A Novel Angular Dioxygenase Gene Cluster Encoding 3-Phenoxybenzoate 1',2'-Dioxygenase in *Sphingobium wenxiniae* JZ-1

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Sphingobium wenxiniae JZ-1 utilizes a wide range of pyrethroids and their metabolic product, 3-phenoxybenzoate, as sources of carbon and energy. A mutant JZ-1 strain, MJZ-1, defective in the degradation of 3-phenoxybenzoate was obtained by successive streaking on LB agar. Comparison of the draft genomes of strains JZ-1 and MJZ-1 revealed that a 29,366-bp DNA fragment containing a putative angular dioxygenase gene cluster (*pbaA1A2B*) is missing in strain MJZ-1. PbaA1, PbaA2, and PbaB share 65%, 52%, and 10% identity with the corresponding α and β subunits and the ferredoxin component of dioxin dioxygenase from *Sphingomonas wittichii* RW1, respectively. Complementation of *pbaA1A2B* in strain MJZ-1 resulted in the active 3-phenoxybenzoate 1',2'-dioxygenase, but the enzyme activity in *Escherichia coli* was achieved only through the coexpression of *pbaA1A2B* and a glutathione reductase (GR)-type reductase gene, *pbaC*, indicating that the 3-phenoxybenzoate 1',2'-dioxygenase belongs to a type IV Rieske non-heme iron aromatic ring-hydroxylating oxygenase system consisting of a hetero-oligomeric oxygenase, a [2Fe-2S]-type ferredoxin, and a GR-type reductase. The *pbaC* gene is not located in the immediate vicinity of *pbaA1A2B*. 3-Phenoxybenzoate 1',2'-dioxygenase catalyzes the hydroxylation in the 1' and 2' positions of the benzene moiety of 3-phenoxybenzoate, yielding 3-hydroxybenzoate and catechol. Transcription of *pbaA1A2B* and *pbaC* was induced by 3-phenoxybenzoate, but the transcriptional level of *pbaC* was far less than that of *pbaA1A2B*, implying the possibility that PbaC may not be the only reductase that can physiologically transfer electrons to PbaA1A2B in strain JZ-1. Some GR-type reductases from other sphingomonad strains could also transfer electrons to PbaA1A2B, suggesting that PbaA1A2B has a low specificity for reductase.

Diaryl ether compounds, such as dibenzo-*p*-dioxin, diaryl ether, dibenzofuran, and their halogenated derivatives, are important environmental contaminants. The existence of a diaryl ether linkage increases the physical, chemical, and biological stabilities of these compounds and reduces their biodegradability (1). Therefore, their metabolic mechanisms are of great interest. 3-Phenoxybenzoate is an important diaryl ether intermediate in the synthesis of most pyrethroids and is also the metabolic product of their degradation.

Microbial metabolism plays a significant role in the dissipation of 3-phenoxybenzoate residues in the environment (2). Until now, two 3-phenoxybenzoate metabolic pathways have been reported. In *Pseudomonas pseudoalcaligenes* POB310, *Pseudomonas* sp. strain NSS2, and *Micrococcus* sp. strain CPN 1, 3-phenoxybenzoate is split into protocatechuate and phenol (3–5), while in *Ochrobactrum tritici* pyd-1, 3-phenoxybenzoate is first transformed to *p*-hydroxy-*m*-phenoxybenzoate and then cleavage of the diaryl ether of *p*-hydroxy-*m*-phenoxybenzoate leads to the production of protocatechuate and *p*-hydroquinone (6). In both pathways, the angular dioxygenation occurs at carbon atoms 1 and 6 of the benzoate moiety of 3-phenoxybenzoate or *p*-hydroxy-*m*-phenoxybenzoate. The genes encoding an angular dioxygenase, PbaAB, which attacks positions 1 and 6 on the benzoate moiety of 3-phenoxybenzoate, resulting in diaryl ether cleavage, were cloned from *P. pseudoalcaligenes* POB310 (3).

Angular dioxygenation is an atypical initial reaction in the bacterial degradation of many aromatic pollutants. Unlike lateral dioxygenation, angular dioxygenation happens at the angular positions, and both the angular position and its adjacent carbon atoms in the aromatic ring are oxidized, resulting in cleavage of the three-ring structure or the diaryl ether structure (7). To date, many angular dioxygenases have been reported. They are all Rieske non-heme iron aromatic ring-hydroxylating oxygenases

(RHOs) and have been categorized as 4 distinct types by the classification system of Kweon et al. (8); e.g., 3-phenoxybenzoate 1,6-dioxygenase from *P. pseudoalcaligenes* POB310 (3) belongs to the type I RHOs, which represent two-component RHO systems consisting of an oxygenase and an FNR_C (ferredoxin-NADP⁺ reductase with the [2Fe-2S] ferredoxin domain connected to the C terminus of the NAD domain)-type reductase; carbazole dioxygenases from *Sphingobium yanoikuyae* XLDN2-5 (9), *Pseudomonas resinovorans* CA10 (10), *Sphingomonas* sp. strain KA1 (11), *Pseudomonas stutzeri* OM1 (12), and *Janthinobacterium* sp. strain J3 (11) belong to the type III RHOs, which are three-component RHO systems that consist of an oxygenase, a [2Fe-2S]-type ferredoxin, and an FNR_N (ferredoxin-NADP⁺ reductase with the [2Fe-2S] ferredoxin domain connected to the N terminus of the flavin-binding domain)-type reductase; another carbazole dioxygenase from *Sphingomonas* sp. strain CB3 (13), dioxin dioxygenase from *Sphingomonas wittichii* RW1 (14), and dibenzofuran dioxygenase from *Terrabacter* sp. strain YK3 (15) belong to the type IV RHOs, which represent three-component RHO systems that consist of a hetero-oligomeric oxygenase, a [2Fe-2S]-type ferredoxin, and a glutathione reductase (GR)-type reductase; and dibenzofuran di-

