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Remarkable Ability of *Pandoraea pnomenusa* B356 Biphenyl Dioxygenase To Metabolize Simple Flavonoids

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Many investigations have provided evidence that plant secondary metabolites, especially flavonoids, may serve as signal molecules to trigger the abilities of bacteria to degrade chlorobiphenyls in soil. However, the bases for this interaction are largely unknown. In this work, we found that BphAE_{B356}, the biphenyl/chlorobiphenyl dioxygenase from *Pandoraea pnomenusa* B356, is significantly better fitted to metabolize flavone, isoflavone, and flavanone than BphAE_{LB400} from *Burkholderia xenovorans* LB400. Unlike those of BphAE_{LB400}, the kinetic parameters of BphAE_{B356} toward these flavonoids were in the same range as for biphenyl. In addition, remarkably, the biphenyl catabolic pathway of strain B356 was strongly induced by isoflavone, whereas none of the three flavonoids induced the catabolic pathway of strain LB400. Docking experiments that replaced biphenyl in the biphenyl-bound form of the enzymes with flavone, isoflavone, or flavanone showed that the superior ability of BphAE_{B356} over BphAE_{LB400} is principally attributable to the replacement of Phe336 of BphAE_{LB400} by Ile334 and of Thr335 of BphAE_{LB400} by Gly333 of BphAE_{B356}. However, biochemical and structural comparison of BphAE_{B356} with BphAE_{p4}, a mutant of BphAE_{LB400} which was obtained in a previous work by the double substitution Phe336Met Thr335Ala of BphAE_{LB400}, provided evidence that other residues or structural features of BphAE_{B356} whose precise identification the docking experiment did not allow are also responsible for the superior catalytic abilities of BphAE_{B356}. Together, these data provide supporting evidence that the biphenyl catabolic pathways have evolved divergently among proteobacteria, where some of them may serve ecological functions related to the metabolism of plant secondary metabolites in soil.

Aryl hydroxylating Rieske-type dioxygenases (ROs) catalyze a *cis*-dioxygenation of aryl compounds to generate a *cis*-dihydrodiol metabolite. ROs are promising biocatalysts that metabolize many substituted benzene or diphenyl rings, as well as bicyclic- or tricyclic-fused heterocyclic aromatics, such as quinoline, dibenzofuran, phenanthridine, and flavonoids (3, 4, 15, 22, 29, 30, 33). The biphenyl dioxygenase (BPDO) which catalyzes the first reaction of the bacterial biphenyl catabolic pathway is an RO that has been extensively studied because of its ability to metabolize several polychlorinated biphenyl (PCB) congeners. The BPDO reaction (Fig. 1) requires three components (10, 12, 13). The catalytic component (BphAE) is an RO protein which is a heterohexamer made up of three α (BphA) and three β subunits (BphE). The ferredoxin (BphF) and the ferredoxin reductase (BphG) are involved in electron transfer from NADH to BphAE. The encoding genes for both *Burkholderia xenovorans* LB400 and *Pandoraea pnomenusa* B356 are *bphA* (BphAE α subunit), *bphE* (BphAE β subunit), *bphF* (BphF), and *bphG* (BphG) (6, 36). The α subunit is the one involved in the catalytic activity. It comprises two domains; the Rieske domain containing a 2Fe-2S Rieske cluster receives the electrons from BphF and transfers them to the catalytic mononuclear iron center of the catalytic domain (7).

Several investigations have shown that BPDO can metabolize flavonoids (4, 15, 29, 30). These plant secondary metabolites (PSMs) are regarded as very promising for the prevention and treatment of cancers (26) and cardiovascular diseases (35). Plants are currently the major source for these chemicals, but the synthesis of novel derivatives exhibiting improved biological properties is often difficult or impractical (22). Furthermore, in the context of the green chemistry concept, new, more selective and more environmentally friendly approaches to manufacture these biologically specific fine chemicals will be required.

Seeger et al. have shown that *B. xenovorans* LB400 BPDO

(BphAE_{LB400}) is able to dihydroxylate several isoflavonoids on ring B (29). BphAE_{LB400} has been extensively investigated because it is regarded as one of the most efficient dioxygenases of natural origin for the degradation of a wide range of chlorobiphenyls. However, in recent years, *P. pnomenusa* B356 BPDO (BphAE_{B356}) was shown to exhibit superior abilities to degrade several biphenyl analogs, including 2,6-dichlorobiphenyl, 2,4,4'-trichlorobiphenyl, and dichlorodiphenyltrichloroethane (DDT), that BphAE_{LB400} metabolizes poorly (9, 20). Furthermore, preliminary unpublished experiments have suggested that BphAE_{B356} metabolizes flavonoids more efficiently than BphAE_{LB400}. On the other hand, a mutant of BphAE_{LB400}, BphAE_{p4}, an evolved BPDO derived from BphAE_{LB400} by the substitutions Thr335Ala Phe336Met, was shown to metabolize a broader range of chlorobiphenyls and dibenzofurans than the parent BphAE_{LB400} (2). The new catalytic properties of this mutant were attributed to the single Thr335Ala substitution. Thr335 exerts constraints on a segment comprised of Val320-Gln322 that lines the catalytic pocket. Replacing Thr335 with Ala releases the constraints on this segment, allowing for more movement during substrate binding (18, 23).

The bacterial metabolism of flavonoids may also have an impact on soil microbiology and on plant-microbe interactions. Many investigations have provided evidence that PSMs may act as signal molecules to trigger the PCB-degrading abilities of soil bacteria (for a review, see reference 34). These signal molecules may have a major impact on the success of rhizoremediation processes

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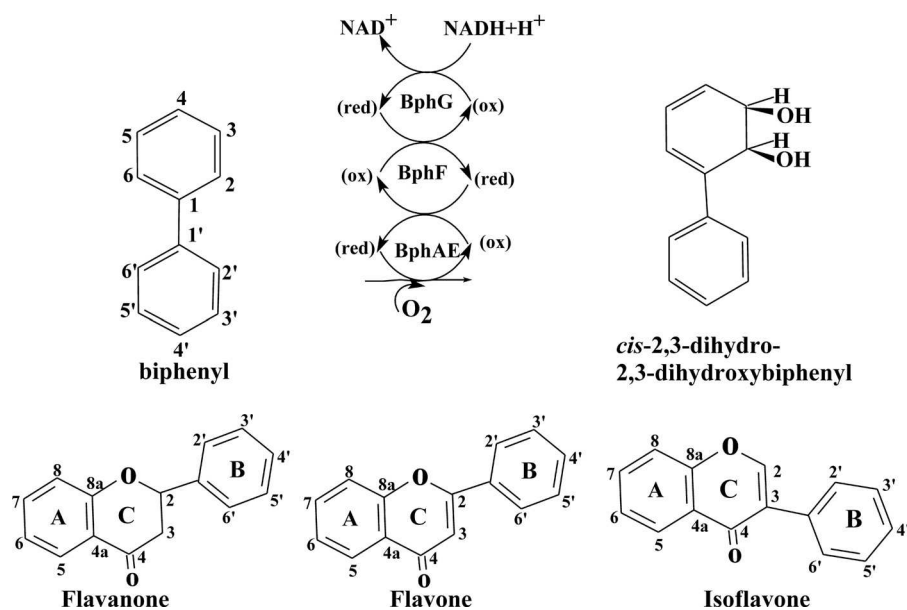


FIG 1 Biphenyl dioxygenase reaction (top) and structures of flavone, flavanone, and isoflavone (bottom).

aiming at the destruction of PCBs in soil. However, the bases for the PCB-degrading bacterium-plant secondary metabolite interactions are largely unknown. In a recent work (37), we showed that *Arabidopsis thaliana* root exudates trigger the PCB catabolic abilities of *Rhodococcus erythropolis* U23A, a rhodococcal rhizobacterium isolated from the rhizosphere of PCB-contaminated-plant roots. Flavanone, one of the major component of these root exudates, was unable to support the growth of strain U23A, but it was metabolized by this strain through its biphenyl catabolic pathway (37). In addition, when used as a cosubstrate with sodium acetate, flavanone was as efficient as biphenyl at inducing the biphenyl catabolic pathway of strain U23A (37). These observations are consistent with the proposed hypothesis whereby flavonoids would act as a signal molecule in soil to modulate the quantity and quality of phenylpropanoids in the rhizosphere (31).

