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Degradation of Bis(4-Hydroxyphenyl)Methane (Bisphenol F) by *Sphingobium yanoikuyae* Strain FM-2 Isolated from River Water▽

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Three bacteria capable of utilizing bis(4-hydroxyphenyl)methane (bisphenol F [BPF]) as the sole carbon source were isolated from river water, and they all belonged to the family *Sphingomonadaceae*. One of the isolates, designated *Sphingobium yanoikuyae* strain FM-2, at an initial cell density of 0.01 (optical density at 600 nm) completely degraded 0.5 mM BPF within 9 h without any lag period under inductive conditions. Degradation assays of various bisphenols revealed that the BPF-metabolizing system of strain FM-2 was effective only on the limited range of bisphenols consisting of two phenolic rings joined together through a bridging carbon without any methyl substitution on the rings or on the bridging structure. A BPF biodegradation pathway was proposed on the basis of metabolite production patterns and identification of the metabolites. The initial step of BPF biodegradation involves hydroxylation of the bridging carbon to form bis(4-hydroxyphenyl)methanol, followed by oxidation to 4,4′-dihydroxybenzophenone. The 4,4′-dihydroxybenzophenone appears to be further oxidized by the Baeyer-Villiger reaction to 4-hydroxyphenyl 4-hydroxybenzoate, which is then cleaved by oxidation to form 4-hydroxybenzoate and 1,4-hydroquinone. Both of the resultant simple aromatic compounds are mineralized.

Bisphenols are a group of chemical compounds that consist of two phenolic rings joined together through a bridging carbon or other chemical structure. They are industrially important chemicals that are widely and abundantly used as the primary raw materials in the production of polycarbonate and epoxy resins. Because of their widespread use, considerable amounts of bisphenols are discharged into a broad range of aquatic and terrestrial environments. Therefore, their behavior in the environment merits close attention.

Biodegradation is a major mechanism for eliminating various environmental pollutants. Studies on the biodegradation of bisphenols have mainly focused on the most abundantly utilized bisphenol, bisphenol A [2,2-bis(4-hydroxyphenyl)propane; BPA]. BPA has been identified as an endocrine-disrupting chemical that may cause adverse effects on human health and wildlife (7, 19, 39). A number of BPA-degrading bacteria have been isolated from enrichments of sludge from wastewater treatment plants (20, 25), aquatic environments (23, 24, 31), soil (30, 33), and samples of compost leachate (43). Spivack et al. (36) revealed that BPA is biodegraded via major and minor pathways, both involving the oxidative skeletal rearrangement of an unactivated aliphatic methyl group in the BPA molecule. Furthermore, Sasaki et al. (33) recently showed that the first step in the biodegradation of BPA is the hydroxylation of the carbon atom of a methyl group or the quaternary carbon in the BPA molecule. Judging from these features of the biodegradation mechanisms, it is possible that the same mechanism used for BPA is used to biodegrade all bisphenols that have at least one methyl or methylene group bonded at the carbon atom between the two phenol groups. However, bisphenol F [bis(4-hydroxyphenyl)methane; BPF], which has no substituent at the bridging carbon, is unlikely to be metabolized by such a mechanism. Indeed, Sakai et al. (31) reported that a BPA-degrading bacterium, *Sphingomonas* sp. strain BP-7, degraded bis(4-hydroxyphenyl)ethane (BPE), 2,2-bis(4-hydroxy-3-methylphenyl)propane (BPP), 2,2-bis(4-hydroxyphenyl)butane (BPB), and 1,1-bis(4-hydroxyphenyl)cyclohexane, but not BPF. Nevertheless, we previously found that BPF is readily degraded by river water microorganisms under aerobic conditions (21). From this evidence, it was clear that a specific mechanism for biodegradation of BPF does exist in the natural ecosystem, although the details were unknown at that time. Potential endocrine disruptive effects of BPF observed in vitro (6, 34, 37) and in vivo (42) also emphasize the importance of understanding the fate in the aquatic environment.

We report here the characterization of a bacterium isolated from river water, *Sphingomonas yanoikuyae* strain FM-2, which is capable of utilizing BPF as its sole source of carbon. The identification of metabolites led to the determination of a biochemical pathway of BPF biodegradation. As expected, the metabolic pathway of BPF is different from that proposed previously for BPA, and BPF appears to be metabolized though a unique pathway by the oxidative cleavage of the two phenol rings via the Baeyer-Villiger reaction (4).

