

Supporting Information

Acceleration of nonylphenol and 4-*tert*-octylphenol degradation in sediment by

Phragmites australis and associated rhizosphere bacteria

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Supporting Information consists of 11 pages, and 6 figures and 1 table.
Analytical procedures (text); phylogenetic characterizations of three isolated strains (Figure S1);
EIMS spectral data of metabolites formed by 4-*tert*-OP degradation (Figure S2); utilization and
degradability of APs by three isolated strains (Table S1); degradation of 4-*tert*-OP and cell growth of
isolated strains under aerobic and anaerobic conditions (Figure S3); cell growth of isolates strains with

23 root extract (Figure S4); degradation of 4-*tert*-OP by isolated strains grown with root extract (Figure
24 S5); and proposed pathway for 4-*tert*-OP degradation by the three isolated strains (Figure S6).

Analytical procedures

26 Bacterial cell density in liquid cultures was measured as OD₆₀₀ and dry-weight of cells. For dry weight
27 measurement, the cells were harvested by centrifugation (9600 × g at 4 °C for 10 min), washed with 50
28 mM potassium phosphate buffer (pH 7.5), and then filtered through a preweighted filter (pore size, 0.2
29 µm; polycarbonate; Millipore, Tokyo, Japan). In this study, OD₆₀₀ = 1 of washed cells of strains IT-1,
30 IT-4, and IT-5 were 0.483 mg dry-weight mL⁻¹, 0.496 mg dry-weight mL⁻¹, and 0.482 mg dry-weight
31 mL⁻¹, respectively. The filter, together with the cells, was dried at 90 °C for 3 h and then weighted.
32 Bacterial cell density in sediments was measured as CFU. One gram wet-weight of sediment sample was
33 added to 10 mL of BSM. The mixture was then shaken and diluted serially with BSM. From appropriate
34 dilutions, 100-µL samples were spread on 4tOP-BSM (2000 mg L⁻¹) agar plates, and the plates were
35 incubated at 28 °C in the dark. After 7 d, CFUs with an extended transparent halo on the plates were
36 counted.

Concentrations of tNP, 4-*tert*-OP, and other APs were determined by high-performance liquid chromatography (HPLC). The metabolites produced by tNP and 4-*tert*-OP degradation were analyzed by gas chromatography-mass spectrometry (GC-MS). In the experiments using tNP-spiked sediments and 4-*tert*-OP-spiked sediments, three vials from each treatment were sampled on each sampling day. The upper parts of the plants (shoots and leaves) were separated from the roots, which were left in the sediment, before extraction of tNP or 4-*tert*-OP. Both types of sediment (i.e., rhizosphere sediment with roots and unvegetated sediment without roots) were acidified with 5 mL of salting-out solution (1 N HCl, 30% [w:v]), shaken with 20 mL of an ethyl acetate-*n*-hexane mixture (2:1, vol/vol) at 300 rpm for 20 min, sonicated in an ultrasonic bath (20 kHz, 200 W, 5-s intervals, 4 °C) for 20 min, and shaken again for 20 min. Then, the organic layer was collected. The extract was dried under flowing nitrogen, dissolved in an equal volume of acetonitrile, and analyzed by HPLC. In addition, the extract was directly analyzed by GC-MS to determine the decrease in levels of NP isomers in the tNP mixture. We confirmed that almost all of the tNP (more than 94%) and 4-*tert*-OP (more than 97%) was recovered from the tNP-spiked or 4-*tert*-OP-spiked sediment by this method.

51 In the liquid culture and whole-cell mixture experiments, the culture sampled at each sampling point
52 was acidified with 1 N HCl to pH 2 to 3, shaken for 3 min with an equal volume of an ethyl acetate-*n*-
53 hexane mixture (2:1, vol/vol), and centrifuged (3200 \times *g* at 4 °C for 10 min); the organic layer was then
54 collected. For the HPLC analysis, the extract sample (500 μ L) was dried under flowing nitrogen, and the
55 dry extract was dissolved in 500 μ L of acetonitrile before analysis. For the GC-MS analysis, the extract
56 (2 to 10 mL) was dried under nitrogen flow, and the dry extract was dissolved in 100 μ L of acetonitrile
57 before analysis. In addition, the extract (2 to 10 mL) was dried under nitrogen flow and then subjected to
58 trimethylsilylation (TMS) at 60 °C for 1 h using 100 μ L of a BSTFA-acetonitrile mixture (1:1, vol/vol).
59 The sample with TMS derivatization was analyzed by GC-MS.

60 HPLC analysis was conducted in a Shimadzu HPLC system with a UV/vis detector and a Shim-pack
61 VP-ODS column (150 mm \times 4.6 mm, particle size 5 μ m; Shimadzu, Kyoto, Japan). In the HPLC
62 analysis, an acetonitrile-water mixture (8:2, vol/vol) was used as the mobile phase, and detection was at
63 the wavelength of 277 nm. The GC-MS analysis was conducted with a Shimadzu GC-MS system
64 (GCMS-QP2010; Shimadzu) using an Rxi-5ms capillary column (30 m, 0.25 mm ID, 1.00 μ m df;
65 Restek, Bellefonte, PA, USA). For the GC-MS analysis, two column temperature programs were used.
66 For the analysis of metabolites without TMS derivatization, the temperature was held at 60 °C for 2 min,
67 increased to 300 °C at 5 °C min⁻¹, and then held at 300 °C for 5 min. For the analysis of metabolites
68 with TMS derivatization, the column temperature was held at 90 °C for 2 min, increased to 150 °C at a
69 rate of 15 °C min⁻¹, increased to 300 °C at 5 °C min⁻¹, and then held at 300 °C for 6 min. The injection,
70 interface, and ion source temperatures were 280 °C, 280 °C, and 250 °C, respectively. Helium
71 (99.995%) was used as the carrier gas at a flow rate of 1.0 mL min⁻¹.

