolated by Strubel *et al.* (60, 61), *Sphingomonas* sp. RW1, isolated from the river Elbe in Germany (71), *Staphylococcus auriculans* DBF 63 characterized by Monna *et al.* (47), *Pseudomonas azelaica* HPB1 (59) and *Terrabacter* sp. DPO360 (58) were shown to transform dibenzofuran to 2,2',3-trihydroxybiphenyl and salicylic acid, all by a novel angular dioxygenation mechanism at the 4,4a position of dibenzofuran. The physiologically, biochemically and genetically best characterized strain (45, 71), RW1, has been shown to use as sole sources of carbon unchlorinated dibenzofuran and dibenzo-*p*-dioxin. It is also able to transform some mono- and dichlorinated congeners such as 1- and 2-chlorodibenzo-*p*-dioxin, 2, 3-dichlorodibenzofuran and 2,3-dichlorodibenzo-*p*-dioxin, but cannot mineralize and grow with these compounds as sole carbon sources (70) due to non-permissive steps in the degradative pathways of this strain for the metabolites formed from these analogues. For example, RW1 is unable to metabolise 4,5-dichlorocatechol, a probable central intermediate in the biodegradation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. It may, however, be possible to enable RW1 to mineralize some chlorinated congeners, either by recruiting of additional enzymatic activities which circumvent the non-permissive steps, or by providing such activities through the development of metabolically complementary mixed cultures. This latter strategy was recently documented with the development of a co-culture, consisting of the dibenzofuran degrading strain *Sphingomonas* sp. RW1 and the 3,5-dichlorosalicylate degrading strain *Burkholderia* sp. JWS, able to completely mineralize 4-chloro-dibenzofuran (2).

The catabolic pathways for dibenzofuran and dibenzo-*p*-dioxin in RW1 proposed by Wittich *et al.* (71) are presented in Fig. 1. The so-called "upper" pathway consists of the transformation of dibenzofuran and dibenzo-*p*-dioxin to salicylate or catechol, respectively, whereas the *meta*-cleavage pathway is responsible of the transformation of these central metabolites to Krebs cycle intermediates. Salicylate may be channelled into either a catechol or a gentisate pathway. The initial reaction of the upper pathway involves the incorporation of two atoms of oxygen in one of the aromatic rings in an angular dioxygenation of dibenzofuran and dibenzo-*p*-dioxin, leading to the formation of phenolic hemiacetals which spontaneously convert to 2,2',3-trihydroxybiphenyl and 2,2',3-trihydroxydiphenyl ether, respectively. The dihydroxylated rings of these two molecules are then *meta*-cleaved by an extradiol dioxygenase yielding 2,2'-dihydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (2-OH-HOPDA) and the 6-(2-hydroxyphenyl)ester of 2-hydroxymuconic acid, respectively. The former is further transformed by a hydrolase to salicylic acid whereas the latter spontaneously converts to catechol. Little information on the lower pathways are presently available. Typically, catechol is subjected to *meta*-cleavage in a dioxygenation reaction leading to the formation of 2-hydroxymuconic acid semialdehyde, which is in turn transformed by a hydrolase to 2-hydroxypenta-2,4-dienoate. This is attacked by a specific hydratase, yielding 4-hydroxy-2-oxovalerate