Given the significant impacts the bacterial metabolism of flavonoids may have on green chemistry and on PCB remediation processes and given the preliminary data showing that the two well-characterized *P. pnomenusa* B356 and *B. xenovorans* LB400 BPDOs metabolized flavonoids differently, we compared the catalytic properties of BphAE_{LB400} and BphAE_{B356} toward the simple flavonoids flavone, isoflavone, and flavanone and we assessed the ability of these flavonoids to induce the biphenyl catabolic pathway of these two organisms. In order to get more insights about structural features of BphAE conferring the ability to metabolize these flavonoids, we also docked these chemicals in these protein structures and compared the structure of the docked enzymes with that of BphAE_{p4}.

MATERIALS AND METHODS

Bacterial strains, plasmids, and chemicals. Wild-type strains *P. pnomenusa* B356 and *B. xenovorans* LB400 were described previously (1, 6). All plasmids used in this study were described previously. pET14b[LB400-*bphAE*] and pET14b[*p4-bphAE*] carry the genes encoding the wild-type BphAE_{LB400} and its mutant BphAE_{p4} (Thr335Ala Phe336Met) (2, 17), plasmid pET14b[B356-*bphAE*] carries the genes encoding BphAE_{B356} (20), and plasmids pET14b[LB400-*bphF*] and

pET14b[LB400-*bphG*] carry the genes encoding strain LB400 BphF and BphG (23). Flavone and flavanone were from Sigma-Aldrich, and isoflavone from Indofine Chemical Company, Inc. They were all 99% pure.

Whole-cell assays to determine the ability of *P. pnomenusa* B356 and *B. xenovorans* LB400 to metabolize flavanone. The metabolism of flavanone by resting-cell suspensions of biphenyl-induced cells of *P. pnomenusa* B356 and *B. xenovorans* LB400 was examined according to a protocol described previously to investigate the metabolism of flavanone by *R. erythropolis* U23A (37). Briefly, each strain was grown overnight on medium MM30 (37) containing 3.4 mM biphenyl, and the cells were harvested, washed, and suspended in M9 medium (37) with no carbon source to an optical density at 600 nm (OD₆₀₀) of 5. This cell suspension was distributed (5-ml amounts) among 50-ml glass tubes, and flavanone was added to a final concentration of 200 μM. The resting-cell suspensions were incubated at either 28°C or 15°C for various periods of between 5 min and 18 h. They were then extracted with ethyl acetate, the organic phase was evaporated, and the residues were treated with *n*-butylboronate (*n*BuB) or *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco, Sigma-Aldrich) as described previously for gas chromatography-mass spectrometry (GC-MS) analyses (37).

Assays to identify the metabolites produced from flavone, flavanone, and isoflavone by BphAE_{B356}, BphAE_{LB400}, and BphAE_{p4} and to determine their kinetic parameters. Reconstituted His-tagged BPDO preparations were used in the experiments to identify the metabolites and kinetics of the enzymes and substrates. His-tagged purified enzyme components were produced in recombinant *Escherichia coli* strains and purified according to published protocols (23). The enzyme assays were performed at 37°C as described previously in a volume of 200 μl in 50 mM morpholinethanesulfonic acid (MES) buffer, pH 6.0, containing 100 nmol substrate (13). The metabolites were extracted at pH 6.0 with ethyl acetate and treated with *n*BuB or BSTFA for GC-MS analyses.

Catalytic activities were determined by monitoring substrate depletion and metabolite production after 10 min of incubation under the conditions described above. GC-MS peak areas were used to quantify substrate depletion and metabolite production. GC-MS analyses were performed using a Hewlett Packard HP6980 series gas chromatograph interfaced with an HP5973 mass selective detector (Agilent Technologies). The mass selective detector was operated in electron impact ionization (EI) mode and used a quadrupole mass analyzer. The steady-state

kinetic parameters of all BphAEs were determined by recording oxygen consumption rates using a Clarke-type Hansatech model DW1 oxygraph (14) for various concentrations of flavonoids between 5 and 150 μ M. The kinetic parameters reported in this work were obtained from the analysis of at least two independently produced preparations tested in triplicate.

Assays to assess the ability of flavone, flavanone, and isoflavone to induce the biphenyl catabolic pathway of strains B356 and LB400. The induction of the biphenyl catabolic pathway of strains B356 and LB400 was assessed on the basis of the amount of 4-chlorobenzoate produced from 4-chlorobiphenyl by resting-cell suspensions previously grown on sodium acetate plus variable concentrations of flavonoids or biphenyl. This assay was performed according to the same method as the previously described assay to assess the ability of flavanone to induce the biphenyl catabolic pathway of *R. erythropolis* U23A (37). Briefly, cells were grown overnight in medium MM30 amended with 30 mM sodium acetate or with 30 mM sodium acetate plus variable amounts (6 mM, 1 mM, 0.01 mM, or 0.001 mM) of flavone, isoflavone, flavanone, or biphenyl. Cells were harvested and washed in M9 medium without carbon source. The suspensions were adjusted to an OD₆₀₀ of 1 with M9 medium and distributed in portions of 200 μ l into 1.5-ml Eppendorf tubes. 4-Chlorobiphenyl was added to a final concentration of 1.25 mM, and the reaction vials were incubated for 120 min at 28°C in an Eppendorf Thermomixer 5436. The suspensions were then acidified with HCl before the metabolites were extracted with ethyl acetate. The extracts were evaporated, and the residues were derivatized with BSFTA for GC-MS analysis (37).

Docking and structure analysis. BphAE_{LB400} (RCSB Protein Databank PDB ID 2XRX), BphAE_{B356} (RCSB Protein Databank PDB ID 3GZX), and BphAE_{p4} (RCSB Protein Databank PDB ID 2XSH) were used as protein targets, and they were prepared as previously described (20). In the case of BphAE_{LB400} and BphAE_{p4}, we used the structural coordinates of dimer AB for the docking. Ligands were all downloaded as sdf files from PubChem (<http://pubchem.ncbi.nlm.nih.gov>) and converted into pdb format in Discover Studio Visualizer 2.5. Both proteins and ligands were processed with AutoDockTools to obtain their proper pdbqt format. The searching space for the ligand was centered on the mononuclear iron and contained 20 Å in each x, y, and z direction. Autodock Vina 1.1.2 (24) with the default parameters was used to perform the automatic docking.

RESULTS

Metabolism of flavanone by biphenyl-induced resting cells of strains B356 and LB400. In a previous report, we showed that although flavanone could not support the growth of *R. erythropolis* U23A, biphenyl-induced cells of strain U23A metabolized this plant metabolite (37). The induced cells of strain U23A produced small amounts of 2-(2,3-dihydro-2,3-dihydroxyphenyl)chromane-4-one and 2-(3,4-dihydro-3,4-dihydroxyphenyl)chromane-4-one when they were incubated in the presence of flavanone, but the major and ultimate metabolite exhibited mass spectral features corresponding to those of 4-oxo-2-chromanecarboxylic acid (37). Neither strain B356 nor strain LB400 could grow on flavone, isoflavone, or flavanone, but biphenyl-induced cells of both converted flavanone to 4-oxo-2-chromanecarboxylic acid as a dead-end metabolite. 4-Oxo-2-chromanecarboxylic acid was identified from the mass spectral features of its trimethylsilyl (TMS) derivative, which exhibited diagnostically important ions at m/z 264 (M^+), 249 ($M^+ - CH_3$), 219 ($M^+ - 3CH_3$), 205 ($M^+ - CH_3 - O - CO$), 174 ($M^+ - COOTMS$), and 131 ($M^+ - COOTMS - O$).

