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Functional Expression of Three Rieske Non-Heme Iron Oxygenases Derived from Actinomycetes in *Rhodococcus* Species for Investigation of Their Degradation Capabilities of Dibenzofuran and Chlorinated Dioxins

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The activity of Rieske non-heme iron oxygenases (aromatic hydrocarbon dioxygenases, AhDOs) is important for the bacterial degradation of aromatic pollutants such as polycyclic aromatic hydrocarbons and dioxins. During our analysis of the role of AhDOs in dioxin bioremediation, some enzymes derived from high G + C Gram-positive actinomycetes were difficult to produce in active form in the *Escherichia coli* protein expression system. In this study, we constructed a heterologous expression system for AhDOs in *Rhodococcus* species using a constitutive expression promoter, P_{dfdB}, and a shuttle vector, pRK401, and analyzed the ability of these enzymes to degrade dibenzofuran and deplete several chlorinated dioxins. Three active AhDOs expressed in *Rhodococcus* strains that were difficult to obtain by the *E. coli* system showed different regiospecificities for dibenzofuran bioconversion as well as different substrate depletion specificities for chlorinated dioxins. Moreover, AhDO derived from *R. erythropolis* TA421 showed relatively diverse depletion-substrate specificity for chlorinated dioxins.

Key words: *Rhodococcus*; Rieske non-heme iron oxygenase; dibenzofuran; biphenyl; dioxins

Environmental pollution by chlorinated dibenzo-*p*-dioxins and dibenzofurans (dioxins) is of increasing concern since these compounds are unintentional by-products of several industrial processes, such as the combustion of garbage and the synthesis of chlorinated organic compounds. They are carcinogenic, mutagenic, and immunotoxic to animals,[1–3] and show endocrine-disrupting activity even at trace concentrations.[4,5] Since dioxins are highly resistant to biological decomposition, they remain in the environment for prolonged periods.[6,7]

Chlorinated dioxins can be microbially degraded (for reviews, see refs. 8–11). In general, degradation proceeds co-metabolically, and it is relatively slow for some congeners, especially of highly chlorinated dioxin. This tendency may be due partially to differences in the substrate specificities of the degrading enzymes. For successful bioremediation of the dioxin-polluted environment, enzymes must be found that can degrade highly chlorinated dioxins more efficiently.

Rieske non-heme iron oxygenases (aromatic hydrocarbon dioxygenases, AhDOs) potentially can attack dioxins. These multi-component enzymes consist of subunits for electron transfer and a terminal oxygenase that contains Rieske-type iron-sulfur clusters as redox centers.[12] They catalyze the hydroxylation of aromatic compounds using reducing equivalents of NAD(P)H and molecular oxygen to convert substrates to dihydrodiol derivatives. Recent findings suggest that some microorganisms harbor multiple copies of various AhDO genes, such as *Rhodococcus jostii* RHA1[13] and *Burkholderia xenovorans* LB400,[14] and these multiple AhDOs might contribute to the degradation of aromatic compounds, even if they are non-native substrates, since AhDOs generally show broad substrate specificities.[15–18] To evaluate the degradation capability of the various AhDOs, it is important to use bacterial strains that are not a wild-type xenobiotic degraders but non-xenobiotic degraders to express the AhDO gene heterologously. Habe *et al.* evaluated the degradation capability of dioxins with two different AhDOs using the *E. coli* protein expression system, and indicated that the AhDOs had different spectra of degradative competence.[19] However, for some AhDOs derived from high-G+C Gram-positive actinomycetes, it has been difficult to obtain active enzymes in the *E. coli* protein expression system (our unpublished results and refs. 20–23).

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Abbreviations: AhDO, aromatic hydrocarbon dioxygenase; DF, dibenzofuran; DD, dibenzo-*p*-dioxin; BP, biphenyl; THBP, 2,2′,3-trihydroxybiphenyl; 2-OHDF, 2-hydroxydibenzofuran; RT, retention time

Although similar problems not limited to the heterologous expression of AhDOs have been reported for the expression of other actinomycetales genes using *E. coli*,[24–27] solutions have not been fully elucidated. A functional expression system for actinomycetales genes is essential to investigate and utilize the diverse catabolic abilities of these organisms.