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source or reference
Strains		
<i>Sphingobium wenxiniae</i> JZ-1 (= DSM 21828 ^T)	Degrades a wide range of pyrethroids and 3-phenoxybenzoate; Sm ^r	2
<i>Sphingobium wenxiniae</i> MJZ-1	Mutant of strain JZ-1; degrades a wide range of pyrethroids but not 3-phenoxybenzoate; Sm ^r	This study
<i>Sphingomonas wittichii</i> RW1	Degrades dibenzo- <i>p</i> -dioxin; Sm ^r	14
<i>Sphingobium jiangsuense</i> BA-3	Degrades 3-phenoxybenzoate; Sm ^r	30
<i>Sphingobium quisquiliarium</i> DC-2	Degrades acetochlor; Sm ^r	31
<i>Sphingomonas</i> sp. strain DC-6	Degrades butachlor; Sm ^r	Our lab
<i>Sphingobium baderi</i> DE-13	Degrades 2-methyl-6-ethylaniline; Sm ^r	31
<i>E. coli</i> DH5α	Host strain for cloning vectors	TaKaRa
<i>E. coli</i> BL21(DE3)	Host strain for expression vectors	TaKaRa
<i>E. coli</i> HB101(pRK600)	Conjugation helper strain	25
Plasmids		
pET-29a(+)	Expression vector; Km ^r	TaKaRa
pBBR1MCS-5	Broad-host-range cloning vector; Gm ^r	24
pBBRA1A2	pBBR1MCS-5 derivative carrying <i>pbaA1A2</i> ; Gm ^r	This study
pBBRA1A2B	pBBR1MCS-5 derivative carrying <i>pbaA1A2B</i> ; Gm ^r	This study
pBBRA1A2BT7	pBBR1MCS-5 derivative carrying <i>pbaA1A2B</i> under the control of the T7 promoter; Gm ^r	This study
pETC	pET-29a(+) derivative carrying <i>pbaC</i> ; Km ^r	This study
pETRedA2	pET-29a(+) derivative carrying <i>redA2</i> ; Km ^r	This study
pETRed3	pET-29a(+) derivative carrying <i>red-3</i> ; Km ^r	This study
pETRed4	pET-29a(+) derivative carrying <i>red-4</i> ; Km ^r	This study
pETRed5	pET-29a(+) derivative carrying <i>red-5</i> ; Km ^r	This study
pETRed6	pET-29a(+) derivative carrying <i>red-6</i> ; Km ^r	This study

oxygenase from *Terrabacter* sp. strain DBF63 (16) belongs to the type V RHOs, which are three-component RHO systems that consist of a hetero-oligomeric oxygenase, a [3Fe-4S]-type ferredoxin, and a GR-type reductase.

Previously, strain JZ-1, which is capable of degrading a wide range of pyrethroids and utilizes them as the sole carbon source for growth, was isolated from activated sludge and was identified as a novel species (*Sphingobium wenxiniae* sp. nov.) (17). Strain JZ-1 degrades cypermethrin, deltamethrin, cyhalothrin, and fenpropathrin by hydrolysis of the carboxylester linkage, yielding chrysanthemic acid derivatives and cyano-3-phenoxybenzyl alcohol. Cyano-3-phenoxybenzyl alcohol is unstable and quickly transforms spontaneously to 3-phenoxybenzaldehyde, which is then oxidized to 3-phenoxybenzoate (2). The gene *pytH*, which encodes the carboxylesterase responsible for the initial hydrolysis of pyrethroids, was cloned from strain JZ-1 (2). In this study, the metabolic pathway of 3-phenoxybenzoate was studied, and a novel angular dioxygenase system responsible for the cleavage of the diaryl ether linkage of 3-phenoxybenzoate was identified. Unlike the previously reported PobAB (3-phenoxybenzoate 1,6-dioxygenase) from *P. pseudoalcaligenes* POB310 (3), the 3-phenoxybenzoate 1',2'-dioxygenase from strain JZ-1 attacks the 1' and 2' positions on the benzene moiety of 3-phenoxybenzoate.

MATERIALS AND METHODS

Chemicals. 2-Phenoxybenzoate, 3-phenoxybenzoate, and 4-phenoxybenzoate (98% purity) were purchased from Sigma (Munich, Germany). Catechol, 3-hydroxybenzoate, 4-hydroxybenzoate, diaryl ether, dibenzofuran, carbazole, fluorene, and dibenzothiophene (98% purity) were obtained from Alfa Aesar, Tianjin, China. Chromatography-grade methanol, acetonitrile, and analytical-grade acetic acid were purchased from the Shanghai Chemical Reagent Co., Ltd., Shanghai, China.

