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NOTE

Biodegradation of bisphenol A and bisphenol F in the rhizosphere sediment of *Phragmites australis*

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The accelerated removal of bisphenols A and F (BPA, BPF) was observed in the rhizosphere sediment of *Phragmites australis*, while they persisted in the absence of *P. australis*. A BPA-degrading bacterium, *Novosphingobium* sp. strain TYA-1, and a BPF-degrading bacterium, *Sphingobium yanoikuyae* strain TYF-1, were isolated from the rhizosphere of *P. australis*. The results suggested that interactions between *P. australis* and these bacteria can accelerate the removal of bisphenols from sediment.

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[**Key words:** Bisphenol A; Bisphenol F; Biodegradation; Rhizosphere; *Phragmites australis*; *Novosphingobium* sp. strain TYA-1; *Sphingobium yanoikuyae* strain TYF-1]

Bisphenols, such as bisphenol A (BPA: 2,2-bis[4-hydroxyphenyl] propane) and its structurally similar analogues, are ubiquitous pollutants in aquatic environments (1). As most bisphenols are endocrine-disrupting chemicals that may adversely affect human health and wildlife (2, 3), their fate in aquatic environments is of great concern. It has been demonstrated that bisphenols are readily biodegradable in surface waters under aerobic conditions (4–6) but not under anaerobic conditions (1, 4, 5, 7); as a result, bisphenols persist in anaerobic sediments for prolonged periods. In fact, bisphenol concentrations in sediments are frequently much higher than in surface waters (1).

We previously found that the biodegradation of surfactants (8) and aromatic compounds (9) is promoted in the rhizosphere of aquatic plants. Rhizosphere oxidation, the ability of plants to release oxygen into the rhizosphere, can stimulate the biodegradation of organic compounds by rhizosphere bacteria (10, 11). Also, it has been reported that the rhizosphere of some aquatic plants accumulate bacterial populations specialized for the degradation of aromatic compounds (9). Although synergies between rhizosphere oxidation by aquatic plants and rhizosphere bacterial activities are potentially essential for the efficient removal of organic pollutants from sediments, the rhizosphere bacteria involved have rarely been studied. In particular, the potential for and mechanisms of bisphenol biodegradation in the rhizosphere of aquatic plants have not been studied thus far.

We therefore investigated the potential for the accelerated biodegradation of BPA and bisphenol F (BPF: bis[4-hydroxyphenyl] methane) in the rhizosphere sediment of the emerged aquatic plant *Phragmites australis*, with a focus on its rhizosphere bacteria. We compared BPA and BPF degradation in sediments with and without *P. australis*, and characterized the behaviors of BPA- and BPF-degrading bacteria in rhizosphere sediment as well as in non-rhizosphere sediment. In addition, we isolated BPA- and BPF-degrading bacteria from the rhizosphere sediment for the first time.

BPA, BPF, 4-hydroxybenzaldehyde (HBAL), 4-hydroxyacetophenone (HAP), 4-hydroxybenzoic acid (4HB), 1,4-hydroquinone (1,4-HQ) and 1,4-benzoquinone (1,4-BQ) were purchased from Wako Pure Chemical Industries (Osaka, Japan). BSTFA (*N*,*O*-bis[Trimethylsilyl]trifluoroacetamide) was purchased from Tokyo Chemical Industry (Tokyo, Japan). The basal salts medium (BSM, pH 7.0) used contained 1.0 g $(NH_4)_2SO_4$, 1.0 g K_2HPO_4, 0.2 g NaH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g NaCl, 0.05 g $CaCl_2$, 8.3 mg $FeCl_3 \cdot 6H_2O$, 1.4 mg $MnCl_2 \cdot 4H_2O$, 1.17 mg $Na_2MoO_4 \cdot 2H_2O$ and 1 mg $ZnCl_2$ per liter of water. BSM containing 0.5 mM BPA (BPA-BSM) or 0.5 mM BPF (BPF-BSM) as the sole carbon source was used for culture of BPA- and BPF-degrading bacteria, respectively. A 1/10 dilution of Luria–Bertani (LB) medium (poly peptone, 10 g L^{-1}; yeast extract, 5 g L^{-1}; NaCl, 10 g L^{-1}; pH 7.0) was used for the culture of heterotrophic bacteria. Agar solid medium was prepared with 2.0% (wt/vol) agar.