We have cloned and analyzed AhDO genes of the dibenzofuran (DF)-utilizing strain *Terrabacter* sp. YK3[20] and the biphenyl (BP)-utilizing strains *Rhodococcus erythropolis* TA421[28] and *R. rhodochrous* K37.[23] In this study, three actinomycetales-derived AhDO genes for which it is difficult to produce functionally active proteins in *E. coli* were functionally expressed in a *Rhodococcus* strain using a constitutive expression promoter for the genus *Rhodococcus*. Their ability to degrade and deplete DF and chlorinated dioxins was analyzed.

Materials and Methods

Bacterial strains, media, and culture conditions. Rhodococcus sp. strain RD2[29] and *R. erythropolis* strains TA422,[30] JCM 2892, and JCM 3201 were used as hosts in the evaluation of AhDO expression, since they do not degrade DF or BP. Strains RD2 and TA422 have spontaneously lost mutant genes for the catabolism of DF and BP in *Rhodococcus* sp. YK2 and *R. erythropolis* TA421 respectively. Strain JCM 2892 (the same as strain IAM 1399) has been used as a host strain for the heterologous expression of the BP dioxygenase gene in *R. jostii* RHA1.[31] Strain JCM 3201 is the type strain of *R. erythropolis*. *Rhodococcus* strains were cultured at 30 °C in Luria-Bertani (LB) medium supplemented with 10 mM glucose (LBG) or MM3Y supplemented with an appropriate carbon source.[29] The carbon sources used in this study were 10 mM glucose, 0.1% DF, and 0.1% BP. Kanamycin (25 μg ml^{-1}, except for strain TA422, at 100 μg ml^{-1}), which were added to the media for the selection of transformants. All the chemicals used in this study were of the highest purity, from Sigma-Aldrich Chemicals (St. Louis, MO), Nacalai Tesque (Kyoto, Japan), Kanto Chemicals (Tokyo), and Wako Pure Chemicals (Osaka, Japan). The chemicals used in the identification of the metabolites are as follows: 2,2′,3-trihydroxybiphenyl (THBP; Wako), 2-hydroxydibenzofuran (2-OHDF; Sigma-Aldrich), and (1S-*cis*)-3-phenyl-3,5-cyclohexadiene-1,2-diol (*cis*-2,3-dihydro-2,3-dihydroxybiphenyl; Sigma-Aldrich).

Construction of AhDO-expression plasmid. Polymerase chain reaction (PCR) was carried out using ExTaq DNA polymerase (Takara Bio, Shiga, Japan) or KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The PCR primers used in this study are listed in Table 1. The nucleotide sequences of PCR-amplified DNA were confirmed prior to use in the experiments. We used a strong, constitutive promoter consisting of the 629-bp promoter region of the dioxygenase gene *dfdB*[29] (P_{dfdB}) with the sequence for *Sfo*I introduced just downstream of the start codon of *dfdB*. Expression constructs of AhDO were ligated to *Rhodococcus-E. coli* shuttle vector pRK401[20] and were used in the transformation of *Rhodococcus* strains. Genetic maps of our AhDO expression constructs are shown in Fig. 1.

A 5.2-kb *Pst*I fragment that encoded *dfdA1A2A3A4* genes derived from *Terrabacter* sp. YK3[20] was used as a wild-type promoter control (pRK401-dfdA). For promoter replacement (*i.e.*, to switch from P_{dfdA} to P_{dfdB}), the *dfdA1-A4* genes with *Eco*RV sites introduced just downstream of the start codon of *dfdA1* were PCR-amplified and ligated downstream of P_{dfdB} (pRK401-P_B+dfdA). A plasmid, pTB1, that encoded *bphA1A2A3A4* of *R. erythropolis* TA421 was used as the PCR template,[28] and the amplified DNA fragment extending from the start codon of $bphA1_{TA421}$ to the stop codon of $bphA4_{TA421}$ was ligated downstream of P_{dfdB} (pRK401-P_B+bphA$_{TA421}$). The two functionally unidentified genes in the *bph* gene cluster of *R. rhodochrous* K37[23] separating the terminal dioxygenase genes *bphA1A2* from the electron transfer protein genes *bphA3A4* were removed. The DNA fragments for *bphA1A2* and *bphA3A4* were ligated downstream of the P_{dfdB}, as illustrated in Fig. 1 (pRK401-P_B+bphA$_{K37}$).