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Strain MJZ-1 is a mutant of strain JZ-1 which lost the ability to degrade 3-phenoxybenzoate.

Culture conditions. *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) broth or on LB agar. Strain JZ-1 was grown at 30°C in LB broth or in mineral salt medium (MSM) supplemented with 0.5 mM 3-phenoxybenzoate. Other bacterial strains were grown aerobically at 30°C in LB broth or on LB agar unless otherwise stated. The LB broth and LB agar were purchased from Difco Laboratories (Detroit, MI). MSM consisted of the following components (in g liter⁻¹): NaCl, 1.0; NH₄NO₃, 1.0; K₂HPO₄, 1.5; KH₂PO₄, 0.5; MgSO₄·7H₂O, 0.2; modified Hoagland trace element solution, 1 ml; and vitamin solution, 1 ml (pH 7.0). Modified Hoagland trace element solution consisted of the following components (in g liter⁻¹): AlCl₃, 1.0; KI, 1.0; KBr, 0.5; LiCl, 0.5; MnCl₂·4H₂O, 7.0; H₃BO₃, 11.0; ZnCl₂, 1.0; CuCl₂, 1.0; NiCl₂, 1.0; CoCl₂, 1.0; SnCl₂·2H₂O, 0.5; BaCl₂, 0.5; Na₂MoO₄, 0.5; NaVO₃·H₂O, 0.1; and Na₂SeO₃, 0.5. The vitamin solution consisted of the following components (in g liter⁻¹): choline chloride, 1.0; D-calcium pantothenate, 1.0; folic acid, 1.0; nicotinamide, 1.0; pyridoxal hydrochloride, 1.0; riboflavin, 1.0; thiamine hydrochloride, 1.0; and *D*-inositol, 2.0.

Identification of metabolites of 3-phenoxybenzoate degradation.

Strain JZ-1 was precultured in LB broth for approximately 2 days, harvested by centrifugation (3,770 × g, 10 min at 4°C), washed twice with fresh MSM, and then resuspended in MSM (the optical density at 600 nm [OD₆₀₀] was adjusted to approximately 2.0). An aliquot of the cells (2%, vol/vol) was inoculated into a 50-ml Erlenmeyer flask containing 20 ml of MSM supplemented with 0.5 mM 3-phenoxybenzoate as the sole carbon source. The cultures were incubated at 30°C and 150 rpm on a rotary shaker. At 12-h intervals, bacterial growth was monitored by measuring the numbers of CFU/ml, and the concentrations of 3-phenoxybenzoate and the metabolites were analyzed by high-pressure liquid chromatography (HPLC) or tandem mass spectrometry (MS/MS) as described below. Each treatment was performed in triplicate, and control experiments without inoculation or without substrate were carried out under the same conditions.

Sequencing, assembly, annotation, and genome comparison. DNA manipulation was carried out as described by Sambrook et al. (18). The genomes of strains JZ-1 and MJZ-1 were sequenced by BGI (Shenzhen, China) using an Illumina HiSeq2000 system (19). The DNA was sequenced as a mixture of shotgun and 350-bp paired-read fragments to provide both uniform genome coverage and a paired-read assembly. Sequencing reads were assembled using the SOAP *de novo* (version 1.05) method (<http://soap.genomics.org.cn/soapdenovo.html>). *De novo* gene prediction was conducted using the Glimmer (version 3.0) system (<http://ccb.jhu.edu/software/glimmer/index.shtml>). The BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) combined with sequences from the KEGG, COG, Swiss-Prot, and nonredundant protein databases was used to accomplish functional annotation using an E-value cutoff of 1E^{-5} . An all-versus-all genome alignment between strain MJZ-1 and strain JZ-1 was performed to identify the deleted DNA fragment in strain MJZ-1 using the Mauve (version 1.2.3) software package (20). Self-formed adaptor PCR (SEFA-PCR) (21) was used for genome walking to determine the whole length and the genomic position of the deleted DNA fragment.

For phylogenetic analysis, all protein sequences were first aligned by use of the ClustalX (version 2.1) program (22) and then imported into MEGA (version 5.0) software (23) to construct the phylogenetic tree by the neighbor-joining method. Distances were calculated using the Kimura two-parameter distance model. Confidence values for the branches of the phylogenetic tree were determined using bootstrap analyses based on 1,000 resamplings.