MATERIALS AND METHODS

Chemicals. BPF, tetramethyl BPF (TMBPF), BPE, BPA, BPP, BPB, benzophenone (BP), 4,4′-dihydroxybenzophenone (DHBP), bis(4-hydroxyphenyl)sulfide (TDP), and bis(4-hydroxyphenyl)sulfone (BPS) were purchased from Tokyo Chemical Industry (Tokyo, Japan). 1,4-Hydroquinone, lithium aluminum hy-

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dride, and diethyl ether were purchased from Wako Pure Chemical Industries (Osaka, Japan). *N*,*O*-bis(trimethylsilyl)acetamide was purchased from Wako Pure Chemical Industries (Osaka, Japan) and Tokyo Chemical Industry (Tokyo, Japan). Silica gel (BW-127ZH [100 to 270 mesh]) was purchased from Fuji Silysia Chemical (Aichi, Japan). All other chemicals were purchased from Kishida Chemical (Osaka, Japan).

Culture media. Artificial river water (ARW) (41), which was used for enrichment of BPF-degrading bacteria from a river water sample, contained (per liter of ultrapure water) 21.8 mg of K_2HPO_4, 8.5 mg of KH_2PO_4, 44.6 mg of Na_2HPO_4, 1.7 mg of NH_4Cl, 22.5 mg of $MgSO_4 \cdot 7H_2O$, 27.5 mg of $CaCl_2$, and 0.25 mg of $FeSO_4 \cdot 6H_2O$. BPF was added to ARW at 0.064 to 0.32 mM as the sole carbon source. A 1/10 dilution of CGY medium (Casitone, 5 g/liter; glycerol, 5 g/liter; yeast extract, 1 g/liter) (28) containing BPF at 0.32 mM (1/10CGYF) was used to isolate BPF-degrading bacteria. The isolates were routinely maintained on CGY medium. Basal salt medium (BSM), which was used for degradation assays of BPF and other aromatic compounds, contained (per liter of ultrapure water) 1.0 g of K_2HPO_4, 1 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeCl_3$, 0.05 g of NaCl, and 0.05 g of $CaCl_2$. BPF at 0.25 to 1.5 mM, glucose at 1.1 mM, or one of the various aromatic compounds at 0.039 to 1.1 mM was added to the BSM as the sole carbon source. The pH of ARW and all media was adjusted to 7.2. Agar was used at 1.5% (wt/vol) for solid media.

Enrichment, isolation, and identification of BPF-degrading bacteria. Enrichment of BPF-degrading bacteria was performed according to a total organic carbon (TOC)-Handai method (21, 41), a kind of river-die-away method, with minor modifications because we had observed aerobic BPF biodegradation by river water microbes with the same method (21). A river water sample collected from the subsurface zone (30- to 50-cm depth) of the downstream reach of the Ai River (Toyonaka, Osaka, Japan) in June 2001 was used as an inoculum. Native microbes were collected from 1.5 liters of the sample by membrane filtration (pore size, 0.45 μm; Millipore, Tokyo, Japan) and dispersed into 150 ml of ARW, resulting in a 10-fold increase in the concentration of river water microbes. Five milliliters of the concentrated microbes was inoculated into 45 ml of ARW in a 70-ml test tube, and 5 ml of BPF solution was added as the sole carbon source to give a final concentration of 0.064 mM. The tube was aerobically incubated at 28°C on a rotary shaker at 120 rpm. Aliquots were periodically sampled to analyze concentrations of BPF and TOC for evaluating the degradation and complete mineralization of BPF, respectively. The control tests without river water microbes and without BPF but with microbes were also performed to confirm the biodegradation of BPF by river water microbes and measure the TOC derived from river water microbes, respectively. After the mineralization of BPF based on TOC analysis was confirmed, 5 ml of the culture was transferred to fresh medium. In the second and third subculturings, BPF was added at 0.26 and 0.32 mM, respectively.

An aliquot of the third subculture was serially diluted by 10-fold steps and plated onto 1/10CGYF agar. The plates were incubated at 28°C, and morphologically different colonies were screened for their ability to degrade BPF in an axenic culture. Consequently, three bacterial strains, designated strains FM-1, FM-2, and FM-3, were isolated as BPF-degrading bacteria.

The isolated bacterial strains were characterized and identified by physiological and phylogenetic analyses. The strains were morphologically and physiologically characterized, classified, and tentatively identified according to the diagnostic tables of bacteria proposed by Cowan and Steel (9) and by using an API 20NE kit according to the instructions of the manufacturer (BioMérieux Japan, Tokyo, Japan). A comparative 16S rRNA gene sequence analysis was performed as follows. Partial 16S rRNA genes were amplified by PCR using primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1392R (5′-ACGGGCGGTGTGT ACA-3′) (3). The determination of the amplified 16S rRNA gene sequence was entrusted to Takara Bio (Shiga, Japan). The 16S rRNA gene sequences were compared to reference sequences by using BLAST similarity searches (2), and closely related sequences were obtained from GenBank. The sequences were aligned by using CLUSTAL W (13). A phylogenetic tree was produced by njplotWIN95 software (27).