Table 1. Primer Sequences Used in This Study

Primer name	Sequence (from 5′ to 3′)[a]
P_{dfdB}-5′	CGGTCGAGGATCTGCTC
P_{dfdB}-3′	GGCGCCCACGGGCCCTCCCCTTTCTC
dfdA1-5′	GATATCACCGTGAATGACAGTGGTC
M13M4	GTTTTCCCAGTCACGAC
$bphA1_{TA421}$-5′	ATGACCAACCAATTGGGTCGCAC
$bphA4_{TA421}$-3′	TCATATGTGGGCACGAGGCGAGC
$bphA1_{K37}$-5′	GATATCACAGTTGATTCGCGC
$bphA2_{K37}$-3′	TCAGAACAGAACGCTGATGT
$bphA3_{K37}$-5′	TCTAGAAAGGAGGCAACACGTGAGCGGGCA-CTCC
$bphA4_{K37}$-3	TCTAGACGTATCGCCCATCATTTC

[a]Restriction endonuclease recognition sequences introduced into the primer sequences are underlined.

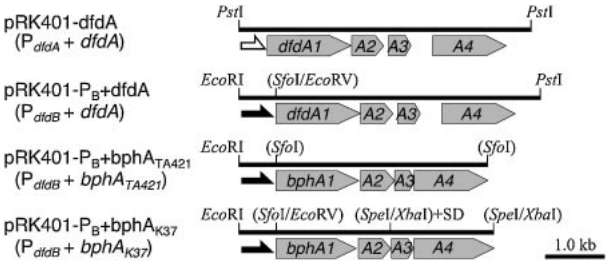

Fig. 1. Structures of AhDO-Expression Constructs Used in This Study.

The size and direction of the open reading frames (ORFs), promoter for *dfdA* (P_{dfdA}), and heterologous promoter P_{dfdB} are indicated by gray, white, and black arrows respectively. Gene names are shown in the arrows. The positions of the restriction endonuclease recognition sites used for construction and the positions of the sites no longer recognized by restriction endonucleases are indicated outside and within parenthesis respectively. The Shine-Dalgarno sequence (SD) introduced upstream of the $bphA3_{K37}$ with PCR is shown.

Resting cultures for the biotransformation of DF. Biotransformation of DF and BP was performed in resting cell cultures prepared from LBG-grown cells.[29] For quantification of DF and its metabolites, resting culture (2 ml adjusted to $OD_{600} = 5.0$ by adding 2 μmol of DF) was incubated for 3 h at 30 °C with 180 rpm rotary shaking and extracted twice with 4 ml of ethyl acetate after acidification to about pH 2.0 with 6 M HCl. The dried extracts dissolved in 2 ml of acetonitrile were analyzed by HPLC. The mobile phase was 0.05% (v/v) trifluoroacetic acid containing 75% (v/v) acetonitrile, and the peak areas of the residual substrates and metabolites were measured at 250 nm in three separate resting culture samples. The lateral dioxygenation products of DF (*i.e.*, *cis*-dihydrodiol metabolites) were measured after acid dehydration as monohydroxydibenzofuran. THBP, 2-OHDF, and DF were quantified by comparison of the areas under the peak for each sample with those for each calibration standard solution (0.0125–1.5 mM) at 250 nm.

