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ORIGINAL PAPER

Isolation and characterization of a novel bacterium, *Sphingomonas bisphenolicum* strain AO1, that degrades bisphenol A

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Abstract Bisphenol A (2,2-bis(4-hydroxyphenyl) propane, BPA), which is used as a synthetic resin material or a plasticizer, is a pollutant that possesses endocrine-disrupting activity. Bioremediation of BPA is used to decrease its polluting effects, and here we report a novel bacterial strain AO1, which is able to degrade BPA. This strain was isolated using enrichment cultivation from a soil sample from a vegetable-growing field; the sample was one of 500 soil samples collected across Japan. Strain AO1 degraded 100 mg/l BPA to an undetectable level within 6 h in MYPG medium (containing malt extract, yeast extract, peptone, and glucose) and within 48 h in minimum medium containing 1% glucose at 30°C. Strain AO1 can utilize BPA as a sole source of carbon and as an energy source under aerobic conditions. The estrogenic activity of BPA in MYPG medium was ultimately reduced by strain AO1, although the activity initially increased. Taxonomical analysis showed that strain AO1 is closely related to *Sphingomonas chlorophenolicum* and *S. herbicidovorans*, neither of which have a capacity for BPA degradation. DNA–DNA hybridization showed that strain AO1 is a novel species of the *Sphingomonas* genus, and we designated AO1 as *S. bisphenolicum*.

Keywords *Sphingomonas bisphenolicum* · Estrogenic activity · Bisphenol A · Biodegradation

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Introduction

Bisphenol A (2,2-bis(4-hydroxyphenyl)propane, BPA) is a compound used in production of polycarbonate plastics, epoxy resins and flame-retardants. These materials are widely utilized as adhesives, protective coatings, paper coatings

for compact disks, developers in dyes, and encapsulation of electrical and electronic parts (Staples et al. 1998). Mass consumption of BPA-based products has led to detection of BPA as a pollutant in rivers and streams (Staples et al. 1998); for example, BPA thought to be derived from waste plastics has been detected in hazardous waste landfill leachates in Japan at a concentration of 17.2 mg/l (Yamamoto et al. 2001). BPA has also been proposed to be an endocrine-disrupting chemical (EDC), and may adversely affect endocrine function in humans and wildlife (Colbone 1995; vom Saal et al. 1998; Howdeshell et al. 1999). Hence, biodegradation of BPA is of importance in elimination of BPA environmental pollution, and this approach has been adapted to highly polluted water, such as leachates from dumps and factories.

Several studies on BPA biodegradation have been reported. Enzymes of white rot fungi, including manganese peroxidases and laccases, degrade BPA (Hirano et al. 2000; Tsutsumi et al. 2001; Fukuda et al. 2001) and eliminate its estrogenic activity (Tsutsumi et al. 2001). A Gram-negative BPA-degrading bacterium, MV1, was isolated from active sludge in a wastewater treatment plant (Lobos et al. 1992; Spivak et al. 1994), and *Sphingomonas paucimobilis* FJ-4 was also obtained from activated sludge (Ike et al. 1995, 2002). A Gram-negative bacterium, WH1, has been isolated from soil contaminated with chemical industry waste, and was subsequently shown to grow through utilization of BPA as a sole carbon and energy source (Ronen and Abeliovich 2000). Metabolic pathways of BPA biodegradation in these strains have been established (Spivak et al. 1994; Sasaki et al. 2005; Ike et al. 1995, 2000), but the mechanism underlying the alteration of BPA estrogen activity by the strains remains unclear.

In this study, we isolated a bacterium possessing a very high BPA biodegradation capacity. We show that the bacterium can use BPA as a sole source of carbon and as an energy source, and we discuss the characteristics and classification of the bacterium.

