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ORIGINAL PAPER

Isolation of bisphenol A-tolerant/degrading *Pseudomonas monteilii* strain N-502

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Abstract Bisphenol A (BPA) is a highly biotoxic compound that kills many microorganisms at a low concentration (1,000 ppm). We isolated BPA-tolerant/degrading *Pseudomonas monteilii* strain N-502 from about 1,000 samples collected from a field, sewage, and pond water. The isolated strain had strong BPA tolerance and high BPA-degrading activity. This strain was able to grow in a minimum medium containing BPA as the sole carbon source. Strain N-502 is an aerobic, motile, gram-negative, nonspore-forming, rod-shaped bacterium and was identified as *P. monteilii,* based on 16 S rRNA gene analysis. Strain N-502 completely degraded BPA 500 ppm in a 10-day, in culture system and was able to degrade BPA 100 ppm in a 2-h resting cell system. This strain also showed potent ability to degrade BPA 500 and 1,000 ppm in the resting cell system. Moreover, the initial BPA degradation rate was accelerated with the addition of Ca^{2+}, Mg^{2+}, and folic acid.

Keywords Bisphenol A · Endocrine-disrupting · BPA-tolerant bacteria · BPA-degrading bacteria · *Pseudomonas monteilii*

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Introduction

Bisphenol A (BPA) is a compound widely used as a raw material in industries. BPA is used in the production of polycarbonate resins, epoxy resins, lacquer coating of food cans, etc. Recently, there have been reports on the dangers of compounds with estrogenic (endocrine-disrupting) activity (Krishnan et al. 1993). Very small amounts of BPA monomer leaching from products result in the detection of BPA compounds throughout the environment. Its effects on ecosystems and human health are thus, a matter of concern.

Several BPA-degrading bacteria, including *Sphingomonas* sp. AO1 (Sasaki et al. 2005), the unidentified gram-negative bacteria strain MV1 (Lobos et al. 1992; Spivack et al. 1994) and strain WH1 (Ronen and Abeliovich 2000), *Sphingomonas paucimobilis* strain FJ-4 (Ike et al. 1995; Ike et al. 2002), and many other studies (Fukuda et al. 2001; Hess et al. 2002; Kang and Kondo 2002a, b; Leitner et al. 2002; Maki et al. 1994; Sakurai et al. 2001; Tanghe et al. 1999; Tsutsumi et al. 2001; Uchida et al. 2001; Yim et al. 2003), have been reported. *S. paucimobilis* strain FJ-4 grows and degrades BPA at a concentration of 228.3 ppm (1.0 mM) or less, but not at a concentration of 342.4 ppm (1.5 mM) or greater. BPA-degrading bacteria with high tolerance to BPA and able to degrade the compound at a concentration of greater than 500 ppm have not yet been isolated.

We attempted to isolate bacteria with high BPA tolerance and degrading activity at concentrations of greater than 500 ppm. Five BPA-tolerant/degrading bacteria were isolated. Among them, *Pseudomonas monteilii* strain N-502 showed high BPA tolerance and the highest degrading activity. This paper describes the

screening for BPA-degrading bacteria and the BPA-degrading activity of *P. monteilii* strain N-502.

Materials and methods

Culture medium, culture conditions, and isolation of bacteria

Approximately 1,000 samples were collected from a field, sewage, and pond. Each sample was suspended in sterilized water and inoculated onto agar plates containing SYP medium consisting of (in grams per liter, pH 7.0) $Na_2HPO_4{\cdot}12H_2O$ 2.13, KH_2HPO_4 1.35, $(NH_4)_2SO_4$ 0.5, KNO_3 0.5, $MgSO_4{\cdot}7H_2O$ 0.2, yeast extract (Difco Laboratories, MI, USA) 0.5, polypeptone (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) 0.5, and 10 ml of trace element solution. The medium was sterilized by autoclaving at 121°C for 15 min and used for the isolation of microorganisms with the ability to degrade BPA, pure culture, and BPA biodegradation experiments.

