Biodegradation (2005) 16: 449–459

Biodegradation of bisphenol A by cells and cell lysate from *Sphingomonas* sp. strain AO1

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Accepted 19 October 2004

Key words: biodegradation, bisphenol A, cytochrome P450, *Sphingomonas* sp.

Abstract

The capacity and pathway of bisphenol A [BPA; 2,2-bis(4-hydroxyphenyl)propane] degradation in *Sphingomonas* sp. strain AO1, which was isolated from the soil of a vegetable-growing field in Japan, were investigated. The bacterial strain was able to grow in a basal mineral salt medium containing BPA as the sole carbon source (BSMB medium), and was able to degrade 115 μg ml^{-1} BPA in 6 h in L medium. Several BPA metabolites were detected in the culture supernatant by HPLC and then identified by GC-MS and LC-MS-MS. These compounds were confirmed to be the same as those reported for other BPA-degrading bacteria. BPA degradation by cells in the basal mineral salt medium was induced by BPA, and activity was detected only in the intracellular soluble fraction in the presence of coenzymes, such as NADH, NAD$^+$, NADPH or NADP$^+$. The addition of metyrapone, a cytochrome P450 inhibitor, to BSMB medium resulted in a decrease in BPA degradation and cell growth. The BPA-degradation activity of the intracellular soluble fraction was also inhibited by the cytochrome P450 inhibitor. Carbon monoxide difference spectra indicated that cytochrome P450 was present in the cells and that the amount of cytochrome P450 corresponded to the cellular BPA-degradation activity. Our results provide evidence that the cytochrome P450 system is involved in BPA metabolism in *Sphingomonas* sp. strain AO1.

Abbreviations: BPA – 2,2-bis(4-hydroxyphenyl)propane; 4-HAP – 4-hydroxyacetophenone

Introduction

Bisphenol A [BPA; 2,2-bis(4-hydroxyphenyl)propane] is an industrially important compound. It is one of the materials necessary for production of polycarbonates, epoxy resins and other plastics, and its worldwide annual consumption is increasing. However, BPA is strongly suspected to be an endocrine disruptor (Olea et al. 1998). Some reports have suggested that the free BPA monomer is detected in foods and beverages packaged in cans coated by lacquer after heat-processing or long-term storage (Brotons et al. 1995; Kawamura et al. 2001; Krishnan et al. 1993). Furthermore, Krishnan et al. (1993) and Gaido et al. (1997) have reported that BPA has estrogenic activity, and demonstrated developmental and reproductive toxicities in rats and mice fed at high-doses, with particularly strong effects in fetuses (Kim et al. 2001; Morrissey et al. 1987; Roy et al. 1997). Sugita-Konishi et al. (2003) recently reported that BPA possesses immunotoxicity and reduces the non-specific host defense to a level that causes acute toxicity in mice. Slight or moderate toxicity

to aquatic organisms has also been reported (Staples et al. 1998). In a major study, Colborn and colleagues reported that BPA is widely distributed in rivers, seas, streams and soils (Colborn et al. 1996). Collectively, these results suggest that it is necessary for BPA to be purged from the environment.

Several systems for BPA removal have been proposed. One such approach involves the use of activated carbon as a BPA absorbent. This method is effective for terminal treatment of drinking water, but not for wastewater, natural environmental water and polluted soil, because coexisting compounds interfere with BPA absorption. Another possible method of BPA removal involves the application of microorganisms or their enzymatic systems to the degradation of BPA. One enzymatic system that has been considered for this purpose is that for the decomposition of lignin by basidiomycetes, in which lignin peroxidase, manganese peroxidase, and/or laccase are involved. It is believed that these enzymes may potentially be used for bioremediation of chemical pollutants (Al-Kassim et al. 1994; Cohen et al. 2002; Gold & Alic 1993; Mayer & Staples 2002). Several investigators have reported that such systems remove significant levels of BPA from solutions (Fukuda et al. 2001; Hirano et al. 2000; Sakurai et al. 2001; Tanaka et al. 2000; Tsutsumi et al. 2001; Uchida et al. 2001). It is unclear, however, whether these systems can degrade BPA into small molecules, because some of them polymerize, rather than decompose, BPA (Sakurai et al. 2001; Tsutsumi et al. 2001; Uchida et al. 2001). Several BPA-degrading bacteria have been reported, including *Sphingomonas paucimobilis* strain FJ-4 and unidentified gram-negative bacteria, strains MV1 and WH1 (Ike et al. 1995; Ike et al. 2002; Lobos et al. 1992; Ronen & Abeliovich 2000; Spivack et al. 1994), and some metabolites derived from BPA have already been identified and metabolic pathways for their formation have been proposed (Ike et al. 1995; Spivack et al. 1994). Furthermore, Ike et al. (2002) also reported that the end products demonstrated no higher activities than BPA in terms of acute toxicity, mutagenicity, and estrogenic activity. These observations support the idea that bacterial systems are superior in the removal of BPA from the environment. However, the BPA degradation activities of the above strains are insufficient to permit their use as a practical BPA removal system, and it also remains unclear which enzymes are involved in the BPA degradation pathway.