Materials and methods

Isolation of a BPA-degrading bacterium

Five-hundred soil and sludge samples were collected from farmlands, riversides, garbage piles and chemical factories across Japan. A basal medium (BSM medium: 1.0 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeCl_3$, 0.05 g NaCl, and 0.05 g $CaCl_2$ per liter, pH 7.2; Ike et al. 1995) was used for the enrichment culture. One gram of each soil sample was incubated in BSM medium containing 20 mg/l BPA as a sole carbon source [BPAM(20)] at 30°C for 7 days in a 100-ml Erlenmeyer flask. Thereafter, 1 ml of the culture medium was transferred in a stepwise manner into fresh BPAM with 40, 60, and 100 mg/l of BPA [BPAM(40), BPAM(60), and BPAM(100), respectively]. Bacterial cells were isolated from the resultant culture by spreading and cultivating on a BPAM(100) agar plate (1.5% agar), and the BPA-degradation capacity of each culture was tested using various incubation times. A BPA-degrading bacterium strain, AO1, was obtained from this procedure, and this strain has been deposited in the International Patent Organism Depositary (IPOD, Tsukuba, Japan), as No. FERM P-17794.

BPA-degradation activity

Strain AO1 was pre-cultured in MYPG medium (3.0 g malt extract, 3.0 g yeast extract, 5.0 g polypeptone, and 10 g glucose per liter, pH 7.0) with shaking at 30°C. The cells were then centrifuged at $1{,}500 \times g$ (TOMY LC-100) for 10 min, washed with 50 mM phosphate buffer twice, and suspended in saline. This bacterial cell suspension was added to fresh BPAM(100) or MYPG medium containing 100 mg/l BPA at an optical density (600 nm) of 0.05, and incubated with shaking at 30°C. Glucose was added to the medium at levels of 0.01%, 0.1%, and 1.0% or not added.

Degradation of aromatic compounds by strain AO1 cells was examined with the same cultivation method, as described above, with supplementation of following aromatic compounds at 100 mg/l substitute for BPA: protocatechuic acid, guaiacol

(*o*-methoxyphenol), catechol (1,2-benzenediol), *o*-chlorobenzoic acid, *m*-chlorobenzoic acid, *p*-chlorobenzoic acid, *p*-hydroxybenzoic acid, *p*-hydroxyacetophenone, phenol, bisphenol B (2,2′-bis(4-hydroxyphenyl)butane), and bisphenol S (4,4′-dihydroxydiphenylsulfone). The medium saturated with BPA (ca. 1.5 mM) was also tested.

The BPA concentration in the medium was measured by high performance liquid chromatography (HPLC, Alliance 2690, Waters Inc.) under the following conditions: column, Mightysil RP18 GP150–4.6 (5 μm) (Kanto Kagaku K.K.); eluate, 70% acetonitrile and 30% water; column oven temperature, 40°C. A photo diode array detector was used to detect absorbance at 217 nm. The detection limit by the HPLC method is 1 mg/l.

Sphingomonas chlorophenolicum ATCC 33790 (X87161) and *S. herbicidovorans* DSM11019 (AB022428) were used as control strains.

Physiological and biochemical characterization

The isolated bacterial strain was identified using a variety of microbiological and biochemical tests. Nutrient agar (1% beef extract, (Difco Laboratories, Detroit, MI, USA), 1% peptone (Difco), 0.5% NaCl, and 1.5% agar, pH 7.2) was used as a basal medium. Unless otherwise stated, all strains tested were cultivated at 30°C. Cell shape, Gram reaction and motility were investigated using cells cultivated for 24 h. Cell morphology was examined using scanning electron microscopy (model JSM-5600LV; JEOL). The cells were fixed with 2% glutaraldehyde and 2% osmic acid in 0.01 M cacodylic acid buffer (NaCl, 0.15 M), and subsequently dried with a series of ethanol treatments. The bacterial cells were lyophilized in *tert*-butyl alcohol and then observed at an accelerated voltage of 10 KV. Gram staining was carried using the Hucker-Conn modification (Hucker and Conn 1923), and motility was investigated by the hanging drop method (Quinn et al. 1994). Reduction of nitrate to nitrite and hydrolysis of starch were tested by the methods of Iizuka and Komagata (1963). Hydrolysis of casein was determined based on the appearance of a clear zone around a colony on nutrient agar containing 12% skimmed milk after 7 days of cultivation at 25°C (Uchino et al. 2000). The oxidation–fermentation (O–F) test was performed using the method of Hugh and Leifson (1953). Catalase activity was detected based on production of bubbles by addition of 3% hydrogen peroxide solution to resting cells. The oxidase test was performed with cytochrome oxidase test paper (Eiken Chemical Co., Ltd, Tokyo, Japan). Other analyses were carried out using general methods (Hasegawa 1985).