The medium was supplemented with BPA (Wako Pure Chemical Industries, Osaka, Japan) at a final concentration of 1,000 ppm, to isolate BPA-tolerant/degrading microorganisms. Cultures in BPA degradation experiments were supplemented with BPA at final concentrations of 100–1,000 ppm. BPA was dissolved in ethanol as stock BPA solution. Liquid cultures contained 100 ml of media in 300-ml Erlenmeyer flasks incubated on a reciprocal shaker (150 rpm at 30°C). Media were solidified by the addition of 1.5% (wt/vol) agar, if necessary. Cultures grown on solid media were also incubated at 30°C.

Colonies that formed halos were subjected to single-colony isolation. The colonies isolated were then inoculated into liquid medium containing BPA. The single colonies in which the degradation of BPA was confirmed were used to determine the bacteria responsible for BPA degradation.

The isolated *P. monteilii* strain N-502 was cultivated at 30°C in a 300-ml Erlenmeyer flask containing 100 ml of medium, with shaking at 150 rpm. For growth and BPA degradation tests of strain N-502, SYP medium was supplemented with BPA at a concentration of 500 ppm.

Measurement of cell growth

Cell density during growth experiments was determined based on the absorbance measured at 660 nm with a model U-2001 spectrophotometer (Hitachi, Tokyo, Japan).

Characterization of the isolates

Biochemical and morphological characterization and identification of strain N-502 were performed by measuring API 20NE (Bio Mérieux S.A., France). 16 S rRNA gene analysis was performed using the extracted DNA of strain N-502 as the template and PCR amplification of the 16 S rRNA gene portion. The sequence reaction was performed using a DYEnamic ET terminator cycle sequence kit (Amersham Biosciences, Tokyo, Japan). The 16 S rRNA gene sequence of N-502 was determined with an ABI PRISM (R) 377 DNA Sequencer (Applied Biosystems Japan Ltd., Tokyo, Japan). The optimal growth temperature and pH of strain N-502 were determined in SYP medium.

BPA-degrading activity in the resting cell system

Strain N-502 was grown in SYP medium to a turbidity of 1.2 at OD_{660}. After collecting the cells by centrifugation, the cell pellet was washed with fresh phosphate buffer (25 mM, pH 7.0). For resting cell assays, the cell pellet was suspended in phosphate buffer to an optical cell density of 1.0 at OD_{660}. Five milliliters of suspended cells was transferred to a test tube, and BPA as a substrate was added. The final concentration of the assay substrate was 100–1,000 ppm unless otherwise noted. Assays were incubated at 30°C on a reciprocal shaker. Then, an equivalent amount of dichloromethane was added to the reaction liquid, and after centrifugation the lower layer, was subjected to gas chromatographic (GC) analysis.

Gas chromatography

Biological removal of BPA compounds was evaluated using GC-flame ionization detection (GC-17A Ver. 3; Shimadzu Corp., Kyoto, Japan). Samples were injected onto an Rtx-5MS (Restec Corp., Bellefonte, PA, USA) fused silica column (5% diphenyl–95% dimethyl polysiloxane; 30 m × 0.32 mm; 0.25-μm film thickness). Helium was used as a carrier gas. The injector and detector temperatures were 275 and 310°C, respectively. The oven temperature program was as follows: 50°C for 1 min; 40°C/min to 200°C; and 10°C/min to 310°C. A Shimadzu C-R8A Chromatopac was used for peak area measurements. The sample injection volume was 1 μl.