When the resting cell suspensions of strain B356 were incubated at 15°C and for less than 5 min, in addition to 4-oxo-2-chromanecarboxylic acid, small amounts of 2-(2,3-dihydro-2,3-dihydroxyphenyl)chromane-4-one and 2-(3,4-dihydro-3,4-dihydroxyphenyl)chromane-4-one were detected. These two metabolites were identified on the basis of their GC-MS spectral features as described below for the

purified enzyme preparation. When the resting cells were incubated at a higher temperature and for longer incubation periods, 4-oxo-2-chromanecarboxylic acid was the only metabolite detected in the culture. This shows that the biphenyl catabolic enzymes of this organism were very efficient in transforming flavanone to 4-oxo-2-chromanecarboxylic acid. In addition, while cells of strain B356 metabolized more than 80% of the added substrate within 5 min at 15°C, cells of strain LB400 metabolized less than 20% of the substrate when they were incubated for 1 h at 28°C. This shows the superiority of strain B356 over strain LB400 in metabolizing flavanone.

Induction of the biphenyl catabolic pathway of strains B356 and LB400 by flavonoids. Flavanone induction was assessed by monitoring the 4-chlorobenzoate produced from 4-chlorobiphenyl which, in both strains B356 and LB400, accumulates as a dead-end metabolite of the biphenyl catabolic pathway. In a recent report, it was shown that the biphenyl catabolic genes are expressed constitutively at low levels during the growth of *B. xenovorans* LB400 on succinate (28). Furthermore, the level of expression of the pathway enzymes appeared to be influenced by posttranscriptional regulation factors and by the physiological state of the cells, which may significantly influence the chlorobiphenyl degradation abilities of cells (28). In spite of these difficulties, we reasoned that the assay monitoring 4-chlorobenzoate should allow us to determine if strains B356 and LB400 respond similarly to the presence of simple flavonoids during growth on sodium acetate and if the enzymes of the upper biphenyl catabolic pathway are induced by flavonoids.

When cells of strain B356 were grown on sodium acetate alone, small amounts of chlorobenzoate were produced in the growth medium (Fig. 2A). However, when cells were grown in the presence of sodium acetate plus variable amounts of biphenyl, the amount of 4-chlorobenzoate varied depending on the amount of biphenyl added to the culture medium (Fig. 2A). This response was similar to that observed for strain U23A grown in the presence of sodium acetate plus biphenyl (37). Cells of strain LB400 grown on sodium acetate alone produced slightly larger amounts of 4-chlorobenzoate than cells of strain B356, and the addition of biphenyl to the growth medium did not induce the biphenyl catabolic enzymes as strongly as for strain B356 (Fig. 2B).

When strain B356 was grown on sodium acetate plus flavanone, for concentrations ranging between 1 mM and 0.01 mM, the amount of 4-chlorobenzoate produced during the assay was not significantly higher than for cells grown on sodium acetate alone (Fig. 2A). Similar results were obtained when cells were grown on sodium acetate plus flavone. However, remarkably, the amounts of 4-chlorobenzoate produced from 4-chlorobiphenyl by resting cells grown on sodium acetate plus isoflavone were significantly higher than those produced for cells grown on sodium acetate plus biphenyl (Fig. 2A). We cannot exclude the possibility that posttranscriptional regulation mechanisms exerted by biphenyl metabolites are responsible for the lower enzyme activity found in cells grown on sodium acetate plus biphenyl. Furthermore, since all three flavonoids are metabolized by whole cells of strain B356, we must exclude the possibility that permeation of flavonoids across the cell membrane/wall had affected the cells' activity for substrate. Therefore, the data show that isoflavone is a good inducer of the biphenyl catabolic enzymes of strain B356. In the case of strain LB400, there was no clear-cut effect for any of the three tested flavonoids that would demonstrate

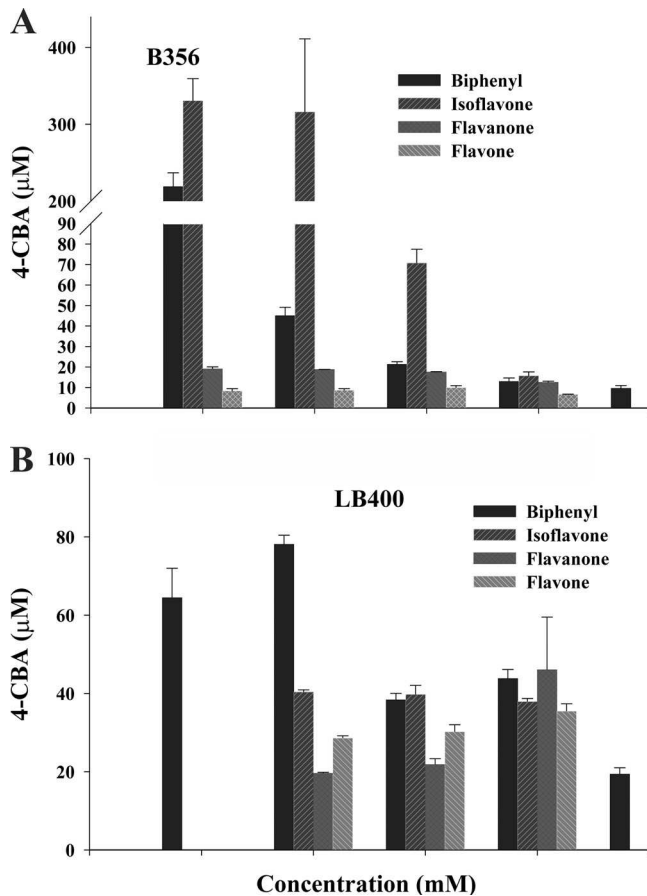


FIG 2 (A) Amounts (μM) of 4-chlorobenzoic acid (4-CBA) produced when standardized resting cell suspensions of strain B356 were incubated with 1,250 μM 4-chlorobiphenyl for 2 h. (B) Amounts (μM) of 4-chlorobenzoic acid produced when standardized resting cell suspensions of strain LB400 were incubated with 1,250 μM 4-chlorobiphenyl for 2 h. Each strain was previously grown overnight at 28°C in MM30 medium amended with 30 mM sodium acetate or with 30 mM sodium acetate plus the indicated concentration of the indicated flavonoid or of biphenyl. Bars represent means ($n = 2$) and standard deviations. The protocol for the standardized 4-chlorobiphenyl assay is described in Materials and Methods. Concentrations of 0.0005 and 0.0001 mM were only used for flavanone.

the ability of these flavonoids to induce the biphenyl catabolic pathway. The amounts of 4-chlorobenzoate produced varied slightly in the presence of flavonoids (Fig. 2B), but the amounts produced were not statistically significantly different from those observed in the absence of flavonoids. Therefore, none of the three flavonoids tested significantly influenced the activity of the biphenyl catabolic enzymes of strain LB400, whereas isoflavone was found to act as an inducer of the biphenyl catabolic pathway of strain B356 when it was added as a cosubstrate with sodium acetate.

Metabolites produced from flavonoids by purified BphAE_{B356}, BphAE_{LB400}, and BphAE_{p4}. Since whole cells of strains B356 and LB400 metabolized flavanone differently and since they responded differently to the presence of flavone, flavanone, and isoflavone in the growth medium, we have compared the ability of purified preparations of their biphenyl dioxygenases to metabolize these three plant metabolites. The purified enzymes were prepared from recombinant *E. coli* cells as described in Ma-

terials and Methods. We have also included BphAE_{p4}, a Thr335Ala Phe336Met mutant of BphAE_{LB400} exhibiting an expanded substrate range compared to that of its parent enzyme (2, 18). In a previous report, we showed that replacing Phe336 of BphAE_{LB400}, which lines the catalytic pocket, with a residue with a smaller side chain (Met336) increases the space inside the catalytic pocket. In a similar manner, the corresponding Ile334 of BphAE_{B356} that lines the catalytic pocket is smaller than Phe336 of BphAE_{LB400} and, thus, allows the enzyme to metabolize bulkier substrates, such as DDT (20). Thr335 is at a remove from the substrate; however, changing this residue to the smaller Ala335 relieves intramolecular constraints on Gly321, allowing for significant movement of this residue during substrate binding and thereby increasing the space available to accommodate bulkier substrates (20). In a manner similar to Ala335 of BphAE_{p4}, Gly333 (corresponding to Thr335 of BphAE_{LB400}) does not interact with Gly319 (corresponding to Gly321 of BphAE_{LB400}). Therefore, although the side chains of Ala335 and Gly333 differ, their effects on the enzyme's structure are likely to be comparable (18).

