Isolation and Characterization of Novel Bisphenol - A-Degrading Bacteria from Soils

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One hundred and seven soil samples were collected from various places in Japan, and their bisphenol-A (BPA, 2,2-bis (4-Hydroxyphenyl) propane) degradative capacities were evaluated. Eighty-five soil samples possessed BPA degradative capacities, and 26 bacterial strains could be isolated as BPA-degrading bacterium. Sequence analysis of their 16S rRNA genes indicated that 22 isolates belonged to proteobacteria groups, and three of four Gram-positive bacterial strains, YA27, NO13, and NO15, were classified as Bacilli. All isolates except strain YA27 completely degraded 115 μg/mL BPA in L medium but strain YA27 degraded only 50 μg/mL BPA. Strain YA27 and three Sphingomonas sp. strains could also grow in basal salt media containing BPA as a sole carbon source (BSMB medium). In HPLC analyses, some isolates, including the three Sphingomonas strains, produced some BPA metabolites in their cultures although the others, including strain YA27, produced no detectable metabolite. Furthermore, the Pseudomonas strains SU1 and SU4 produced some BPA metabolites that were different from the metabolites detected in the degradation of BPA by the S. bisphenolicum strain AO1. These results suggested that all isolates could be applicable to the bioremediation of BPA-polluted soil and water. Furthermore, we suggest that Bacillus sp. YA27 and Pseudomonas SU1 and SU4 may exhibit novel BPA metabolism pathways that are distinct from that of S. bisphenolicum AO1.

Key words: Endocrine disruptor / Biodegradation / Sphingomonas / Bacillus / Cytochrome P450.

INTRODUCTION

Bisphenol A (BPA, 2,2-Bis(4-Hydroxyphenyl) propane) is a material used in the production of polycarbonate and epoxy resins. Its annual production and use have been increasing. BPA is also known as a putative endocrine disruptor. The endocrine disruption potential of BPA in vitro and in vivo in human and animal cells has been reported and re-

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viewed (Bonefeld-Jorgensen, 2007). BPA was detected at ≥ 0.1 μg/L in 95% of human urine samples (Calafat et al., 2005) and altered some cell functions at concentrations between 1 pm and 1 nM (2.28 × 10⁻³ to 10⁻⁵ μg/L) (Welshons et al., 2006). The other important concern regarding BPA is that this compound is often detected at relatively low concentrations in many soil and water environments (Kang et al., 2006b; Loos et al., 2007; Kitada et al., 2008). It has also been reported that BPA-polluted water from some landfills and industrial disposal facilities contains BPA at concentrations more than
1,000-times higher than in other general water samples from the environment (Furhacker et al., 2000; Yamamoto et al., 2001). It is suspected that BPA pollution in the environment is caused by discarding or dumping BPA itself and/or BPA-containing products without any treatment, such as in the case of incineration or by release from wastewater treatment facilities. Indeed, BPA and some other endocrine disruptors have been detected in effluent from sewage treatment facilities (Harries et al., 1996; Pettersson et al., 2006; Pothitou et al., 2008), and the application of photocatalytical technology with a TiO₂ catalyst was recently proposed to remove and degrade some endocrine disruptors (Zhang and Zhou, 2008). These observations suggest the need for highly safe and effective systems for BPA removal or degradation.