Biodegradation assays. Unless otherwise indicated, cultivation was carried out on a rotary shaker (120 rpm) at 28°C. In the BPF biodegradation assays, cells were grown to the late logarithmic phase in BSM containing BPF (0.5 mM) or glucose (1.1 mM) or in CGY medium (only during utilization assays for various aromatic compounds). The cells were harvested by centrifugation (21,000 × *g*, 4°C, 10 min) and washed twice with a 50-mg/liter sodium tripolyphosphate solution. The cells were then added to a final cell density of 0.01 (i.e., the optical density at 600 nm [OD_{600}]) to BSM containing either BPF or one of the following compounds as the sole carbon source: TMBPF, BPE, BPA, BPP, BPB, DHBP, TDP, or BPS (as other bisphenols); BP (as a structurally similar compound); or phenol, *o*-cresol, *m*-cresol, *p*-cresol, benzoic acid, catechol, 1,4-hydroquinone, 2-hydroxybenzoate (2HB), 3HB, 4HB, protocatechuic acid, gentisic acid, phthalic acid, or phenoxyacetic acid (as simple aromatic compounds). At appropriate intervals, the cell density and the concentrations of TOC and substrate were measured.

Analytical procedures. Bacterial growth was monitored by recording the increase in OD_{600} with a model UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan).

Substrate concentrations were determined with a Shimadzu LC-10A*vp* high-performance liquid chromatography (HPLC) system consisting of an SIL-10AF automatic sampler, LC-10AD*vp* solvent delivery units, a CTO-10A*vp* column oven, and an SPD-10A*vp* UV/VIS detector (Shimadzu). The instrument was equipped with a Shim-Pack VP-ODS column (150 by 4.6 mm [inner diameter]; particle size, 5 μm; Shimadzu). Acetonitrile and water at a ratio of 1:1 was used as the mobile phase with a flow rate of 1.0 ml/min. The detection was carried out at 280 nm. TOC was analyzed by using a TOC analyzer (TOC-5000A; Shimadzu). For analyses of substrates and TOC, samples were centrifuged (19,000 × *g*, 4°C, 10 min), and a portion of the supernatant was used.

For isolation of metabolites produced by BPF biodegradation, a culture of strain FM-2 on BPF was freeze-dried and dissolved in 1 ml of acetonitrile. After filtration (DISMIC 13HP045AN; pore size, 0.45 μm; Advantec, Tokyo, Japan), the solution was subjected to HPLC analysis as described above but with a Shim-Pack VP-ODS column (250 by 4.6 mm [inner diameter]; particle size, 5 μm; Shimadzu). If necessary, acetonitrile and 10 mM sodium phosphate buffer (pH 3) at a ratio of 1:1 was used as the mobile phase, and peaks that represented metabolites were collected. Isolated metabolites were dried under a nitrogen atmosphere, redissolved in 500 μl of acetone, and subjected to gas chromatography-mass spectrometry (GC-MS) analysis. If necessary, metabolites were trimethylsilylated by treatment with *N*,*O*-bis(trimethylsilyl)acetamide solution at room temperature for 1 h. The GC-MS analysis of metabolites with or without derivatization was conducted on an Agilent 6890N network gas chromatograph (Agilent Technology, Santa Clara, CA) coupled with a JEOL JMS-700 high-resolution mass spectrometer (Nihondenshi, Tokyo, Japan). A 25-m (0.25 mm [inner diameter]; film thickness, 0.25 μm) fused silica capillary column CP-Sil 8 CB-Low Bleed/MS (GL Sciences, Tokyo, Japan) was used. A 1-μl quantity of the metabolites with or without derivatization was injected in splitless mode for 30 s. Helium gas (99.9999%) was used as the carrier gas at a flow rate of 1.2 ml/min. The oven temperature programs were as follows: (i) 50°C isothermal for 2 min, 50 to 100°C at 10°C/min, and 100 to 260°C at 20°C/min for analyses of 4HB and 1,4-hydroquinone and (ii) 90°C isothermal for 1 min and increasing to 300°C at 15°C/min for analyses of other metabolites. The MS analysis was performed at 70 eV. The structures of the metabolites were confirmed by comparing the fragmentation patterns of the mass spectra with those of authentic compounds [bis(4-hydroxyphenyl)methanol, DHBP, 4HB, 1,4-hydroquinone, and 1,4-benzoquinone] or those predicted for known compounds.