The metabolites of the $BphA_{K37}$ sample were further analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The sample (20 μl) was injected into a reverse-phase Inertsil ODS-3 column (1 × 100 mm; GL Sciences, Tokyo) connected to a model 1100 series liquid chromatography system (Agilent Technologies, Waldbronn, Germany). The peaks were eluted at a flow rate of 50 μl min^{-1} by isocratic elution with 0.08% (v/v) trifluoroacetic acid in 36% (v/v) acetonitrile, and 10-μl fractions were collected. Selected fractions were subjected to MALDI-TOF MS (Ultraflex MALDI-TOF; Bruker-Franzen Analytik, Bremen, Germany) in reflector mode using 2,5-dihydroxybenzoic acid as matrix. The observed spectra were recalibrated internally using matrix related ions (Na form 177.01638u, K form 192.99031u).

Assay for depletion of chlorinated dioxins. Depletion of chlorinated dioxins was assayed with cells prepared as described above, which were suspended in MM3Y medium with 5 mM glucose to a density of $OD_{600} = 15$. Chlorinated dioxins dissolved in *N,N*-dimethylformamide were added to 2 ml of cell suspension (final concentration, 10 μg ml^{-1}) and incubated for 48 h at 30 °C with 200 rpm rotary shaking. After incubation, the remaining substrates were extracted twice with 2 volumes of ethyl acetate, and the dried extract was re-dissolved in 1.0 ml of toluene. Chlorinated dioxins were quantified in three individual extracts of each substrate with GC-MS, as described previously.[32] Substrates extracted from the resting cell cultures of RD2 transformed with vector plasmid pRK401 were used as negative control. The substrate depletion rates were calculated by comparing the amount of substrate remaining in the AhDO-expressed resting cell cultures, the mean being the amount of substrate remaining in the negative control.

Results and Discussion

Heterologous expression of DfdA in Rhodococcus *species*

There are several examples of heterologous expression of genes derived from actinomycetes in host strains other than *E. coli*, such as the genera *Pseudomonas*,[22] *Streptomyces*,[25,27,33] and *Rhodococcus*.[20,21,23,24,26] The genus *Rhodococcus* has been extensively investigated, especially for its ability to degrade diverse organic compounds.[34] In addition, the presence of a hydrophobic cell envelope[35,36] might provide *Rhodococcus* with advantages for the degradation of hydrophobic xenobiotics. Hence we decided to use strains of the genus *Rhodococcus* as hosts for the heterologous expression of actinomycetales AhDOs that could not be obtained in active form by means of the *E. coli* protein expression system.

The activity of DF-dioxygenase (*dfdA1-A4*) expressed under the control of the wild promoter (P_{dfdA}) was compared with that expressed under the control of a constitutive strong promoter (P_{dfdB}) (Fig. 1). In strain RD2, we found that a regulatory gene involved in the transcriptional activation of P_{dfdA} was lacking, and we observed low level, constitutive expression of P_{dfdA} (our unpublished results). Resting cells of RD2 transformed with pRK401-dfdA (P_{dfdA}-*dfdA*) or pRK401-P_B+dfdA (P_{dfdB}-*dfdA*) were incubated with DF, and the accumulation of THBP (a DF metabolite) in the culture supernatants after 2 and 5 h was determined by HPLC (Table 2). The switch from P_{dfdA} to P_{dfdB} increased THBP accumulation about 12 times (2 h) and 10 times (5 h). Next we examined the ability of different host strains with the DfdA-expression construct to degrade DF (Table 2). The highest activity was detected in strain RD2. Hence we decided to use strain RD2 as the host in further analysis.

Heterologous expression of two different BphAs in Rhodococcus *sp. RD2*

The *bphA* genes, $bphA_{TA421}$ and $bphA_{K37}$, were derived from two BP-utilizing *Rhodococcus* strains, *R. erythropolis* TA421 and *R. rhodochrous* K37, respectively. We have reported that both of these genes were involved in BP utilization.[23,28] The amino acid sequences of their respective BphA subunits had only from 35% (BphA4) to 44% (BphA3) identity. Our preliminary attempts to express the two BphAs in *E. coli* failed (data not shown for $BphA_{TA421}$, see ref. 23 for $BphA_{K37}$). In this study, resting cell cultures of RD2-transformants of both $bphA_{TA421}$ and $bphA_{K37}$ expressed under the control of P_{dfdB} converted BP to *cis*-2,3-dihydro-2,3-dihydroxybiphenyl (data not shown).