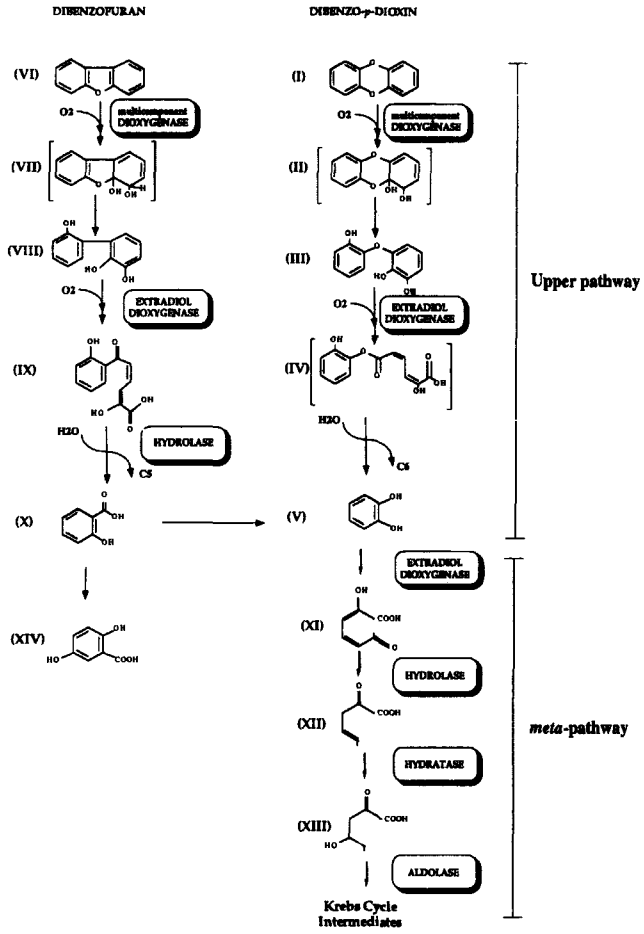

Fig. 1. Proposed catabolic pathways for dibenzofuran and dibenzo-*p*-dioxin in *Sphingomonas* sp. RW1. Chemical designations are (I) dibenzo-*p*-dioxin, (II) dibenzo-*p*-dioxin-*cis*-dihydrodiol, (III) 2,2',3-trihydroxydiphenyl ether, (IV) 2-hydroxy-6-oxo-6-(2-hydroxyphenoxy)-hexa-2,4-dienoate, (V) catechol, (C6) 2-hydroxy-*cis*,*cis*-muconate, (VI) dibenzofuran, (VII) dibenzofuran-*cis*-dihydrodiol, (VIII) 2,2',3-trihydroxybiphenyl, (IX) 2-hydroxy-6-oxo-6-(2-hydroxyphenyl-hexa-2,4-dienoate), (X) salicylate, (C5) 2-hydroxypentadienoate, (XI) 2-hydroxymuconate semialdehyde, (XII) 2-hydroxypenta-2,4-dienoate, (XIII) 4-hydroxy-2-oxovalerate and (XIV) gentisate. Unstable compounds which spontaneously transform to other products are indicated in brackets.

which is then transformed by an aldolase to products that enter the Krebs cycle (Fig. 1). Such a pathway is expected to occur in *Sphingomonas* sp. RW1.

Some of the enzyme of these pathways have now been purified and characterized biochemically, and in some cases genetically. First, we shall present and discuss data concerning the initial stereospecific angular dioxygenation of one of the aromatic rings by a dioxin dioxygenase, and then provide information on the other steps of the upper pathway.

The initial dioxygenase is a three-component enzyme with an atypical electron supply system

The stereospecific angular dioxygenation of dibenzofuran and dibenzo-p-dioxin is carried out by a dioxin dioxygenase, which has been purified from RW1 and characterized by Bünz and Cook (13). Crude cell extracts exhibited relatively low levels of enzyme which was unstable (80% loss of activity after storage at 4°C for 24 h). A nonlinear activity: protein concentration relationship was observed, suggesting that the enzyme is a multicomponent complex. The properties of the five polypeptides purified by Bünz and Cook (13) are presented in Table 1. In fact, the enzymatic complex consists only of four of these polypeptides, as is the case for other three-component dioxygenases acting as ring hydroxylases of monocyclic or bicyclic aromatics (15). The initial dioxygenase itself is a $\alpha_2\beta_2$ heterodimer of two of these polypeptides, which requires electrons in order to ring hydroxylate its substrate. These electrons derive from NADH plus H^+ which is oxidized to NAD^+ by a specific reductase, which in turn transfers them to a ferredoxin, which then reduces the large subunit of the initial dioxygenase (Fig. 2).

Two reductases (components A1 and A2), which are monomeric flavoproteins of about 44 kDa containing a labile flavin adenine dinucleotide cofactor, were purified and shown to be able to reduce the ferredoxin. Neither reductase contained an [Fe-S] cluster which clearly distinguishes them from reductases associated with Class III ring hydroxylating oxygenases, typified by the naphthalene dioxygenase (22) and from those associated with Class IA and Class IB enzymes (9). The N-terminal sequences of these two proteins were determined by Edman degradation (13); no significant similarity to other known flavoproteins was detected using these sequences as queries. The electron transport system associated with the dioxin dioxygenase therefore appears unusual.