Recently, some bacteria, most of which were Gram-negative, that were independently able to degrade BPA, were isolated from soil, river, and sludge of wastewater treatment facilities (Kang et al., 2006a). The metabolic pathway was investigated extensively in Strain MV1 (Lobos et al., 1992, Spivack et al., 1994), Sphingomonas paucimobilis FJ4 (Ike et al., 2000), S. bisphenolicum AO1 (Sasaki et al., 2005b), and Achromobacter xylosoxidans B-16 (Zhang et al., 2007). These bacterial cells could metabolize BPA by two pathways. In both pathways, BPA was oxidized and, subsequently, degraded to two phenol compounds, 4-hydroxybenzaldehyde (4-HBAH) / 4-hydroxyacetophenone (4-HAP) or 4-hydroxybenzoic acid (4-HBA) / 4-hydroxyphenacetyl alcohol (4-HPA). Finally, BPA was degraded to an undetectable level. 4-HBAH, 4-HAP, and 4-HBA were converted into cell components, CO₂, and H₂O. It was also reported that the endocrine-disruptor activity of BPA in culture finally became undetectable although its activity had temporally increased. Furthermore, in S. bisphenolicum AO1, the cytochrome P450 monooxygenase system, P450oxid monooxygenase, was involved in the first reaction in both of the two pathways, and converted BPA to 1,2-bis(4-hydroxyphenyl)-1-propanol and 2,2-bis(4-hydroxyphenyl)-1-propanol (Sasaki et al., 2005a). These observations indicate that Gram-negative BPA degrading bacteria can be applied to the bioremediation of BPA-polluted environments, although small amounts of 2,2-bis(4-hydroxyphenyl)propanoic acid and 4-HPA could not be metabolized (Spivack et al., 1994).

On the other hand, the Gram-positive bacteria, Streptomyces sp. from river-water (Kang et al., 2004) and Bacillus pumilus from traditionally fermented food (Yamanaka et al., 2007) were isolated as a BPA-degrading bacterium and had relatively high BPA degrading activities. However, BPA metabolic pathways in these bacteria were unclear and no metabolites from BPA were detected in the culture. It is suspected that these strains did not degrade BPA, but converted it to insoluble compounds (Uchida et al., 2001) or adsorbed it (Endo et al., 2007).

In this report, many different kinds of bacterial strains, including three Gram-positive strains, capable of degrading BPA were isolated from soil samples in Japan. Their capacities for BPA biodegradation were evaluated. We also will discuss their BPA degradation pathways.

**MATERIALS AND METHODS**

**Bacterial strains, media, and growth conditions**

Bacterial strains isolated from the soil samples are listed in Table 1. These bacteria and soil samples were cultivated at 30°C for 24 h and 48 h at 120 rpm in a glass Erlenmeyer flask and a glass test tube containing L medium (10 g Bacto Tryptone, 5 g Bacto-Yeast extract, and 5g NaCl per liter, adjusted to pH 7.3 with NaOH) and basal salt medium (BSM, consisting of 1 g (NH₄)₂SO₄, 1 g K₂HPO₄, 0.05 g NaCl, 0.2 g MgSO₄, 7H₂O, 0.01 g FeCl₃, and 0.05 g CaCl₂ per liter) containing BPA or BAP/ethanol as carbon sources. BPA was dissolved in distilled-desterilized water and added to L medium and BSM at a final concentration of 115 μg/mL (L-BPA and BSMB, respectively). In some experiments, BPA (1 g/mL) was dissolved in ethanol to improve its solubility, and 0.03% of this BPA-ethanol solution was added to BSM (BSMB(300)); BPA and ethanol final concentrations were 300 μg/mL and 0.03%, respectively. Escherichia coli strain TOP10F’ and strain JM109 were used as a host strain and pCR2.1 was used as a vector for the gene cloning. Recombinant cells were cultivated in LB medium containing 100 μg/mL ampicillin. 15% agar was added to a medium for the construction of agar plates.

**Isolation of BPA degrading bacteria**

Each soil sample (0.5g) was suspended in 1 mL deionized water and vortexed for 30 seconds. A 10 mL volume of BSMB(300) medium was inoculated with 0.1 mL of the supernatant and cultivated at 30°C for 24 h. The cultures in which bacterial-cell growth was confirmed were re-inoculated into fresh medium at 30°C for 24 h. After four cycles of cultivation, the culture was spread on a BSMB(300) agar plate and was grown at 30°C for 24 h. The largest colony on the plate was selected and subsequently stored in 15% glycerol solution at -80°C. Soil samples were used...
within 3 days and stored at 4°C until their use.