Table 2. Bioconversion of Dibenzofuran to 2,2′,3-Trihydroxybiphenyl with Different Expression Promoter and Host Strains

Strains and expression constructs	Cultivation periods and activity[a]	
	2 h	5 h
Rhodococcus sp. RD2 (pRK401-dfdA)	26.4	54.4
Rhodococcus sp. RD2 (pRK401-P_B+dfdA)	333	538
R. erythropolis TA422 (pRK401-P_B+dfdA)	239	351
R. erythropolis JCM 2892 (pRK401-P_B+dfdA)	247	328
R. erythropolis JCM 3201 (pRK401-P_B+dfdA)	140	203

[a]Activity is the amount of 2,2′,3-trihydroxybiphenyl (μM) produced by 1 OD_{600} unit of cells. Quantification of 2,2′,3-trihydroxybiphenyl was performed as described in "Materials and Methods."

Biotransformation of DF with AhDO expressed in Rhodococcus *sp. RD2*

This study compared the DF dioxygenation ability of three AhDO enzymes using AhDO-expressing resting cells of RD2. We dehydrated the *cis*-dihydrodiols of DF to monohydroxy derivatives by acid catalysis prior to extraction, since some of the dihydrodiols of DF gradually dehydrated spontaneously during sample preparation and HPLC analysis. The HPLC chromatograms of the extracts at 250 nm and within UV absorption spectrum from 200 to 360 nm are shown in Fig. 2A and B.

DF was added at a final concentration of 1 mM to the resting cultures, and we detected 0.88 mM of DF in the negative control cultures (a pRK401-transformed RD2 strain). No metabolite of DF was observed in the negative control cultures (Fig. 2A, top), suggesting that the missing 0.12 mM of DF had been lost during the experimental process or was incorporated into the cells. Compared to the negative controls, the resting cultures of the *dfdA*-expressing strain degraded 94% of DF (0.83 mM) after 3 h, but two *bphA*-expressing strains degraded only 19% ($BphA_{TA421}$) and 28% ($BphA_{K37}$) of the DF (0.17 and 0.25 mM respectively). In the extracts of resting cultures of the *dfdA*-expressing strain, a major peak and a minor peak with retention times (RT) of 3.3 and 4.6 were identified as THBP and 2-OHDF respectively (Fig. 2A and B). The 2-OHDF is probably derived from *cis*-1,2-dihydro-1,2-dihydroxydibenzofuran by acid dehydration[37] (Fig. 2C), since the *cis*-1,2-dihydrodiol of DF rather than the *cis*-2,3-dihydrodiol has frequently been identified as a metabolite of DF formed by AhDOs.[16,33,38] The amount of 2-OHDF accumulated in the media was 0.014 mM (this amount was only 1.7% of that of the THBP, 0.84 mM), suggesting that DfdA oxygenates DF specifically at position 4, 4a.

Of the two major peaks found in the culture supernatants of the BphA-expressing strains (Fig. 2A, two bottom chromatograms), one (RT = 4.6) was identified as 2-OHDF. MALDI-TOF-MS analysis of the other peak (RT = 4.8) revealed the monoisotopic mass to