The electron transfer activities of the reductases could be easily measured by reduction of some redox indicators such as dichlorophenolindophenol, or in a coupled reaction with the ferredoxin by a cytochrome *C* reduction assay. The two reductases were found to be isofunctional as they could both reduce the ferredoxin which transfers electrons to the dioxin dioxygenase in an *in vitro* reconstituted system (13). However, the A2 reductase was slightly more active (1.6-fold) than the A1 reductase in the dichlorophenolindophenol assays and, from the purification data presented by Bünz and Cook (13), seems to be present at twice the concentration of

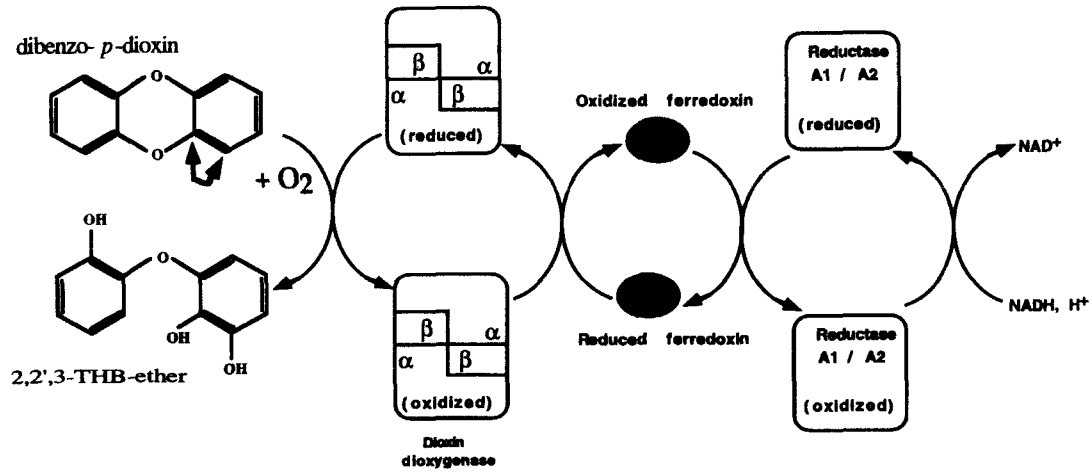

Fig. 2. The dioxin dioxygenase and its electron supply system. The reaction carried out by the multicomponent ring-hydroxylating dioxin dioxygenase and its electron transfer chain is shown. A flavoprotein reductase, RedA2, accepts electrons from NADH and transfers them *via* the ferredoxin Fdx1 to the terminal oxygenase. The reduced terminal oxygenase catalyses the angular oxidation of dibenzo-*p*-dioxin and some other aromatic substrates.

the A1 reductase in RW1 cells grown under inducing conditions for the dioxin dioxygenase. These aspects favour the A2 flavoprotein as the main (but not necessarily exclusive) reductase constituant of the electron transfer chain of the dioxin dioxygenase.

The second component of the redox chain is a 12 kDa protein which exhibits all the usual characteristics of ferredoxins: small size, acidic behaviour and presence of an [Fe-S] prosthetic group (12). From the N-terminal sequence determination of the purified ferredoxin, it was deduced that it shares some similarities with a group of electron carriers comprising the putidaredoxin of *Pseudomonas putida* (42, 54), a ferredoxin from a photosynthetic bacterium (3, 50), a ferredoxin from *Escherichia coli* (65) and the mammalian adrenodoxin (16). All these ferredoxins contain a [2Fe-2S] cluster coordinated in the polypeptide by four cysteine residues arranged in the pattern Cys-Xaa_5-Cys-Xaa_2-Cys-Xaa_n-Cys. Some of these proteins have been identified as the electron donors for monooxygenases involved in the catabolism of aromatic compounds, namely FdxP from *Caulobacter crescentus* (69), the terpredoxin from *Pseudomonas* sp. (53), the putidaredoxin from *Pseudomonas putida* (54) and the rhodocoxin from *Rhodococcus* sp. strain NI86/21 (48), which are involved in the hydroxylation of *p*-hydroxybenzoate, α-terpineol, camphor and S-ethyldipropylcarbamothioate, respectively. From a phylogenetic point of view, these ferredoxins are unrelated to the Fe-S proteins which usually function as electron donors to Class IIB ring hydroxylating dioxygenases and which contain a Rieske-type [2Fe-2S] cluster coordinated by two cysteines and two histidines. It has therefore been proposed (13) that the new multiple component dioxin dioxygenase of *Sphingomonas* sp. RW1 is a class IIA ring hydroxylating dioxygenase, according to the classification scheme of Batiè *et al.* (9) which is essentially based on the characteristics of the electron supply system. The dioxin dioxygenase is the second representative of this class, the first one being the pyrazon dioxygenase described by Sauber *et al.* (56).

