Review

Molecular Bases of Aerobic Bacterial Degradation of Dioxins: Involvement of Angular Dioxygenation

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In the last decade, extensive investigation has been done on the bacterial degradation of dioxins and its related compounds, because this class of chemicals is highly toxic and has been widely distributed in the environment. These studies have revealed the primary importance of a novel dioxygenation reaction, called angular dioxygenation, in the aerobic bacterial degradation pathway of dioxin. Accompanied by the electron transport proteins, Rieske nonheme iron oxygenase catalyzes the incorporation of oxygen atoms to the ether bond-carrying carbon (the angular position) and an adjacent carbon, resulting in the irreversible cleavage of the recalcitrant aryl ether bond. The 2,2',3-trihydroxydibiphenyl or 2,2',3-trihydroxydiphenyl ether derivatives formed are degraded through meta cleavage. In addition to the degradation system of dibenzofuran and dibenzo-p-dioxin (the nonchlorinated model compounds of dioxin), those of fluorene and carbazole were shown to function in dioxin degradation. Some dioxin degradation pathways have been studied biochemically and genetically. In addition, feasibility studies have shown that some dioxin-degrading strains can function in actual dioxin-contaminated soil. These studies provide useful information for the establishment of a bioremediation method for dioxin contamination. This review summarizes recent progress on molecular and biochemical bases of the bacterial aerobic degradation of dioxin and related compounds.

Key words: angular dioxygenation; bacterial degradation; carbazole; dioxin; fluorene

called "dirty dozen", those PCDDs and PCDFs having a 2,3,7,8-substitution pattern, but also low-chlorinated and unchlorinated dibenzo-p-dioxins (DD) and dibenzofuran (DF), as well as their brominated analogues. Figure 1 illustrates the chemical structure and numbering system of the dioxins and its related compounds cited in this review. Complex mixtures of congeners of PCDDs and PCDFs are frequently detected in environmental samples such as sediments.1-3 The dioxins are recalcitrant molecules that are extremely persistent in soil sediments. For example, the half-life of 2,3,7,8-tetrachloro-DD in soils and sediments is 1 to 10 years.4,5

Recently, bioremediation, the exploitation of the biodegradative activities of microorganisms to

Polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), called dioxins, are produced by incineration processes and, unintentionally, as by-products during the synthesis of pesticides and herbicides. Many of their congeners are highly toxic compounds with undesirable effects on the environment and living beings, but have been widely distributed in the environment. The dioxin-like compounds should not only comprise the so-

Fig. 1. Chemical Structure of Polychlorinated Dibenzo-p-dioxins (PCDDs) and Polychlorinated Dibenzofurans (PCDFs) and Their Structural Analogues, and Their Numbering Conventions.

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Abbreviations: CAR, carbazole; DD, dibenzo-p-dioxin; DF, dibenzofuran; FN, fluorene; IS, insertion sequence; ORF, open reading frame; PAH, polycyclic aromatic hydrocarbon; PCDD, polychlorinated dibenzo-p-dioxin; PCDF, polychlorinated dibenzofuran; TEQ, toxicity equivalency quantity; Tn, transposon
remove environmental pollutants and recalcitrant xenobiotics, has been attempted. It has become more popular as an alternative to physical and chemical methods, because of the low cost and small impact on the environment. Among the bioremediation techniques, bioaugmentation, the addition of bacteria to a contaminated environment to increase the degradation of pollutants, is attracting attention, because in situations where indigenous microorganisms cannot degrade these xenobiotics, this may be the only method for successful bioremediation. Hence, many researchers have focused their studies on the isolation and identification of the bacteria that can degrade xenobiotics, including dioxins.

The initial step in the aerobic microbial degradation of various aromatic compounds is usually the introduction of two hydroxyl groups into the benzene ring, forming cis-dihydrodiols (Fig. 2(A)). The enzymes catalyzed the incorporation of both atoms of dioxygen into aromatic substrates are called dioxygenases, and this type of dioxygenation is termed the lateral dioxygenation or cis-dihydroxylation. cis-Dihydrodiols are dehydrogenated by cis-dihydrodiol dehydrogenase, resulting in the formation of a catechol moiety. Because the fission of the aromatic ring occurs at a catechol moiety in meta or ortho fashion, the formation of a catechol moiety is a critical step in the degradation pathways of aromatic compounds. On the other hand, the elucidation of the bacterial degradation pathways of dioxin-related compounds found a new type of oxidative attack with high regioselectivity and specificity for the angular position (Fig. 2(B)). In this dioxygenation, one carbon that is bonded to the oxygen atom in DD and DF, and its adjacent carbon in the aromatic ring, are both oxidized. This reaction, termed angular dioxygenation, is catalyzed by the Rieske nonheme iron oxygenases, which are termed angular dioxygenases.

The angular dioxygenation has been found to be involved in the degradation pathways for dioxins (DF and DD), and its structural analogues, fluorene (FN), carbazole (CAR), diphenyl ether and its derivatives, and dibenzothiophene. Other biodegradation pathways for these compounds via lateral dioxygenation or monooxygenation has been well investigated and reviewed. Diphenoxy ether and dibenzothiophene degradation pathways containing angular dioxygenation have not been thoroughly described, but the FN- and CAR-degradation systems function in the degradation of dioxins. As summarized below, the degradation pathways of FN and CAR are similar to those of DF and DD, and the FN- and CAR-degrading bacteria can decompose dioxins through an identical pathway by DF (DD)-mineralizing bacteria. Among the degradation steps of dioxins, angular dioxygenation is the most important, because this single-step oxygenation destroys the planar structure of dioxin from which the dioxin toxicity derives.

In this paper, to outline the progress in the biochemical and molecular studies on the bacterial degradation of dioxins, we describe the degradation pathway of dioxin and related compounds via angular dioxygenation, and the features of their degradative genes and enzymes.