Molecular genetic analysis aasd

Cells were cultivated in nutrient broth for 24 h with shaking, and then collected by centrifugation. DNA was extracted by the method described by Saito and Miura (1963). A partial 16S rDNA fragment was amplified by PCR, using a universal primer set corresponding to positions 8–27 (forward primer) and 1492–1510 (reverse primer), based on the *Escherichia coli* numbering system (Weisburg et al. 1991). Direct sequencing of the amplified DNA fragment was carried out, as described by Satomi et al. (1997). The similarity of 16S rDNA sequences from strain AO1 and other species registered in the GenBank, EMBL and DDBJ databases was evaluated using the BLAST algorithm (Altschul et al. 1990). Data analysis was performed with Genetyx software (Genetyx Corp.). Nucleotide substitution rates (Knuc values) were calculated using the method of Kimura (1980), and a phylogenetic tree was constructed using the neighbor-joining method of Saitou and Nei (1987). The accession numbers of genes used in the phylogenetic analysis are shown in Fig. 1.

DNA–DNA hybridization experiments were also carried out using the microplate hybridization method (Ezaki et al. 1989).

Estrogenic activity transition in BPA degradation process

Strain AO1 cells were cultivated in MYPG medium at 30°C for 17 h and collected by centrifugation at 1,500 × *g* (TOMY LC-100). Thereafter, cells were washed and then were incubated in fresh MYPG medium containing 100 mg/l BPA at 30°C for 8 h with sampling a culture supernatant

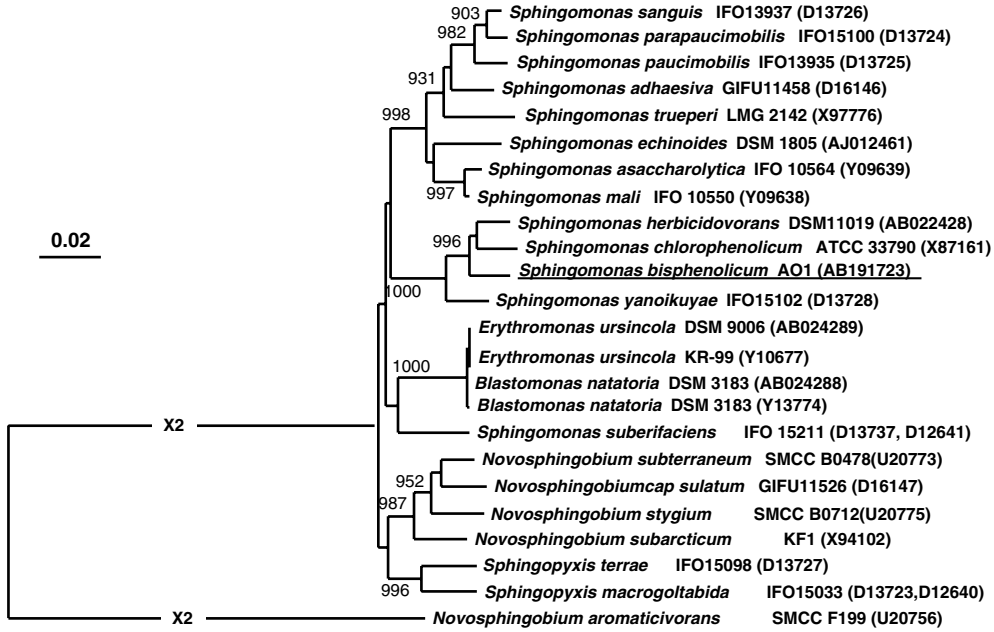

Fig. 1 Phylogenetic tree constructed by the neighbor-joining method based on 16S rDNA sequences of AO1 and related bacteria. Scale bar represents an evolutionary distance (Knuc) of 0.02

every one hour for measurements of the BPA and 4-hydroxyacetophenone (4-HAP) concentrations. 4-HAP was detected by GC-MS (Shimadzu QP-5050A) equipped with a DB-5MS capillary column (0.25 μm × 0.25 mm × 30 m; J&W Scientific) under the following chromatographic condition: the injector temperature is 250°C, and the sequential elution condition is 40°C for 2 min, 40–100°C at 5°C/min, 100–200°C at 10°C/min, and 200–300°C at 20°C/min. Helium at 14 psi was used as the carrier gas. The interface was kept at 270°C. The mass spectra were obtained by the electron impact positive mode at 70 eV in the full scan mode. Estrogenic activities were measured five times using the two-hybrid method (Tsutsumi et al. 2001) and were represented as relative activities to a control value which is without the bacterium.