In the case of the dioxygenase itself, the size of the two components, 45 kDa for the large subunit and 23 kDa for the small subunit, as well as the presence of a Rieske-type [2Fe-2S] cluster in the large subunit as revealed by ultra-violet/visible spectroscopy of the purified native protein, indicate that the structure of the dioxin dioxygenase is similar to that observed for other three-component dioxygenases (15). However, the 20 amino acid N-terminal sequence of the α subunit of the dioxin dioxygenase does not exhibit any significant similarities with other known dioxygenases (Table 1).

The activity of the dioxin dioxygenase was assayed in a reconstituted *in vitro* system. It transformed dibenzofuran to trihydroxybiphenyl, and dibenzo-*p*-dioxin to trihydroxydiphenyl ether, with concomittant consumption of one molecule of oxygen per molecule of product formed. Therefore, it appears that the same enzymatic complex initiates the catabolic pathways of both substrates (Fig. 1) in RW1. In contrast to all other known multi-ring dioxygenases, such as the toluene dioxygenase (30) and the biphenyl dioxygenase (5, 23), *in vitro* the purified dioxin dioxygenase specifically attacks the dibenzo-*p*-dioxin molecule by introducing two atoms

of oxygen at a pair of vicinal carbon atoms, one of which is involved in one of the bridges between the two aromatic rings, rather than the neighboring carbon atoms not involved in the bridges (13). These results confirmed the first descriptions of this angular attack presented by Engesser *et al.* (21) and by Wittich *et al.* (71). In addition, the dioxin dioxygenase can attack biphenyl, xanthene, fluoren-9-one and dibenzothiophene. In the latter case, the products of the reaction are presumably the sulfoxide and the sulfone of dibenzothiophene. As one of the major sources of acid rain is sulphur emission from coal combustion (31), the microbial degradation of this organosulphur compound is of interest for the potential desulphurization of coal and fossil fuels.

The dioxin dioxygenase complex appears to be very unstable due to its sensitivity to oxygen and temperature. Its purification and characterization by Bünz and Cook (13) therefore represents a significant advance and has provided important insights into its structure and activity.

Characterization of the electron transport system associated with the dioxin dioxygenase

Initial efforts to genetically analyse the dioxin catabolic pathway of RW1 met with only limited success which was restricted to the readily scored *meta*-cleavage enzymes. However, the purification of the polypeptides of the dioxin dioxygenase, the determination of their N-terminus sequences and the growing database of sequences of degradative enzymes, opened the way for new strategies. Sequence similarity searches and alignment of related protèins reveal consensus sequences in functionally critical regions. This in turn permits the design of PCR amplification strategies to generate specific oligonucleotide probes for the corresponding structural genes, for example with a degenerate forward primer based on the experimentally-determined N-terminal sequence of the purified protein and a degenerate reverse primer based on a consensus sequence found in its C-terminal region (Fig. 3A). Such a strategy was used to clone the genes specifying the reductase A2 and the ferredoxin. However, in the latter case, no well-conserved sequence at the C-terminus of this class of small proteins is found. Comparison of the sequences of the four most closely-related ferredoxins revealed, however, a stretch of six amino acids, four of which are conserved in all the proteins. From this sequence, a primer was designed which was short (17 nt) and highly degenerate (4). The PCR strategy was therefore improved by addition of a second, more selective, amplification step using a third primer.

This nested-PCR strategy is schematically presented in Fig. 3B. It involved the generation of a rather unspecific group of PCR fragments using one forward primer based on the N-terminal sequence of the protein and the highly degenerate primer based on the short conserved C-terminal sequence, the isolation of these PCR fragments, and the use of these as a template for a second PCR amplification with the same reverse primer and a new forward primer corresponding to a second re-