Based on the GC-MS peak area of the remaining substrate, purified preparations of BphAE_{B356} incubated with 100 nmol flavanone oxidized more than 90 nmol this substrate within 10 min. Under identical conditions, BphAE_{p4} metabolized approximately 20 nmol flavanone and BphAE_{LB400} metabolized less than 10 nmol of this substrate (Fig. 3A). Consistent with these results, the amount of metabolites generated by BphAE_{B356} after 10 min of incubation was significantly higher than for the two other enzymes (Fig. 3A). In addition, the pattern of metabolites generated by the three enzymes differed significantly. BphAE_{B356} produced two metabolites in approximately equal amounts. They both exhibited a very similar mass spectral fragmentation pattern (Table 1). The presence of ions at m/z 147 ($M^+ - n\text{-BuBO}_2 - C_6H_5$) and 120 ($M^+ - n\text{-BuBO}_2 - C_6H_5 - CH - CH_2$) was consistent with a dihydroxylation occurring on ring B. BphAE_{LB400} generated only one of the two dihydrodiol metabolites, whereas BphAE_{p4} produced four dihydrodiol metabolites from flavanone. It produced the two metabolites resulting from the oxidation of ring B, but in addition, it produced two other dihydrodiols that could only result from a hydroxylation of ring A. The mass spectral fragmentation patterns of their butylboronate derivatives are shown in Table 1. The ions at m/z 192 ($M^+ - C_6H_5 - C_3H_3 - O$) and 176 ($M^+ - C_6H_5 - C_3H_3 - O_2$) resulting from the loss of the non-hydroxylated ring B (C_6H_5) provide evidence that the hydroxylation occurred on ring A. These data were confirmed by the GC-MS analysis of the trimethylsilyl derivatives of the metabolites, which evidenced the formation of two dihydrodiol metabolites from BphAE_{B356}, a single one from BphAE_{LB400}, and four dihydrodiol metabolites from BphAE_{p4} (not shown).

BphAE_{B356} and BphAE_{p4} metabolized, respectively, 70 nmol and 50 nmol of flavone when they were incubated with 100 nmol of this substrate for 10 min. However, BphAE_{LB400} performed very poorly on flavone, where less than 5% of the added substrate was degraded. As with flavanone, the pattern of metabolites produced from flavone differed significantly among the enzymes (Fig. 3B). Two metabolites were produced when the reaction was catalyzed by BphAE_{B356}, but their proportion differed considerably. Based on the mass spectral fragmentation features of their butylboronate derivatives shown in Table 1, they were identified as 2-(2,3-dihydro-2,3-dihydroxyphenyl)chromene-4-one and 2-(3,4-dihydro-3,4-dihydroxyphenyl)chromene-4-one. On the basis of the

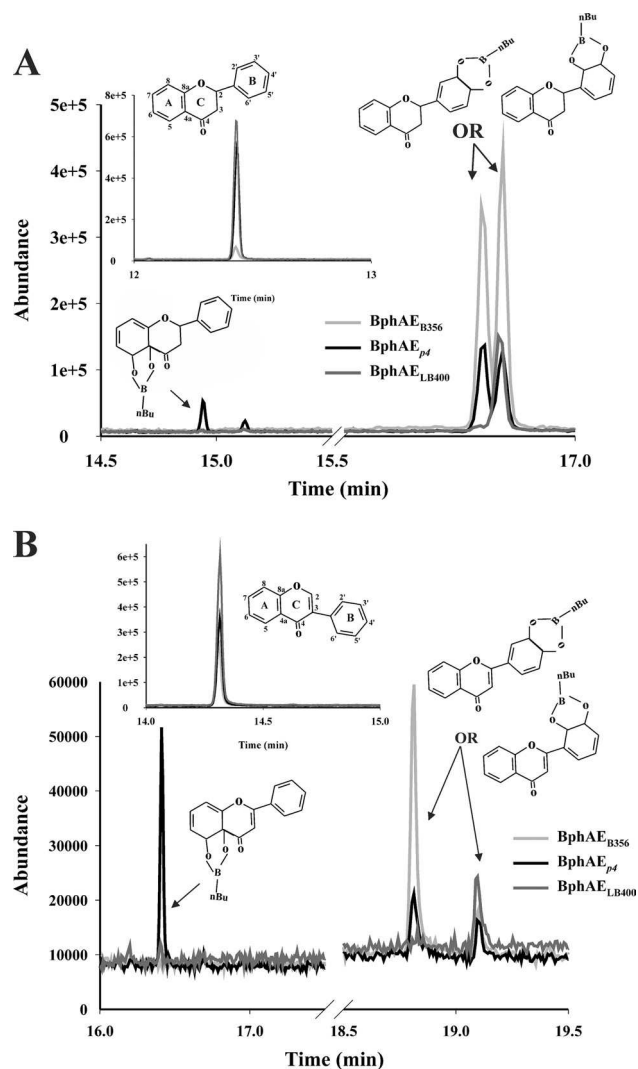


FIG 3 (A) Total ion chromatogram showing the peaks of metabolites produced from flavanone after 10 min by reconstituted His-tagged BphAE_{B356} (gray curve), BphAE_{LB400} (dark gray curve), and BphAE_{p4} (black curve). The inset shows the peak of the substrate remaining in the reaction vial. (B) Total ion chromatogram showing the peaks of metabolites produced from flavone after 10 min by reconstituted His-tagged BphAE_{B356} (gray curve), BphAE_{LB400} (dark gray curve), and BphAE_{p4} (black curve). The inset shows the peak of the substrate remaining in the reaction vial.

TABLE 1 Mass spectral features of the butylboronate-derived metabolites produced from flavanone and flavone by BphAE_{B356}, BphAE_{LB400}, and BphAE_{p4}

Substrate	No. of metabolites produced by:			Metabolite structure ^a	Oxidized ring	M ⁺	Other ions
	BphAE _{B356}	BphAE _{LB400}	BphAE _{p4}				
Flavanone	2	1	2	2-(2,3-Dihydro-2,3-dihydroxyphenyl)chromane-4-one or 2-(3,4-dihydro-3,4-dihydroxyphenyl)chromane-4-one	B	324	308, 267, 240, 224, 147, 120
			2	4a,5-Dihydro-4a,5-dihydroxy-2-phenylchromane-4-one	C	324	308, 267, 240, 224, 192, 176, 131
Flavone	2	1	2	2-(2,3-Dihydro-2,3-dihydroxyphenyl)chromene-4-one or 2-(3,4-dihydro-3,4-dihydroxyphenyl)chromene-4-one	B	322	306, 265, 238, 222, 210, 181, 120
			1	4a,5-Dihydro-4a,5-dihydroxy-2-phenylchromene-4-one	C	322	306, 265, 238, 222, 192, 163, 129

^a Structures were tentatively identified on the basis of their mass spectral fragmentation features and on the orientation of the docked substrates in the enzyme catalytic pocket.

docking experiments described below, the major metabolite would result from a hydroxylation of carbons 2'-3' to generate the 2-(2,3-dihydro-2,3-dihydroxyphenyl)chromene-4-one. BphAE_{p4} produced three metabolites from flavone (Fig. 3B). The GC-MS features of their butylboronate derivatives are shown in Table 1. Two of the metabolites are identical to those produced by BphAE_{B356}. The mass spectral features of the third one, which is a major metabolite, comprise ions at m/z 192 ($M^+ - C_6H_5 - C_3H - O$) and 163 ($M^+ - n\text{-BuB} - C_6H_5 - C_2H$) which are consistent with a hydroxylation on ring A. On the basis of the docking experiments described below, this metabolite would be 4a,5-dihydro-4a,5-dihydroxy-2-phenylchromene-4-one. BphAE_{LB400} produced trace amounts only of the metabolite corresponding to the peak of 2-(3,4-dihydro-3,4-dihydroxyphenyl)chromene-4-one.