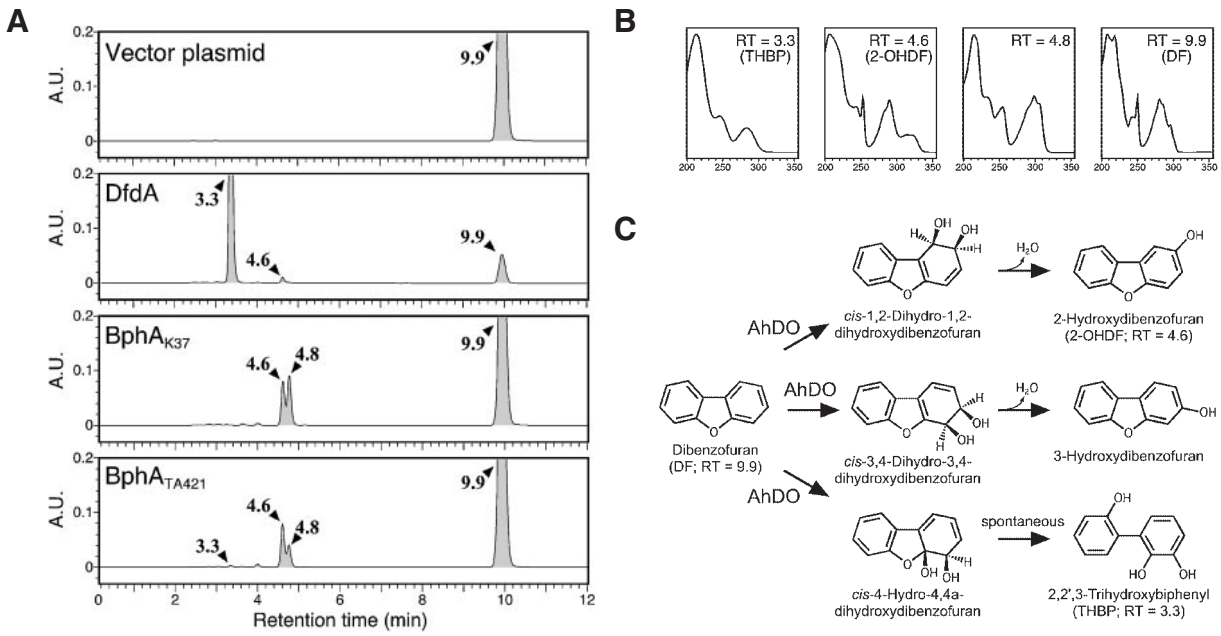

Fig. 2. Degradation of DF with Three AhDO-Expressing RD2 Strains.

Extract of resting cell cultures of strain RD2 expressing the DfdA, BphA$_{K37}$, and BphA$_{TA421}$ enzymes and a negative control (strain RD2 transformed with vector plasmid pRK401) were analyzed by HPLC. A, A 250-nm chromatogram (representative of three experiments) showing separation of DF metabolites from cultures of cells transformed with the expression constructs and vector plasmid. Significant peaks (arrowheads) detected on HPLC analysis with retention times are indicated. The UV-absorption spectra of the peaks are shown in B. Peaks identified with authentic chemicals are 2,2′,3-trihydroxybiphenyl (RT = 3.3), 2-hydroxydibenzofuran (2-OHDF; RT = 4.6), and dibenzofuran (DF; RT = 9.9). The peak with RT = 4.8 was identified as mono-hydroxydibenzofuran, and was perhaps 3-hydroxydibenzofuran judging by the spectrum features. Specific AhDO-catalyzed and spontaneous or acid-catalyzed dehydration of DF is illustrated in C.

be 184.051u, in good agreement with the calculated monoisotopic mass for $C_{12}H_8O_2$ (184.052u), suggesting that this is a derivative of monohydroxyl DF. The UV absorption maxima of the product (215, 232, 255, 298, and 305; Fig. 2B, RT = 4.8) closely resembled those of 3-hydroxydibenzofuran,[37,39] leading to the speculation that it was 3-hydroxydibenzofuran and was derived from the *cis*-2,3- or *cis*-3,4-dihydrodiol of DF. The latter compound (Fig. 2C) is more likely, since it is a known lateral dioxygenation product of DF by AhDOs.[16,38] A small amount of THBP accumulation was observed in the BphA$_{TA421}$ samples (2.4% of the degraded DF), but not in the BphA$_{K37}$ samples. These results suggest that the two BphA enzymes oxygenate at the lateral rather than angular positions of DF, and yet oxygenate at different lateral positions.