Degradation of the Dioxins, FN and CAR via Angular Dioxygenation

(1) Microbial degradation of DF and DD

Over the last decade, as listed in Table 1, a number of bacteria capable of mineralizing DF, DD, or both have been isolated by enrichment culture methods using a carbon-free mineral medium with DF or DD added as the sole source of carbon and energy. Fortnagel et al. and Engesser et al. established that initial angular attack could occur on DD at the 4 and 4a carbon atoms (Fig. 3). This angular dioxygenation for DF produces highly unstable hemiacetal products, which have not been observed directly. A compound analogous to DF, 9-fluorenone, was incubated with DF-grown bacteria, and the chemical structure of a dead-end product accumulated in the reaction mixture was identified. Based on the mass spectrum and nuclear magnetic resonance analytical data, the resultant product was identified as 1-hydroxyfluoren-9-one (chemical structure is shown in Fig. 3). Thus, formation of hemiacetals from DF/DD was inferred.

Although, in early works using various DF-degrading bacteria, the formation of a trihydroxyl compound was not observed in the culture supernatant, Fortnagel et al. reported that a UV-derived mutant strain (designated HH69-II), which could no longer grow with DF as the carbon source, accumulates 2,2',3-trihydroxybiphenyl as a metabolic intermediate of DF (Fig. 3). From the culture supernatant of
Table 1. Aerobic Bacteria Able to Degrade or Co-Oxidize Dioxins, 9-Fluorenone, or CAR via Angular Dioxygenase

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Initial dioxygenase</th>
<th>Substrate*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brevibacterium sp. strain DPO220</td>
<td>DF, 4,4a-dioxygenase</td>
<td>DF, FN</td>
<td>Engesser et al., 11] Strubet al. 12]</td>
</tr>
<tr>
<td>Terrabacter sp. strain DPO360</td>
<td>DF, 4,4a-dioxygenase</td>
<td>DF</td>
<td>Schmid et al. 15]</td>
</tr>
<tr>
<td>Sphingomonas sp. strain HH69</td>
<td>DF, 4,4a-dioxygenase</td>
<td>DF</td>
<td>Fortnagel et al., 16,17] Harms et al., 18,20] Schreiner et al. 21]</td>
</tr>
<tr>
<td>Terrabacter sp. strain DBF63</td>
<td>DF, 4,4a-dioxygenase</td>
<td>DF, FN</td>
<td>Monna et al., 38] Kasuga et al., 39,40] Habe et al., 41,42] Nojiri et al. 43]</td>
</tr>
<tr>
<td>Sphingomonas sp. strain HH19k</td>
<td>DF, 4,4a-dioxygenase</td>
<td>DF</td>
<td>Harms and Zehnder 44,45]</td>
</tr>
<tr>
<td>Sphingomonas sp. strain RW16</td>
<td>DF, 4,4a-dioxygenase</td>
<td>DF</td>
<td>Wittich et al. 46]</td>
</tr>
<tr>
<td>Pseudomonas sp. strain F274</td>
<td>9-Fluorenone 1,1a-dioxygenase</td>
<td>9-Fluorenone</td>
<td>Selifonov et al., 47] Grifoll et al. 48,49]</td>
</tr>
<tr>
<td>P. mendocina strain MC2</td>
<td>CAR, 1,9a-dioxygenase</td>
<td>CAR, 9-Fluorenone</td>
<td>Casellas et al. 50]</td>
</tr>
<tr>
<td>Ralstonia sp. strain RGI1123</td>
<td>CAR, 1,9a-dioxygenase</td>
<td>CAR</td>
<td>Grosser et al., 51] Schneider et al., 52]</td>
</tr>
<tr>
<td>Pseudomonas sp. strain CA06</td>
<td>CAR, 1,9a-dioxygenase</td>
<td>CAR, DF, DD</td>
<td>Ouchiya et al., 53]</td>
</tr>
<tr>
<td>P. stutzeri strain ATCC 31258</td>
<td>CAR, 1,9a-dioxygenase</td>
<td>CAR</td>
<td>Hisatsuka and Sato 60]</td>
</tr>
<tr>
<td>Pseudomonas sp. strain LD2</td>
<td>CAR, 1,9a-dioxygenase</td>
<td>CAR</td>
<td>Gieg et al. 60]</td>
</tr>
<tr>
<td>P. stutzeri strain OM1</td>
<td>CAR, 1,9a-dioxygenase</td>
<td>CAR</td>
<td>Ouchiya et al. 55]</td>
</tr>
<tr>
<td>Sphingomonas sp. strain CDH-7</td>
<td>CAR, 1,9a-dioxygenase</td>
<td>CAR</td>
<td>Kirmura et al. 60]</td>
</tr>
<tr>
<td>Burkholderia sp. strain JB1</td>
<td>2-chlorinated DF, 2-chlorinated DSM 4</td>
<td>2-chlorinated DSM 4</td>
<td>Parsons et al. 67]</td>
</tr>
<tr>
<td>Burkholderia sp. strain LB400</td>
<td>Biphenyl 2,3-dioxygenase</td>
<td>DF, DD</td>
<td>Seeger et al. 60]</td>
</tr>
<tr>
<td>P. pseudoalcaligenes strain KF707</td>
<td>Biphenyl 2,3-dioxygenase</td>
<td>DF, DD</td>
<td>Suenaga et al. 60]</td>
</tr>
</tbody>
</table>

* Only the parental compounds which were found to be metabolized via angular dioxygenation are shown, if not otherwise noted.

The large (α) subunit of the terminal oxygenase component was mutated by random priming recombination.