To confirm the effect of *P. australis* on BPA and BPF degradation in the sediment, degradation experiments of BPA and BPF were conducted in sediments with and without *P. australis*. *Phragmites australis*, sediment and surface water samples were obtained from Inukai pond in Osaka University (Suita, Osaka). *Phragmites australis* was collected from the *P. australis* planting zone and rhizosphere sediment samples were collected from a 20 to 40 cm depth around roots. Non-rhizosphere sediment samples were collected from the same depth at locations at least 1 m away from the nearest *P. australis*. About 100 g of each sediment sample was placed in 150-mL vials, and BPA or BPF

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was added to a final concentration of 25 mg kg^{-1}. The samples were mixed by shaking at room temperature for 3 h. One *P. australis* plant was planted in each vial containing the rhizosphere sediment. Finally, 10 mL of pond water was added to all vials. We set up 12 identical sediment microcosms for each treatment. These microcosms were statically incubated in an incubation chamber (30 °C, 10,000 lx, 16-h light/8-h dark). Sterile control tests, using autoclaved sediment (121 °C, 20 min×3) and 1-month-old sterile *P. australis*, were also performed for BPA and BPF. To obtain sterile *P. australis*, seeds were sterilized by a 1-min wash in 70% ethanol and a 5-min wash in sodium hypochlorite solution (5% available chorine), rinsed twice with sterile water and germinated on sterile Hoagland's nutrient (9) 0.25% (w/v) gellan gum-solid medium. Each young plant was aseptically transferred to a 500-mL flask containing 200 mL of sterile Hoagland's nutrient solution, and maintained in an incubation chamber (30 °C, 10,000 lx, 16 h light/8 h dark).Three vials for each treatment were taken periodically for the analyses of BPA and BPF and the determination of the densities of BPA-degrading and BPF-degrading bacteria. The shoots and leaves were removed prior to the extraction of bisphenols from rhizosphere sediments. Sediments with and without the roots were acidified with 5 mL of salting-out solution (1 N HCl, 30% NaCl), shaken with 20 mL of 1:1 (vol/vol) dichloromethane–methanol at 300 rpm for 20 min, sonicated in an ultrasonic bath (20 kHz, 200 W, 5 s interval, 4 °C) for 20 min and shaken again for 20 min; then, the organic layer was collected. This extraction was repeated three times for each sample. The extract was dried under a nitrogen flow and analyzed by high-performance liquid chromatography (HPLC) as described previously (12). The numbers of BPA-degrading, BPF-degrading and heterotrophic aerobic bacteria were determined by the plate count method under aerobic conditions and using the media mentioned above. The plates were incubated at 28 °C for 7 d, and colonies which were capable of growing on BPA-BSM or BPF-BSM were counted as BPA-degrading and BPF-degrading bacteria, respectively, in this study. The densities of BPA-degrading, BPF-degrading and heterotrophic aerobic bacteria were expressed as CFU per g of dry sediment. The abundance of BPA-degrading or BPF-degrading bacteria was also expressed relative to the density of heterotrophic bacteria.

BPA and BPF declined slowly in the sediment microcosms without *P. australis*; 21% of the initial BPA and 24% of the initial BPF had disappeared from the sediment microcosms within 42 d (Fig. 1A, C). BPA and BPF levels declined more rapidly in the rhizosphere sediment microcosms with *P. australis*, leading to a removal of 90% of the initial BPA and 92% of the initial BPF within 42 d (Fig. 1A, C). At the start of the experiments, the densities and relative abundances of BPA-degrading and BPF-degrading aerobic bacteria were practically identical in all treatments (Fig. 1B, D). The densities remained at same levels over the duration of the experiments in the sediment microcosms without *P. australis* (Fig. 1B, D). In contrast, the densities and the relative abundances of BPA- and BPF-degrading aerobic bacteria gradually increased over time when *P. australis* was present in the sediment microcosms (Fig. 1B, D). After 42 d, the densities of BPA- and BPF-degraders were 930- and 150-fold higher in the sediment microcosms with plant roots than in those without plant roots, respectively. These results indicated that BPA-degrading and BPF-degrading aerobic bacteria are ubiquitous not only in surface waters (4–6), but also in sediments. However, these bacteria cannot fully exhibit their metabolic potential nor grow under anaerobic conditions (1, 4, 5, 7) and, as a result, BPA and BPF persisted in the anaerobic sediment (Fig. 1A, C). On the other hand, in the rhizosphere sediment of *P. australis*, higher rates of BPA and BPF degradation were shown to be correlated with higher densities of BPA-degrading and BPF-degrading aerobic bacteria. Thus, it was suggested that the release of oxygen by *P. australis* into the rhizosphere may promote the aerobic degradation of BPA and BPF by rhizosphere bacteria. About 3.5% of the