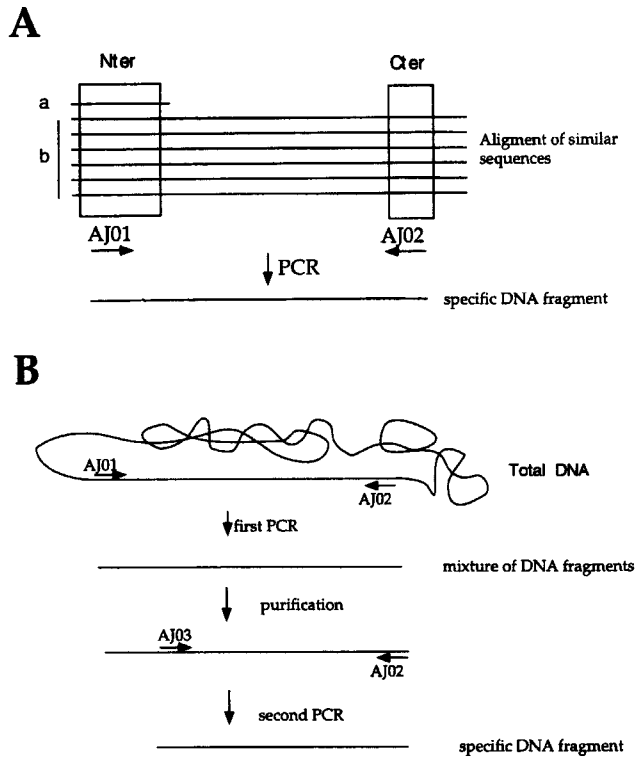

Fig. 3. PCR-based strategy developed to clone the genes involved in the dibenzofuran and dibenzo-*p*-dioxin pathways. Panel A: The N-terminal sequence of each purified polypeptide (a), determined by Edman degradation, was aligned with the sequences of similar polypeptides (b). From this alignment, a C-terminal consensus sequence was defined. A degenerate forward primer was then designed from the N-terminal consensus sequence (AJ01) and a degenerate reverse primer from the consensus C-terminal sequence (AJ 02). Panel B: To render more specific the amplification of the desired fragment, a third primer can be used in a nested-PCR reaction. Degenerate PCR primers AJ01 and AJ 03 were designed according to the N-terminal sequence of the protein while the reverse-primer AJ02 is designed from a C-terminal consensus sequence. The first PCR reaction is carried out with the AJ01 and AJ02 primers using total genomic DNA from the bacterial strain, giving a mixture of DNA fragments as a result of the high degeneracy of the primers. After purification, these products are used as template in a second, more specific reaction using degenerate primers AJ03 and AJ02.

gion of the known N-terminal sequence of the protein. This yielded a specific probe for the ferredoxin, designated *fdx1*, which was then used to screen a previously constructed pLAFR3-based cosmid library of RW1 DNA (34). A 4.6 kb DNA fragment encompassing the ferredoxin gene was thereby identified (4). Whereas the genes encoding the α and β subunits of multi-component dioxygenases have thus far always been found to be contiguous with the gene of the cognate electron carrier (Fig. 4), the *fdx1* gene of RW1 is not linked with the dioxin dioxygenase genes. Rather, it is clustered with genes apparently encoding two atypical decarboxylases/isomerases and a glutathione S-transferase (Fig. 5). A role for such proteins in the proposed dibenzo-*p*-dioxin and dibenzofuran degradative pathways of RW1 (71) has not thus far been suggested, so their functions and possible involvement in these pathways is presently unclear. It may be noted, however, that glutathione S-transferase genes are often found in operons encoding catabolic pathways for multiring aromatic compounds, as exemplified by the *bphK* gene of *Burkholderia* LB400, a strain able to degrade biphenyl and some polychlorinated biphenyls (Hofer *et al.*, 1994), although the functional role of such enzymes in these pathways remains to be established (68).

In order to verify that the *fdx1* gene encodes a functional electron carrier, it was hyperexpressed in *Escherichia coli* and the resulting protein was pu-