DF-using bacteria, salicylic acid was identified.12,17,38 This indicates that 2,2',3-trihydroxybiphenyl is meta cleaved, and the resultant meta cleavage compound is hydrolyzed to salicylic acid and 2-hydroxypent-2,4-dienoic acid.12,21 In the DF-degradation pathway reported in Pseudomonas sp. strain HH69,17 which was reassigned to the genus Sphingomonas,20 S. wittichii strain RW1,22 and Staphylococcus auricularis strain DBF63,8 which was reassigned to the genus Terrabacter,19 the metabolic intermediate, salicylic acid, was branched into catechol and gentisic acid degradation pathways.

Initial angular dioxygenation for DD followed by spontaneous ring cleavage resulted in the formation of 2,2',3-trihydroxydiphenyl ether (Fig. 3).22 Catechol has also been identified by GC-MS analysis as a metabolite of DD. Because the meta cleavage activity for 2,3-dihydroxydiphenyl ether could be detected, Wittich and coworkers proposed that catechol was formed from 2,2',3-trihydroxydiphenyl ether via meta cleavage and subsequent hydrolysis.22 However, the possible product formed by the hydrolysis, 2-hydroxymuconic acid, has not been identified as a metabolite of DD. Pfeifer et al.70 reported that the meta cleavage of 2,3-dihydroxydiphenyl ether by 2,3-dihydroxybiphenyl 1,2-dioxygenase leads to the formation of phenol and 2-pyrene-6-carboxylate as products of ring fission and ether cleavage without participation of free intermediates. Thus, it is likely that only a meta cleavage enzyme is necessary for the production of catechol from 2,2',3-trihydroxydiphenyl ether as shown in Fig. 3. Similar to the catechol metabolism in the DF degradation pathway, catechol formed from DD was mineralized through meta, ortho, or both cleavage pathways.22

(2) Microbial degradation of FN

FN is an abundant constituent of coal tar and creosote, and one of the simplest polycyclic aromatic hydrocarbons (PAHs). FN is one of the 16 PAHs in the list of priority pollutants compiled by the U.S. Environmental Protection Agency.71 FN-using bacteria, Pseudomonas sp. strain F274,48 and 9-fluorenone-using bacteria, P. mendocina strain MC2,50 were isolated from soil severely contaminated with creosote and PAHs, respectively. Several DF-using strains can also grow on FN,14,38

Based on the identification of 9-fluorenone and...
9-fluorenol as metabolic intermediates of FN, monooxidation at the C9 position to give 9-fluorenol followed by the dehydrogenation to 9-fluorenone was shown to be the initial conversion before angular dioxygenation of the aromatic ring (Fig. 3). Because 1-hydro-1,1a-dihydroxy-9-fluorenone was identified as the degradation product of FN or 9-fluorenone, angular dioxygenation has been established as the crucial step in FN degradation (Fig. 3). Unlike the hemiacetal intermediates produced by angular dioxygenation of DF and DD, the angular dioxygenation product of 9-fluorenone is chemically stable. Dehydrogenation of the 1-sec-hydroxyyl group of 1-hydro-1,1a-dihydroxy-9-fluorenone would yield a β-diketone-type compound, hydrolysis of which at the C9–C1a bond would give 2'-carboxy-2,3-dihydroxybiphenyl. 8-Hydroxy-3,4-benzo-coumarin was identified as a metabolite of FN, but this compound is in fact the β-lactone of 2'-carboxy-2,3-dihydroxybiphenyl and could be formed as a result of a reversible dehydration reaction. 2'-Carboxy-2,3-dihydroxybiphenyl was meta cleaved at the C1–C2 bond, and subsequent hydrolysis resulted in the formation of phthalic acid and 2-hydroxypenta-2,4-dienoic acid, as shown in Fig. 3. Phthalic acid is further degraded by Pseudomonas sp. strain F274 via protocatechuic acid.

**Fig. 3. Overview of Bacterial Degradative Pathways for Dibenzofuran (DF), Dibenzo-p-dioxin (DD), Fluorene (FN), and Carbazole (CAR) via Angular Dioxygenation.**

The structures shown in brackets have not been characterized. The red, blue, and green arrows show angular dioxygenation, meta cleavage, and hydrolysis, respectively. The production of catechol and 2-pyron-6-carboxylic acid from the meta cleavage compound of 2,2',3-trihydroxybiphenyl ether in the DD degradation pathway was proposed by the results reported by Pfeifer et al. 2-Pyron-6-carboxylic acid is a by-product. The arrows with solid and broken lines indicate the enzymatic and spontaneous conversions, respectively.

(3) Microbial degradation of CAR

CAR is a nitrogen heterocycle released into the environment with fossil fuels or their products such as creosote. CAR is used as a chemical feedstock for the production of dyes, medicines, and plastics, and is known to possess mutagenic and toxic activities and also to be a recalcitrant molecule. As shown in Table 1, several CAR-using bacteria were isolated by enrichment culture, with CAR as a sole source of carbon and energy, or of carbon, nitrogen, and energy.
OuchiAmy et al.53) isolated Pseudomonas sp. strain CA06 and P. resinovorans strain CA10 from soil and activated sludge samples, respectively, which use CAR as the sole source of carbon, nitrogen, and energy. Anthranilic acid and catechol were identified as the main metabolites of CAR by HPLC and GC-MS. In addition, meta cleavage activity for 2,3-dihydroxybiphenyl was strongly induced in strain CA10 cell grown with CAR. These results suggested that strain CA10 degrades CAR to anthranilic acid through angular dioxygenation, meta cleavage, and hydrolysis. This degradation pathway of CAR by strain CA10 is homologous to that of DF degradation as shown in Fig. 3. From the extract of culture medium of various CAR-using bacteria, anthranilic acid was identified as a main metabolite,52,63-66 suggesting that these CAR-using bacteria degrade CAR through a pathway similar to that of strain CA10.