As was the case for the previous two substrates, BphAE_{B356} performed better than the other two enzymes toward isoflavone. However, in this case, all three enzymes produced the same two metabolites from this flavonoid (Fig. 4). Both of them exhibited a fragmentation pattern comprising ions at m/z 181 ($M^+ - n\text{-BuBO}_2 - CO - CH$), 165 ($M^+ - n\text{-BuBO}_2 - CO - CH - O$), and 120 ($M^+ - n\text{-BuBO}_2 - C_6H_5 - C_2H$) that was consistent with a dihydroxylation of ring B.

Kinetic parameters of purified BphAE_{B356}, BphAE_{LB400}, and BphAE_{p4} toward flavone, flavanone, and isoflavone. The steady-state kinetic parameters of purified preparations of BphAE_{B356}, BphAE_{p4}, and BphAE_{LB400} toward each of the three flavonoids were calculated from the initial oxygen consumption using a Clark-type oxygraph. Notably, for the three substrates, the k_{cat} and k_{cat}/K_m values for BphAE_{B356} were in the range reported (20) for biphenyl (respectively, 4.3 s^{-1} and $63 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$) when this enzyme was used under the same reaction conditions (Table 2). Consistent with the whole-cell assays described above, flavanone was the best substrate, exhibiting k_{cat} and k_{cat}/K_m values that were significantly higher than those previously reported for biphenyl (Table 2). However, flavone and isoflavone were also good substrates for the enzyme since their kinetic parameters were in same range as those reported for biphenyl. Furthermore, for all substrates, the steady-state kinetic parameters of BphAE_{B356} were significantly higher than those for BphAE_{p4}. The reported k_{cat} and k_{cat}/K_m values of BphAE_{p4} toward biphenyl (1.0 s^{-1} and $31 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$) (23) were higher than for all three flavonoids. Therefore, although BphAE_{p4} performed well on these substrates, unlike

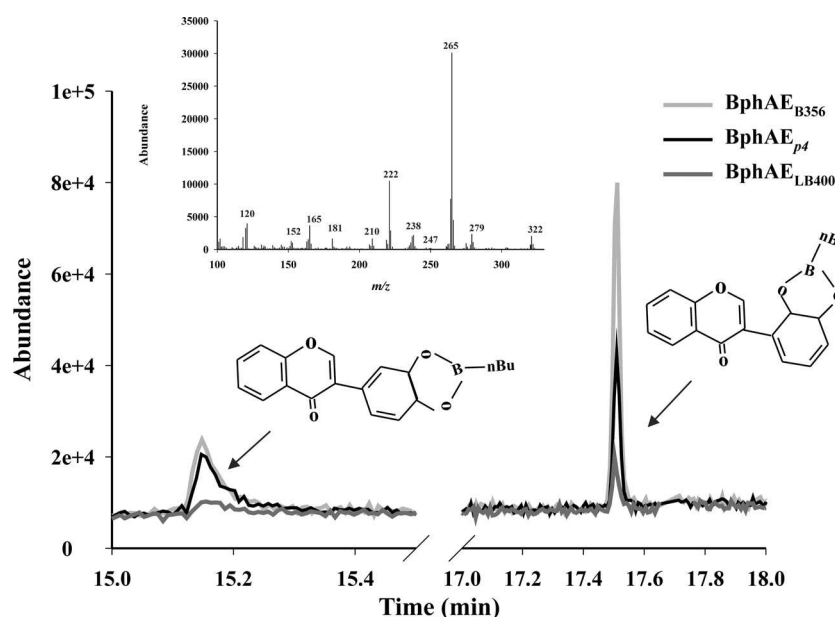


FIG 4 Total ion chromatogram showing the peaks of metabolites produced from isoflavone after 10 min by reconstituted His-tagged BphAE_{B356} (gray curve), BphAE_{LB400} (dark gray curve), and BphAE_{p4} (black curve). The inset shows the mass spectrum of the metabolite exhibiting a retention time of 17.5 min. The mass spectrum for the second metabolite is almost identical (not shown).

BphAE_{B356}, biphenyl remains a better substrate than the flavonoids. Consistent with the time point measurement experiments, BphAE_{LB400} was poorly active toward the three flavonoids. The steady-state kinetic parameters obtained with flavone and isoflavone were too low to be determined accurately and therefore are not reported here. Flavanone was the best substrate; the k_{cat} and k_{cat}/K_m values obtained with this substrate were significantly lower than the values reported when BphAE_{LB400} was used to metabolize biphenyl under identical conditions (respectively, 0.9 s^{-1} and $41 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$) (23). Together, these data show that in comparison to the activity of BphAE_{LB400}, the double Thr335Ala Phe336Met substitution in BphAE_{p4} contributed to enhancement of the catalytic activity toward the simple flavonoids. However, these mutations did not allow the enzyme to reach the level of activity of the naturally occurring BphAE_{B356}.

TABLE 2 Steady-state kinetic parameters of BphAE_{B356}, BphAE_{LB400}, and BphAE_{p4} toward flavanone, flavone, and isoflavone^a

Substrate, enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($10^3 \text{ M}^{-1} \text{ s}^{-1}$)
Flavanone			
BphAE _{B356}	77.5 ± 4.8	9.0 ± 0.4	116.1 ± 8.9
BphAE _{p4}	27.5 ± 5.7	0.60 ± 0.1	21.8 ± 4.0
BphAE _{LB400}	32.1 ± 3.9	0.36 ± 0.0	11.1 ± 1.3
Flavone			
BphAE _{B356}	121.4 ± 7.2	4.0 ± 1.3	32.9 ± 11.2
BphAE _{p4}	21.4 ± 1.4	0.08 ± 0.0	3.8 ± 0.1
Isoflavone			
BphAE _{B356}	15.8 ± 1.0	1.2 ± 0.0	75.9 ± 4.7
BphAE _{p4}	27.6 ± 0.3	0.59 ± 0.0	21.3 ± 0.0

^a The \pm standard deviations of the results for two independently produced enzyme preparations are shown.

Structural analysis of docked flavonoids at active sites of BphAE_{B356}, BphAE_{LB400}, and BphAE_{p4}. In order to identify the structural features of BphAE_{B356} and BphAE_{LB400} that explain why the two enzymes catalyze flavone, isoflavone, and flavanone oxidation differently, we docked these flavonoids at their active sites. Since previous reports showed that an induced-fit mechanism was required to bind the substrate productively inside the BPDO catalytic pocket (23), we docked the flavonoids in the substrate-bound form of the enzymes after removing biphenyl (or 2,6-dichlorobiphenyl in the case of BphAE_{p4}). When flavone was docked into BphAE_{B356}, consistent with the biochemical data, the conformation of the top-ranked docked molecule exhibited an orientation that would enable an oxygenation of ring B. Carbons 2' and 3' of ring B closely aligned with carbons 2 and 3 of the oxidized ring of biphenyl in the complexed form (Fig. 5A). This suggested that the major metabolite produced from flavone when BphAE_{B356} catalyzed the reaction would be 2-(2,3-dihydro-2,3-dihydroxyphenyl)chromene-4-one. Therefore, the regiospecificity of BphAE_{B356} toward flavone would be similar to that of the previously reported BphA1A2(2072) which was obtained by shuffling *bphA1* from *Pseudomonas alcaligenes* with *bphA* from *B. xenovorans* LB400 (32). In the case of BphAE_{LB400}, none of the docked substrate conformations exhibited a productive orientation toward the catalytic iron. This is consistent with the fact that the catalytic activity of BphAE_{LB400} toward this flavonoid was very low. When BphAE_{B356} docked with flavone was superposed to the biphenyl-bound form of BphAE_{LB400} (without biphenyl), the chromene oxo group of the docked molecule was at less than 3 Å from both Phe336 and Gly321 of BphAE_{LB400} (not shown). Therefore, the proximity of the chromene oxo group to these two residues probably prevents productive binding to BphAE_{LB400}.