**Analysis of BPA and its metabolites with HPLC**

A full description of the methods used to identify and analyze BPA and its metabolites can be obtained in our previous paper (Sasaki et al., 2005a). In this study BPA and its metabolites were analyzed by HPLC using a TSKgel OD-4PW (TOSOH Co., Tokyo Japan), installed in a Hitachi HPLC model D-7100 system containing a L-7450H diode array detector (Hitachi Hi-Tech Science Systems Co., Hitachinaka, Japan). A sample was collected from the culture by centrifugation at 9,200g for 10 min at 4°C and subsequently filtered through a 0.20 μm Millex filter (Millipore Co., Billerica, MA) to remove insoluble compounds. When necessary, this sample underwent ethyl acetate extraction. The recovered ethyl acetate fraction was dried with a centrifugal evaporator CVE-2000 (Tokyorikakai Co., Tokyo, Japan) and re-suspended in 20% acetonitrile. The BPA concentration in the sample was calculated from the BPA peak area, compared with authentic BPA dissolved in 20% acetonitrile solution.

**BPA degradation capacity of resting cells**

Bacterial cells cultivated in L medium at 30°C until the early stationary phase (OD₆₅₀ = approx. 8.0) were collected by centrifugation at 4°C (9,200g, for 10 min), and washed twice with 50 mM Tris-HCl buffer (pH 8.0). The cells at a final OD₆₅₀ of 4.0 were re-suspended in a reaction solution of 50 mM Tris-HCl buffer (pH 8.0) containing 100 μg/mL BPA. The suspension was incubated at 30°C for 20 min and, thereafter, kept on ice at least for 10 min to stop the reaction. The supernatant of the resultant reaction solution was collected by centrifugation at 9,200g for 10 min at 4°C. The remaining BPA concentration in the reaction solution was measured by the HPLC method described above. The BPA degraded in the reaction over 20 min was represented as the BPA degradation activity of the resting cells.

**16S rRNA gene analysis**

Sequences of 16S rRNA genes were determined for classification of the isolated bacteria. Chromosomal DNA was isolated and purified with a Wizard Genomic DNA Purification System (Promega Co., WI). A partial region of the 16S rRNA gene was
amplified with the PCR method, as follows. 50 μL reaction solution contained 0.5 μg chromosomal DNA, 50 pmole of each of the primer sets, 0.2 mM dNTPs, the buffer appropriate for the DNA polymerase, and 1 U of Elongase Enzyme Mix (Invitrogen Co., CA). The primer set consisted of 5’-GAGAGTTGATCTGGCTCAG-3’ (27f) and 5’-AAGAGGTTGATCCANCRCRA-3’ (1525r) (Lane, 1991). The reaction conditions were as follows: one cycle of 94°C for 30 s, 30 cycles of 94°C for 30 s, 57°C for 30 s, and 74°C for 45 s, and one cycle of 74°C for 10 min. The amplified fragment was separated on an agarose gel by electrophoresis and purified with a QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden Germany). The purified fragment was cloned into pCR2.1 with a TOPO TA Cloning system (Invitrogen Co., CA), and sequenced with a BigDye Terminator v3.1 cycle sequencing kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Co., CA). Homologous gene sequences were collected from the Genbank database with a Blast program. Phylogenetic trees were constructed with an N-J method and represented using a TreeView program. The nucleotide sequences determined in this study have been registered in the DDBJ/EMBL/GenBank (accession nos. are AB453283 [strain SU1], AB453284 [strain NE2], AB453285 [strain SU3], AB453286 [strain SU4], AB453287 [strain SU5], AB453288 [strain HYO6], AB453289 [strain OIT7], AB453290 [strain HI9], AB453291 [strain HI10], AB453292 [NAR11], AB453293 [strain FU12], AB453294 [strain NO13], AB453295 [strain NO14], AB453296 [strain NO15], AB453297 [KA16], AB453298 [OS17], AB453299 [HA18], AB453300 [SU19], AB453301 [FU20], AB453302 [HUK21], AB453303 [HUK22], AB453304 [SO11], AB453305 [SO1a], AB453306 [SO4a], and AB453307 [YA27]).