When anthranilic acid was used for a growth substrate by strain CA10, catechol and a small amount of cis,cis-muconic acid were detected.53) These results indicate the possibility that anthranilic acid is converted to catechol by anthranilate 1,2-dioxygenase, and that the catechol formed is mineralized via ortho cleavage by strain CA10. On the other hand, P. stutzeri strain OM1 was found to metabolize catechol via meta cleavage.65)

Biochemical and Genetic Characterization of the Dioxin Degradation Pathway

The genes for the meta cleavage enzyme involved in DF and DD degradations were isolated by shotgun cloning with the enzyme activity used to screen for the positive clone.15,25,39) This method has been used to clone the aromatic compound degradation genes, because a colony of Escherichia coli cells having the meta cleavage enzyme gene has a bright yellow color from the meta cleavage compound when an appropriate substrate having the catechol moiety is sprayed on. In addition, angular dioxygenase, meta cleavage enzyme, and hydrolyase were purified from the DF-degrading bacteria.15,23,24) The degradative genes involved in the degradation pathways of easily degradable aromatic compounds, such as naphthalene, biphenyl, toluene, and phenol, often have a well-clustered gene organization. Thus, based on the genetic information on the meta cleavage enzyme and purified enzyme, attempts to clone the other degradative genes from DF- and DD-degrading bacteria by the gene-walking method have been done. However, these attempts almost completely failed, because the DF- and DD-degradative genes reported so far are dispersed in the genome and do not constitute a simple gene cluster.32,40,43) This is why the dioxin degradation pathway has not been well characterized. In contrast, CAR and dioxin degradation pathway of P. resinovorans strain CA10 has been characterized reasonably well at the genetic level.55,56,58,62)

(1) Dioxin degradation pathway of S. wittichii strain RW1

S. wittichii strain RW1 is the most well-characterized DF/DD-degrading bacterium. Since Wittich et al.23 reported the isolation of the DF and DD-degrader, strain RW1, there have been intensive attempts to isolate the enzymes and genes involved in the DF and DD degradation. Two isofunctional meta cleavage compound hydrolases (hydrolyase H1 and H2), and respective components of the initial angular dioxygenase system, DF 4,4a-dioxygenase (dioxin dioxygenase) were purified from strain RW1 cells by Cook and coworkers.23,24) The hydrolases H1 and H2 were presumed to be monomeric. There was a 50% identity between the N-terminal amino acid sequences of the two hydrolases, but no similar protein sequences were observed by database search analysis.23) The DF 4,4a-dioxygenase system was found to consist of a terminal oxygenase that has a heterotetrameric structure (α2β2) and an electron-transfer system.24) An atypical electron transfer chain interacting with the terminal oxygenase component of the dioxin dioxygenase system, consisting of a 12-kDa putidaredoxin-type [2Fe–2S] ferredoxin and two isofunctional monomeric flavoproteins (ferredoxin reductase A1 and A2), has been purified, and the sequences of the N-termini of the polypeptides have been analyzed.24) The characteristics of the electron transport chain indicate that the dioxin dioxygenase system of strain RW1 is a class II A dioxygenase system according to the classification scheme of Batie et al.24)

As described above, the dbfB gene encoding 2,2',3-trihydroxybiphenyl 1,2-dioxygenase, the meta cleavage enzyme involved in DF degradation, was cloned from the genomic cosmid library of strain RW1.25) Comparison of the amino acid sequence of DbfB showed significant similarities to the meta cleavage enzyme involved in the biphenyl/polychlorinated biphenyl degradation pathway. Unlike most meta cleavage enzymes, which have an oligomeric quaternary structure, the DbfB of strain RW1 is a monomeric protein.18) NMR analysis of purified DbfB showed that the high spin Fe(II) iron present in the active form of the enzyme is coordinated by at least two His residues.26) Using the N-terminal sequence of purified putidaredoxin-type [2Fe–2S] ferredoxin and the conserved stretch of only four amino acids at the C-terminal of the ferredoxin, Armengaud and Timmis30) designed a nested-PCR strategy to obtain a specific probe for the ferredoxin gene. With this probe, the authors identified the ferredoxin gene, fdxI. Ferredoxin FdxI of strain RW1 shares about 40% identity with several [2Fe–2S] ferredoxins involved in elec-
tron transfer to bacterial monooxygenase systems. UV and visible spectrophotometry and electron paramagnetic resonance spectroscopy using the hyperexpressed Fdx1, it was found that Fdx1 contains a putidaredoxin-type [2Fe-2S] cluster. Fdx1 is thus related to the group of ferredoxins that typically donate electrons to monooxygenases, named cytochrome $P_{550}$, and that are an atypical electron transfer system for the Rieske nonheme iron oxygenase system. The redA2 gene encoding the ferredoxin reductase A2 was also cloned by PCR. From the nucleotide and deduced amino acid sequences, as well as from the biochemical data obtained from a recombinant form of this reductase, it is evident that this flavoprotein is similar to class-1 cytochrome $P_{550}$ type reductase. Indeed, the two proteins of the electron transport system, RedA2 and Fdx1, are similar to their counterparts supplying electrons to the well-characterized cytochrome $P_{550}$, a monooxygenase from *P. putida*, which is able to oxidize camphor. The genes encoding the terminal oxygenase component of dioxin dioxygenase were also isolated by a PCR-based technique. The *dxnA1* and *dxnA2* cistrons, encoding the large ($\alpha$) and small ($\beta$) subunits of the terminal oxygenase component, were just upstream of the hydrolase gene *dxnB* encoding hydrolase H1 (Fig. 4). In the 4.5 kb upstream region of the *dxnA1* gene, the *dbfB* gene was located in the opposite direction (Fig. 4). Recently, Armengaud et al. reported that there are nine additional genes, *dxnC* to *dxnI*, in the downstream region of *dxnB*, constituting the same operon with *dxnA1A2B* (Fig. 4). DxnDFEGHI were found to form a complete 4-hydroxyxalicylic acid/hydroxyquinol-degradative pathway. A homology search analysis found an additional putidaredoxin-type ferredoxin gene (*fdx3*) in this operon (Fig. 4). Fdx3 functions as an electron transport protein from RedA2 to DxnA1A2 in the dioxin dioxygenase system. Thus, *S. wittichii* degrades DF and DD by the enzymes as shown in the Fig. 5, and the respective enzymes was encoded in the physically distinct loci, *dxnA1A2Bfdx3, fdx1, redA2*, and *dbfB* in the RW1 genome. Armengaud et al. reported that, while the expression of the *dbfB* gene was constitutive, that of *dbfA1A2* is modulated according to the available carbon source. However, the detailed transcriptional control of dioxin degradation genes of strain RW1 has not been reported.