In the present study, the capacity and pathway of BPA degradation of the newly isolated *Sphingomonas bisphenolica* strain AO1 were investigated, and the possible enzymes involved in BPA degradation are discussed.

Materials and methods

Chemicals

BPA was purchased from Wako Pure Chemical Industries (Osaka, Japan). Bacto Tryptone and Bacto Yeast Extract were supplied by Difco Laboratories (Detroit, MI). NADH, NAD^+, NADPH, and $NADP^+$ were obtained from Oriental Yeast Industries Co. (Tokyo, Japan). *N*,*O*-Bis(trimethylsilyl)acetamide and metyrapone were purchased from GL Sciences Inc. (Tokyo, Japan) and Sigma Chemical Co. (St. Louis, MO), respectively. All other reagents used were products of Wako Pure Chemical Industries. Solvents for HPLC analysis were of HPLC grade.

Strain and growth conditions

Sphingomonas sp. strain AO1 (K. Oshiman, personal communication) was used. This strain was isolated from the soil of vegetable field in Tsukuba, Japan, and classified as a new species by 16S rDNA sequence homology, DNA–DNA hybridization at the genome level, and some conventional physiological tests (K. Oshiman, personal communication). Two types of media were used in the present study, L medium (10 g of Bacto Tryptone, 5 g Bacto Yeast Extract, and 5 g NaCl per liter, pH 7.2) and a basal mineral salt medium (BSM medium, 10 g $(NH_4)_2SO_4$, 10 g K_2HPO_4, 0.5 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g $FeCl_3$, and 0.5 g $CaCl_2$ per liter, pH 7.2). BPA (BSMB medium), 4-hydroxyacetophenone (4-HAP) (BSMH medium), or glucose (BSMG medium) at 115 μg ml^{-1} was added to BSM medium as the sole carbon source. BPA at 115 μg ml^{-1} was also added to L medium (L-BPA medium). Cells were cultivated at 30 °C in a 500 ml Erlenmeyer flask containing 100 ml of medium, with shaking at 120 rpm. Cell growth was monitored at an optical density of

650 nm (OD_{650}) by a U-2001 double beam spectrophotometer (Hitachi High-Technologies Co., Tokyo, Japan). Media were solidified, if necessary, by addition of 1.5% (wt vol^{-1}) agar.

HPLC analysis of BPA and its metabolites

Samples were centrifuged at 9200 × g at 4 °C for 10 min and the resultant supernatant was immediately filtrated through a 0.20 μm Millex filter (Japan Millipore Co., Tokyo, Japan) for removal of insoluble compounds. The resultant filtrate was used for detection and measurement of BPA and its metabolites. A Hitachi HPLC model D-7100 system, consisting of a D-7300 column oven, L-7100 pump, L-7450H diode array detector, D-7100 interface module, and D-7000 software (Hitachi Ltd., Tokyo, Japan), and installed with a TSK gel OD-4PW (C18 reverse-phase) column (length, 15 cm; inside diameter, 4.6 mm; Tosoh Co. Ltd., Tokyo, Japan), was used in the analysis. The column was equilibrated with 20% acetonitrile solution. Samples were applied to the equilibrated column, and eluted with a linear gradient of acetonitrile-water (20–100%) for 10 min at 1.0 ml min^{-1}, with absorbance monitoring at 280 nm. The concentrations of BPA and 4-HAP were calculated from the integration values of the peaks corresponding to the known peaks for these compounds. Metabolites were collected and concentrated by evaporation, and the structures of the metabolites were then identified by either of the methods described below.

GC-MS analysis

If necessary, samples were silylated by treatment with *N*,*O*-bis(trimethylsilyl)acetamide solution with heating at 76 °C for 3 h. A gas chromatography-mass spectrometer (GC-MS) Q-MASS910 system (Perkin Elmer Japan Co., Ltd., Yokohama, Japan) installed with a TCWAX column (length, 30 m; inside diameter, 0.25 mm; GL Sciences Inc., Tokyo, Japan) was used for structure determination. The initial temperature was set at 150 °C and maintained for 3 min. The temperature was then increased to 260 °C at a rate of 10 °C min^{-1}, and then kept at 260 °C for 10 min. MS analysis was performed at an electron energy of 70 eV. The structures of metabolites were confirmed from the fragmentation patterns of the mass spectra, through comparison with those predicted for known compounds.