Synthesis of bis(4-hydroxyphenyl)methanol. Bis(4-hydroxyphenyl)methanol, one of the possible metabolites of BPF biodegradation, was synthesized as follows. Under a nitrogen atmosphere, 76 mg of lithium aluminum hydride and 20 ml of diethyl ether were placed in a 200-ml flask. Then, DHBP (428 mg) in 20 ml of diethyl ether was slowly added, and the mixture was stirred for 15 min. The mixture was heated at 50°C in a water bath for approximately 20 min under ether reflux. After the mixture was acidified with dilute hydrochloric acid, the ether layer was separated. The target compound in the aqueous layer was extracted with 20 ml of diethyl ether, and the organic layer was dried over anhydrous Na_2SO_4. After the insoluble material was removed by filtration, the ether was evaporated, and the resultant residue was purified by silica gel column chromatography (*n*-hexane–ethyl acetate, 95:5 to 30:70 [gradient elution]). The fraction of 30% *n*-hexane and 70% ethyl acetate was isolated and, finally, the organic solvent was evaporated to obtain bis(4-hydroxyphenyl)methanol. The compound was identified by comparing the GC-MS spectrum of its trimethylsilylated derivative with the previously reported one (GC purity, >90%) (12).

Nucleotide sequence accession numbers. The partial 16S rRNA gene sequences of strains FM-1, FM-2, and FM-3 were registered in GenBank/EMBL/DDBJ as accession numbers AB331237, AB331238, and AB331239, respectively.

RESULTS

Isolation and identification of BPF-degrading bacteria. River water microbes reduced 78% of the TOC in 7 days when incubated in ARW and 0.064 mM BPF. In the control experiment without river microbes, no decrease in TOC concentration was observed, indicating that BPF was biodegraded in the

TABLE 1. Taxonomic characteristics of BPF-degrading bacteria isolated from river water

Characteristic	FM-1	FM-2	FM-3
Shape	Sphere	Rod	Rod
Gram stain	–	–	–
Motility	–	+	–
OF test[a]	Oxidative	Oxidative	Oxidative
Catalase	+	+	+
Oxidase	–	+	–
Urease	–	–	–
β-Galactosidase	–	+	+
Arginine dehydrolase	–	–	–
Indole production	–	–	–
Esculin hydrolysis	+	+	+
Gelatin hydrolysis	–	–	–
Nitrate reduction	–	–	–
Growth on:			
Glucose	+	+	+
L-Arabinose	–	+	+
D-Mannose	–	+	+
D-Mannitol	–	–	–
N-Acetyl-D-glucosamine	–	+	–
Maltose	+	+	+
Gluconate	–	+	+
n-Caprate	–	–	–
Adipate	+	–	–
D,L-Malate	–	–	–
Citrate	–	–	–
Phenylacetate	–	–	–

[a] OF, oxidation/fermentation test.

test experiments. To obtain pure cultures, we enriched BPF-degrading bacteria by successive transfers to fresh ARW containing BPF at 0.064, 0.26, and 0.32 mM. In each subculture, 75 to 89% of the initial TOC disappeared without any lag period. Finally, three morphologically different colonies which were capable of growing on BPF were isolated and designated strains FM-1, FM-2, and FM-3.

Morphological and biochemical analysis (Table 1) in combination with phylogenetic analysis based on partial 16S rRNA gene sequence indicated that the three bacteria isolated were members of the family *Sphingomonadaceae*. Strain FM-1 was allocated within the *Novosphingobium* cluster and showed the highest sequence identity (99.8%) with *Novosphingobium aromaticivorans*. Strains FM-2 and FM-3 were members of the genus *Sphingobium*, and the closest phylogenetic relative of these strains was *Sphingobium yanoikuyae* NBRC 15102^T with a sequence similarity of 99.1%. The morphological and biochemical characteristics of strain FM-1 and strains FM-2 and FM-3 (Table 1) were almost identical to those of the type strains of *N. aromaticivorans* (5) and *S. yanoikuyae* (40), respectively. Based on these results, we identified strain FM-1 as *N. aromaticivorans*, while strains FM-2 and FM-3 were identified as *S. yanoikuyae*.