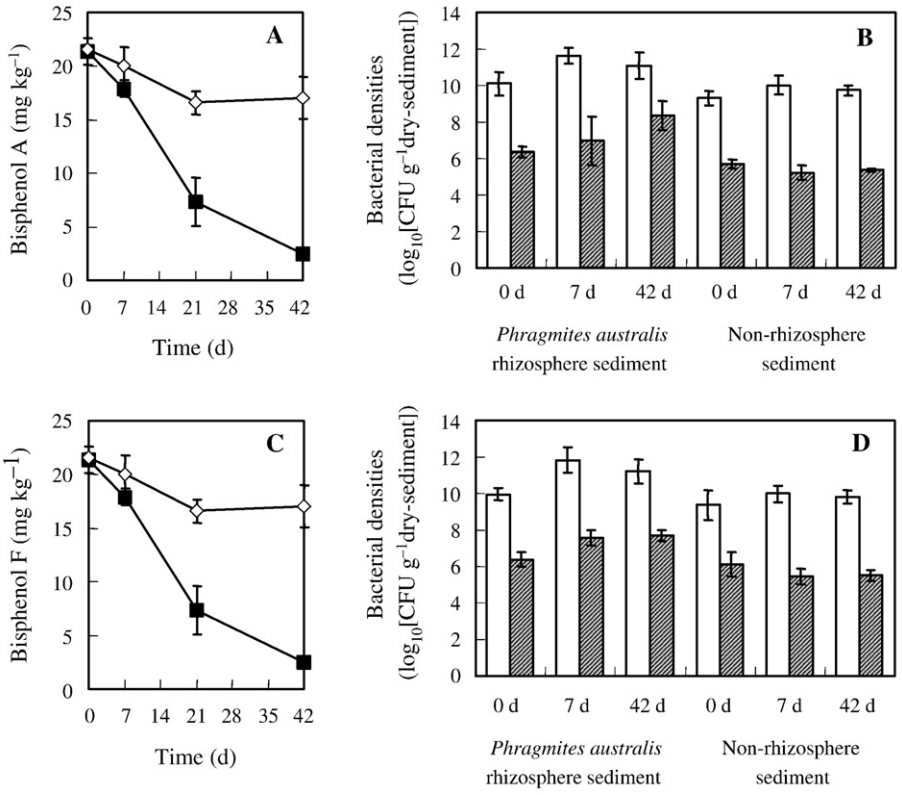

FIG. 1. Removal of BPA (A) and BPF (C) from BPA- or BPF-spiked (25 mg kg^{-1}) sediments with (closed squares) and without (open diamonds) *P. australis*. In B and D, the densities of heterotrophic aerobic bacteria (open bars), BPA-degrading aerobic bacteria (closed bars in B), and BPF-degrading aerobic bacteria (closed bars in D) in BPA-spiked (B) and BPF-spiked sediments (D) are shown. Data represent means of triplicate experiments, and error bars indicate 95% confidence intervals.

initial BPA and 1.7% of the initial BPF had disappeared from the sterile sediment with sterile *P. australis* within 42 d. Although certain plants can absorb BPA from water through their roots and metabolize it (13), the plant uptake of BPA and BPF adsorbed to sediment particles was probably limited. Together with the correlation of degradation rates and bacterial densities, it can be suggested that the removal of BPA and BPF from the *P. australis* rhizosphere is largely attributable to biodegradation by the rhizosphere bacteria.