RESULTS

Isolation of BPA degrading bacteria from soil samples

One hundred and seven soil samples were collected mainly from the Kansai district in Japan, including those of a landfill contaminated with BPA (in Tsuruga, Fukui, in Table 1) and a garbage waste facility previously contaminated with dioxins (in Nose, Osaka, in Table 1). Each sample was inoculated into BSMBE(300) medium and cultivated at 30°C for 24 h. Bacterial cell growth was observed in 85 samples and BPA reduction in their culture supernatants was confirmed in all cultures by HPLC analysis (data not shown). These results indicate that BPA is biodegradable and that it can be metabolized by some bacterial strains in soils either alone or by the combined activity of numerous BPA-degrading bacteria.

After an additional three cycles of cultivation in fresh BSMBE(300) medium, the bacterial cells were isolated on a BSMBE(300) agar plate. Each colony from each plate was picked and used for further experiments, described below; it was considered that all the colonies on each plate were derived from one bacterial strain on the basis of the colony morphology. Consequently, 26 strains were selected as possessing a high BPA-degrading ability. Their BPA degradation capacities in BSMBE(300) were also listed as the amount of BPA degraded after 48 h of cultivation in Table 1.

Classification of BPA-degrading bacteria

The 26 bacterial strains, which were highly active in BPA degradation as described above, were classified with the 16S rDNA sequence analysis. Each partial 16S rRNA gene region in the chromosomal DNA was amplified with the PCR method as described in the Materials and Methods. However, strain HN8 could not be classified because no fragments from its 16S rRNA gene region(s) were amplified using this method. Using 25 of the 16S rRNA gene sequences and the Genbank database, the phylogenetic tree is illustrated in Fig. 1 and their bacterial names are listed in Table 1. Twenty-two strains were Gram-negative bacteria; three isolates belonged to α-proteobacteria, three isolates to β-proteobacteria, and 16 isolates to γ-proteobacteria. The other three strains were Gram-positive bacilli.

BPA degradation in BSM medium

Strain AO1 could grow in BSM medium containing 115 μg/mL BPA as a sole carbon source (Oshiman et al., 2006). It was subsequently confirmed whether the isolated strains were able to metabolize BPA as a sole carbon source. Only three Sphingomonas sp. strains SO11, SO1a, and SO4a were able to grow and metabolize BPA in BSM medium (Fig. 2). Consequently, these strains completely degraded the BPA within 48 h. The other strains could not grow in BSM medium except for Bacillus sp. strain YA27, which could grow and metabolize BPA in BSM medium containing 50 μg/mL BPA (BSM(50)) (Fig. 2). This result indicates that the four strains are able to utilize BPA as a carbon source and may metabolize it to CO₂, H₂O, and cell components, similar to strain MV1 (Lobos et al., 1992).

BPA degradation in L-BPA medium

The previous sections indicate that all isolates, except for three Sphingomonas sp. strains and Bacillus
sp. YA27, could not grow in BSMB medium but did grow in BSMBE medium. The growth and the BPA-degradation capacity in L-BPA medium were subsequently verified in all isolates. With the exception of strain YA27 (Fig. 3D), the other strains were able to grow in L-BPA medium. Only three *Sphingomonas* sp. strains were able to completely degrade BPA in 20 h or less (Fig. 3A-C). Strain YA27 was able to grow in L medium containing 50 μg/mL BPA and to degrade BPA completely. The other isolates degraded only approximately 10 % of the BPA in L-BPA medium in 24 h (data not shown).