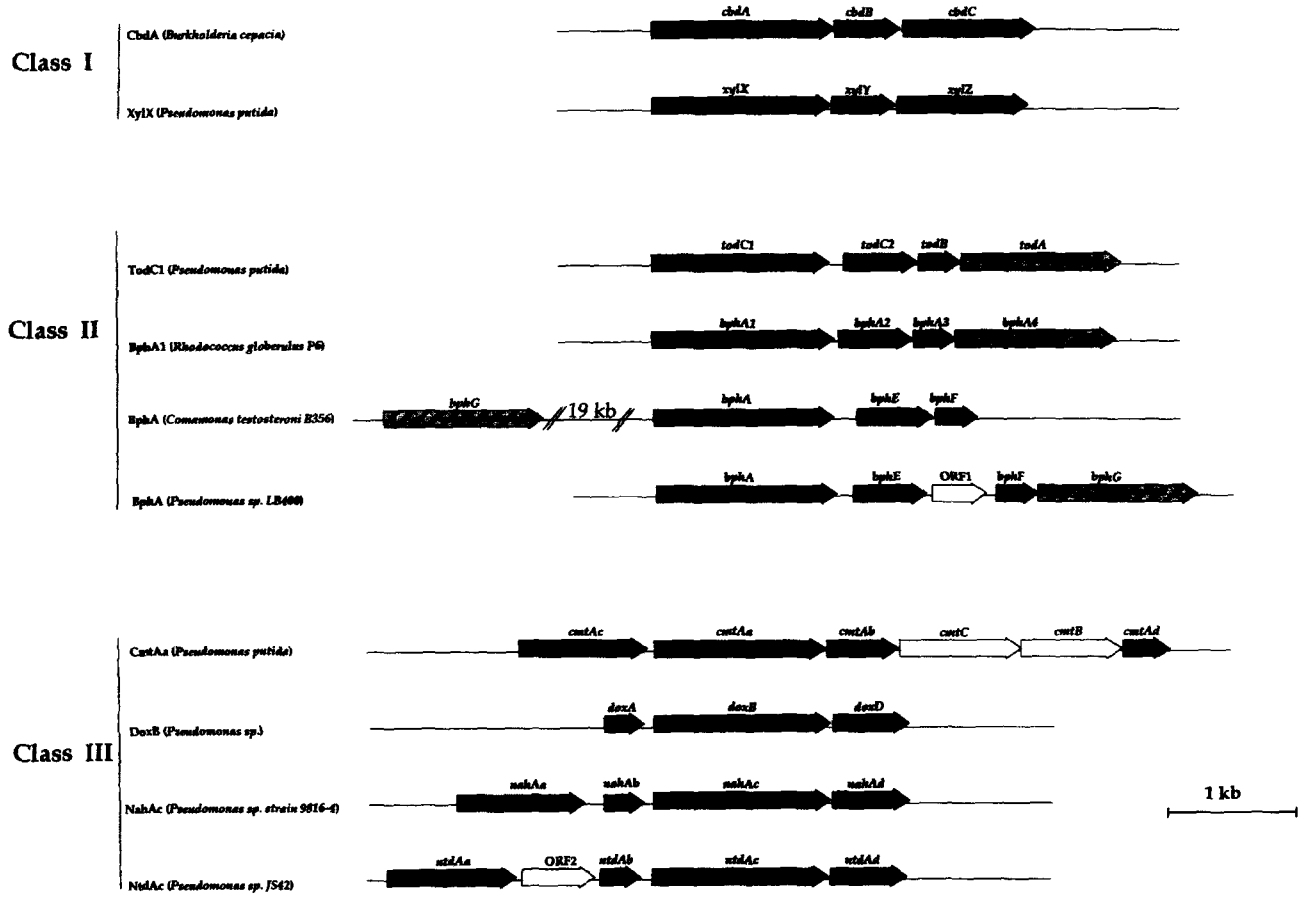

Fig. 4. Usual genetic organization of three-component dioxygenases. The organisation of the genes encoding the different polypeptides of representative hydroxylating enzymes is shown. The general name of each cluster, the name of the microorganism containing the enzymes, as well as the class (according to Batie *et al.* [9]) to which they belong, are indicated in front of the clusters. Gene names are given above each ORF and the arrows indicate the direction of transcription. Each cluster was aligned with the others, taking the gene specifying the α subunit dioxygenase as a reference. Genes are drawn according to their size and relative position. Abreviations for genes and references are: *cbdABC* for plasmid-encoded 3-chlorobenzoate-4,5-dioxygenase of *Alcaligenes* sp. BR60 (49), *xylXYZ* for benzoate and toluate dioxygenase of *Pseudomonas putida* mt-2 (51), *todC1C2BA* for toluene dioxygenase of *P. putida* F1 (72), *bphA1A2A3A4* for biphenyl dioxygenase from *Rhodococcus globerulus* P6 (5), *bphGAEF* for biphenyl dioxygenase from *Comamonas testosteroni* sp. B356 (64), *bphAEFG* for biphenyl dioxygenase from *Burkholderia* sp. LB400 (23), *doxABD* for dibenzothiophene dioxygenase from *Pseudomonas* sp. (17), *nahAabcd* for naphthalene dioxygenase from *Pseudomonas* sp. strain 9816-4 (Parales, unpublished) , and *ntdAabcd* for 2-nitrotoluene dioxygenase from *Pseudomonas* sp. JS42 (52).