To obtain bisphenol-degrading aerobic bacteria in the microcosm rhizosphere sediments, we enriched them from these sediments after completing the experiments. One gram of rhizosphere sediment sample was transferred to a 300-mL flask containing 100 mL of BPA-BSM or BPF-BSM, and incubated at 28 °C on a rotary shaker at 120 rpm for 14 d. After confirmation of bacterial growth, 1 mL of each culture was transferred to fresh BPA-BSM or BPF-BSM. The diluted third subcultures were spread onto BPA-BSM or BPF-BSM agar plates, and the plates were incubated at 28 °C.

We isolated a bacterial strain capable of growing on BPA, designated strain TYA-1, and another one growing on BPF, designated strain TYF-1. Both strains were Gram-negative, motile, and rod-shaped. Phylogenetic analysis (12) based on partial 16S rRNA gene sequences indicated that the two isolates were members of the *Sphingomonadaceae* (Fig. 2). Strain TYA-1 showed the highest sequence identity (98.1%) with *Novosphingobium subarcticum* DSM 10700^T, while strain TYF-1 showed the highest sequence identity (99.1%) with *Sphingobium yanoikuyae* NBRC 15102^T. Thus, we identified strain TYA-1 as *Novosphingobium* sp. and strain TYF-1 as *S. yanoikuyae*. Gram-negative bacterial strain MV-1 (14,15), *Sphingomonas paucimobilis* strain FJ-4 (16), *Sphingomonas bisphenolicum* strain AO1 (17) and *Sphingomonas* sp. strain BP-7 (18) have been identified as BPA-degrading bacteria, while *S. yanoikuyae* strain FM-2 (12) has been characterized as a BPF degrader. These strains were isolated from wastewater treatment plants, surface waters or soils. Our strain TYA-1 is a novel rhizosphere-derived BPA-degrading bacterium belonging to the genus *Novosphingobium*, whose members were not previously known to degrade BPA, while strain TYF-1 is closely related to strain FM-2.

The abilities of strains TYA-1 and TYF-1 to degrade BPA and BPF were investigated through degradation tests. Cells grown in BPA-BSM (TYA-1) or BPF-BSM (TYF-1) were harvested by centrifugation (9600×*g*, 4 °C, 10 min), washed twice with 50 mM potassium phosphate buffer (pH 7.5), and incubated at a cell density of an OD_{600} of 0.02 in BPA-BSM or BPF-BSM, at bisphenol concentrations of 0.1 mM, 0.2 mM, 0.5 mM and 1.0 mM. Cultivation was carried out on a rotary shaker (120 rpm) at 28 °C. The cell density (OD_{600}) and the concentrations of substrates and metabolites produced by BPA or BPF degradation were monitored periodically as described previously (12).

Strain TYA-1 grown on BPA completely degraded 0.1 to 1.0 mM BPA within 24 h (Fig. 3A) and showed growth curves that depended on initial BPA concentration (Fig. 3B). Three metabolites were detected by HPLC analysis (12) at retention times (RTs) of 2.30 min (metabolite I), 2.34 min (metabolite II) and 1.75 min (metabolite III), along with a corresponding decrease in BPA at a RT of 4.41 min. The metabolite peaks vanished within 36 h. The metabolites were identified by gas chromatography–mass spectrometry (GC–MS; 12). The mass spectrum of metabolite I (m/z of fragment ions [% relative intensity, characterization]; m/z 122 [94, M^+], 121 [100, M^+ – H] and 93 [56, M^+ – COH]) agreed well with that of HBAL, and that of metabolite II (m/z 136 [34, M^+], 121 [100, M^+ – CH_3] and 93 [47, M^+ – $COCH_3$]) corresponded to that of HAP. The mass spectrum of metabolite III (m/z 280 [20, M^+], 267 [98, M^+ – CH_3], 223 [70, M^+ – CH_3, CO_2], 193 [42, M^+ – OTMS] and 73 [100, TMS]) corresponded to that of ditrimethylsilyl-4HB. In addition, 4HB, HBAL, and HAP could be utilized for growth by strain TYA-1. Corresponding to the degradation of HBAL, 4HB was produced (data not shown). Based on these results, we propose the metabolic pathway of BPA mineralization by strain TYA-1 as follows: BPA is first transformed into HAP and HBAL. Thereafter, HAP is directly mineralized, while HBAL is further transformed into 4HB which is then mineralized. Strains MV-1 and FJ-4 degrade BPA mainly through the cleavage of the bridging propane-part by oxidation to form HAP and HBAL: subsequently, HAP is mineralized directly while HBAL is mineralized via 4HB (15, 16). Therefore, the BPA degradation pathway proposed for strain TYA-1 seems to resemble that proposed for strains MV-1 and FJ-4 and involves oxidations as key steps (15, 16).