LC-MS-MS analysis

The liquid chromatography combined with tandem mass spectrometer Model D-7100 LC/3DQMS system (Hitachi Ltd) was used in the analysis. A Hydrosphere C18 reverse-phase column (length, 15 cm; inside diameter, 4.6 mm; YMC Co. Ltd., Kyoto, Japan) was installed for HPLC, and the conditions for the HPLC analysis were the same as those described above. Mass spectra were obtained with a three-stage particle beam liquid chromatography-MS interface using the atmospheric pressure chemical ionization (APCI) method. MS analysis (m/z 100–800) was carried out in either positive or negative ionization mode. The temperature of the ion source was maintained at 180 °C and the ionizing temperature was set at 350 °C. The cone and capillary voltages were ±30 V and 3.5 kV, respectively. The structures of the metabolites were confirmed from the fragmentation patterns of the mass spectra, through comparison with those predicted for known compounds.

Preparation of the cell fraction and the culture supernatant fraction

The culture was harvested at the early-stationary phase, during which the BPA-degradation activity reaches a maximum, and centrifuged at 9200 × g at 4 °C for 10 min. The proteins in the resultant supernatant were concentrated by addition of ammonium sulfate at a final saturated concentration of 80%, with stirring at 4 °C overnight. The precipitate was collected by centrifugation (18,000 × g) at 4 °C for 20 min and suspended in 50 mM Tris–HCl (pH 8.0) buffer. The resultant solution (the extracellular fraction) was dialyzed in 50 mM Tris–HCl (pH 8.0) buffer using a dialysis membrane (Seamless Cellulose Tubing, Wako Pure Chemical Industries, small size 8). The cell sediment was washed twice with 50 mM Tris–HCl (pH 8.0) buffer and resuspended in fresh buffer (the whole cell fraction). This cell suspension was sonicated in an ice-water bath (20 W, 5 × 1 min with each 1-min interval) with a Sonifier (model 250, Branson Ultrasonics Co., Danbury, CT) to disrupt the

cells. The resultant suspension was separated into the supernatant (the intracellular soluble fraction) and the precipitate by ultracentrifugation (165,000 × g at 4 °C for 1 h). The precipitate was resuspended in 50 mM Tris–HCl (pH 8.0) buffer (the membrane fraction). The protein concentration was determined by a BCA protein assay reagent kit (Pierce Chemical Co., Rockford, IL).

Analysis of BPA-degradation activity

Samples were suspended in 50 mM Tris–HCl (pH 8.0) buffer containing BPA at 100 μg ml^{-1} and the suspension was incubated at 30 °C. The suspension was, if necessary, supplemented with a metal salt ($CaCl_2$, $CuCl_2$, $FeCl_2$, $FeCl_3$, KCl, $MgCl_2$, $MnCl_2$, NaCl, $NiCl_2$, $ZnCl_2$, or $CuSO_4$) or a coenzyme (FMN, FAD, NADH, NAD^+, NADPH, or $NADP^+$) at a concentration of 0.1 or 1 mM. Degradation of BPA was ascertained by HPLC, on the basis of a decrease in the BPA peak and the appearance of new peaks associated with metabolites. One unit of the enzyme activity was defined as the amount of enzyme required to degrade 1 μmol of BPA per min.

To examine the stability of the BPA-degradation activity, the cell samples were kept at 4 °C in the presence of EDTA, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), 2-mercaptoethanol, or sodium dithionite at a concentration of 0.1 or 1 mM.

Spectrophotometric analysis of cytochrome P450

Reduced cytochrome P450 carbon monoxide difference spectra were determined as described by Omura and Sato (Omura & Sato 1964). The intracellular fraction reduced with 1 mg ml^{-1} sodium dithionite was used in the spectral analysis. The difference in spectra acquired before and after purging with carbon monoxide was monitored with a U-2001 double beam spectrophotometer. The amount of cytochrome P450 was calculated from the absorbance at 450 nm, using the cytochrome P450 extinction coefficient, 91 mM^{-1} cm^{-1} (Omura & Sato 1964).

Results

Properties of strain AO1

A novel strain with BPA-degradation activity was isolated from farm soil in Tsukuba by the enrichment method, using BSMB medium containing BPA as the sole carbon source (K. Oshiman, personal communication). This strain, which formed yellow colonies, is a gram-negative, non-sporulating, rod-shaped, strictly aerobic, motile, and catalase-positive bacterium. Its 16S rDNA sequence (accession No. AB191723) is very similar to that of *Sphingomonas chlorophenolica* ATCC 33790 (97% homology) and that of *Sphingomonas herbicidovorans* DSM11019 (97% homology). Other physiological and phylogenetic data indicate that the strain belongs to the *Sphingomonas* species (K. Oshiman, personal communication), and it has been designated as *Sphingomonas* sp. AO1.