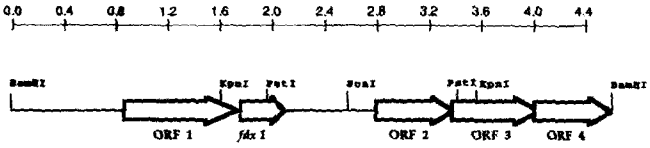

Fig. 5. Genetic organization of the locus encompassing the ferredoxin gene. The position and orientation of the different ORFs detected within the 4.5 kb *Bam*HI fragment are shown by full arrows. Transcription of the genes is from left to right. A scale bar on the bottom of the figure indicates the absolute gene sizes in kilobase pairs (kb). ORF1 and ORF2 encode two putative decarboxylases whereas ORF3 and ORF4 specify a glutathione S-transferase and a polypeptide showing no homology to known proteins, respectively.

rified and shown by ultraviolet-visible absorption spectrophotometry and EPR spectroscopy to contain a putidaredoxin-type [2Fe-2S] cluster (4). The redox potential of the cluster was determined to be -245 (± 5) mV, some 100 mV lower than the values observed for electron shuttles acting with Class IIB or Class III dioxygenases (44, 62).

A similar strategy was employed to amplify part of the gene of the reductase component of the electron supply system. In this case, the degenerate primers were designed from the N-terminal sequence of the protein (13) and from a well-conserved motif assumed to be located at its C-terminus. The probe obtained was used to screen the cosmid library of RW1 DNA and a large DNA fragment encompassing the reductase gene was identified and characterized. Comparison of the predicted amino acid sequence of the reductase with sequences in the EMBL/GENBANK/DDBJ data banks revealed some marked similarities with putidaredoxin reductase (42, 54), terpredoxin reductase (53) and rhodocoxin reductase (48), and some less well defined similarities with reductases acting with Class IIB dioxygenases. Few similarities with reductases of other classes of multicomponent non-heme iron mono- and dioxygenases were found, which is consistent with the biochemical and functional characteristics of these proteins: the stereospecificity of removal of the hydride ion from NADH by Class II A and B reductases are *pro*-S whereas all others remove the *pro*-R hydride ion (57). The cloned reductase gene was hyperexpressed in *E. coli* and the resulting reductase was purified and shown to donate electrons to the *fdx1* ferredoxin in a cytochrome c reduction assay. The availability of the two genes specifying the electron supply system of the dioxin dioxygenase will permit in the near future the construction of optimized cassettes for the heterologous expression of functional dioxin dioxygenase.

The genes specifying the different components of the initial dioxygenase are not clustered

The genes specifying the different polypeptides of three-component dioxygenase systems (Class IIB and Class III) are usually clustered (Fig. 4). In some cases, such as in the polychlorinated biphenyl degrader *Pseudomonas* sp. KKS102, the reductase gene is not contiguous with the three other genes, but nethertheless present in the same locus (39). The localization of the ferredoxin and the reductase genes on different DNA fragments, neither of which encodes the dioxin dioxygenase (4), is thus unusual. Whether or not this unusual genetic organization will turn out to be common for Class IIA ring-hydroxylating dioxygenases, whether *Sphingomonas* sp. RW1 generally does not have its catabolic determinants clustered in operons, or whether such genetic organization is rare and limited to the dioxin catabolic pathway, remain to be determined.

Earlier unsuccessful attempts to clone the dioxin dioxygenase genes by constructing an expression library and screening for ring-hydroxylating activity are now explained as there is no linkage of the genes encoding the components of the dioxygenase. Moreover, problems to coexpress a similar ferredoxin and its specific reductase have been experienced (54). The presence of both putidaredoxin reductase and putidaredoxin in the same *E. coli* cell line might perhaps result in cell death due to leakage of electrons from NADH through the reductase to putidaredoxin, which has a greater sensitivity to oxidation by molecular oxygen than do the other components of this electron transfer system. Therefore, efforts to co-express all components of the dioxygenase may need to rely on the use of an inducible expression system.