Expression of *pbaA1A2B* in mutant MJZ-1, *Sphingomonas wittichii* RW1, and *E. coli*. A 2,424-bp fragment containing *pbaA1A2* and a 2,825-bp fragment containing *pbaA1A2B* were amplified from the genomic DNA of strain JZ-1 with the primers A1A2F/A1A2R and A1A2F/A1A2BR (see Table S1 in the supplemental material), respectively. Both fragments contain a 561-bp native promoter region flanking the upstream region of *pbaA1A2B*. Then, the two fragments were digested with HindIII and SacI and cloned into the corresponding sites of broad-host-range plasmid pBBR1MCS-5 (24), yielding pBBRA1A2 and pBBRA1A2B, respectively. Subsequently, the recombinant plasmids were transformed into *E. coli* DH5 α and validated by sequencing. The constructs were then introduced into strain MJZ-1 and strain RW1 using triparental mating with pRK600 as a helper (25). The abilities of *E. coli* DH5 α , strain MJZ-1, and strain RW1 harboring pBBRA1A2 or pBBRA1A2B to degrade 3-phenoxybenzoate were determined by whole-cell transformation according to the method described by Liu et al. (26) with some modifications. Briefly, the strains harboring pBBRA1A2 or pBBRA1A2B were precultured to post-log phase in LB, harvested by centrifugation, washed, and resuspended in 20 ml MSM to a final OD₆₀₀ of 1.0; and then 3-phenoxybenzoate was added to the cell suspensions at a final concentration of 0.5 mM. The cell suspensions were incubated aerobically at 30°C (for strain MJZ-1 and strain RW1) or 37°C (for *E. coli*) and 150 rpm on a rotary shaker. Samples were collected at appropriate intervals to monitor reaction progress by HPLC, as described below.

Furthermore, to express *pbaA1A2B* in *E. coli* BL21(DE3) using the pET-29a(+) T7 promoter expression system, a 112-bp DNA fragment containing the T7 promoter and ribosome-binding site from pET-29a(+) was introduced into the 5' end of *pbaA1A2B* by overlap extension PCR using the primer set T7F/T7R/A1A2BT7F/A1A2BT7R (see Table S1 in the supplemental material). The HindIII-SacI-digested fusion PCR product was cloned into the corresponding sites of pBBR1MCS-5 to produce pBBRA1A2BT7. *E. coli* BL21(DE3) harboring pBBRA1A2BT7 was grown in 100 ml LB broth at 37°C to an optical density at 600 nm of 0.6, and 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and 0.5 mM FeCl₃ were then added (27). After 12 h of incubation at 16°C, the cells were harvested by centrifugation and subjected to whole-cell transformation according to the method described above.

RNA isolation and quantitative real-time PCR. An aliquot of the cells of strain JZ-1 was inoculated at a level of 2% (vol/vol) into a 250-ml Erlenmeyer flask containing 100 ml of MSM supplemented with 10 mM

glucose or 1 mM 3-phenoxybenzoate. The cultures were incubated at 30°C and 150 rpm on a rotary shaker. When approximately 50% of the 3-phenoxybenzoate was degraded, the cultures were harvested by centrifugation ($3,770 \times g$, 10 min at 4°C). Total RNA was extracted using an RNA isolation kit (TaKaRa, China) and treated with gDNA Eraser (TaKaRa, China) according to the manufacturer's instructions. A reverse transcription (RT) reaction was performed using a PrimeScript RT reagent kit (TaKaRa, China). Then, 5 μ l of 1:10-diluted cDNA samples was used as the template for quantitative real-time PCR with 0.5 μ M gene-specific primers (RT-A1F/RT-A1R, RT-A2F/RT-A2R, RT-BF/RT-BR, RT-CF/RT-CR, or RT-16SF/RT-16SR, as shown in Table S1 in the supplemental material) and 10 μ l SYBR Premix *Ex Taq* II (TaKaRa, China) in a total volume of 20 μ l. All samples were investigated in triplicate. Quantitative real-time PCR was performed in a Realplex2 system (Eppendorf, Germany) with the following thermal cycling profile: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Each quantitative real-time assay was tested in a dissociation protocol to ensure that each amplicon was a single product. The $2^{-\Delta\Delta C_T}$ threshold cycle (C_T) method was used to calculate relative changes in gene expression (28). The 16S rRNA gene was used as the internal control since it was transcribed similarly in both the presence and absence of 3-phenoxybenzoate, as demonstrated by reverse transcription-PCR (data not shown).

Coexpression of *pbaA1A2B* with *pbaC* or GR-type reductase genes from other sphingomonad strains in *E. coli*. To investigate if PbaA1A2B could functionally combine with 6 putative GR-type reductases, the genes encoding the reductases PbaC (strain JZ-1), RedA2 (*Sphingomonas wittichii* RW1), Red-3 (*Sphingobium jiangsuense* BA-3), Red-4 (*Sphingomonas quisquiliarum* DC-2), Red-5 (*Sphingomonas* sp. strain DC-6), and Red-6 (*Sphingomonas baderi* DE-13) were amplified from the genomic DNA of the corresponding strains using primer pairs CF/CR, RA2F/RA2R, R3F/R3R, R4F/R4R, R5F/R5R, and R6F/R6R, respectively (see Table S1 in the supplemental material). An NdeI restriction site was introduced into the 5' end of all the forward primers, and a HindIII (for *pbaC*, *red-3*, *red-5*, and *red-6*), SalI (for *redA2*), or XhoI (for *red-4*) restriction site was introduced into the 5' end of the reverse primers. The amplified products were digested with NdeI and HindIII (or SalI or XhoI) and ligated into the corresponding sites of plasmid pET29a(+), and the recombinant plasmids were then transformed into *E. coli* BL21(DE3) harboring pBBRA1A2BT7. The abilities of the recombinants to transform 3-phenoxybenzoate were determined by whole-cell transformation according to the method described above.