Results

Isolation of a BPA-degrading bacterium

One gram of each of 500 soil and sludge samples collected in Japan was inoculated directly into BPAM(20) liquid medium. After incubation for 7 days at 30°C, 144 samples showed increased turbidity, suggesting inhabitation of these samples by BPA-degrading bacteria. We then attempted to obtain bacterial strains with higher BPA-degradation activity using the enrichment cultivation method, which resulted in 123, 110, and 92 samples with increased turbidities in BPAM(40), BPAM(60) and BPAM(100) liquid media, respectively. Finally, 92 isolates from the BPAM(100) cultures were isolated by spreading and cultivating on BPAM(100) agar plates, and their BPA-degrading activity was individually tested using various incubation times in BPAM(100) liquid medium. Almost all isolates showed weak and unstable BPA degradation, but strain AO1, which was isolated from the soil of a vegetable field, showed strong and stable BPA degradation, especially in MYPG medium.

Physiological and biochemical characterization

Strain AO1 was shown to be a Gram-negative, non-spore forming and aerobic rod-shaped bacterium of 0.5–0.7 μm in diameter and 0.7–2.2 μm

in length. The AO1 colony that formed on nutrient agar plates was yellow, convex and circular, and had a smooth surface. Strain AO1 was positive in motility, catalase and oxidase tests, and positive for reduction of nitrate, liquefaction of gelatin and utilization of citrate. The strain was negative in O-F and V-P tests, and negative for growth under anaerobic conditions, hydrolysis of aesculin, hydrolysis of starch, hydrolysis of Tween 80, hydrolysis of casein, decomposition of DNA, degradation of urea, degradation of tyrosine, degradation of arginine, production of indole, production of hydrogen sulfide, growth in the presence of 2, 5, and 7% NaCl, and growth in the presence of 0.01% lysozyme. The pH and temperature ranges for growth were 5.5–8.5 and 10–40°C, respectively. A comparison of physiological and biological characteristics of the strain AO1 and type strains of *S. chlorophenolicum* and *S. herbicidovorans* are shown in Table 1. Acid production levels from organic compounds and some other characteristics of strain AO1 differed from the two type strains. Based on these results, AO1 was considered to belong to the *Sphingomonas* genus (formerly the *Flavobacterium* genus) (Holt et al. 1994).

Molecular genetic analysis aasd

To identify the species of strain AO1, the partial 16S rDNA fragment was amplified by PCR. The 16S rDNA sequence of strain AO1 was registered in the DDBJ database (accession no. AB191723), and compared with other 16S rDNA sequences of *Sphingomonas* sp. and neighboring species. Based on this analysis, strain AO1 was clustered with *S. chlorophenolicum* and *S. herbicidovorans*, while other *Sphingomonas* species were clustered in other groups (Fig. 1). Strain AO1 showed a high similarity to *S. yanoikuyae* (96.3%), *S. chlorophenolicum* (97.8%) and *S. herbicidovorans* (96.6%), indicating that AO1 is a member of the genus *Sphingomonas*. However, the highest similarity is only 97.8%, suggesting that AO1 may be a novel species.

DNA–DNA hybridization experiments (Stackebrandt and Goebel 1994) between genomes isolated from strain AO1 bacteria and from type strains of the *S. chlorophenolicum* and *S. herbicidovorans* species were performed for the purpose of classification. The strain AO1 genomes showed 5% and 17% similarities to *S. chlorophenolicum* ATCC 33790 and 4% and

Table 1 Physiological and biological characteristics of strain AO1 and two type strains, *Sphingomonas chlorophenolicum* and *Sphingomonas herbicidovorans*. Data of the two type strains were cited from Nohynek et al. (1995), Takeuchi et al. (2001), and Yabuuchi et al. (2002)

Characteristic	Strain AO1	*S. chlorophenolicum*	*S. herbicidovorans*
Motility	+	–	+
Behavior to oxygen	Aerobic	Aerobic	Aerobic
Colony color	Yellow	Yellow	Deep yellow
Catalase test	+	+	+
Oxidase test	+	+	–
Reduction of nitrate	+	–	–
Hydrolysis of			
Aesculin	–	+	+
Gelatin	+	+	+
Starch	–	–	–
Tween 80	–	–	–
Utilization of citrate	+	–	–
DNase	–	+	–
Acid production from			
Glucose	+	+	+
Maltose	+	–	+
Sucrose	+	+	–
Ribose	–	–	–
Xylose	+	+	+
Sorbitol	–	–	–
Mannitol	–	+	+
Inositol	–	–	–

Characters scored as + : positive, – : negative

30% similarities to *S. herbicidovorans* DSM11019. These results also indicate that strain AO1 belongs to a new species.