Unlike the result for BphAE_{B356}, the conformation of the top-ranked docked flavone molecule in BphAE_{p4} was consistent with the observation that its major metabolite resulted from a dihy-

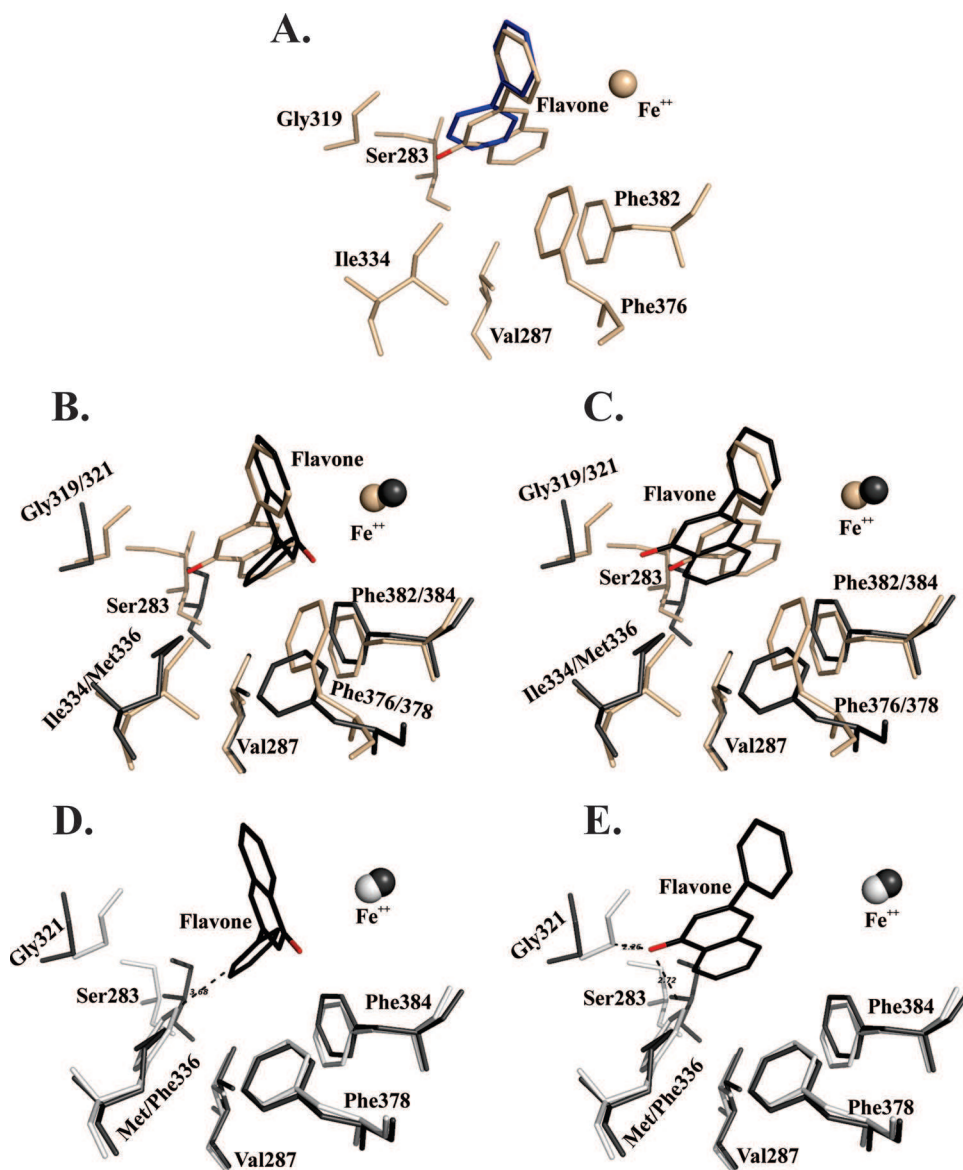


FIG 5 (A) Superposition of catalytic center residues of the flavone-docked (wheat) and biphenyl-bound (blue) forms of BphAE_{B356}. (B and C) Superposition of catalytic center residues of two flavone-docked forms of BphAE_{p4} (black) and the top-ranked flavone-docked form of BphAE_{B356} (wheat). (D and E) Superposition of catalytic center residues of two flavone-docked forms of BphAE_{p4} (black) and the biphenyl-bound form of BphAE_{LB400} (white) after removal of biphenyl. The oxygen of the flavone oxo group is in red.

droxylation on ring A. On the basis of the orientation of ring A toward the catalytic Fe²⁺ in the docked form of BphAE_{p4}, the hydroxylation should occur onto carbons 4a and 5 to produce 4a,5-dihydro-4a,5-dihydroxy-2-phenyl chromene-4-one (Fig. 5B). Another of the 10 top-ranked conformations of flavone in BphAE_{p4}'s catalytic pocket was similar to but did not superpose exactly with the molecule docked in BphAE_{B356} (Fig. 5C). In a previous report, the ability of BphAE_{p4} to oxidize 2,6-dichlorobiphenyl on the *meta-para* and *ortho-meta* carbons of biphenyl was attributed to the fact that none of the C-2'-C-3' or C-3'-C-4' pairs of carbons were equidistant from the catalytic Fe²⁺ and they all were within 4.5 Å from it (18). A similar situation was obtained when flavone was docked in BphAE_{p4}. Carbons C-2' and C-3' of ring B were not equidistant from the catalytic Fe²⁺, and they were

more distant from it than the corresponding atoms of flavone docked in BphAE_{B356} (Fig. 5C). This may explain why both 2-(2,3-dihydro-2,3-dihydroxyphenyl)chromene-4-one and 2-(3,4-dihydro-3,4-dihydroxyphenyl)chromene-4-one were produced and why these metabolites were produced in lesser amounts than when BphAE_{B356} catalyzed the reaction.

As shown in Fig. 5B, when flavone takes an orientation enabling an oxidation of ring A (in black), Phe376 of BphAE_{B356} is too close (1.5 Å) to the chromene oxo group to allow productive binding of this substrate. Therefore, this residue or others that modulate its conformation exert a strong influence on the regio-specificity of the enzyme toward flavone. In order to confirm that Phe336 and Gly321 prevent the binding of flavone to BphAE_{LB400}, we superposed both docked conformations of flavone in BphAE_{p4}

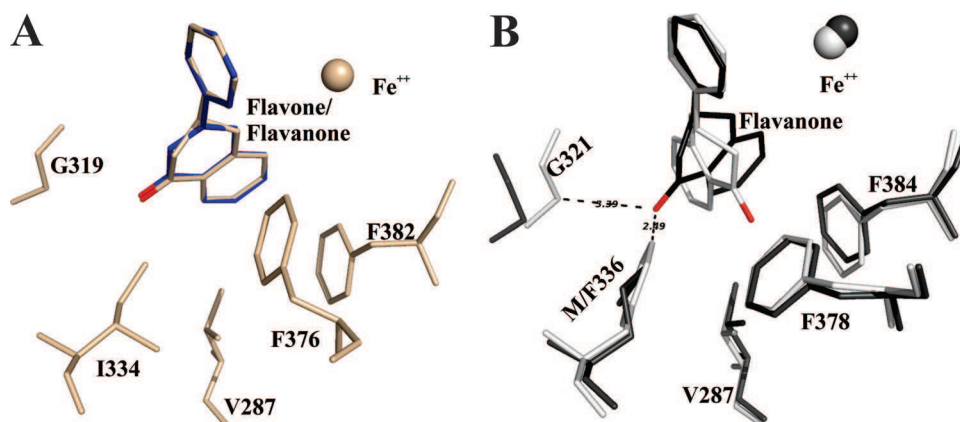


FIG 6 (A) Superposition of catalytic center residues of the flavanone-docked (wheat) and flavone-docked (blue) forms of BphAE_{B356}. (B) Superposition of catalytic center residues of the top-ranked flavanone-docked form of BphAE_{p4} (black) enabling the oxidation of ring B and the top-ranked flavanone-docked form of BphAE_{LB400} (white). The oxygen of the flavanone oxo group is in red.

with the biphenyl-bound form of BphAE_{LB400} after biphenyl removal. This is shown in Fig. 5D and E, where it is clear that for both conformations of the docked substrate in BphAE_{p4}, residues Gly321 and Phe336 of BphAE_{LB400} are both too close to the substrate to allow productive binding. Therefore, the Phe336Met and the Thr335Ala substitution are both required to facilitate flavone binding to BphAE_{p4}. However, as shown from the steady-state kinetic parameters of the enzymes, although BphAE_{p4} can metabolize flavone, its turnover rate of reaction is significantly lower than that of BphAE_{B356}. Therefore, although the two mutations that occurred in BphAE_{p4} enhanced its catalytic activity toward flavone, other structural features of BphAE_{B356} that are not present in BphAE_{p4} are required to facilitate the chemical steps in the catalytic oxygenation reaction. A structural comparison of the catalytic pockets of BphAE_{B356} and BphAE_{p4} identified Phe376/Phe378 and Ser283/Ile283 as likely candidates to explain the different catalytic properties of the two enzymes (Fig. 5).