Analytical methods. The samples were freeze-dried, dissolved in 1 ml of methanol, and filtered through a 0.22- μ m-pore-size Millipore membrane. For HPLC analysis, a separation column (internal diameter, 4.6 mm; length, 250 mm) filled with Kromasil 100-5-C₁₈ was used. The mobile phase was acetonitrile-water (50:50, vol/vol) with 0.5% acetic acid, and the flow rate was 0.8 ml/min. The detection wavelength was 280 nm, and the injection volume was 20 μ l. The metabolites were further identified by MS/MS (Finnigan TSQ Quantum Ultra AM thermal triple-quadrupole mass spectrometer). In MS/MS, the metabolites were separated, confirmed by standard MS, and ionized by electrospray with a positive polarity. Characteristic fragment ions were detected using second-order MS.

Nucleotide sequence accession numbers. The GenBank accession number of DNA fragment F1 (containing the *pbaA1A2B* gene cluster and the catechol-degrading gene cluster *catFJIBCAD*) is [KJ009324](#), the GenBank accession number of catechol-degrading gene cluster *catBCAIFD* in the genome of strain JZ-1 is [KJ620836](#), the GenBank accession number of DNA fragment F2 (containing the *pbaC* gene) is [KJ009325](#), and the GenBank accession numbers of the reductase genes *red-3*, *red-4*, *red-5*, and *red-6* are [KJ009326](#), [KJ020538](#), [KJ020540](#), and [KJ020539](#), respectively.

RESULTS AND DISCUSSION

Identification of metabolites of 3-phenoxybenzoate degradation. Strain JZ-1 can degrade and utilize 3-phenoxybenzoate as

FIG 1 Degradation of 3-phenoxybenzoate (■) by strain JZ-1 and its growth (○) along with the yield of 3-hydroxybenzoate (▲) in MSM supplemented with 0.5 mM 3-phenoxybenzoate as the carbon source under aerobic conditions. The data are represented as the mean $\pm$ standard deviation for triplicate experiments.

the carbon source for growth (Fig. 1). One metabolite appeared during 3-phenoxybenzoate degradation and was identified as 3-hydroxybenzoate on the basis of HPLC and MS/MS analyses (see Fig. S1 in the supplemental material). Approximately 0.48 mM 3-hydroxybenzoate (almost equivalent to the initial molar concentration of 3-phenoxybenzoate) was formed upon the complete dissipation of 3-phenoxybenzoate. Prolonged incubation did not cause a decline in the 3-hydroxybenzoate level, suggesting that 3-hydroxybenzoate could not be further transformed. Thus, based on our present data, we propose a new 3-phenoxybenzoate degradation mechanism in strain JZ-1 that differs from that described in previous reports, in which 3-phenoxybenzoate is con-

verted to 3-hydroxybenzoate and catechol by angular dioxygenation at the 1' and 2' positions on the benzene moiety and catechol can be completely degraded (Fig. 2).

Occasionally, we found that a few colonies of strain JZ-1 lost the ability to degrade 3-phenoxybenzoate after successive streaking on LB agar. One such mutant was designated MJZ-1; strain MJZ-1 was able to grow on LB agar but not on MSM agar supplemented with 3-phenoxybenzoate as the carbon source (see Fig. S2 in the supplemental material). The metabolite analysis also showed that strain MJZ-1 could not degrade 3-phenoxybenzoate, indicating that the gene responsible for the angular dioxygenation of 3-phenoxybenzoate was deleted or disrupted. However, strain

FIG 2 Degradation pathway of 3-phenoxybenzoate in strain JZ-1 and organization of the genes involved in the pathway. (A) Proposed degradation pathway of 3-phenoxybenzoate by strain JZ-1; (B) structure of 3-phenoxybenzoate showing the position of each carbon atom; (C) pattern of 3-phenoxybenzoate cleavage by PobAB from *P. pseudoalcaligenes* POB310 (3); (D) organization of the genes involved in 3-phenoxybenzoate catabolism in strain JZ-1. CoA, coenzyme A.

TABLE 2 Deduced function of each ORF within the missing 29,371-bp fragment of mutant MJZ-1