The gene encoding the *meta*-cleavage enzyme is also not directly linked to the dioxin dioxygenase genes

The second step of the dioxin catabolic pathway, the cleavage of the dihydroxylated aromatic ring by the introduction of two further oxygen atoms, is carried out by DbfB, a *meta*-cleavage enzyme, 2,2',3-trihydroxybiphenyl-1,2-dioxygenase (Fig. 1). The structural gene coding for this enzyme has been cloned from a cosmid expression library using a functional colorimetric assay, and sequenced (34). The *dbfB* gene does not appear to be directly linked with the dioxin dioxygenase genes nor with the genes specifying the other enzymes of the path-

ways. The amino acid sequence of this enzyme is typical of those of extradiol dioxygenases, as was shown recently by a structure-validated alignment (20, 33). After hyperexpression of its structural gene in *E. coli*, the protein has been purified anaerobically and characterized. 2,2',3-trihydroxybiphenyl and 2,3-dihydroxybiphenyl are substrates of the enzyme and, like the substrates of many *meta*-cleavage enzymes (19), inhibit the enzyme when present at high concentration (34). Although the size of the polypeptide is close to that of other *meta*-cleavage enzymes, it was found to be active as a monomer in contrast to all other extradiol dioxygenases thus far studied which are active as homotetrameric or homooctameric proteins (34). This feature of DbfB makes nuclear magnetic resonance studies feasible and has allowed identification of the ligands of ferrous iron in the catalytic centre of this enzyme (10).

RW1 has been shown to contain at least three *meta*-cleavage enzymes (13, 34) and another dibenzofuran degrader, *Terrabacter* sp. strain DPO 360, also contains several distinct extradiol dioxygenases (58). One PCB-degrader, *Rhodococcus globerulus* P6, has been shown to possess up to eight different *meta*-cleavage enzymes (6, 43). The reason for the multiplicity of such isoenzymes has not yet been definitively explained. While the protein DbfB is thought to be the *meta*-cleavage enzyme involved in the "upper" pathway of dibenzo-*p*-dioxin and dibenzofuran degradation, the two other proteins may be involved in the ring-cleavage of catechol or chlorocatechol of the *meta* pathway. One of the isoenzymes of P6 has been shown to be less sensitive to substrate-mediated inactivation by chlorinated substrates, suggesting that it may be important for the catabolism of certain chlorinated congeners (Prucha, M. and K.N. Timmis, unpublished results).

The hydrolytic step of the dibenzofuran pathway

Although the ring cleavage product in the dioxin pathway, 2-hydroxy-6-oxo-6-(2-hydroxyphenoxy)-hexa-2,4-dienoate, spontaneously decomposes to catechol and hydroxy-*cis,cis*-muconate, the corresponding product in the dibenzofuran pathway, 2-hydroxy-6-oxo-6-(2-hydroxyphenyl-hexa-2,4-dienoate (2-OH-HOPDA), is enzymatically hydrolysed to salicylate and 2-hydroxypentadienoate (Fig. 1). Bünz *et al.* (14) have purified from *Sphingomonas* sp. RW1 two different hydrolases able to convert the substrate 2-OH-HOPDA. The N-terminal sequences of these two hydrolases are quite different from known hydrolases. However, the two proteins are isoenzymes and exhibit 50% similarity within their 20 first amino acids. Such diversity of hydrolases which apparently have the same substrate range, if not the same specific activity (14), is unusual. The reason is unclear but possibly one of the hydrolases play a role in a second degradative pathway, such as for biphenyl since biphenyl-utilizing mutants of this organism arise spontaneously (71). Preliminary data indicate that the RW1 strain may contain at least four different genes encoding such hydrolases (D'Enza, M., B. Happe, and J. Armengaud, unpublished results). It is known that different hydrolases exhibit different specificities towards bicyclic or monocyclic aromatic substrates (28) and, it will thus be of interest to study theses isoenzymes in terms of substrate range and kinetic parameters.

Conclusions

The present review highlights the atypical genetic organization of the degradative pathways of dibenzo-*p*-dioxin, dibenzofuran and their chloroderivatives in *Sphingomonas* sp. RW1, which seem to be significantly different from those of other aromatic compounds, such as naphthalene, cumene, toluene, benzene and biphenyl. The recent cloning of the genes specifying the enzymatic components of the enzymes of the initial steps in the dibenzofuran and dibenzo-*p*-dioxin pathways, and analysis of the genes and their products, have opened new perspectives for the degradation of such compounds and represent an important base from which to obtain a more complete description of the genetical, biochemical and regulational levels of the different actors involved in the catabolism of these compounds. This will provide the knowledge base for development of rational strategies for improving catabolic activities towards chlorinated-derivatives, and for bioremediation applications.

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