Gas chromatography/mass spectrometry

The metabolic intermediary compounds of BPA were evaluated using gas chromatography/mass spectrome-

try (GC/MS) (HP6890/HP5973; Agilent Technologies, Inc., USA). Samples were injected onto an HP-5MS (Agilent Technologies) fused silica column (5% phenyl–95% methyl polysiloxane; 30 m × 0.25 mm; 0.25-μm film thickness). Helium was used as a carrier gas. The injector and detector temperatures were 275 and 310°C, respectively. The oven temperature program was as follows: 50°C for 1 min; 20°C/min to 200°C; and 15°C/min to 310°C. The sample injection volume was 1 μl.

Results

Influence of BPA on growth of type strains

The effects of BPA on the growth of microorganisms are not well understood. The effects of BPA toxicity on the growth of microorganisms were examined using the type strains *Escherichia coli* IFO 3366, *Staphylococcus aureus* IFO 3183, *Micrococcus roseus* IFO 3764, *Rodococcus erythropolis* ATCC 12320, and *Corynebacterium ammoniagenes* ATCC 12072. These strains were cultivated with shaking (150 rpm) at 30°C in a test tube (18φ × 130 mm) containing 5 ml of SYP liquid medium, with BPA added at final concentration of 0–10,000 ppm. The growth of *R. erythropolis* ATCC 12320 was inhibited at a BPA concentration of 100 ppm. The growth of the other type strains was almost completely inhibited at low BPA concentrations of less than 1,000 ppm (Fig. 1). It was thus clarified that BPA potently inhibits the growth of microorganisms at low concentrations.

Isolation and characterization of *P. monteilii* N-502

We attempted to isolate BPA-tolerant/degrading bacteria from approximately 1,000 samples collected from a field, sewage, and pond water. BPA-degrading bacteria that formed a halo around the colony were isolated with the addition of solid agar to culture medium containing BPA 1,000 ppm. Five BPA-degrading bacteria, isolated from soil samples were able to degrade BPA. They were all aerobic bacteria and rod-shaped. Strain AC-10, which formed red colonies was gram-negative and its 16 S rRNA gene sequence is similar to those of *Serratia* sp. (99% homology). Strain N-502 and N-1 were gram-negative and similar to *Pseudomonas* sp. (99% homology). Strain N-502 and N-1formed yellow and green colonies, respectively. Strains AL-1 and AL-5 were gram-positive and similar to *Bacillus* sp. (99% homology), respectively. Among them, *Pseudomonas* sp. strain N-502 showed stable ability to degrade BPA. It was able to grow with BPA as the sole carbon source at pH 5–9. The optimal growth temperature and pH value in SYP medium were 30°C and 7.0, respectively. Therefore, strain N-502 was selected for further study.

Strain N-502 is an aerobic, motile, gram-negative, nonspore-forming, rod-shaped (0.7–0.8 × 1.5–2.0 μm) bacterium. It reacts passively in the catalase and oxidase tests, as well as being oxidative in the OF test. Its 16S rRNA gene sequence (accession No. AF094737) is very similar to those of *Pseudomonas putida* (100% homology) and *P. monteilii* (100% homology). Strain N-502 had assimilation of glucose, gluconate potassium, *n*-capric acid, DL-malic acid, sodium citrate and phenyl acetate. Identification code number by API 20NE kit was No. 0140457. In addition, this strain exhibited the ability to utilize inositol and produced fluorescent pigment in King's medium (Barrow et al. 1993; Elomari et al. 1997; Krieg and Holt 1984; Stackebrant et al. 2002; Wayne et al. 1987). Therefore, on the basis of these biochemical and physiological findings and the results of 16S rRNA analysis, the isolate was identified as