Gene name	Proposed product	Position in F1	Product size (no. of amino acids)	Homologous protein (GenBank accession no.), source	% identity
<i>tnp1</i>	Transposase for insertion sequence IS6100	1032–1826	264	Transposase for insertion sequence IS6100 (YP_003108355), <i>Escherichia coli</i>	99
<i>orf1</i>	TonB-dependent receptor	1935–4313	792	TonB-dependent receptor (YP_004556027), <i>Shingobium chlorophenolicum</i> L-1	43
<i>orf2</i>	Fumarylacetate (FPA) hydrolase	4382–5215	277	Fumarylacetate (FPA) hydrolase (YP_001260466), <i>Shingomonas wittichii</i> RW1	59
<i>pbaA1</i>	3-Phenoxybenzoate dioxygenase α subunit	5263–6570	435	DxmA1 (YP_001260286), <i>Shingomonas wittichii</i> RW1	65
<i>pbaA2</i>	3-Phenoxybenzoate dioxygenase β subunit	6570–7100	176	DxmA2 (YP_001260285), <i>Shingomonas wittichii</i> RW1	52
<i>pbaB</i>	[2Fe-2S] ferredoxin	7122–7442	106	CarAcl (YP_717977), <i>Shingomonas</i> sp. KA1	43
<i>tnp2</i>	Transposase for insertion sequence IS6100	8280–9059	259	Transposase for insertion sequence IS6100 (YP_003108355), <i>Escherichia coli</i>	88
<i>catD</i>	3-Oxoacidate enol-lactone hydrolase	11318–12113	270	3-Oxoacidate enol-lactone hydrolase (YP_717971), <i>Shingomonas</i> sp. KA1	100
<i>catA</i>	Catechol 1,2-dioxygenase	12216–13106	295	Catechol 1,2-dioxygenase (YP_717970), <i>Shingomonas</i> sp. KA1	100
<i>catC</i>	Muconolactone δ -isomerase	13132–13422	95	Muconolactone isomerase (YP_717969), <i>Shingomonas</i> sp. KA1	98
<i>catB</i>	Muconate cycloisomerase	13424–14581	384	Muconate cycloisomerase (YP_717968), <i>Shingomonas</i> sp. KA1	100
<i>catR1</i>	Transcriptional regulator	14672–15580	301	Transcriptional regulator CatR (YP_717967), <i>Shingomonas</i> sp. KA1	100
<i>catR2</i>	Transcriptional regulator	15584–16357	256	Transcriptional regulator, IclR family (YP_717966), <i>Shingomonas</i> sp. KA1	100
<i>catI</i>	3-Oxoacidate CoA ⁺ transferase subunit A	16480–17151	222	3-Oxoacidate CoA transferase subunit A (YP_717965), <i>Shingomonas</i> sp. KA1	100
<i>catF</i>	3-Oxoacidate CoA transferase subunit B	17193–17822	208	3-Oxoacidate CoA transferase subunit B (YP_717964), <i>Shingomonas</i> sp. KA1	100
<i>catF</i>	β -Ketoadipyl-CoA thiolase	17822–19030	401	Acetyl-CoA acetyltransferase (YP_717963), <i>Shingomonas</i> sp. KA1	99

^a CoA, coenzyme A.

MJZ-1 still maintained the ability to degrade pyrethroids and used them as the sole carbon source for growth (data not shown), suggesting that strain JZ-1 can utilize chrysanthemic acid derivatives, the other products of pyrethroid hydrolysis, to grow. The genomic analysis also showed that pyrethroid-hydrolyzing carboxylesterase-encoding gene *pytH* still exists in the genome of MJZ-1.

Genome comparison of strains JZ-1 and MJZ-1. The draft genome of strain JZ-1 was 4,766,968 bp in length, and the total number of predicted genes was 4,887. The length of the draft genome of strain MJZ-1 was 4,641,033 bp, and the total gene number was 4,620. By comparing the draft genomes of the two strains, a 6,434-bp fragment of strain JZ-1 was not found in the draft genome of strain MJZ-1. The absence of the 6,434-bp fragment was confirmed by PCR. Subsequently, the genomic regions flanking the 6,434-bp fragment were determined by DNA walking. Finally, a 59,859-bp fragment (F1) was assembled. Sequence comparison and PCR analysis revealed that a 29,366-bp portion of this fragment was found to be missing in strain MJZ-1 (Fig. 2D).

ORF analysis of the missing fragment in strain MJZ-1. Using an open reading frame (ORF) search and BLAST analysis, a dioxygenase gene cluster consisting of *pbaA1*, *pbaA2*, and *pbaB* was found in the missing fragment (Fig. 2D; Table 2). *pbaA1* encodes a putative 48-kDa protein consisting of 435 amino acids, *pbaA2* encodes a putative 21-kDa protein consisting of 176 amino acids, and *pbaB* encodes a putative 11-kDa protein consisting of 106 amino acids. PbaA1 and PbaA2 exhibit moderate levels of identity to the corresponding α (36 to 65%) and β (30 to 52%) subunits of some angular dioxygenases, respectively. These dioxygenases are responsible for the angular dioxygenation of dioxin in *Sphingomonas wittichii* RW1 (14), carbazole in *Sphingomonas* sp. CB3 (13), and dibenzofuran in *Terrabacter* sp. YK3 (15). Alignment of PbaA1 with the α subunits of some angular dioxygenases revealed that PbaA1 contained conserved sequences for a Rieske [2Fe-2S] domain (CXHX₁-CX₂H) and a nonheme Fe(II) domain (EX₄DX₂H X₄H) (see Fig. S3 in the supplemental material), suggesting that PbaA1 is the oxygenase component of an RHO. PbaB is a [2Fe-2S]-type ferredoxin and shares 43% identity with CarAcl, the ferredoxin of carbazole dioxygenase from *Sphingomonas* sp. KA1 (11). All of these analyses indicated that *pbaA1A2B* is most likely responsible for the angular dioxygenation of 3-phenoxybenzoate. However, interestingly, there was no evidence of a gene coding for a reductase in the immediate vicinity of *pbaA1A2B*, whereas all reported angular dioxygenase systems involved in aromatic degradation require a reductase to transfer electrons.