Strain TYF-1 grown on BPF completely degraded 0.1 to 1.0 mM BPF within 18 h (Fig. 3C) and showed growth curves that depended on initial BPF concentration (Fig. 3D). Three metabolites were detected by

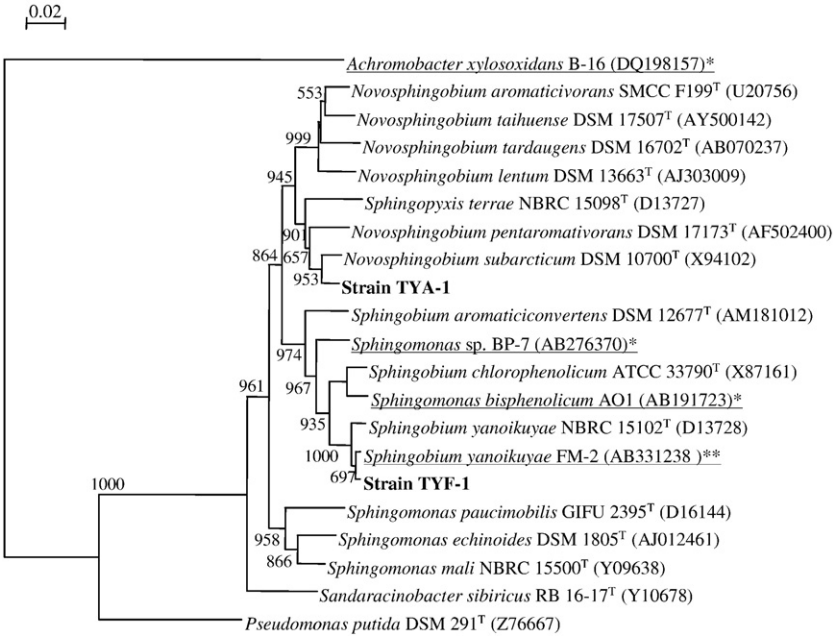

FIG. 2. Phylogenetic relationships established by the neighbor-joining method based on 16S rRNA gene sequences of strains TYA-1 and TYF-1, type strains of *Sphingomonadaceae*, and previously isolated BPA-degrading (*) or BPF-degrading (**) bacterial strains. Numbers on the branches indicate bootstrap confidence estimates obtained with 1000 replicates. The scale bar represents an evolutionary distance (K_{nuc}) of 0.01.