BPA-degradation activity

Strain AO1 could metabolize BPA in BPAM(100) medium in the absence of glucose, but the addition of glucose improved cell growth and shortened the BPA degradation time (Fig. 2). BPA at 100 mg/l was reduced to a level undetectable by HPLC within 44 h in BPAM(100) medium containing 0.1% or 1.0% glucose, and within 6 h in MYPG medium (data not shown), whereas neither of the neighboring species *S. chlorophenolicum* nor *S. herbicidovorans* degraded BPA in either medium (data not shown). BPA degradation by strain AO1 cells was also confirmed by the following observations: boiled AO1 lysate did not decrease the BPA concentration (Fig. 2) and an extract of the collected AO1 lysate did not contain BPA.

The following aromatic compounds were degraded by strain AO1 at 30°C for 120 h both in BSM medium containing 0.1% glucose and MYPG medium: catechol, *p*-hydroxybenzoic acid, *p*-hydroxyacetophenone, phenol, bisphenol B, and a saturated solution of BPA. However, the following compounds were not degraded by strain AO1: protocatechuic acid, guaiacol, *o*-chlorobenzoic acid, *m*-chlorobenzoic acid, *p*-chlorobenzoic acid, and bisphenol S. *p*-Hydroxybenzoic acid and phenol were degraded in MYPG medium but not in BSM medium containing 0.1% glucose.

Estrogenic activity transition in BPA degradation process

To examine if strain AO1 could eliminate BPA estrogenic activity by BPA degradation, the estrogenic activity of MYPG medium containing BPA at 100 mg/l was monitored during cultivation with strain AO1. The BPA concentration in culture supernatants decreased to an undetectable level after incubation for 5 h, but the relative estrogenic activity increased by 3.6 times after 4 h before gradually decreasing to an undetectable level by 12 h; although the activity of the control remained at the initial concentration (Fig. 3, and data not shown). 4-HAP, a BPA metabolite, was not detected at the start of the incubation, but gradually increased to 31 mg/l after incubation for 6 h and then decreased. Correlation between the time courses of estrogen activity and 4-HAP concentration was not observed.

Discussion

Strain AO1, which had the highest BPA-degradation capacity among the strains obtained in this

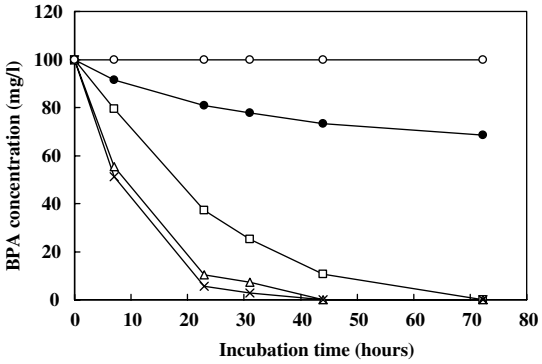

Fig. 2 Typical time courses for the degradation of 100 mg/l BPA in BPAM medium by the strain AO1. Glucose was added to the medium at levels of 0.01% (open squares), 0.1% (open triangles), and 1.0% (crosses), or not added (solid circles). Boiled AO1 was used as a control (open circles)