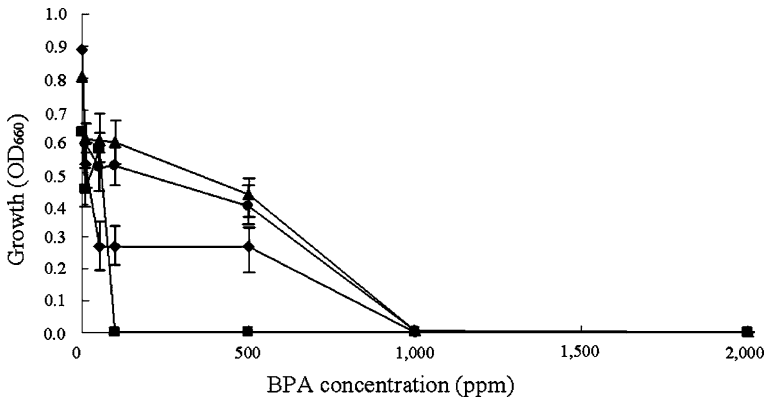

Fig. 1 Effect of bisphenol A (BPA) concentration on growth of type strains. Rhombus, *E. coli* IFO 3366; *square*, *Rodococcus erythropolis* ATCC 12320; *triangle*, *S. aureus* IFO 3183; *circle*, *Micrococcus roseus* IFO 3764. *Error bars* indicate that standard deviation obtained in five independent experiments

P. monteilii. *P. monteilii* is non-pathogenic and registered risk assessment 1 with the Deutsche Sammlung von Mikroorganismen (DSM).

BPA tolerance of strain N-502

The effects of BPA on the growth of strain N-502 were examined in SYP liquid medium containing BPA 0–20,000 ppm. Strain N-502 was cultivated at 30°C, pH 7.0 with shaking at 150 rpm for 24 h in a test tube (18$\varphi \times 130$ mm) containing 5 ml of liquid medium. This strain was able to grow at a BPA concentration of greater than 1,000 ppm (Fig. 2). Strain N-502 thus has good tolerance to high concentrations of BPA.

Degradation activity of strain N-502

The BPA-degrading activity of strain N-502 was examined in both a culture system and resting cell system. First, the BPA-degrading activity of strain N-502 was measured in the culture system. It was cultured in a 300-ml Erlenmeyer flask containing 100 ml of SYP liquid medium at 30°C with shaking at 150 rpm. BPA was then added to the SYP medium at a concentration of 500 ppm. Strain N-502 was able to grow at the high BPA concentration of 500 ppm and degraded BPA 468 ppm (93.6%) during the 6-day culture period. After 10 days of culture, BPA was degraded completely (Fig. 3).

Next, the BPA-degrading activity of strain N-502 was determined in the resting cell system. The optimal temperature and pH for BPA degradation by resting cells were 30°C and 7.0, respectively (data not shown). Physical adsorption of BPA to cells was evaluated using cells that had been boiled for 30 min. The amount of BPA adsorption at concentrations of 100, 500, and 1,000 ppm was 2.7, 1.4, and 6.3 ppm, respectively.

Strain N-502 cultures were next incubated at 30°C on a rotary incubator shaker at 150 rpm. Its BPA-degrading activity was then examined at BPA concentrations of 100, 500, and 1,000 ppm in the resting cell system. Figure 4 shows the 2-h time course of BPA degradation by strain N-502 in the resting cell system. When BPA 100 ppm was added, strain N-502 degraded the compound completely after 2 h. When BPA at concentrations of 500 and 1,000 ppm was added, it was degraded by 86.4 and 53.1%, respectively, after 2 h.

The initial BPA-degrading rate of strain N-502 at BPA concentrations of 100, 500, and 1,000 ppm in the resting cell system was 83.2, 275.8, and 487.3 ppm/min, respectively (mean values of experiments repeated five times). The initial BPA-degrading rate means the amount of BPA degraded in 1 m. Strain N-502 thus had potent degradation activity at high BPA concentrations of 500 or 1,000 ppm.