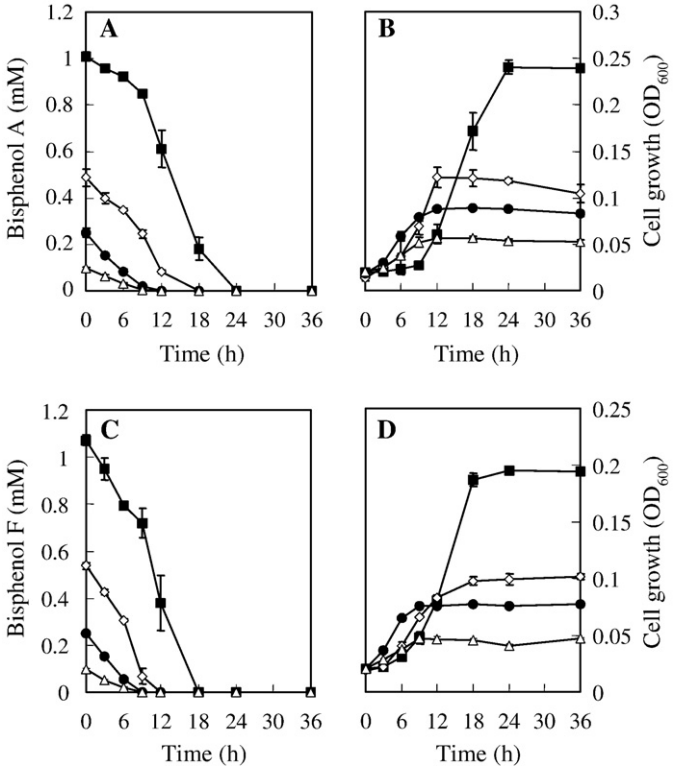

FIG. 3. BPA degradation (A) and cell growth (B) of strain TYA-1 grown on BPA, and BPF degradation (C) and cell growth (D) of strain TYF-1 grown on BPF. Initial bisphenol concentrations were 0.1 mM (open triangles), 0.2 mM (closed circles), 0.5 mM (open diamonds), and 1.0 mM (closed squares). Data represent the means of triplicate experiments, and error bars indicate 95% confidence intervals.

HPLC analysis at RTs of 1.75 min (metabolite I), 1.86 min (metabolite II) and 2.38 min (metabolite III), along with a decrease in BPF (RT 4.41 min). The mass spectrum of metabolite I (*m/z* 280 [22, M^+], 267 [100, $M^+ - CH_3$], 223 [78, $M^+ - CH_3, CO_2$], 193 [48, M^+ – OTMS] and 73 [83, TMS]) corresponded to that of ditrimethylsilyl-4HB. The mass spectra of metabolite II (*m/z* 110 [100, M^+], 82 [19, $M^+ - C_2H_4$] and 81 [31, M^+ – COH]) and metabolite III (*m/z* 108 [64, M^+], 82 [29, M^+ – CO], 80 [26, $M^+ - C_2H_4$] and 54 [100]) agreed well with those of 1,4-HQ and 1,4-BQ, respectively. 4HB supported growth of strain TYF-1, while 1,4-HQ and 1,4-BQ did not. 1,4-HQ and 1,4-BQ accumulated in culture at low concentrations ($\leq$0.1 mM) when strain TYF-1 cells were incubated with 1.0 mM BPF (data not shown). Therefore we propose that strain TYF-1 metabolizes BPF into 4HB, 1,4-HQ, and 1,4-BQ. Thereafter, 4HB is directly mineralized, while 1,4-HQ and 1,4-BQ remain. The pathway from BPF to 4HB followed by mineralization seems to be the major route. Strain FM-2 degrades BPF through the hydroxylation of the α-carbon atom, followed by oxidation and a Baeyer–Villiger reaction to 4HB and 1,4-HQ, which subsequently are mineralized (12). The degradation pathway of BPF proposed for strain TYF-1 seems to be partly similar to that proposed for strain FM-2 and involves oxidations as key steps (12). However, the utilization of 1,4-HQ differs between the two strains. Thus, TYF-1 seems to represent a new type of BPF-degrading bacteria.

Strain FJ-4, which exhibits the highest BPA-degrading activity among the previously reported BPA-degrading strains, can degrade 0.5 mM BPA within 12 h (16), while strain FM-2 can degrade 0.5 mM BPF within 9 h (12). The BPA-degrading and BPF-degrading activities of our strains are considered comparable or superior to those of previously reported bisphenol-degrading bacteria.

In summary, *P. australis* stimulated the activities of BPA-degrading and BPF-degrading bacteria in the rhizosphere sediment, while bacterial activities were restricted in anaerobic sediment in the absence of *P. australis*. We isolated a BPA-degrading bacterium, *Novosphingobium* sp. strain TYA-1, and a BPF-degrading bacterium, *S. yanoikuyae* strain TYF-1, from the rhizosphere sediment of *P. australis*. The degradation pathways of BPA or BPF proposed for the isolated strains seem to involve oxidations as key reactions. The combination of bisphenol-degrading bacteria and this aquatic plant can promote the removal of bisphenols from sediments. The 16S rRNA gene sequence data of strains TYA-1 and TYF-1 have been submitted to the DDBJ/EMBL/GenBank databases under accession nos. AB491194 and AB491195, respectively.

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