**Detection of BPA metabolites**

In the isolates, including three *Sphingomonas* sp. strains SO11, SO1a, and So4a, and *Bacillus* sp. YA27, BPA metabolites were analyzed by HPLC. Bacterial cells were cultivated in L-BPA or BSMBE medium at 30°C for 48 h. In almost all the strains except for *Sphingomonas* sp. strains and strains SU1 and SU4, no obvious metabolites were detected from their cultures (data not shown). In the cultures of three *Sphingomonas* sp. strains three metabolites were detected (Fig. 4). All metabolites were also detected in the culture of the *S. bisphenolicum* strain AO1 cultivated in L-BPA medium (Sasaki et al., 2005b), suggesting that peaks 1, 2, and 3 were 4,4'-dihydroxy-α-methylstilbene, 1,2-bis (4-hydroxyphenyl)-2-propanol and 2,2-bis (4-hydroxyphenyl)-1-propanol, and 4-HAP, respectively. This result suggested that the BPA metabolic pathways in *Sphingomonas* sp. strains might be the same as those in strains AO1 and MV1. Furthermore, other obvious metabolites were observed in the cultures of

![Phylogenetic tree](image-url)
*Pseudomonas* strains SU1 and SU4 (Fig. 4), indicating that additional bacterial BPA metabolic pathways might exist in nature.

**The Cytochrome P450 monooxygenase system was involved in the BPA biodegradation of the *Sphingomonas* strains**

In strain AO1, the cytochrome P450 monooxygenase system, which consisted of P450<sub>biad</sub>, Fd<sub>biad</sub>, and Red<sub>biad</sub>, was involved in the degradation of BPA and catalyzed BPA hydroxylation. This hydroxylation was inhibited by a cytochrome P450 inhibitor, metyrapone (Sasaki et al., 2005a). The inhibitory effects of metyrapone on BPA degrading activities in resting cells of isolates were subsequently investigated. Metyrapone partially inhibited the BPA degradation in the *Sphingomonas* sp. strains (Table 2). The BPA degradation activities in other isolates were too low to detect any inhibition by metyrapone. We also detected *bisdAB*, which encoded P450<sub>biad</sub> and Fd<sub>biad</sub>, by Southern hybridization analysis, only in the genomes of *Sphingomonas* sp. strains (data not shown). These results indicated that the cytochrome P450 monooxygenase system was involved in the BPA degradation by *Sphingomonas* sp..

**DISCUSSION**

BPA is a putative endocrine disruptor and is suspected to cause disturbance or disorder in the ecosystem through its function as an estrogen. This research indicated that many bacterial strains that could degrade and metabolize BPA were present in the environment. Three isolates of *Sphingomonas* sp. metabolized BPA as the sole carbon source, likely via their own cytochrome P450 monooxygenase system. *Bacillus* sp. YA27 also metabolized BPA as a sole carbon source. These results suggested that these four strains might prove useful to remove BPA from a BPA-polluted environment.

Recently, it was reported that BPA was present at a relatively low concentration in many soil (0.5 μg/kg), sediment (1-100 μg/kg), and water (0.01-1 μg/L) environments in Japan (http://www.safe.nite.go.jp/risk/kenkyukai.html, in Japanese) and in some waste landfills at high concentrations (Yamamoto et al., 2001). Isolates identified in this study might be useful for the bioremediation and bioaugmentation of soil and water environments pol-

![FIG. 2. BPA degradation by strains SO11, SO1a, SO4a, and YA27 in BSMB or BSMB(50) medium. Strains SO11 (circles), SO1a (triangles), and SO4a (squares) were cultivated in BSMB medium at 30°C, and strain YA27 (diamonds) was grown in BSMB (50) medium. Growth was monitored by the OD<sub>650</sub> (closed symbols) of the culture and the remaining concentrations of BPA in the culture (open symbols) were measured by HPLC analysis.](image)