The large subunit of the terminal dioxygenase of AhDO contributes to substrate specificity.[12] The large subunits of the BP dioxygenases involved in BP-utilization in the two *Rhodococcus* strains, BphA1$_{TA421}$ in *R. erythropolis* TA421 and BphA1$_{RHA1}$ in *R. jostii* RHA1, showed 79.5% identity, suggesting that the two AhDOs have similar substrate specificities. Recently, biotransformation of several aromatic compounds by resting cells of *R. erythropolis* IAM 1399 expressing BphA$_{RHA1}$ has been reported.[18] The results reported and obtained in this study indicate that the AhDOs for BP-utilization expressed in heterologous *R. erythropolis* host strains are able to oxygenate DF, but that the oxygenation-position specificities of DF are different: the relative amount of angular DF-dioxygenation activity was greater in BphA$_{RHA1}$ (42% of total DF-oxygenation activity) than in BphA$_{TA421}$ (only about 2.4% of total DF-oxygenation activity). The clear difference in the specificity of the DF oxygenation position between the two relatively similar AhDOs is interesting, and indicates that these two AhDOs can be used to investigate enzymatic control of the oxygenation position specificity for DF by AhDOs.

AhDO-expressing Rhodococcus *sp. RD2 depleted chlorinated dioxins*

To determine the depletion-substrate specificities of the three AhDOs for chlorinated dioxins, this study observed the ability of AhDO-expressing RD2 strains to deplete chlorinated dioxins. The amount of residual chlorinated dioxins after 48 h of cultivation was compared among cultures of the three AhDO-expressing RD2 strains and the vector plasmid-transformed RD2 strain (Fig. 3A–C).

The three AhDO-expressing strains almost completely depleted 10 ppm of 2-chlorodibenzo-*p*-dioxin under the culture conditions used in this study (data not shown). The DfdA and BphA$_{TA421}$ strains depleted 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) by only 29% and 18% respectively, while the BphA$_{K37}$ strain depleted it by 99%. The BphA$_{TA421}$ strain (but not the DfdA- or BphA$_{K37}$ strains) depleted 98% of 2,7-DCDD, the DfdA- and BphA$_{TA421}$ strain (but not BphA$_{K37}$) depleted 2,8-dichlorodibenzofuran (2,8-DCDF) almost completely, and the two BphA strains (but not the DfdA) depleted 2,3-DCDD. The DfdA and BphA$_{TA421}$ strains weakly depleted 1,4-DCDD, but none of the strains depleted 1,6-DCDD. Only the BphA$_{TA421}$ strain depleted 2,3,7-trichlorodibenzo-*p*-dioxin (TrCDD), by about 40%.

The dichlorinated dioxins that were almost completely depleted by AhDO-expressing resting cells included 2,8-DCDF by DfdA, 1,3-DCDD by BphA$_{K37}$, and 2,3-

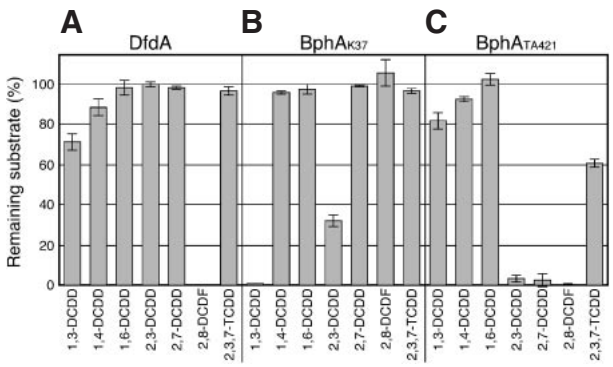

Fig. 3. Depletion of Chlorinated Dioxins with Three AhDO-Expressed RD2 Strains.

Residual chlorinated dioxins in RD2 cultures expressing genes of (A) DfdA, (B) $BphA_{K37}$, and (C) $BphA_{TA421}$, compared to the residual substrates in negative control cultures (RD2 cells transformed with vector plasmid). Bars represent the percentages of substrates remaining in culture. The amounts in the negative controls were arbitrarily set to 100%. The data are the means with standard deviations from three determinations.