Characterization of BPA degradation by strain AO1

Strain AO1 completely degraded BPA at 115 μg ml^{-1} in 6 h, when cultivated in L-BPA medium at 30 °C (Figure 1). Three novel peaks (peaks 1, 2 and 3 in Figure 1a) were observed by HPLC at 2 h-cultivation. The levels of these metabolites gradually decreased and they became undetectable after 8 h. BPA at 115 μg ml^{-1} did not inhibit the cell growth of strain AO1 in L medium (Figure 1b). Supplementation of the L-BPA medium with glucose at 0.1, 1.0 and 10 g l^{-1} did not provide an advantage for either growth or for the BPA-degradation capacity of strain AO1 (data not shown).

On cultivation in BSMB medium, BPA at 115 μg ml^{-1} was degraded to an undetectable level in 117 h, and the metabolites detectable in our system disappeared by 140 h (Figure 2). The turbidity of the culture (OD_{650}) increased from 0.01 to 0.17 over this period without lag, but a remarkable delay in BPA degradation was observed in BSMB medium, in contrast to the results in L-BPA medium. To attempt to shorten this delay, we added 100 μg ml^{-1} glucose to the BSMB medium. Glucose addition stimulated the growth rate and the rate of BPA degradation after the lag, but a delay in BPA degradation (for *ca* 20 h) was still observed.

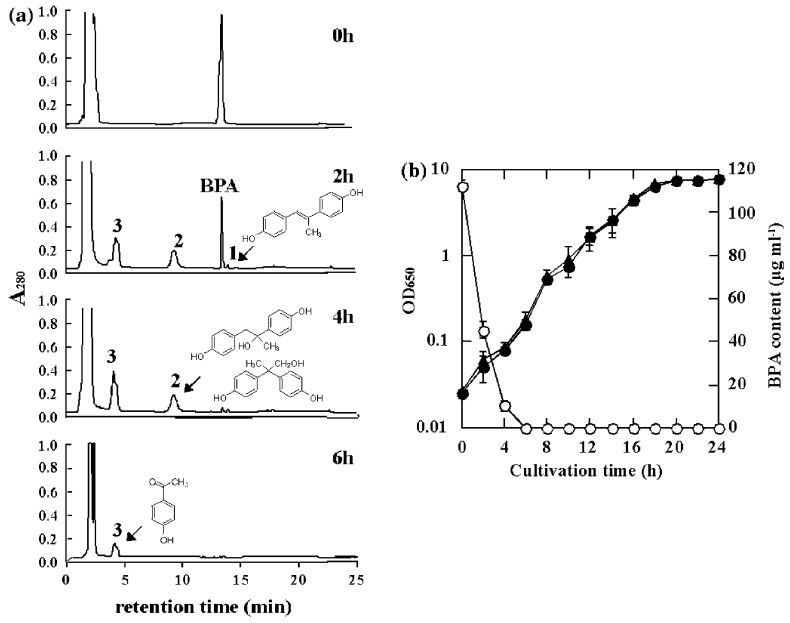

Figure 1. HPLC analysis of BPA degradation by strain AO1 in L-BPA medium. Strain AO1 was cultivated in L-BPA medium. (a) A_{280} chromatograms of the culture supernatants from HPLC analysis. Peaks 1, 2 and 3 correspond to 4,4′-dihydroxy-α-methyl-stilbene, 1,2-bis(4-hydroxyphenyl)-2-propanol and 2,2-bis(4-hydroxyphenyl)-1-propanol, and 4-HAP. (b) The OD_{650} for growth (closed circles) and the BPA content (open circles) remaining in L-BPA medium. The closed triangles, which overlap with the closed circles, indicate the OD_{650} for growth of strain AO1 cultivated in L medium alone. Error bars indicate the standard deviation obtained in three independent experiments.

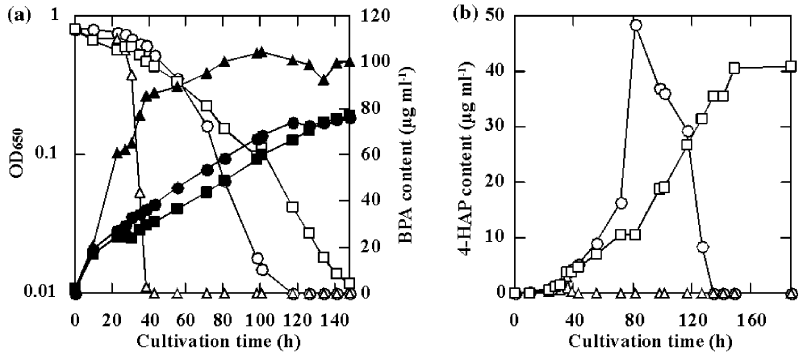

Figure 2. BPA degradation by strain AO1 in BSM supplemented with BPA and/or glucose. Cells were pre-cultivated in BSMB (circles, triangles) and BSMG (squares) and then cultivated in BSMB with (triangles) or without (circles and squares) 100 μg ml^{-1} glucose. (a) Growth (OD_{650}, closed symbols) and BPA levels (open symbols) in the culture supernatants. (b) 4-HAP levels in the culture supernatants.