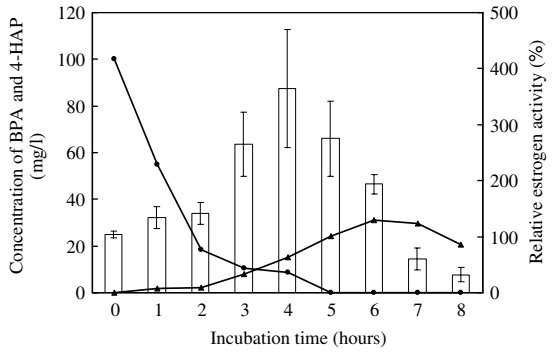

Fig. 3 Typical time courses for the degradation of 100 mg/l BPA in MYPG medium by strain AO1. The relative estrogen activity was defined as percentage of the initial activity (open boxes with standard error bars) at each time point. BPA and 4-HAP concentrations are represented by solid circles and solid triangles, respectively

study, was isolated using enrichment culture of a soil sample from a vegetable-growing field. In total, 92 strains of BPA-degrading bacteria were obtained from 500 soil samples. Although some of these samples may not have been contaminated with BPA, our results indicate that BPA-degrading bacteria are widely distributed in the environment, as found by Ike et al. (1995) and Dorn et al. (1987).

Although BPA-degrading bacteria such as the MV1 strain have been reported previously, strain AO1 appears to possess some unique properties, as described below. The BPA metabolites produced by AO1 cells in the medium decreased to an undetectable level (Sasaki et al. 2005), whereas several metabolites, including 2,3-bis (4-hydroxyphenyl)-1,2-propanediol and *p*-hydroxyphenacyl alcohol, remained in the culture after treatment with MV1 cells (Lobos et al. 1992; Spivak et al. 1994). Culturing of MV1 cells in the presence of BPA under conditions of vigorous aeration caused 4-HAP accumulation in the culture at levels inhibitory for cell growth (Lobos et al. 1992). In contrast, strain AO1 can degrade BPA at saturation BPA concentration (about 1.5 mM) and can also degrade 100 mg/l bisphenol B. *S. paucimobilis* FJ-4 cells can degrade BPA at a concentration up to 1.0 mM, but cannot grow in a medium containing BPA at a saturated concentration, and cannot degrade BPA at this concentration or degrade 100 mg/l bisphenol B. Lobos et al. (1992) reported difficulties in colony formation and maintenance of strain MV1 on solid minimal medium containing BPA as a sole carbon source. However, strain AO1 cells can easily be preserved both on solid medium and in a rich liquid medium. Hence, the properties of strain AO1 cells appear to be advantageous for bioremediation.

Estrogenic activity of strain AO1 metabolites increased after incubation for 4 h and a 4-HAP peak was observed after 6 h, before the estrogenic activity finally disappeared. The ability of strain AO1 to eliminate estrogenic activity provides a great benefit for bioremediation, but the initial increase in activity is a disadvantage. This observation indicates that some BPA metabolites formed by strain AO1 have relatively high estrogenic activities and remain in the culture for some time. For strain MO6, Ohtani et al. (2003) have suggested that 4,4′-dihydroxy-α-methylstilbene (DHMS), an intermediate formed during BPA degradation, probably contributes to a rise in estrogenic activity, since its structure is similar to diethylstilbestrol (DES), a synthetic estrogen. Strains MV1 and FJ-4 are thought to have the same BPA degradation pathway as that of strain MO6, the first step of which is transformation of BPA to DHMS, and the degradation pathway of strain AO1 may also be similar (Sasaki et al. 2005). Hence, BPA degradation by bacterial cells may temporarily increase estrogenic activity. However, Ike et al. (2002) found that the end products of BPA degradation by strain FJ-4 cells, including 4-HAP, had no or very low acute toxicity, mutagenicity and estrogenic activity, compared with BPA. Further investigation of the BPA degradation pathway in strain AO1 will be required to confirm these results for AO1.

Strain AO1 was shown to belong to a new species in the *Sphingomonas* genus, based on DNA–DNA hybridization analysis. Hence, we propose that strain AO1 should be designated as a new species, *S. bisphenolicum*. The genus *Sphingomonas* was defined by Yabuuchi et al. (1990) as comprising strictly aerobic, chemoheterotrophic, yellow-pigmented, Gram-negative, rod-shaped bacteria that contain glycosphingolipids as cell envelope components. Takeuchi et al. (2001) have proposed that the genus *Sphingomonas* can be divided into four individual genera based on phylogenetic analyses of 16S rDNA gene sequences. However, Yabuuchi et al. (2002) insisted that genus *Sphingomonas* should be undivided by the phenotypic, biochemical and genetic analyses. According to the classification by Takeuchi et al. (2001), strain AO1 is classified into the genus *Sphingobium*, in which *Sphingobium chlorophenolicum* and *S. herbicidovorans* are members.

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