Effects of metal ions, coenzymes, and folic acid on the BPA-degrading activity of strain N-502

Metal ions, coenzyme, and the vitamin folic acid were added to phosphate buffer 25 mM containing BPA at final concentrations of 100, 500, and 1,000 ppm in the resting cell system. The metal ions Zn^{2+}, Ba^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, and Mg^{2+}, coenzymes FAD, FMN, NAD, and NADP, and folic acid were then added singly at concentrations of 1 and 10 mM, and BPA-degrading activity was measured at 30°C. The degradation rate test was repeated five times, and approximately the same results were obtained in each. The addition of Ca^{2+}, Mg^{2+}, and folic acid increased the BPA-degrading activity of strain N-502. Other metal ions and coenzymes did not affect the activity.

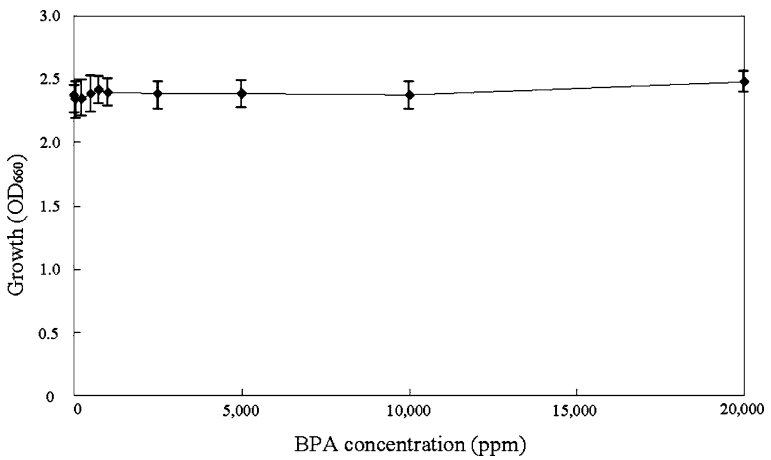

Fig. 2 Effect of BPA concentration on the growth of strain N-502. *Error bars* indicate that standard deviation obtained in five independent experiments

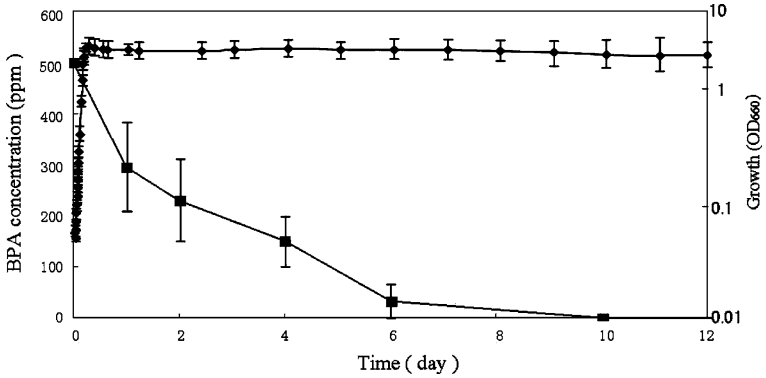

Fig. 3 Degradation of BPA by strain N-502 in culture medium containing BPA 500 ppm *square*, BPA concentration (ppm) in liquid medium; rhombus, growth of strain N-502. *Error bars* indicate that standard deviation obtained in five independent experiments

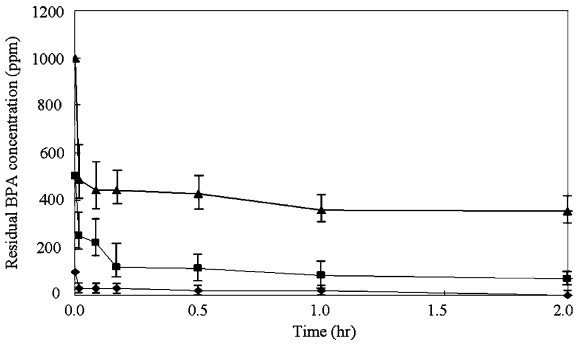

Fig. 4 BPA degradation by strain N-502 in the resting cell system. Initial BPA concentrations of rhombus, 100 ppm; *square*, 500 ppm; *triangle*, 1,000 ppm. *Error bars* indicate that standard deviation obtained in five independent experiments