Accumulation of 4-HAP, one of the metabolites of BPA, was undetectable during cultivation with glucose (Figure 2b). The effect of glucose on pre-cultivation was also examined. The rate of BPA degradation for cells pre-cultivated in BMSG medium was slower than that for cells pre-cultivated in BSMB medium, and the rate of accumulation of 4-HAP for BSMG-pre-cultivated cells was also slower than that for BSMB-pre-cultivated cells (Figure 2b).

Identification of metabolites

Metabolites of BPA were separated by HPLC and collected and identified by GC-MS or LC-MS-MS.

Peak 1 in Figure 1A, which eluted with a retention time of 14.2 min, showed GC-MS peaks at m/z (% relative intensity in parentheses) of 371(100), 205(7), 117(28) and 103(31), suggesting it to be 4,4′-dihydroxy-α-methylstilbene. Other metabolites were identified by LC-MS-MS. Peaks 3 and 2 in Figure 1A were predicted to be 4-HAP [m/z: 135(100) (M–H), 120(21) (M–H–CH_3)] and either 1,2-bis(4-hydroxyphenyl)-2-propanol [m/z: 243(93) (M–H), 225(100) (M–H–H_2O)] or 2,2-bis(4-hydroxyphenyl)-1-propanol [m/z: 243(100) (M–H), 225(7) (M–H–H_2O), 211(37) (M–H–H_2O–CH_3)], or a mixture thereof, respectively, based on a comparison with the mass spectra of known compounds (Spivack et al. 1994).

BPA degradation by subcellular fractions

Like other BPA-degrading strains that have been reported previously (Ike et al. 1995; Spivack et al. 1994), the strain AO1 metabolites of BPA were detected in the culture supernatant but not in cells (data not shown). To determine the location of the BPA-degrading enzyme(s) in strain AO1, the degradation activities of the extracellular and whole cell fractions, which were prepared from the culture in L medium, were examined. Only the whole cell fraction had substantial activity, indicating that the BPA-degrading enzyme(s) is located in the cell. The activity of the disrupted cells was, however, markedly low, compared with that of the whole cell fraction (Table 1). Furthermore, when divided into the membrane and intracellular soluble fractions, the BPA-degradation activities became very low (Table 1). Assuming a requirement for metal ions or coenzymes for the enzyme activity, we added these into the reaction solution, and measured the BPA-degradation activity. The activity of the intracellular soluble fraction was measurable following addition of NADH, NAD^+, NADPH, or $NADP^+$ at 1 mM (Table 2). 4,4′-Dihydroxy-α-methylstilbene was found to be a product of this reaction by LC-MS-MS analysis (data not shown). However, FMN, FAD and metal ions (Ca^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, K^+, Mg^{2+}, Mn^{2+}, Na^+, Ni^{2+} and Zn^{2+}) had no effect on the BPA-degradation activity. No activity was detectable in the membrane fraction, both with or without the supplements in the reaction.

Table 1. BPA degradation by subcellular fractions obtained from strain AO1 cells

Fraction[a]	BPA degradation (%)[b]
Whole cell	78.9 ± 8.0
Mixture of membrane and intracellular soluble	40.9 ± 4.0
Membrane	4.9 ± 1.7
Intracellular soluble	<2.4

[a] The samples for the reaction were prepared from a cell suspension (1 ml) corresponding to an OD_{650} of 4.0. The reaction was performed for 30 min.
[b] The initial concentration of BPA was 100 μg ml^{-1} and complete degradation corresponds to 100%. All values are shown as means ± standard deviations for three trials.

Table 2. The effects of various cofactors on BPA degradation by the intracellular soluble fraction

Cofactor[a]	Activity (U g^{-1})[b]
None	<0.6
NADH	10.4 ± 1.1
NAD^+	4.6 ± 0.8
NADPH	8.6 ± 0.7
$NADP^+$	2.5 ± 1.2

[a] The final concentration of each cofactor in the reaction was 1 mM.
[b] All values are shown as means ± standard deviations for three trials.