(2) Dioxin degradation pathway by *Terrabacter sp.* strain DBF63

Two genes encoding meta cleavage enzymes (*dbfB* and open reading frame K1 [ORFK1]) were cloned from *Terrabacter sp.* strain DBF63 by shotgun cloning on the basis of the expressed activity of meta cleavage enzymes. Based on the comparison of the substrate specificity, it was suggested that, at least DbfB is involved in the DF degradation by strain DBF63. Nucleotide sequence analysis showed *dbfB* and *dbfC* cistrons encoding a meta cleavage enzyme and a meta cleavage compound hydrolase, respectively.

Recently, Kasuga et al. succeeded in cloning the *dbfA1* and *dbfA2* genes, which encode the large ($\alpha$) and small ($\beta$) subunits of the terminal oxygenase component of the DF 4,4a-dioxygenase system, respectively, from strain DBF63 genome by a PCR-based method. DbfA1 and DbfA2 showed moderate homology to the respective subunits of other ring-hydroxylating dioxygenases (less than 40%), and some motifs such as the Fe(II) binding site and the
Involvement of Angular Dioxygenation in Dioxin Degradation

![Diagram](https://via.placeholder.com/150)

**S. wittichii** strain RW1

**Terrabacter sp.** strain DBF63

**P. resinovorans** strain CA10

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Fig. 5. DF Degradation by the Enzyme Systems Harbored by *S. wittichii* Strain RW1, *Terrabacter* sp. Strain DBF63, or *P. resinovorans* Strain CA10.

The reactions done by the multi-component ring-hydroxylating dioxygenase (iron-sulfur protein; terminal oxygenase component) and their electron transfer system, *meta* cleavage enzyme, and hydrolase are shown. Chemical designations: I, dibenzofuran (DF); II, 2,2′,3-trihydroxybiphenyl; III, 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid; IV, salicylic acid.

Rieske-type [2Fe–2S] cluster ligands\(^{77}\) were conserved in DbfA1. DF 4,4a-dioxygenase activity was confirmed by the resting cell reaction of *E. coli* cells containing the cloned *dbfA1* and *dbfA2* genes with the complementation of nonspecific ferredoxin and ferredoxin reductase components of *E. coli*. It was also found that DbfA1A2 can catalyze the angular dioxygenation for 9-fluorenone (Habe et al., unpublished results) and 9-hydroxylation for FN.\(^{40}\) Phylogenetic analysis showed that DbfA1 formed a branch with a recently reported large (α) subunit of PAH dioxygenase from Gram-positive bacteria.\(^{40}\) The citron *dbfA1A2* is a part of an about 7.3-kb-long operon consisting of 8 ORFs. Although a gene encoding a putative [3Fe–4S] ferredoxin was observed in this operon, the ferredoxin reductase gene has not been identified (Habe et al., unpublished results). The enzymes involved in the dioxin degradation by strain DBF63 are summarized in Fig. 5.

By the pulsed field gel electrophoresis and Southern hybridization, two linear plasmids, pDBF1 and pDBF2, about 160 kb and 190 kb long, respectively, were detected in strain DBF63, and the *dbfA1A2* citron was shown to be located on these two plasmids.\(^{43}\) On the other hand, *dbfBC* genes were located on the chromosome.\(^{43}\) Although the regulator protein and effector molecule have not been identified, reverse transcription-PCR analysis showed that *dbfA1A2* and *dbfBC* genes are strongly expressed in the DF-grown strain DBF63 cells.\(^{43}\) These results suggest that *dbfA1A2* and *dbfBC* genes are involved in the DF degradation, and that these degradative genes are dispersed on the strain DBF63 genome.

(3) Degradation pathway for CAR and dioxin of *P. resinovorans* strain CA10

The transposon mutagenesis of *P. resinovorans* strain CA10 indicated a putative gene cluster involved in the CAR degradation including the *meta* cleavage enzyme gene.\(^{59}\) On the basis of these results, we cloned the intact CAR-degrading *car* gene cluster from the wild-type strain CA10 by shotgun cloning, using the *meta* cleavage activity for 2,3-dihydroxypiphenyl.\(^{55,56}\) As a result of the nucleotide sequence analysis of the cloned DNA fragment, homology search analyses, and the measurement of the enzyme activities, the genes encoding angular dioxygenase for CAR (CAR 1,9a-dioxygenase; CarAaAcAd), *meta* cleavage enzyme (CarBaBb), and *meta* cleavage compound hydrolase (CarC) were identified, as shown in Fig. 6(A). It was found that the 1,263-bp DNA region containing the entire *carAa* gene was tandemly duplicated except for one base, and that there were only three bases (GCG) between these tandemly-linked DNA regions.\(^{56}\) To our knowledge, such gene structure has never been observed in other degradative gene clusters. The significance of this duplication of *carAa* genes in strain CA10 cells is unknown. Recently, we succeeded in isolating several CAR- and dioxin-degrading bacteria, some of which have a homologous *car* gene cluster containing a single copy of the *carAa* gene (Widada et al., unpublished results). Comparison of the CAR- and dioxin-degrading capacities between bacteria having different copy numbers of the *carAa* gene might provide us insight on the significance of this gene duplication. In addition, the genes encoding the terminal oxygenase component (CarAa) of CAR 1,9a-dioxygenase are separated from the genes encoding the ferredoxin component (CarAc) and ferredoxin reductase com-
component (CarAd) by a DNA fragment of about 2-kb encoding carBaBbC (Fig. 6A). These first three enzymes in CAR degradation have been found to function in dioxin degradation as the counterparts of the corresponding enzymes in DF (DD)-degrading bacteria.\(^{39,41,56,57}\) The functions of the Car enzymes of strain CA10 in DF degradation may be as shown in Fig. 5.