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Metyrapone (mM)</th>
<th>BPA degradation (%)&lt;sup&gt;*&lt;/sup&gt;</th>
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<tr>
<td>Strain</td>
<td></td>
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<tr>
<td>SO1a</td>
<td>0</td>
<td>54.6±3.7</td>
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<tr>
<td></td>
<td>0.5</td>
<td>48.7±1.9</td>
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<td></td>
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<td>42.0±1.3</td>
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<tr>
<td>SO4a</td>
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<td>58.3±5.5</td>
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<td></td>
<td>0.5</td>
<td>50.7±1.7</td>
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<tr>
<td></td>
<td>5.0</td>
<td>41.6±3.4</td>
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<tr>
<td>SO11</td>
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<td>42.8±2.9</td>
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<td></td>
<td>5.0</td>
<td>39.4±3.9</td>
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<sup>*</sup>The reaction solution containing bacterial cells (OD<sub>650</sub>=4.0) and 100 μg/mL BPA was incubated at 30°C for 20 min with or without metyrapone. Remaining BPA concentration was measured by HPLC analysis. Values represent means ± standard deviations from three independent experiments.
FIG. 3. BPA degradation in L-BPA medium. Strains SO11 (A), SO1a (B), SO4a (C), and YA27 (D) were cultivated in L-BPA and L media at 30°C. Growth in L-BPA and L medium (closed circles and triangles, respectively) was monitored by the OD<sub>600</sub> and the remaining concentrations of BPA in the culture supernatant (open circles) were measured by HPLC analysis.

FIG. 4. Analysis of BPA and its metabolites. Strains SO11 (A), SO1a (B), and SO4a (C) were cultivated in L-BPA medium at 30°C for 12, 8, and 16 h, respectively. Strains SU1 and SU4 were cultivated in BSMBE(300) medium at 30°C for 48 h. BPA and its metabolites were extracted in ethyl acetate and analyzed by HPLC analysis.
luted with BPA.

Some researchers have reported that BPA is biodegradable and was essentially degraded in soil (Ying et al., 2003; Sarmah and Northcott, 2008) and water environments (Klecka et al., 2001; Kang and Kondo, 2005; Ike et al., 2006). Furthermore, many BPA-degrading bacteria have been previously isolated (Kang et al., 2006a). In this study, many soil samples exhibited BPA degradation capacities and 26 bacterial strains were isolated as a BPA-degrading strain from these samples. It was also clear that several soil samples did not exhibit detectable BPA-degradation capacities. Furthermore, a few bacterial isolates degraded BPA solely, but many isolates might degrade BPA partially and cooperatively. These results indicate that BPA is potentially biodegradable and some bacteria exhibited BPA degradation capacities, but many soil environments in nature might lack sufficient microbial capacity to degrade BPA. Partial degradation of BPA may create new environmental pollution concerns, because some reports have indicated that some BPA metabolites exhibited obvious estrogenic activities (Oshiman et al., 2007; Ike et al., 2002).

Little information has been collected about BPA degradation by Gram-positive bacteria and only one strain of Streptomyces sp. (Kang et al., 2004) from the river and three strains of Bacillus pumilus from kimchi (Yamanaka et al., 2007), a traditional Korean food, have been reported. In this study, three Gram-positive bacteria, which exhibited BPA-degradation capacity, were also isolated from soil samples. These observations indicate that Gram-positive bacteria are also involved in BPA degradation in the environment and may be applicable to the removal of BPA from polluted soil. However, no BPA metabolites were detected (data not shown) and their metabolic pathway was unclear. In this study, the BPA-degradation capacity of Bacillus sp. YA27 was not inhibited by the cytochrome P450 monooxygenase inhibitor, metyrapone, indicating that this strain might express a unique BPA degradation pathway.

In the Pseudomonas sp. SU1 and SU4, unique BPA-metabolites were observed and their BPA degradation capacities were not inhibited by metyrapone. These results suggest that additional BPA metabolic pathways exist in bacteria.

This study also indicated that the three isolated strains of Sphingomonas sp. exhibited the same BPA-degradation pathway as strain AO1 by HPLC analysis of BPA metabolites and the metyrapone inhibition test. We have already cloned bisdAB, encoding the cytochrome P450 monooxygenase components involved in BPA degradation (Sasaki et al., 2008), and it is clear that these gene fragments were embedded in a plasmid (data not shown). These observations suggest that comparison of the bisd AB genes of four Sphingomonas sp. strains in their nucleotide and amino acid sequences and comparison of their plasmids might reveal the gene transfer from cell-to-cell, the molecular evolution of the gene, and the mechanism of acquisition of new functions in bacterial cells.

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