BPF degradation by strain FM-2. Strain FM-2 was selected for further studies because it exhibited the highest BPF-degrading ability among the three BPF-degrading isolates. A typical time course of cell growth and BPF degradation (BPF concentration, 0.5 mM) of strain FM-2 is shown in Fig. 1. After preincubation in BSM containing BPF as the sole carbon source, strain FM-2 completely degraded 0.5 mM BPF within 9 h without any lag period. The TOC concentration decreased in proportion to the decrease in BPF beginning at 6 h and reached a plateau within 12 h, the final TOC removal efficiency being 93%. The turbidity of the culture (OD_{600}) increased from 0.01 to 0.15 over the period of BPF degradation. When preincubated on glucose instead of BPF, a lag period of 8 h was observed prior to the onset of BPF degradation. Thereafter, nearly 95% of the initial BPF was degraded within 16 h. The onset of TOC removal and cell growth were also delayed, although their maximal values were almost the same as those of the culture pregrown on BPF. These results indicate that the BPF-metabolizing system of strain FM-2 was inducible.

Utilization and degradability of other aromatic compounds. Among the bisphenols and the structurally similar compounds tested, only DHBP and BP were utilized by strain FM-2 for growth. In contrast, TMBPF, BPE, BPA, BPP, BPB, TDP, and BPS were neither utilized for growth nor degraded by strain FM-2. These results suggest that strain FM-2 specifically degrades bisphenols that consist of two phenolic rings joined together through a bridging carbon without any methyl substituents on the rings or on the bridging structure.

Among the simple aromatic compounds tested, catechol, 1,4-hydroquinone, and protocatechuic acid were able to support the growth of strain FM-2. In addition, although benzoic acid and 4HB did not support growth as the sole carbon source, strain FM-2 precultivated on BPF could degrade these compounds.

Identification of metabolites and BPF biodegradation pathway. During degradation of BPF by strain FM-2 precultivated on BPF at 28°C (the standard conditions in the present study), HPLC analysis detected two metabolites with retention times of 2.2 min (metabolite I) and 2.6 min (metabolite II) that occurred concurrently with the decrease of BPF. The amounts of these metabolites peaked at 6 h and thereafter decreased to an undetectable level by 12 h. To detect other metabolites and understand the transformation pattern of all metabolites, we also carried out biodegradation assays at 37, 40, and 45°C. Although strain FM-2 is capable of growing at 37 and 40°C, the strain loses the proliferation activity at 45°C. At 45°C the BPF-degrading enzymes produced during the precultivation period enable limited degradation activity prior to their inactivation. At 37°C, two additional metabolites with retention times of 2.0 min (metabolite III) and 2.7 min (metabolite IV) appeared after metabolites I and II were produced. At 40°C,

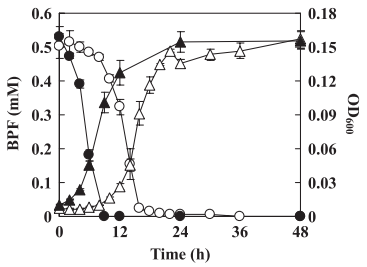

FIG. 1. Time course of BPF degradation by strain FM-2 after preincubation on BPF (closed symbols) or glucose (open symbols). The BPF concentration (circles) and OD_{600} (triangles) were monitored. Error bars indicate the standard deviation obtained from three independent experiments.

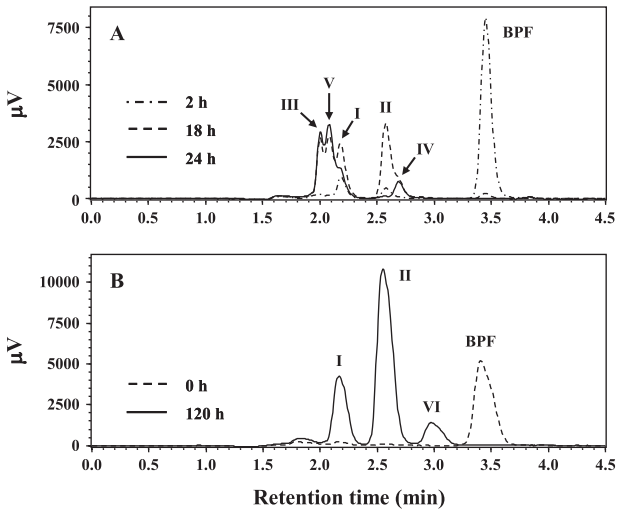

FIG. 2. HPLC chromatograms showing the generation of metabolite peaks along with BPF degradation by strain FM-2 at 40°C (A) and 45°C (B). Numbers in the chromatograms represent the metabolite numbers defined in the text.