On the basis of the identification of the oxygenation product after a biotransformation experiment, CAR 1,9a-dioxygenase was shown to catalyze the angular dioxygenation for CAR, DF, DD, xanthene, and phenoxathiin, but not for 9-fluorenone or dibenzothiophene,\(^{56,57}\) indicating that the angular dioxygenation occurs at the angular position adjacent to an oxygen or nitrogen atom more preferably than that adjacent to a sulfur or carbon atom. The three components of the CAR 1,9a-dioxygenase system have been purified (Nam \textit{et al.}, in preparation). Based on gel-filtration analyses, CarAa, CarAc, and CarAd were trimer, monomer, and monomer proteins, respectively. In the purified His-tagged CarAd, 0.6 mole FAD per mole of protein was detected. UV and visible absorption spectroscopy indicated that CarAa, CarAc, and CarAd proteins have the Rieske-type,\(^{78}\) Rieske-type,\(^{78}\) and plant-type\(^{79}\) [2Fe-2S] clusters, respectively. These facts are in agreement with the functions suggested from the deduced amino acid sequences.\(^{56}\)

The Rieske nonheme iron oxygenase systems are classified into three classes, classes I, II, and III, in terms of the number of constituent components and the nature of the redox centers.\(^{74,80}\) Further subdivision is based on the type of flavin in the reductase (Ia and IB), or the coordination of the [2Fe–2S] cluster in the ferredoxin (IIa and IIb). Based on this classification system, CAR 1,9a-dioxygenase seems to have a class III electron transport chain like a well-known naphthalene dioxygenase. On the other hand, Nam \textit{et al.}\(^{39}\) proposed a new classification scheme for the oxygenase components based on their amino acid sequence similarity in the α subunits of terminal oxygenase component, because the Batie classification system could not cover all of the dioxygenase systems. On the basis of Nam’s scheme, terminal oxygenases were classified into four groups, I–IV. The oxygenase components included by group I in Nam’s classification have only the identical subunit, and most of the terminal oxygenase components of class IA oxygenases in the Batie classification belong to this group I. The terminal oxygenase components of class IB, class II, and class III oxygenase systems in the Batie classification are mainly classified into group II, group IV, and group III, respectively, in Nam’s classification system. The phylogenetic study found that CarAa has a closer relation to the terminal oxygenase contained by class IA dioxygenase systems, and CarAa was classified into group I in Nam’s classification.\(^{39}\)

Recently, Schmidt and Shaw\(^{81}\) suggested that the terminal oxygenase and ferredoxin components containing a Rieske-motif form a superfamily with one ancestor. To investigate the evolutionary relationships of CarAa and CarAc with other Rieske-type
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Fig. 7. Phylogenetic Tree of Ferredoxin and Terminal Oxygenase Components of Oxygenase Systems.

The scale bar denotes 0.1 substitution per site. The tree was constructed with the Clustal W package. The name of each bacterial strain and its DDBJ/EMBL/GenBank or SWISSPROT accession number are shown after the enzyme name in parentheses. Grouping into Groups I-IV of oxygenase proteins is based on the scheme of Nam et al. This figure is adapted from Nam et al. (in preparation). The terminal oxygenase (CarAa) and ferredoxin (CarAc) of CAR 1,9a-dioxygenase from P. resinovorans strain CA10 are shaded.

proteins, a phylogenetic tree was established (Fig. 7, Nam et al., in preparation). As shown in Fig. 7, the ferredoxins involved in the monoxygenase system and dioxygenase system constitute the distinctive clusters, and CarAc was contained in the ferredoxin cluster of dioxygenase system. As for the oxygenase components, the distant affiliations of the oxygenases were observed between group I and groups II, III, and IV in Nam’s classification. These phylogenies indicate that group I oxygenases are evolutionally different from group II, III, and IV oxygenases. In addition, among the oxygenases classified into group I, CarAa of strain CA10 and OxoO of strain 86 were phylogenetically distant from other group I oxygenases (Fig. 7). On the other hand, the phylogenetic tree of ferredoxin-NADP+ reductase-type reductases showed that CarAd of strain CA10 is more closely related to the reductase components of some monooxygenase systems, such as toluene 4-monooxygenase, toluene/o-xylene monooxygenase, and alkene monooxygenase, and alkene monooxygenase systems (Fig. 8) (Nam et al., in preparation). The distant phylogenetic affiliations of respective components of CAR 1,9a-dioxygenase indicate the possibility that some of these three components have been recruited from different oxygenase systems.

We have analyzed the nucleotide sequence of the 27,939-bp-long upstream and 9,448-bp-long downstream regions of carAaAaBbCcAaCc(ORF7)Ad
Thirty-two ORFs were identified, and the car gene cluster was found to consist of ten genes (carAAaBbBaBbCacAdDFe), as shown in Fig. 6(A), encoding the enzymes for the first three-step conversions of CAR to anthranilic acid and the degradation of 2-hydroxypenta-2,4-dienoic acid. The unusual gene structure and the higher phylogenetic relatedness of CarFE to the enzymes involved in 3-(3-hydroxyphenyl)propionic acid degradation indicated that carFE has been recruited from the other locus. The open reading structure and detailed expression mechanism of car genes have not been reported, but the strong expression of these genes as several transcriptional units was observed in CAR- and anthranilate-grown strain CA10 cells (Urata et al., unpublished results). In the 21-kb upstream region from carAA, the genes encoding Rieske nonheme iron oxygenase (ORF26, ORF27, and ORF28) were found (Fig. 6(A)). Inductive expression in CAR- and anthranilate-grown cells and the results of homology searching indicate that these genes encode the anthranilate 1,2-dioxygenase involved in CAR degradation. Therefore, these ORFs were designated antABC.