The initial degrading rate at BPA concentrations of 100, 500, and 1,000 ppm was 83.2, 275.8, and 487.3 ppm/min, respectively, without metal ions or the vitamin. With the addition of Ca^{2+} 1 mM to the resting cell system containing BPA 1,000 ppm, a 14% increase in BPA degradation was observed (Table 1). With the addition of Mg^{2+} 1 mM, an approximately 32% increase in the initial degradation rate was observed when the resting cell system contained a BPA concentration of 500 ppm. At a BPA concentration of 500 ppm, the addition of folic acid 10 mM to the resting cell system resulted in an approximately 36% increase in BPA degradation, and at a BPA concentration of 1,000 ppm, an approximately 42% increase was observed. Thus the addition of folic acid was the most effective in increasing the BPA degradation by strain N-502.

Analysis of the metabolic intermediary compounds of BPA of strain N-502

Strain N-502 cultures were next incubated at 30°C on a rotary incubator shaker at 150 rpm. The metabolic intermediary compounds of BPA were then examined at BPA concentrations of 500 ppm in the resting cell system. The optimal temperature and pH by resting cells were 30°C and 7.0, respectively. The metabolic intermediary compounds of BPA were evaluated using GC/MS.

Figure 5 shows the 2-h time course of BPA degradation by strain N-502 in the resting cell system. Peaks 1 and 2 appeared 2 h later with decreased the amount of BPA in the resting cell system. Peaks 1 and 2 in Fig. 5,which eluted with retention time of 7.8 and 8.3, suggested them to be 4-hydroxybenzaldehyde (HBAL) and 4-hydroxyacetophenone (4-HAP) by GC/MS, respectively. Peaks 1 and 2 were predicted to be HBAL and 4-HAP, respectively. These were based on a comparison with the mass spectra of known compounds (Spivak et al. 1994).

Table 1 Influence of metal and folic acid on initial BPA degradation rate

		Initial BPA degradation rate (ppm/min)		
		BPA conc.		
		100 ppm	500 ppm	1,000 ppm
Strain N-502 alone	–	83.2	275.8	487.3
Ca^{2+}	1 mM	89.4	284.3	553.6
	10 mM	87.9	291.5	543.3
Mg^{2+}	1 mM	90.7	363.8	609.1
	10 mM	87.6	303.7	543.3
Folic acid	1 mM	82.1	280.2	525.6
	10 mM	85.3	374.7	693.8