five metabolites—metabolites I, II, III, IV, and a novel metabolite V with a retention time of 2.1 min—appeared as follows (Fig. 2A). Metabolite I first appeared and increased, which was followed by the appearance of metabolite II. Then, metabolites III and V appeared at the same time, and subsequently metabolite IV emerged. Metabolites I and II completely disappeared within 48 h. In contrast, metabolites III, IV, and V persisted in the culture suspension even after 72 h, although metabolite V decreased more rapidly than metabolites III and IV. The concentrations of metabolites were determined for commercially available compounds as described below. The measurements revealed that metabolites III (1,4-hydroquinone), IV (1,4-benzoquinone), and V (4HB) generated from 0.5 mM BPF were 0.22, 0.20, and 0.12 mM at 24 h, respectively. This meant that detected metabolites accounted for more than half of the BPF added.

At 45°C, a sixth metabolite with retention time of 3.0 min (metabolite VI) appeared together with metabolites I and II (Fig. 2B). All three metabolites persisted without notable decreases even after 120 h. The maximal accumulation of metabolite II, which was identified as DHBP by GC-MS analysis as described below, was 0.17 mM at 120 h. In control cultures not inoculated with strain FM-2 at 28, 37, 40, and 45°C, no depletion of BPF or generation of metabolites was observed (data not shown). This confirmed that the metabolites detected here resulted not from chemical oxidation but from biodegradation by strain FM-2.

The six metabolites detected were isolated by HPLC and identified by GC-MS. GC-MS spectra of the metabolites are shown in Fig. 3. Metabolites I, II, III, IV, and V were identi-

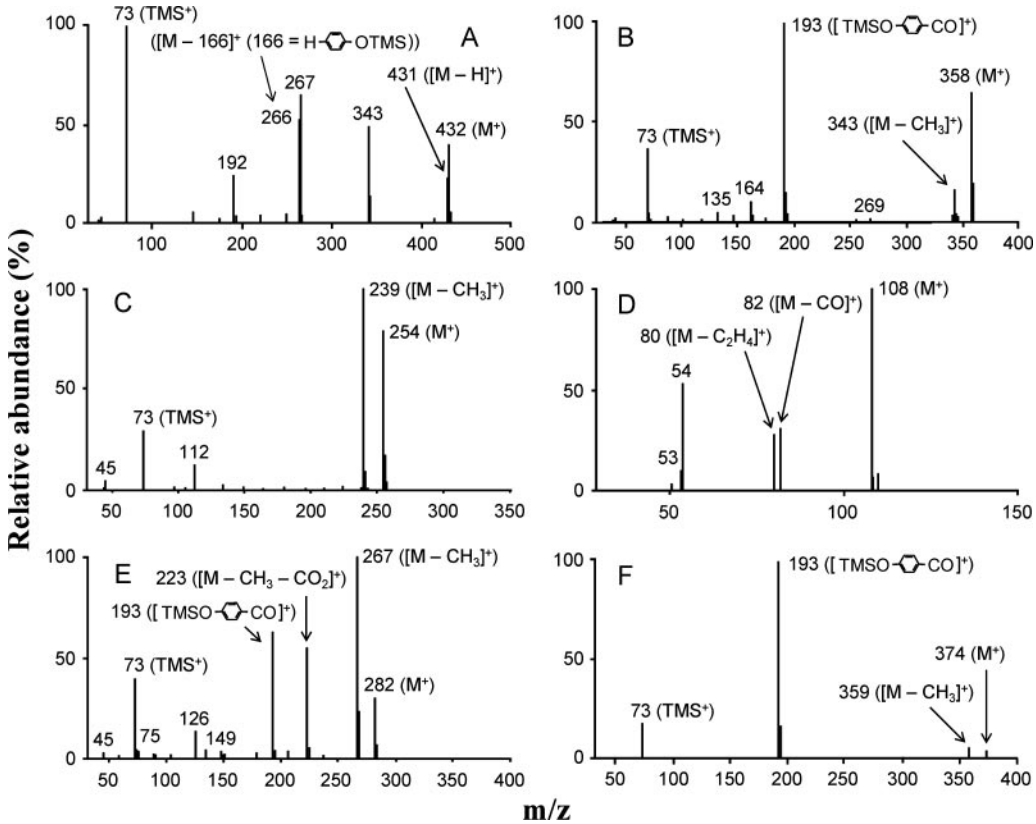

FIG. 3. Mass spectra of metabolites formed during degradation of BPF by strain FM-2. (A) Metabolite I identified as bis(4-hydroxyphenyl)-methanol with trimethylsilylation. (B) Metabolite II identified as DHBP with trimethylsilylation. (C) Metabolite III identified as 1,4-hydroquinone with trimethylsilylation. (D) Metabolite IV identified as 1,4-benzoquinone. (E) Metabolite V identified as 4HB with trimethylsilylation. (F) Metabolite VI tentatively identified as 4-hydroxyphenyl 4-hydroxybenzoate with trimethylsilylation.