Notably, *pbaA1A2B* is located in an 8,059-bp region between two transposase genes, *tnp1* and *tnp2* (Fig. 2D). Tnp1 and Tnp2 exhibit high levels of identity (99% and 88%, respectively) to the IS6100 transposase-like protein from *Escherichia coli* (9, 26). Furthermore, a gene cluster, *catFJBCAD*, which shows high identity (98% to 100%) and shares the organization of the catechol-degrading gene cluster involved in the catabolism of carbazole in *Sphingomonas* sp. KA1 (11) is located 2,194 bp downstream of the transposable element (Fig. 2D). Interestingly, another putative catechol-degrading gene cluster, *catBCAIIJFD* (see Table S2 in the supplemental material), exists in the genomes of both JZ-1 and MJZ-1. A substrate utilization study revealed that mutant MJZ-1 still maintains the ability to degrade and utilize catechol, and an enzyme assay also showed that strain MJZ-1 has catechol 1,2-dioxygenase activity but not catechol 2,3-dioxygenase activity

(data not shown). These results indicate that gene cluster *catBCAIIJFD* is involved in catechol degradation in strain MJZ-1.

Functional expression of *pbaA1A2B*. To further confirm the function of the gene cluster *pbaA1A2B*, *pbaA1A2* and *pbaA1A2B* were introduced into strain MJZ-1, strain RW1, and *E. coli* DH5 α . The whole-cell transformation experiments revealed that strain MJZ-1 and strain RW1 harboring pBBRA1A2B but not pBBRA1A2 acquired the ability to degrade and grow on 3-phenoxybenzoate, and the end metabolite was identified as 3-hydroxybenzoate (data not shown); thus, we confirmed that PbaA1A2B are the oxygenase and ferredoxin components of the angular dioxygenase (3-phenoxybenzoate 1',2'-dioxygenase) responsible for the angular dioxygenation at the 1' and 2' positions on the benzene moiety of 3-phenoxybenzoate and that the ferredoxin PbaB is indispensable for the angular dioxygenase. However, *E. coli* DH5 α harboring either pBBRA1A2B or pBBRA1A2 could not degrade 3-phenoxybenzoate. The failure might be caused by the absence of a proper reductase for electron transfer or the low efficiency of the native promoter of *pbaA1A2B* in *E. coli* DH5 α (29). To exclude the latter possibility, *pbaA1A2B* was placed under the control of a T7 promoter from the vector pET-29a(+) and introduced into *E. coli* BL21(DE3) (29). Whole-cell transformation assay results showed that the IPTG-induced suspension of *E. coli* BL21(DE3) harboring pBBRA1A2BT7 was still unable to degrade 3-phenoxybenzoate (data not shown), indicating that the absence of a suitable reductase is the actual reason for the failed expression of *pbaA1A2B* in *E. coli*.

Identification of the gene coding the reductase that transfers electrons to PbaA1A2B in strain JZ-1. Since PbaA1A2 shows moderate similarity to the corresponding α and β subunits of the dioxin dioxygenase (DxnA1A2), which needs a GR-type reductase, RedA2 (14), it is possible that the reductase transferring electrons to the 3-phenoxybenzoate 1',2'-dioxygenase from strain JZ-1 is homologous to RedA2. Therefore, the amino acid sequence of RedA2 was aligned with the genome of strain JZ-1, and only one putative GR-type reductase, PbaC, which showed 58% identity with RedA2, was retrieved. PbaC is a 44-kDa protein that consists of 408 amino acids and contains a consensus motif for a flavin adenine dinucleotide-binding (ADP-binding) site (GXGX₂GX₃AX₆G) (15). To determine if PbaC could act as the reductase for PbaA1A1B, *pbaC* was ligated into pET-29a(+) to generate pETC. A whole-cell transformation assay showed that *E. coli* BL21(DE3) harboring both pBBRA1A2BT7 and pETC acquired the ability to degrade 3-phenoxybenzoate, producing equimolar amounts of 3-hydroxybenzoate and catechol (see Fig. S4 in the supplemental material). These results suggest that PbaC can transfer electrons to PbaA1A2B and the 3-phenoxybenzoate 1',2'-dioxygenase is a type IV RHO consisting of three components: a hetero-oligomer oxygenase, a [2Fe-2S] ferredoxin, and a GR-type reductase. In addition to 3-phenoxybenzoate, *E. coli* BL21(DE3) harboring both pBBRA1A2BT7 and pETC could also convert 4-phenoxybenzoate to 4-hydroxybenzoate and catechol, and 4-phenoxybenzoate was degraded a little faster than 3-phenoxybenzoate (see Fig. S5 in the supplemental material). However, this strain could not degrade 2-phenoxybenzoate, diaryl ether, dibenzofuran, carbazole, fluorine, or dibenzothiophene.