DCDD, 2,7-DCDD, and 2,8-DCDF by $BphA_{TA421}$. Clearly, the three AhDO enzymes have different substrate depletion specificities for dichlorinated dioxins. Habe *et al.* investigated the degradation activity of dioxins by resting cells of *E. coli* that heterologously expressed AhDO.[19] They used two AhDOs having angular dioxygenation activity for DF, DbfA in *Terrabacter* sp. DBF63 (DFDO, a DF and fluorene-degrading AhDO) and CarA in *Pseudomonas* sp. CA10 (CARDO, a carbazole-degrading AhDO), and reported that DFDO-expressing resting cells degraded over 95% of 2,3-DCDD and 2,8-DCDF, but no 2,7-DCDD, and that CARDO-expressing resting cells degraded 60–80% of 2,3-DCDD and 2,8-DCDF and 20–40% of 2,7-DCDD. These and our findings confirm that the three angular dioxygenases DfdA, DFDO, and CARDO have strong ability to degrade 2,8-DCDF.

We found several reports on co-metabolic degradation of chlorinated dioxins by aromatic compound-degrading bacteria, wild-type strains that used catabolic pathways to degrade aromatic compounds for co-metabolism. In one study, a Gram-negative DF- and DD-utilizing bacterium, *Sphingomonas wittichii* RW1, was found to degrade 2,3- and 2,8-DCDF efficiently and 2,3-DCDD and 2,7-DCDF relatively slowly.[40] Fukuda *et al.* reported that DF-utilizing *Sphingomonas* sp. strain HL7 was similar to strain RW1 in degradation abilities as to 2,3-DCDD and 2,8-DCDF.[41] Moreover, a Gram-positive actinomycete, *Rhodococcus opacus* SAO101, utilizing DF and DD for growth, was found to deplete 2,3-, 2,7- and 2,8-DCDD by 16–23%.[42]

It has been reported that *S. wittichii* RW1 has multiple copies of the terminal dioxygenase subunit gene for AhDO,[43] and there is a possibility that these uninvestigated AhDOs affect dioxin degradation in RW1 cells. Also, it has been shown that *R. opacus* SAO101 can utilize diverse aromatic compounds,[44] and therefore might have multiple AhDO genes with different specificities in addition to *narAaAb* genes,[45] as reported for some diverse aromatics-degrading actinomycetes, such as *Rhodococcus jostii* RHA1,[46] *R. erythropolis* TA421,[28] and *Mycobacterium vanbaalenii* PYR-1.[47,48] In contrast, our results here and those of Habe *et al.* clearly indicate that individual AhDOs can specifically deplete chlorinated dioxins. Thus the experimental approach used in this study can facilitate the development of AhDOs that effectively degrade chlorinated dioxins.

The AhDO-expressing strains used in this study had limited ability to degrade 1,4-DCDD and 1,6-DCDD. Schreiner *et al.* analyzed the influence of substitution position on the biodegradation of all 210 congeners of polychlorinated DDs and DFs by aromatic compound-degrading bacteria, and found that chlorine substitution at position 1, 4, 6, or 9 generally retarded degradation.[49] Since we could not find other specific reports on the degradation of 1,4-, 1,6- or 1,9-dichlorinated dioxin congeners by aromatic compound-degrading bacteria, further investigation of these congeners is needed. These results and findings also suggest that AhDOs should be screened for degrading activity against these dichlorinated dioxins to isolate unusual substrate specificities for chlorinated dioxins.

The $BphA_{TA421}$ enzyme has wider specificity for dichlorinated dioxins and can deplete 2,3,7-TrCDD, which closely resembles the most toxic dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. These results suggest that the $BphA_{TA421}$ is a better dioxin-degrading enzyme. The $BphA_{TA421}$ enzyme has angular dioxygenation activity, while the $BphA_{K37}$ enzyme does not. This suggests that enzymes acting on non-chlorinated substrates at many different positions can catalyze the oxygenation of dioxins chlorinated at diverse positions. Further analysis of metabolites of chlorinated dioxins with AhDO-expressing *Rhodococcus* strains is required to demonstrate the relationships of degradative abilities and regiospecificities.

In conclusion, we report the expression of active AhDOs of actinomycetales that are difficult to obtain using the *E. coli* expression system. We determined their ability to transform and deplete DF and several chlorinated dioxins. The experimental system used in this study, consisting of a *Rhodococcus* host strain and the constitutive expression promoter P_{dfdB} active in *Rhodococcus* strains, may provide a way to study genes that are difficult to evaluate in the *E. coli* system.

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