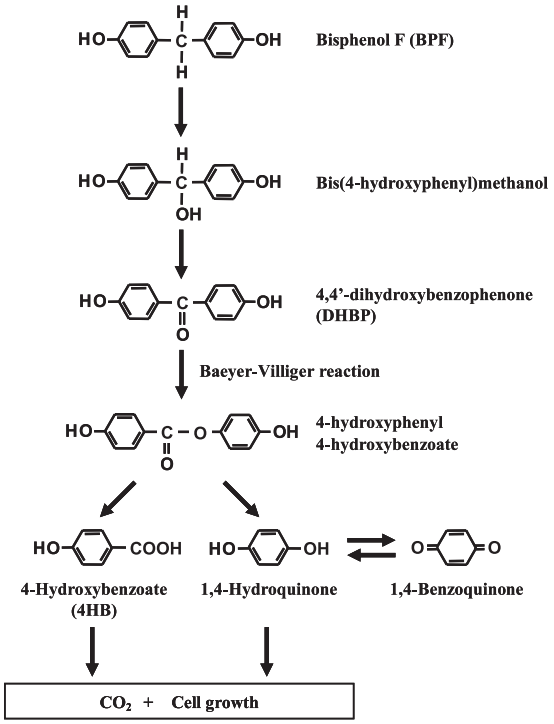

FIG. 4. Proposed pathway of BPF metabolism by strain FM-2.

fied, respectively, as bis(4-hydroxyphenyl)methanol, DHBP, 1,4-hydroquinone, 1,4-benzoquinone, and 4HB by comparing their mass spectra to those of authentic compounds (Fig. 3A, B, C, D, and E). Metabolite VI, with a retention time of 2.8 min, was tentatively identified as 4-hydroxyphenyl 4-hydroxybenzoate by interpreting its mass spectral pattern (Fig. 3F), because no authentic reference compound was available.

On the basis of the metabolites identified and their patterns appearing during BPF degradation assays, we proposed the following pathway for the metabolism of BPF by strain FM-2 (Fig. 4). The bridging carbon of BPF is initially hydroxylated to form bis(4-hydroxyphenyl)methanol, which is further oxidized to DHBP. Subsequently, DHBP is transformed to 4-hydroxyphenyl 4-hydroxybenzoate by insertion of an oxygen atom between the carbon atoms of the ketone group and one of the phenolic rings in DHBP (the Baeyer-Villiger reaction). The 4-hydroxyphenyl 4-hydroxybenzoate is cleaved by oxidation to 4HB and 1,4-hydroquinone. The 4HB is directly mineralized. In contrast, some 1,4-hydroquinone is mineralized, and some portion undergoes reversible auto-oxidation to 1,4-benzoquinone (14). Under dark conditions, 1,4-benzoquinone was not detected during BPF biodegradation (data not shown), which supports the occurrence of the reversible, photochemical conversion between 1,4-hydroquinone and 1,4-benzoquinone. As described above, detected metabolites accounted for a large proportion of the initially added BPF despite the inability to quantify bis(4-hydroxyphenyl)methanol and 4-hydroxyphenyl 4-hydroxybenzoate due to a lack of commercially available compounds. Furthermore, the accumulation of metabolites during degradation was only transient, thereby precluding the quantification of the maximum amounts of these compounds that were formed. These observations suggest that the proposed pathway is a major route for BPF degradation by strain FM-2.

DISCUSSION

We successfully isolated three BPF-degrading bacterial strains—*N. aromaticivorans* strain FM-1 and *S. yanoikuyae* strains FM-2 and FM-3—from a river water sample. Considering that most of the BPA-degrading bacteria previously isolated also belong to the family *Sphingomonadaceae* (20, 31, 32), this family seems to play a major role in the depletion of various bisphenols in the aquatic environment.

Our results indicated that BPF degradation by strain FM-2 proceeded through (i) an initial rearrangement of the bridging structure of BPF to release two simple aromatic compounds, 4HB and 1,4-hydroquinone, and (ii) the complete degradation of 4HB and 1,4-hydroquinone. Because the peaks representing the metabolites of BPF degradation by strain FM-2 were also detected during degradation of BPF by strains FM-1 and FM-3 (data not shown), the BPF biodegradation pathway proposed here may be common among different bacteria.