The presence of cytochrome P450 in the intracellular soluble fraction

It is known that bacterial cytochrome P450s that play a central role in the oxidative metabolism of organic or xenobiotic compounds are generally linked to NADH or NADPH oxidation (Lewis & Hlavica 2000; Wong 1998). In the putative BPA-metabolic pathway in bacteria, some oxidative steps, such as hydroxylation, are involved (Spivack et al. 1994). Considering also that NADH or NADPH was shown to be necessary for the degradation of BPA in strain AO1, we investigated the effect of metyrapone, a specific inhibitor of cytochrome P450 (Testa & Jenner 1981), on the growth of strain AO1, in order to investigate a possible relationship between cytochrome P450 and degradation of BPA. Metyrapone significantly inhibited the degradation activity in BSMB medium

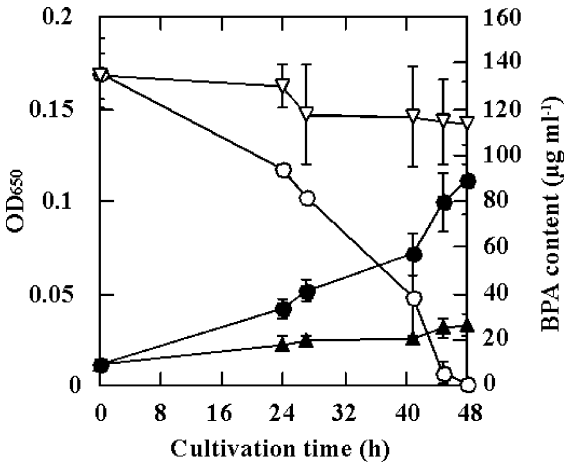

Figure 3. Influence of metyrapone on the growth of strain AO1 in BSMB medium. Strain AO1 was cultivated in BSMB medium. OD_{650} values (closed symbols) of the culture with (triangles) or without (circles) metyrapone (0.5 mM) are indicated. The BPA content (open symbols) remaining in the medium during growth under each condition is also shown. Error bars indicate the standard deviation obtained in three independent experiments.

(Figure 3), but not in BSMG medium (data not shown). We also tested the effect of metyrapone on BPA degradation by the intracellular soluble fraction from cells cultivated in L medium, and showed that metyrapone inhibited BPA degradation in a concentration-dependent manner (by 18.4% at 0.5 mM and by 71.9% at 5.0 mM). These data suggest the involvement of the cytochrome P450 system in the degradation of BPA by strain AO1.

To examine the presence of cytochrome P450 in the cells, carbon monoxide difference spectra of the intracellular soluble fractions prepared from cells cultivated in BSMB, BSMG, and BSMH media were recorded. The maximum BPA-degradation activity of cells cultivated in BSMB medium was 8.1 ± 1.2 U g^{-1} of protein ($n = 3$), whereas the activity of those cultivated in BSMG and BSMH media were undetectable (<0.6 U g^{-1} of protein). The absorbance peak at ca. 450 nm was observed only in the spectrum of the intracellular soluble fraction from BSMB-grown cells (Figure 4). This peak shifted from 450 to 420 nm after incubation at room temperature (data not shown). This is a common feature of cytochrome P450s (Martinis et al. 1996), and collectively these observations indicate that the cytochrome P450 system is involved in BPA degradation in strain AO1.

BPA-degradation activity of cells grown in L medium

The BPA-degradation activities of the whole cell and the intracellular soluble fractions were monitored during the cultivation. These activities increased gradually until the stationary growth

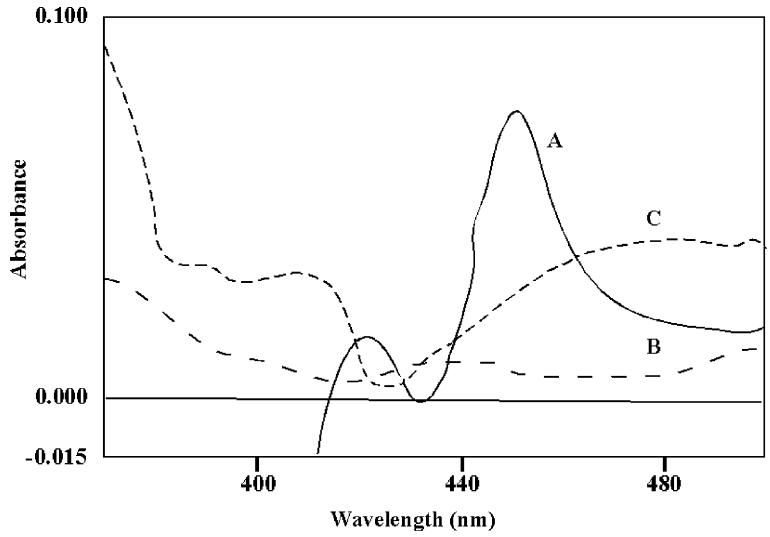

Figure 4. Carbon monoxide difference spectra of the intracellular soluble fractions from the cells grown in a basal salt medium. Strain AO1 was cultivated in BSMB (A), BSMG (B), or BSMH (C) medium. Intracellular soluble fractions were purged with CO for 30 s. The protein concentrations were 9.0 mg ml^{-1}.