PobAB is the only reported 3-phenoxybenzoate dioxygenase found in *P. pseudoalcaligenes* POB310. PobAB could also degrade 3-phenoxybenzoate and 4-phenoxybenzoate but not 2-phenoxybenzoate, which is the same as the 3-phenoxybenzoate 1',2'-di-

oxygenase from strain JZ-1. Nevertheless, 3-phenoxybenzoate 1',2'-dioxygenase could be clearly distinguished from PobAB. First, 3-phenoxybenzoate 1',2'-dioxygenase is a type IV RHO, whereas PobAB is a type I RHO (see Fig. S6 in the supplemental material). Second, PbaA1 shows very low similarity (only 8%) with PobA, the oxygenase component of PobAB. Third, 3-phenoxybenzoate 1',2'-dioxygenase catalyzes the hydroxylation at the 1' and 2' positions of the benzene moiety of 3-phenoxybenzoate, producing catechol and 3-hydroxybenzoate, whereas dioxygenation of 3-phenoxybenzoate by PobAB happens at the 1 and 6 positions of the benzoate moiety of 3-phenoxybenzoate, yielding protocatechuate and phenol. These differences clearly demonstrate that the 3-phenoxybenzoate 1',2'-dioxygenase differs from PobAB in structure as well as catalytic mechanisms. The phylogenetic tree of PbaA1 with the large subunits of 71 characterized RHOs showed that PbaA1 is clustered with the oxygenase component DxnA1 of dioxin dioxygenase, which is also a type IV RHO (see Fig. S6 in the supplemental material). However, 3-phenoxybenzoate 1',2'-dioxygenase differs from dioxin dioxygenase in some essential genetic and biochemical characteristics. PbaA1, PbaA2, and PbaB share only 65%, 52%, and 10% identity with DxnA1, DxnA2, and Fdx1, respectively. 3-Phenoxybenzoate 1',2'-dioxygenase is unable to degrade dibenzofuran, carbazole, and dibenzothiophene, which are the substrates of dioxin dioxygenase; and 3-phenoxybenzoate and 4-phenoxybenzoate, the preferred substrates of the 3-phenoxybenzoate 1',2'-dioxygenase, cannot be degraded by the dioxin dioxygenase (unpublished data).

Transcriptional levels of *pbaA1A2B* and *pbaC* of strain JZ-1 under 3-phenoxybenzoate induction. The relative changes in the transcription of *pbaA1*, *pbaA2*, *pbaB*, and *pbaC* of strain JZ-1 under 3-phenoxybenzoate-induced and non-3-phenoxybenzoate-induced conditions were investigated by real-time PCR. The data in Fig. 3 show 206-fold, 431-fold, 409-fold, and 4.7-fold changes in gene transcription of *pbaA1*, *pbaA2*, *pbaB*, and *pbaC*, respectively, indicating that the transcription of *pbaA1*, *pbaA2*, *pbaB*, and *pbaC* was induced by 3-phenoxybenzoate. However, the transcriptional level of *pbaC* was only 1 to 2% of that of *pbaA1*, *pbaA2*, and *pbaB*, implying the possibility that PbaC may not be the only reductase that can physiologically transfer electrons to PbaA1A2B in strain JZ-1.

Coexpression of *pbaA1A2B* with some GR-type reductase genes from other sphingomonad strains in *E. coli*. The results presented above showed that *Sphingomonas wittichii* RW1 harboring *pbaA1A2B* acquired the ability to convert 3-phenoxybenzoate; and our unpublished data also revealed that some other sphingomonad strains, such as *Sphingobium jiangsuense* BA-3 (30), *Sphingomonas quisquiliarum* DC-2 (31), *Sphingomonas* sp. DC-6, and *Sphingomonas baderi* DE-13 (31), could also transform 3-phenoxybenzoate when harboring *pbaA1A2B*, which suggests that these strains had at least one reductase to serve PbaA1A1B. To find these reductases, the amino acid sequence of PbaC was aligned with the draft genomes of strains BA-3, DC-2, DC-6, and DE-13 (the draft genomes of these strains have been sequenced; unpublished data), and four putative GR-type reductases, Red-3 (strain BA-3, 68% identity), Red-4 (strain DC-2, 69% identity), Red-5 (strain DC-6, 69% identity), and Red-6 (strain DE-13, 92% identity), were retrieved. PbaC was also aligned with the genome of *E. coli*, but no protein that showed homology with PbaC was retrieved. The genes coding the four reductases mentioned above

FIG 3 Transcriptional levels of *pbaA1A2B* and *pbaC* of strain JZ-1 under 3-phenoxybenzoate induction.

and RedA2 were coexpressed with *pbaA1A2B* in *E. coli* BL21(DE3). The whole-cell transformation experiments showed that after 2 days of incubation, all of the transformants completely transformed the added 0.5 mM 3-phenoxybenzoate, indicating that all the reductases tested could transfer electrons to PbaA1A2B. The phenomenon that the oxygenase component of RHO has a low specificity for electron transport components was also found in other strains (29, 32, 33). Possibly, this kind of gene arrangement and organization facilitates microbe adaptation in different environments (9, 14, 26, 34–36). In this way, the location of the oxygenase component on a transposable element enables bacteria to acquire the ability to degrade different aromatic substrates quickly by horizontal gene transfer. The nonstringent combination of an oxygenase component with reductase increases gene utilization efficiency and saves genetic resources, which may be helpful for the evolution of new catabolic functions.

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