Four similar IS5-related insertion sequences (ISs; three copies of ISPre1 and one copy of ISPre2, formerly designated as IS5car1 to IS5car4) were identified in neighboring regions of car and ant loci (Fig. 6(A)). The nucleotide sequence of ISPre2 showed 81.6% identity with that of ISPre1, although it was 98% identical with the IS-like sequence containing tmPAl from P. putida strain AN10. Comparison of the nucleotide sequences indicated that the one-ended transposition of ISPre1 together with the 5’-portion of antA into the immediate upstream of carAA had resulted in the formation of additional copies of ISPre1 and ORF9. In addition to the IS-dependent recombination, gene duplications (ORFs 11 and 12, and two copies of carAA gene) had occurred.

In addition, the car gene clusters were found on the 199-kb circular plasmid, pCAR. Because catechol formed from CAR is degraded through the ortho cleavage pathway encoded by the chromosomal cat
operon (Fig. 6(B)),\textsuperscript{42} it can be considered that strain CA10 had acquired a metabolic capacity for dioxin and CAR by recruitment of pCAR1. In other bacteria having the ability to degrade both dioxin and CAR, we have also detected the pCAR1-like plasmid containing the car gene cluster homologue, although chromosomally encoded car gene clusters were found as well (Widada et al., unpublished results). This phenomenon suggests that pCAR1 is important in the distribution of the degradation capacity for dioxin and CAR in the environment. Very recently, the entire nucleotide sequence (199,035 bp) of pCAR1 was analyzed (Maeda et al., unpublished results). The presence of the homologous genes to the trh and tra genes involved in the conjugal transfer of the plasmids Rts1 from Proteus vulgaris\textsuperscript{83} and R27 from Salmonella strains\textsuperscript{84} suggested the possibility that pCAR1 is a self-transmissible plasmid. Interestingly, it was also shown that the entire car and anti loci are contained in a 73-kb-long transposon, Tn4676. The transposase and cointegrate resolution proteins are closely related to those of the toluene- and xylene-degrading transposon, Tn4651,\textsuperscript{85} identified from the TOL plasmid pWW0, although the replication and maintenance systems of pCAR1 has no relationship to those of pWW0. Because several bacterial strains have Tn4676 inserted on their chromosome (Shintani et al., unpublished results), both the plasmid pCAR1 and Tn4676 are important in the distribution of the degrading capacity for dioxin and CAR in the environment.

### Microbial Degradation of Chlorinated DF and DD

#### (1) Aerobic degradation of chlorinated DFs and DDs

Although the nonhalogenated basic structure DF and DD is effectively mineralized by appropriate bacterial strains as mentioned above, their polychlorinated derivatives are not. The abilities of DF (DD)- or CAR-mineralizing bacteria to oxidize mono- to trichlorinated congeners of DD and DF were analyzed.\textsuperscript{19,27,35,41} Harms et al.\textsuperscript{19} reported that a mutant strain Pseudomonas (Sphingomonas) sp. \textit{strain HH69-II,} which is defective in 2,3-dihydroxybiphenyl 1,2-dioxygenase, led to the formation of an equal amount of 4'-chloro-2,2',3-trihydroxybiphenyl and 4-chloro-2,2',3-trihydroxybiphenyl from 3-chloro-DF. Wilkes et al.\textsuperscript{27} demonstrated that most mono- and dichlorinated DFs and DDs tested were degraded to the corresponding mono- and dichlorinated salicylic acids and catechols, respectively, together with salicylic acid and catechol. Wittich et al.\textsuperscript{46} also reported that the conversion of 3-chloro-DF in the presence of an inhibitor of \textit{meta} cleavage by Sphingomonas sp. \textit{strain RW16} led to the accumulation of two metabolites that are likely to be 4'-chloro-2,2',3,5,6-pentachloro- and 4-chloro-2,2',5,6-tetrachloro-DF.

These results indicated that angular dioxygenase from these bacteria attacked both the substituted and the nonsubstituted aromatic nuclei of monochlorinated DFs and DDs nonspecifically. However, in these studies, the concentrations of the target substrates were from 100 to 1,000 ppm. On the other hand, Parsons et al.\textsuperscript{57} proposed that the initial dioxygenation took place on the nonchlorinated ring during the degradation of a low concentration (0.3 ppm) of 2-chloro-DF and 2-chloro-DD by the biphenyl-utilizing \textit{Burkholderia} sp. \textit{strain JBI,} since 5-chlorosalicylic acid and 4-chlorocatechol as well as only one isomer each of chlorinated 2,2',3,4-tetrachloro- and 2,2',3,5,6-pentachloro-DFs were detected in respective biotransformation experiments. In our experiments on dioxin degradability by the CAR 1,9a-dioxygenase from \textit{strain CA10} and the DF 4,4a-dioxygenase from \textit{strain DBF63,} we found that these two angular dioxygenases act mainly on the nonsubstituted aromatic nucleus of chlorinated DFs and DDs (10 ppm), although both enzymes were able to act on the chlorine-substituted aromatic rings.\textsuperscript{41} Wilkes et al.\textsuperscript{27} also suggested that at least the initial conversion rates for the chlorinated DFs and DDs decreased with increasing chlorine substitution. In addition, based on a biotransformation experiment by using parental bacterial strain, \textit{strains CA10} and DBF63, and \textit{E. coli} transformants expressing the \textit{meta} cleavage enzyme and hydrolase in addition to the initial angular dioxygenase, Habe et al.\textsuperscript{41} suggested that the influence of the chlorine substitution pattern of mono- to trichlorinated DFs and DDs on the formation of corresponding chlorocatechol and chlorosalicylic acid may not only depend on the substrate specificity of the initial angular dioxygenases but also on those of subsequent acting enzymes, especially hydrolase. Two DF (DD)-degrading bacteria, \textit{S. wittichii} \textit{strain RW1} and \textit{Sphingomonas} sp. \textit{strain RW16}, can transform monochlorinated DF by angular dioxygenation, \textit{meta} cleavage, and hydrolysis, but the resultant products, corresponding monochlorinated salicylic acids, were accumulated in the culture.\textsuperscript{27,29,40} Wittich and co-workers reported the complete mineralization of monochlorinated DF by the consortium consisting of a DF-degrader and a chlorosalicylic acid-degrading bacteria.\textsuperscript{29,40}