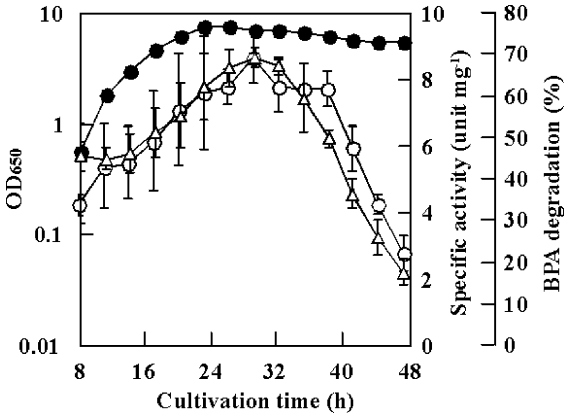

Figure 5. The BPA-degradation activities of the whole cell and intracellular soluble fractions from strain AO1 cells cultivated in L medium. The whole cell and the intracellular soluble fractions prepared from a cell suspension (1 ml) corresponding to an OD_{650} of 4.0 were incubated in reaction buffer containing 115 μg ml^{-1} BPA for 15 min. The symbols represented optical density for growth (closed circles), BPA degradation by the whole cell fraction (open circles), and specific activity (open triangles) in the intracellular soluble fraction. Error bars indicate the standard deviation obtained in three independent experiments.

phase and reached a maximum level after about 20 h. Thereafter, the specific activities rapidly decreased (Figure 5).

Stability of BPA-degradation activity

It was assumed that the BPA-degradation activity of the intracellular soluble fraction would be unstable, and this activity actually completely disappeared in 4 h at 4 °C. We also examined the effects of protease inhibitors and antioxidants on the stability of the BPA-degradation activity. Only the addition of sodium dithionite at 1 mM led to retention of the activity of the intracellular soluble fraction after a 48-h-incubation at 4 °C (data not shown). Other test compounds (PMSF, EDTA, DTT and 2-mercaptoethanol) were unable to improve stability.

Discussion

In this paper, we have reported the properties of a novel BPA-degrading bacterium that was isolated in Japan, *Sphingomonas* sp. strain AO1. This strain has high activity, even following cultivation in L medium without BPA, whereas the activity of other BPA-degrading strains, such as *Sphingomonas paucimobilis* strain FJ-4 and strain MV1, were lower in medium without BPA (Ike et al. 1995; Lobos et al. 1992). In BSMB medium, a glucose supplement improved cell growth and BPA degradation in strain AO1, and our results suggest that degradation of BPA by strain AO1 might be increased by more active cell growth. The results also suggest that strain AO1 may be useful for the bioremediation of BPA, particularly because complete degradation of BPA occurs without the accumulation of metabolites. Hence, strain AO1 cultivated in L-BPA medium was able to completely degrade BPA and the metabolites remaining in the culture were below the HPLC detection level after 8 h. With strains FJ-4 and MV1, on the contrary, many BPA metabolites remained during the cultivation, including 2,3-bis(4-hydroxyphenyl)-1,2-propanediol and *p*-hydroxyphenacyl alcohol. Ike et al. (2002) indicated that the end products of BPA degradation by strain FJ-4 had neither remarkable acute toxicity, estrogenic activity, nor mutagenic toxicity, and suggested that BPA degradation by bacterial cells seem to be environmentally safe, as well as beneficial for human health, although it is also necessary to examine the toxicities of metabolites that disappear during the cultivation. In particular, Yoshihara et al. (2001) has warned that one BPA metabolite, 4,4′-dihydroxy-α-methylstilbene, is structurally similar to diethylstilbestrol, a synthetic estrogen, and has a high estrogenic activity that is 100 times that of BPA. Hence, further knowledge of the BPA degradation pathway in strain AO1 is needed to develop an approach for rapid removal of metabolites with estrogenic activity.

In the case of strains FJ-4 and MV1, BPA degradation by cells cultivated in medium with BPA as the sole carbon source has been reported to be faster than that by cells cultivated with glucose (Ike et al. 1995; Lobos et al. 1992). Based on our results for cultivation in a basal mineral salt medium containing different carbon sources, the BPA degradation system is induced by BPA in strain AO1. A delay of ca. 20 h or more was, furthermore, observed before BPA degradation was detectable. It is unclear why such a prolonged delay was observed. One possibility is that the BPA-degradation activity was reduced after the

pre-cultivation, but the data indicate that BPA activity increased until the early-stationary phase, and then decreased markedly thereafter. The instability of BPA-degrading enzyme(s) might be responsible for the reduced activity, as mentioned in the Results section. A further possibility is that 4-HAP accumulated during pre-cultivation might inhibit the initial step of BPA degradation, since the BPA-degradation rate seemed to be lowered by 4-HAP accumulation (Figure 2).