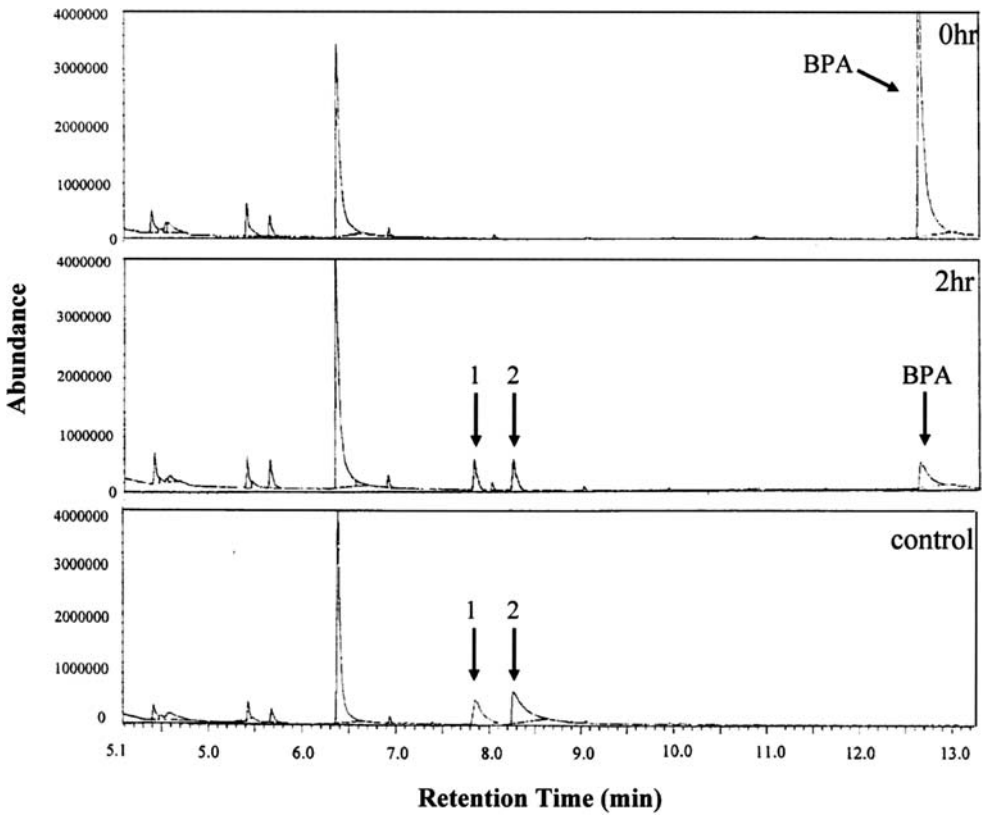

Fig. 5 The metabolic intermediary compounds in BPA degradation in the resting cell using GC/MS analysis. Peaks 1 and 2 correspond to HBAL and 4-HAP, respectively

Discussion

To the best of our knowledge, this is the first report to confirm that BPA inhibits the growth of microorganisms at the low concentration of 1,000 ppm. *P. monteilii* strain N-502 was the first strain found with high tolerance to BPA and the ability to degrade the compound when present in high concentrations in the growth medium.

The BPA tolerance of microorganisms can be estimated according to the limiting log *P* value for growth based on the toxicity index log *P* value proposed by Inoue and Horikoshi (1989). That is, if the limit log *P* value is lower than the log *P* value of the compound, the microorganism has tolerance to the compound. The log *P* value, which is defined as the logarithm of a solvent's partition coefficient in a standard octanol: water mixture, is used as a quantitative index of solvent polarity in quantitative structure-activity relationship analyses.

To examine the BPA tolerance of strain N-502, it was placed on SYP solid medium and overlain with several solvents that had various log *P* values. The limiting log *P* value for the growth of strain N-502 was 3.1 according to the toxicity index-based log *P* value (Table 2). Strain N-502 was able to grow in the presence of a solvent (*p*-xylene) with a log *P* value greater than 3.1. It was thus confirmed that strain N-502 had tolerance for high concentrations of BPA and was capable of growth at BPA concentrations of greater than 1,000 ppm because, the log *P* value of BPA is 3.3.

On the other hand, when the limiting log *P* values for the growth of type strains *E. coli* IFO 3366, *S. aureus* IFO 3183, *M. roseus* IFO 3764, and *R. erythropolis* ATCC 12320 were examined, it was evident that *E. coli* IFO 3366 could grow in solvents with log *P* values greater than or equal to 3.8, such as propylbenzene (log *P* = 3.8), diphenylether (log *P* = 4.2), and isooctane (log *P* = 4.8) but not in *o*-dichlorobenzene, ethylbenzene, and *p*-xylene with log *P* values of 3.6, 3.3, and 3.1, respectively (Table 2). It therefore appears that a critical point is reached between log *P* values of 3.8 and 3.6, at which, the solvent is sufficiently polar to prevent growth. *S. aureus* IFO 3183 and *R. erythropolis* ATCC 12320 grew in solvents with log *P* values greater than or equal to 4.8, such as isooctane (log *P* = 4.8). *M. roseus* IFO 3764 grew in solvents with log *P* values greater than or equal to 4.5, such as cyclohexane (log *P* = 4.5). The limiting log *P* values for the growth of *E. coli* IFO 3366, *S. aureus* IFO 3183, *M. roseus* IFO 3764, and *R. erythropolis* ATCC 12320 were 3.8, 4.8, 4.5, and 4.8, respectively. The effects of BPA on these microorganisms were similar to those of ethylbenzene from log *P* value 3.3 of BPA. BPA is a very biotoxic compound to the