In the flavanone docking experiment, both flavanone and flavone are placed at the same position in BphAE_{B356} where carbon 2' and 3' of ring B superposed almost perfectly with the reactive carbons of biphenyl (Fig. 6A). Biochemical analysis showed that BphAE_{B356} oxidized both the 2',3' and 3',4' carbons of ring B (Fig. 3A). Therefore, the binding of BphAE_{B356} to flavanone can induce other conformations of the substrate that the docking experiment could not reproduce. The docking experiment with BphAE_{p4} showed that flavanone can take an orientation where ring B superposes exactly with ring B of the docked molecule in BphAE_{B356} (not shown). However, similar to flavone docking and consistent with the biochemical data, flavanone can also be docked in BphAE_{p4} in an orientation that would enable a hydroxylation of ring A (not shown). As seen by the observations described above, although BphAE_{LB400} is not as efficient as BphAE_{p4} in oxidizing flavanone, its activity toward this substrate is more efficient than that toward flavone. Consistent with the biochemical data, automatic docking placed flavanone in an orientation that would allow an oxygenation of ring B by BphAE_{LB400}. Structural analysis shows that, unlike the results of the docking experiment done with BphAE_{p4}, in the case of BphAE_{LB400}, the chromane moiety of flavanone is oriented such that the oxo group of ring C is distanced from Phe336 and pulled toward Phe378 and Phe384 (Fig. 6B).

This shows that the chromane moiety of the molecule reacts differently than the chromene moiety of flavone with the surrounding atoms of the catalytic pocket of BphAE_{LB400}. However, the docking experiment has limitations, since it did not allow identification of the protein atoms of BphAE_{LB400} that interact with the chromane moiety of flavanone.

The isoflavone docking experiments are also in agreement with the biochemical data. Ring B of isoflavone superposes well with ring B of flavone in the top-ranked isoflavone-docked form of BphAE_{B356}. The docking experiment suggests that the major metabolite generated by the oxygenation of isoflavone would be 3-(2,3-dihydro-2,3-dihydroxyphenyl)chromene-4-one (Fig. 7A). In the case of BphAE_{p4}, it is not clear why the oxo group is flipped in the opposite orientation for the top-ranked form of isoflavone-docked BphAE_{p4} (Fig. 7B). There are no apparent constraints that would prevent isoflavone from taking the same conformation as in BphAE_{B356}. This shows that as for flavanone, other structural features that the docking experiment could not identify are likely to be involved in the binding process for this flavonoid. It is also interesting that automatic docking places isoflavone in a productive orientation for BphAE_{LB400} (Fig. 7C). However, in this case, unlike the case for BphAE_{p4}, the oxo group is in an orientation similar to that found in the isoflavone-docked BphAE_{B356}. Since isoflavone was a poor substrate for BphAE_{LB400}, it is likely that unidentified structural features that place isoflavone in the opposite orientation in BphAE_{p4} are required to enable the chemical reactions to proceed. Since in the docked form of BphAE_{B356}, the oxo group is at a distance of approximately 4 Å from Fe²⁺ and from the two His that coordinate it, the proximity of the oxo group to the iron may hinder the catalytic activity in BphAE_{LB400}. The iron is at the interface between two α subunits, and it was shown in a previous work that protein structures surrounding the catalytic iron move during binding and that these structures appeared to be involved in maintaining the integrity of the $\alpha_3\beta_3$ conformation of the enzyme (23). However, more structural analyses of the substrate-bound enzymes will be required to determine more precisely why BphAE_{LB400} has poor activity on isoflavone.

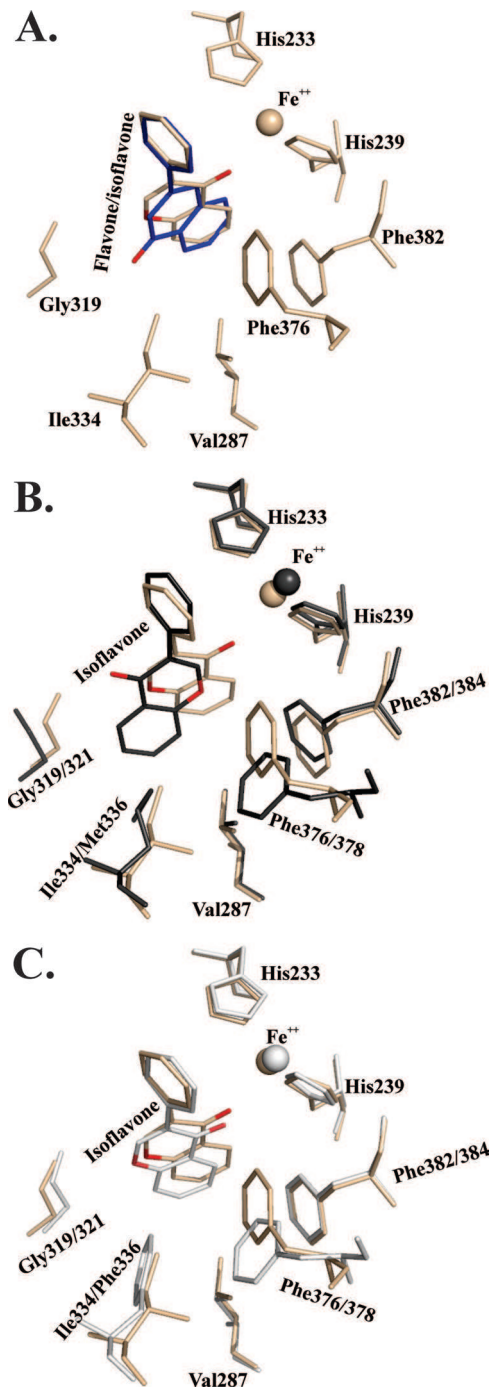


FIG 7 (A) Superposition of catalytic center residues of the top-ranked isoflavone-docked (wheat) and flavone-docked (blue) forms of BphAE_{B356}. (B) Superposition of catalytic center residues of the top-ranked isoflavone-docked forms of BphAE_{p4} (black) and of BphAE_{B356} (wheat). (C) Superposition of catalytic center residues of the top-ranked isoflavone-docked forms of BphAE_{LB400} (white) and of BphAE_{B356} (wheat). The oxygen of the flavanone oxo group is in red.

DISCUSSION

The perception of flavonoids by plant pathogens and their function as signals in the initiation of legume-rhizobium symbiosis have been well characterized (31). However, the potential impacts

of flavonoids on soil and rhizosphere bacteria that do not interact with plants directly in a host-pathogen or symbiotic interaction remain largely unknown. Better insight into this mechanism will help in understanding how plants promote PCB degradation in soil. Many investigations have identified plant secondary metabolites (flavonoids or terpenes) as likely candidates to trigger microbial degradation of PCBs in soil (34). Shaw et al. (31) have hypothesized that PSMs act to shape rhizosphere microbial community structure and, thus, they may have an impact on the rhizosphere function by triggering microbial pathways that can influence the quality and quantity of PSMs in soil. However, this hypothesis remains to be demonstrated.