In the biodegradation of BPA, the oxidative skeletal rearrangement in the bridging aliphatic group is a crucial process for cleaving the phenolic rings to form simple aromatic compounds and for the subsequent complete mineralization (36). In contrast, in the degradation of BPF by strain FM-2, the conversion from DHBP to 4-hydroxyphenyl 4-hydroxybenzoate appears to be the critical step leading to the release of simple aromatic compounds. This reaction is the so-called Baeyer-Villiger reaction that involves the insertion of an oxygen atom between the keto carbon and an adjacent carbon in ketones or aldehydes to form an ester or a lactone. Biologically, the Baeyer-Villiger reaction is catalyzed by two types of flavoenzymes (Baeyer-Villiger monooxygenases), FAD- and NADPH-dependent enzymes and FMN- and NADH-dependent enzymes, and has exquisite substrate-, enantio-, and regioselectivity (1). Numerous studies have demonstrated that the reaction is involved in the conversion of a broad range of aromatic compounds such as acetophenone and its derivatives (10, 11, 17, 18, 22, 38), benzaldehyde and its derivatives (22, 26), and aliphatic phenols (10, 11). In the established Baeyer-Villiger reaction, insertion of the oxygen atom commonly occurs at the substituent with greater electron-releasing potential of the ketone group. Nevertheless, in the metabolism of BPF by strain FM-2 the apparent substrate of the Baeyer-Villiger reaction is DHBP, a compound which has the same structure on both sides of the ketone group. To our knowledge, this is the first report suggesting that the Baeyer-Villiger reaction occurs on a symmetric compound.

Results of the biodegradation assays for various bisphenols and structurally similar compounds indicated that the BPF-metabolizing system of strain FM-2 has high substrate specificity. The initial reaction in BPF biodegradation targets the bridging structure. Nevertheless, even TMBPF, which has the same bridging structure as BPF, was not degraded by strain FM-2 precultivated on BPF. Therefore, the enzyme catalyzing the initial hydroxylation appears to be highly specific.

Cleavage of BPF by strain FM-2 resulted in the generation of two single aromatic compounds: 4HB and 1,4-hydroquinone. It has been previously shown that 4HB is aerobically

degraded by bacteria via the protocatechuic acid or gentisic acid pathways (16). After accumulation of 4HB in the BPF degradation assays, a compound assumed to be protocatechuic acid was observed in the GC-MS analysis (data not shown); therefore, 4HB may be degraded through the protocatechuic acid pathway. Utilization of protocatechuic acid for growth would support this indication. Although we did not detect metabolites, the degradation of 1,4-hydroquinone by strain FM-2 may occur as shown in studies of *Pseudomonas* sp. and *Moraxella* sp. by Darby et al. (11) and Spain and Gibson (35), respectively. In these bacteria, 1,4-hydroquinone is converted to β-ketoadipic acid through γ-hydroxymuconic semialdehyde and maleylacetic acid.

The long-term accumulation of 1,4-hydroquinone and 1,4-benzoquinone and the transient accumulation of 4HB at 40°C, which were not detected below 37°C, indicate that at the higher temperature enzymes for 1,4-hydroquinone degradation are inactivated and enzymes for 4HB degradation are less active. In addition, the transient accumulation of 1,4-hydroquinone even at 28 and 37°C suggested that enzyme acting on this substrate is not as effective as that which hydroxylates 4HB. The ferrous ion concentration in the culture medium could affect the relevant oxygenase activities (35). Nevertheless, rapid decrease of both BPF and the metabolites at 28 and 37°C, resulting in TOC removal of >95% within 24 h, indicated that all of the enzymes involved in BPF mineralization could effectively work to some extent under these temperature conditions. The accumulation of bis(4-hydroxyphenyl)methanol, DHBP, and 4-hydroxyphenyl 4-hydroxybenzoate at 45°C indicated that this temperature inactivates the enzyme(s) catalyzing the formation of single aromatic compounds.

Members of the family *Sphingomonadaceae* have been previously found to possess unique systems for metabolizing various organic contaminants such as BPA (33, 36), alkylphenols (8, 15), and polycyclic aromatic hydrocarbons (29), which other organisms cannot attack, although these contaminants are ubiquitous in the environment. Our study suggests that strain FM-2 of *S. yanoikuyae* can also metabolize BPF by an intriguing strategy: via symmetric Baeyer-Villiger rearrangement. BPF-degrading enzymes including the Baeyer-Villiger-type monooxygenase from strain FM-2 should show interesting properties. Further study to gain a more detailed understanding of the properties of the BPF-degrading enzymes from strain FM-2 is now under way.

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