Table 2 Solvent tolerance of type strains

	log *P*	Strain N-502	*E. coli* IFO 3366	*R. erythropolis* ATCC 12320	*S. aureus* IFO 3183	*M. roseus* IFO 3764
Dodecane	7.0	+	+	+	+	+
Decane	6.0	+	+	+	+	+
n-Nonane	5.5	+	+	+	+	+
Octane	4.9	+	+	+	+	+
Isooctane	4.8	+	+	+	+	+
Cyclooctane	4.5	+	+	–	–	+
Diphenylether	4.2	+	+	–	–	–
n-Hexane	3.9	+	+	–	–	–
n-Propylbenzene	3.8	+	+	–	–	–
o-Dichlorobenzene	3.6	+	–	–	–	–
Cyclohexane	3.4	+	–	–	–	–
Ethylbenzene	3.3	+	–	–	–	–
p-Xylene	3.1	+	–	–	–	–
Styrene	2.9	–	–	–	–	–
Limiting log *P* value for growth		3.1	3.8	4.8	4.8	4.5

+ Growth, – no growth

microorganisms examined because its log *P* value is 3.3. The limiting log *P* values for the growth of these type strains are greater than 3.8. This suggests that the growth of the type strains was inhibited at low BPA concentrations because they lack tolerance to the compound.

This was also the first report showing that the addition of Ca^{2+}, Mg^{2+}, and folic acid to the resting cell system increases the BPA degradation rate of strain N-502. It was previously shown that Ca^{2+} and Mg^{2+} stabilize the growth of microorganisms when cultured with growth-inhibitory solvents (Inoue et al. 1991). It is assumed that Ca^{2+} and Mg^{2+} act to stabilize the growth and catabolism of strain N-502. The role of folic acid in BPA degradation should be examined in detail in future studies.

Strain N-502 was able to grow with BPA as the sole carbon source. Both 4-hydroxybenzaldehyde and 4-hydroxyacetophenone were confirmed to be the metabolic intermediary compounds in BPA degradation in liquid culture using GC/MS. Both were also degraded in the resting cell system. It was reported that the pathway for the bacterial metabolism of BPA is oxidation to the triol 1,2-bis(4-hydroxyphenol)-2-propanol and methylhydroxylated 2,2-bis(4-hydroxy-phenyl)-1-propanol. The 1,2-bis(4-hydroxyphenol)-2-propanol is then dehydrated to 4,4′-dihydroxy-α-methylstilbene, which is cleaved by oxidation to 4-hydroxybenzaldehyde and 4-hydroxyacetophenone. 4-Hydroxybenzaldehyde is oxidized to hydroxybenzoic acid. 2,2-Bis(4-hydroxyphenyl)-1-propanol is oxidized to form both 2,2-bis(4-hydroxyphenyl)propanic acid and 2,3-bis(4-hydroxyphenyl)-1,2-propane-diol (Lobos et al. 1992; Spivack et al. 1994). 2,3-Bis(4-hydroxyphenyl)-1,2-propane-diol is then oxidized to both 4-hydroxybenzoic acid and 4-hydoxyphenacyl alcohol. Therefore, we hypothesize that, strain N-502 utilizes a similar pathway for the metabolism of BPA, in which the compound is oxidized to 1,2-bis(4-hydroxyphenol)-2-propanol, 4-hydroxybenzaldehyde, and 4-hydroxyacetophenone.

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