In a previous report, we showed that the *R. erythropolis* U23A biphenyl catabolic pathway was induced by flavanone (37). In this work, we showed that isoflavone instead of flavanone was an inducer for the biphenyl catabolic pathway of *P. pnomenusa* strain B356, whereas none of the three flavonoids induced the biphenyl catabolic pathway of strain LB400. The observation that strains U23A, B356, and LB400 responded differently to simple flavonoids is consistent with divergent regulation mechanisms for their respective biphenyl catabolic operons. Strain LB400's *bph* operon is controlled by *orf0*, producing a positive regulator belonging to the GntR family, and by *bphR2*, producing a LysR-type regulator (5), whereas the biphenyl operons of *Rhodococcus globululus* P6 and *Rhodococcus jostii* RHA1 are regulated through a two-component regulatory system (19, 21). The regulation of strain U23A's *bph* operon is likely to be very similar to that of other rhodococci. The regulatory system of strain B356's *bph* operon has not yet been elucidated. However, a gene (*orf0*B356, GenBank accession number [JQ322530](https://www.ncbi.nlm.nih.gov/nuccore/JQ322530)) coding for a protein exhibiting 48% homology with BphS of *Cupriavidus oxalaticus* A5 (previously called *Cupriavidus necator* A5, *Alcaligenes eutrophus*, or *Ralstonia eutropha* A5) (25) and exhibiting homology with other members of the GntR family was found in the genome of strain B356 just upstream of *bphG*. Sequence alignment of this protein with other members of GntR family proteins showed it clustering with BphS of strain A5 and of *Pseudomonas* sp. strain KKS102 (not shown) which, unlike Orf0 of strain LB400, were found to be negative regulators (5, 27). The phylogenetic tree obtained when sequences of known BphAs from cultured and uncultured bacteria are aligned (38) shows three branches: two comprise principally Gram-negative proteobacteria, and one comprises exclusively high-GC-content Gram-positive bacteria of the rhodococcal group. It is noteworthy that BphAE_{B356} clusters with BphA1A2 of strain A5 and of strain KKS102, whereas BphAE_{LB400} belongs to a separate branch. The fact that a similar clustering of the phylogenetic tree is obtained for the deduced amino acid sequences of the regulatory protein and of the first enzyme of the biphenyl catabolic pathway of these strains highlights the possibility that the three biphenyl catabolic pathway clusters may have evolved to serve distinct functions in the environment.

In this work, in order to get more insight about how these pathways interact with simple flavonoids, we compared the metabolism of flavanone, flavone, and isoflavone by BphAE_{B356} and BphAE_{LB400}. Biochemical data showed that, unlike BphAE_{LB400}, BphAE_{B356} is well fitted to metabolize these PSMs. Structural analysis identified two features of BphAE_{LB400} that are responsible for the poor ability of the enzyme to metabolize these flavonoids. As observed in the case of 2,6-dichlorobiphenyl (9, 18) and in the case of DDT (20), Phe336 is too large to enable productive binding

with large substrates. In addition, the fact that Gly321 is constrained through a network of hydrogen bonding significantly hinders binding with these substrates (18). In a previous report, we showed that replacing Thr335 with Ala335 in BphAE_{LB400} relieved constraints on the Val320-Gly321-Gln322 segment, allowing for more movement during substrate binding. This feature enables BphAE_{p4} to accommodate bulkier substrates, such as 2,6-dichlorobiphenyl (18). Similar to Ala335 of BphAE_{p4}, the corresponding residue Gly333 of BphAE_{B356} is too short to form any contact with this segment (not shown). Therefore, Gly319 of BphAE_{B356} is more relaxed than Gly321 of BphAE_{LB400}, which explains in part why BphAE_{B356} can metabolize substrates such as 2,6-dichlorobiphenyl (9) or flavonoids that BphAE_{LB400} metabolizes poorly. However, other unidentified structural features influence binding to flavonoids. For example, it is noteworthy that BphAE_{LB400} was found to catalyze the oxidation of flavanone more efficiently than flavone. The superior properties of the enzyme toward flavanone were attributed to the fact that the oxo group of the chromane moiety was placed away from Phe336. This indicates that protein structures involved in the binding process interacted differently with the chromene and chromane moieties of the molecule.

This is also supported by the superior catalytic abilities of BphAE_{B356} compared to those of BphAE_{p4} toward flavanone, flavone, and isoflavone in spite of the fact that both BphAE_{B356} and BphAE_{p4} contain a smaller amino acid than Phe336 of BphAE_{LB400} at position 336 and, in addition, the fact that the correspondence of Gly321 of BphAE_{p4} to Gly319 of BphAE_{B356} is less constrained than that of Gly321 of BphAE_{LB400}. The docking experiments did not allow us to identify precisely the BphAE_{B356} structural features that conferred to the enzyme an ability superior to that of BphAE_{p4} to catalyze the reaction. In a previous report, it was shown that the helix between residues 282 and 288 moved considerably more toward the substrate during substrate binding with BphAE_{p4} than with BphAE_{LB400}. This movement was attributed to the Thr335Ala substitution that altered the intramolecular hydrogen bonding networks involving residues of this helix (18). It was suggested (9) that the mobile character of this helix may influence binding to substrates larger than biphenyls. Furthermore, residue 283 is at the entranceway of the catalytic pocket and both residue Ile283 of BphAE_{B356} and residue Ser283 of BphAE_{p4} are very close (less than 3 Å) to ring A of flavone (Fig. 5) in the flavone-docked enzyme. In the biphenyl-bound form of BphAE_{LB400}, Ser283 is far from the substrate. Although its precise role in substrate binding is not clear, residue 283 and the helix to which it belongs appear to be likely candidates for engineering enzymes exhibiting altered substrate specificity and regiospecificity toward flavonoids. However, we cannot exclude other residues that are not in contact with the substrate but that may influence substrate binding by other mechanisms. For example, in a recent report, residues 338 and 409 of BphAE_{LB400} were found to act synergistically to influence the catalytic properties of the enzyme by interacting with residues that are involved in subunit assembly and electron transport (23).

In previous reports, *E. coli* cells producing *P. alcaligenes* KF707 BPDO were found to catalyze the hydroxylation of flavone (16) and of flavanone (11). BphA1A2 from strain KF707 is more than 95% homologous to BphAE_{LB400}, except that like BphAE_{B356}, residue 335 (corresponding to Phe336 of BphAE_{LB400}) is an Ile and residue 334 (corresponding to Thr335 of BphAE_{LB400}) is an Ala. Therefore, with respect to their catalytic properties toward fla-

vonoids, the structural features of BphA1A2_{KF707} and BphAE_{p4} are expected to be comparable. However, a BphA1A2_{KF707} variant was obtained which exhibited enhanced activity toward flavonoids (15). This variant was obtained by the substitutions His255Gln, Val256Ile, Gly266Ala, and Phe277Tyr. It is not clear whether all these residues together or a combination of some of them were required to enhance the activity toward flavonoids. The likely involvement in substrate specificity and selectivity of the mobile loop between residues 240 and 260 that overhang the entranceway of the catalytic pocket has been discussed previously (18). In addition, the likely involvement of residues 266 and 267 in the catalytic properties of BphAE_{B356} toward DDT has also been discussed (20). Nevertheless, our data with BphAE_{B356} and BphAE_{LB400} and the data related to BphA1A2_{KF707} variants show the complexity of the substrate binding process, which involves interaction between the substrate and many protein atoms that either contact the substrate or modulate the conformation of protein structures that are required to enable a productive binding.

Altogether, our investigation identified residues that are involved in substrate binding with simple flavonoids and provided evidence that BphAE_{B356} has evolved to be better fitted than BphAE_{LB400} to metabolize these PSMs. Moreover, the fact that the biphenyl catabolic pathway of strain B356 was induced by isoflavone provides additional evidence supporting the hypothesis brought forward by Focht (8) and others (31) that the biphenyl catabolic pathways have evolved in bacteria to serve ecological functions, perhaps related to the metabolism of plant secondary metabolites in soil.

In this work, by singling out simple flavonoids and comparing the ability of two well-characterized biphenyl-degrading bacteria to metabolize them, we have shown that both the metabolism of flavonoids and the response to them as signal molecules to trigger the biphenyl catabolic pathway vary considerably among bacteria. This conclusion is significant for the development of more rational approaches for designing efficient rhizoremediation processes. Hence, our data imply that the efficiency of the process will depend on the choice of appropriate bacterial strains responding to the specific PSMs produced by the plants with which they are associated.

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