An extensive study of the influence of the substituted pattern on the depletion of many of the 210 congeners of polychlorinated DFs and DDs by several bacterial strains was reported by Schreiner et al.\textsuperscript{21} In the study, the DF-degrader \textit{Sphingomonas} (formerly \textit{Pseudomonas}) sp. \textit{strain HH69} degraded 31% of 2,3,7,8-tetrachloro-DF and 15% of 2,3,7,8-tetrachloro-DD within 84 days. Surprisingly, 1,2,4,5-tetrachlorobenzene-mineralizing \textit{Burkholderia}
(formerly Pseudomonas) sp. strain PS12 degraded 64% of 2,3,7,8-tetrachloro-DF in the same time, and 100% of 2,3,7,8-tetrachloro-DD within 25 days. Chlorine substitution in the 1-, 4-, 6-, or 9-position generally retarded the biodegradation.21)

(2) Bacterial biodegradation of dioxins in soils and sediments

Polychlorinated DDs/DFs are often bound to soil organic matter and surfaces of particles. The reduced bioavailability of halogenated DD/DF, therefore, is of interest and was examined by Harms and Zehnder.44,45 Megharaj et al.29 reported that culturing the S. wittichii strain RW1 first in soil extract medium prolonged the survival of this strain in soil. Halden et al.34 reported the removal of DF, DD, and 2-chloro-DD (10 ppm each) from a soil microcosm by the addition of strain RW1. The density of strain RW1 cells and the contents of soil organic matter influenced the rate and extent of removal. Recently, we tried experimentally to bioremediate an actual dioxin-contaminated soil that was contaminated mainly by tetra- to octachlorinated dioxins, by using the soil slurry system and P. resinovorans strain CA10 cells.60 The soil slurry (ratio of soil:water, 1:5, wt/vol) prepared with dioxin-contaminated soil collected at an incinerator site was incubated with CAR-grown strain CA10 cells. During the 7-day incubation, the total amount of chlorinated DD and DF congeners and toxicity equivalency quantity (TEQ) decreased from 725 to 665 ng/g soil and 11 to 9.4 ng TEQ/g soil, respectively, by a single inoculation of the CAR-grown strain CA10 cells (1011 CFU/g dry soil). Although the degradation rate of total dioxin was 8.3%, strain CA10 had a potential to transform tetra- to heptachlorinated congeners including the most toxic compound, 2,3,7,8-tetrachlorinated DD. With a similar slurry system, a preliminary bio-remediation experiment with Terrabacter sp. strain DBF63 cells was done.49 From this experiment, we detected approximately 10% depletion of tetra- to hexachlorinated congeners by 7 days of incubation with DF-grown strain DBF63 cells (109 CFU/g dry soil).

Conclusions and Prospects

Because angular dioxygenation for dioxin results in the spontaneous cleavage of the three-ring structure, the single-step oxidation destroys the planar structure from which the toxicity of dioxin originates. This fact indicates the primary importance of initial angular dioxygenation in decontamination of dioxin. Recently, biphenyl 2,3-dioxygenase, the initial dioxygenase in the biphenyl degradation pathway by Burkholderia sp. strain LB400, has been reported to catalyze the angular dioxygenation for DD and DF.68 Suenaga et al.69 also reported that the biphenyl 2,3-

dioxygenase with point mutation(s) introduced of P. pseudoalcaligenes strain KF707 catalyzed the angular dioxygenation for DD and DF. These results indicate that this novel dioxygenation is one of the reactions originating from the “relaxed” substrate specificity of the Rieske nonheme iron oxygenase super family. Recently, the three-dimensional structure of the terminal oxygenase component of naphthalene 1,2-dioxygenase has been reported.77,78 The structure of the angular dioxygenase will provide useful information on the “relaxed” substrate recognition and activation of the reaction.

On the basis of biochemical and genetic information, the extension of the substrate range of dioxin-degrading bacteria, higher expression of dioxin degrading enzyme(s), and the construction of the hybrid metabolic pathway allowing the improved mineralizing capacity for dioxins will probably be achieved in the near future. Although, to optimize the biodegradation conditions, a laboratory scale-microcosm study, a feasibility study with a pilot plant, and a field trial on a long-term basis are still necessary, bacteria harboring the dioxin-degrading capacity are promising for the bioremediation of dioxin contamination.

The well-clustered genetic organization of entire pathways or independently functioning pathway segments developed through exposure to corresponding compounds for a long time. On the other hand, the degradative genes involved in the degradation of higher recalcitrant compounds seemed to be disordered or separated. In fact, dioxin-degrading genes were separated from each other on the genomes of S. wittichii strain RW1 and Terrabacter sp. strain DBF63.43 Dioxin- and CAR-degrading car genes of P. resinovorans strain CA10 are disordered and have a novel mosaic structure.58 Perhaps the different levels of maturation in genetic structures resulted from the differences in the length of exposure to xenobiotics or rarity of the gene(s) indispensable for degradation. That is, the genetic analysis of the degradative genes for dioxin and CAR will provide useful information on the maturation (evolution) of xenobiotic-degrading gene clusters. Acknowledgments

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