Cytochrome P450 system is likely to be involved in BPA degradation by strain AO1, for the following reasons. The cytochrome P450 inhibitor, metyrapone, is classified as a heterocyclic ligand of cytochrome P450, and has the potential not only to compete with substrate binding to ferricytochrome P450, but also to compete with oxygen for binding to the substrate-ferrocytochrome P450 complex (Testa & Jenner 1981). Metyrapone has been shown to inhibit many bacterial cytochrome P450 systems, including the pyrrolidine and morpholine catabolic enzymes from *Mycobacterium* sp. (Poupin et al. 1998; Schrader et al. 2000), the biotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) to 4-nitro-2,4-diazabutanol by *Rhodococcus* sp. (Bhushan et al. 2003; Coleman et al. 2002; Seth-Smith et al. 2002), and the dechlorination of pentachlorophenol (PCP) by *Rhodococcus chlorophenolicus* PCP-I (Uotila et al. 1991). In our study, metyrapone inhibited both growth and BPA degradation of strain AO1 in BSMB medium, and also inhibited BPA degradation by the intracellular soluble fraction. These results are supportive of the hypothesis that cytochrome P450 is involved in BPA degradation by strain AO1.

It was reported that BPA degradative bacteria, strains FJ-4 and MV1, had two pathways in BPA metabolism from structural analyses of metabolites (13, 33). In strain AO1, two of BPA metabolites identified, 1,2-bis(4-hydroxyphenyl)-2-propanol and 2,2-bis(4-hydroxyphenyl)-1-propanol, which were the initial reaction product of each BPA pathway of strains FJ-4 and MV1, were observed. Furthermore, metyrapone decreased the productions of these two molecules in BPA degradation by resting cells. Therefore, it is proposed that strain AO1 has two pathways in BPA metabolism and that cytochrome P450(s) is involved in both pathways.

The presence of a soluble cytochrome P450 was clarified by the results of the spectrophotometric assay. The amount of cytochrome P450 in the cells cultivated in the basal mineral salt medium corresponded to the BPA-degradation activity. The CO difference spectrum of the intracellular soluble fraction of cells grown in BSMB medium, which had the highest activity, showed a typical peak at 450 nm, but this peak was not present for cells grown in BSMH and BSMG media.

It is also known that bacterial cytochrome P450 systems are involved in the hydroxylation, dehalogenation, or epoxidation of xenobiotics, many of which have toxic and carcinogenic properties (Munro & Lindsay 1996; Wong 1998). These reaction systems are composed of three components and electrons are transferred from NAD(P)H *via* an FAD-containing reductase and a small iron–sulfur protein to the cytochrome P450 (Lewis & Hlavica 2000). The bacterial cytochrome P450 systems are very unstable because they are composed of individual enzymes, and their activities are easily lost upon disruption of the native cell structure (Omura 1999). In this report, we have shown that $NAD(P)^+$ or NAD(P)H was necessary for the early step of BPA degradation, and we also found that the BPA-degradation activity of strain AO1 was very unstable. Hence, we conclude that strain AO1 cells may possess at least one soluble cytochrome P450 involved in BPA degradation.

Recently, it was reported that the rat liver S9 fraction can metabolize BPA and that SKF 525-A, an inhibitor of the cytochrome P450 system, inhibited this metabolism (Yoshihara et al. 2001), and Atkinson & Roy (1995a; 1995b) have previously reported that cytochrome P450s of male rat microsomes were involved in the formation of a bisphenol-*o*-quinone from BPA, *via* 5-hydroxy bisphenol and a bisphenol semiquinone. These BPA metabolites were not detected in metabolism of BPA by strain AO1, and it is of interest that BPA metabolism differs between bacteria and animals, although it is likely that cytochrome P450 systems play a common role in the initial step of BPA degradation across species. To confirm this, it will be necessary to obtain further information on the biodegradation pathways in bacteria and animals and to identify the BPA-degrading enzymes in strain AO1. However, the present report represents, to our knowledge, the first to describe

the enzyme and coenzymes which play a part in BPA degradation by bacteria.

Acknowledgements

We are grateful to Mr. Iwasaki for technical assistances with some of the experiments. We thank Dr. Yasuhiro Yamashita and Dr. Hidehisa Kawahara of the Department of Biotechnology, Kansai University, for GC-MS analysis and helpful advice regarding the data analysis. We also thank Hitachi High-Technologies Corporation for the LC-MS-MS analysis.

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