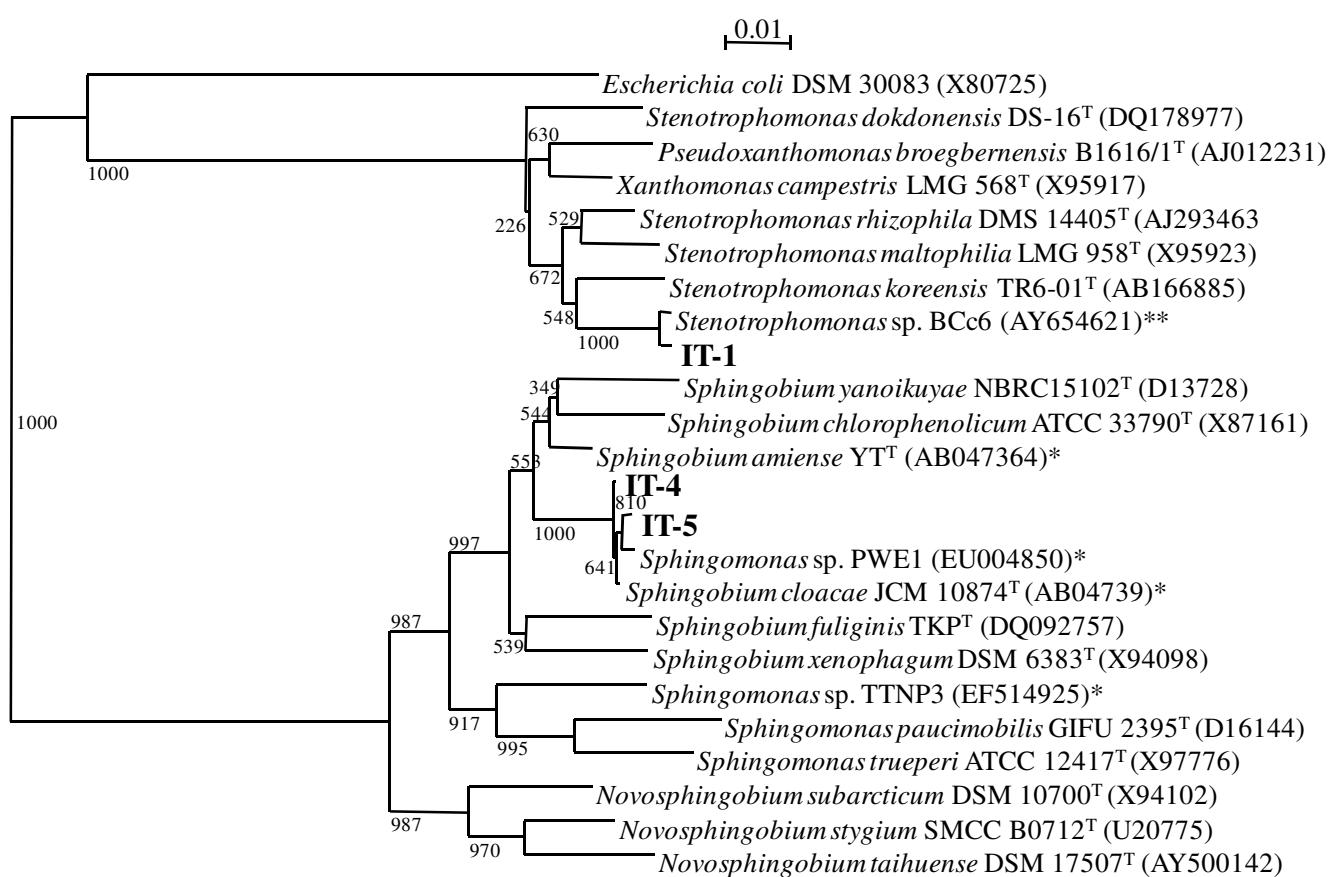


FIGURE S1. Phylogenetic relationships among strains IT-1, IT-4, and IT-5, type strains of Sphingomonadaceae and *Stenotrophomonas* sp., and previously isolated 4-NP-degrading (*), 4-*tert*-OP-degrading (*), and NPEO-degrading (**) bacterial strains, established by the neighbor-joining method using 16S rRNA gene sequences. Numbers on branches indicate bootstrap confidence estimates obtained with 1000 replicates. The scale bar represents an evolutionary distance (Knuc) of 0.01.

Strain IT-1 showed high sequence identity with *Stenotrophomonas* sp. BCc6 (99.3%), a known NPEO-degrading bacterium, and *Stenotrophomonas koreensis* TR6-01^T (97.6%). Strain IT-4 showed high sequence identity with *Sphingomonas* sp. PWE1 (99.8%), a known 4-*tert*-OP-degrading bacterium; and *Sphingobium* cloacae S-3^T (99.8%), a known branched 4-NP-degrading bacterium. Strain IT-5 also showed high sequence identity with *Sphingomonas* sp. PWE1 (99.2%) and *S. cloacae* S-3^T (99.1%). Thus, we identified strain IT-1 as *Stenotrophomonas* sp. and strains IT-4 and IT-5 as *Sphingobium* sp.

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EIMS spectral data of 4-*tert*-OP and its metabolites

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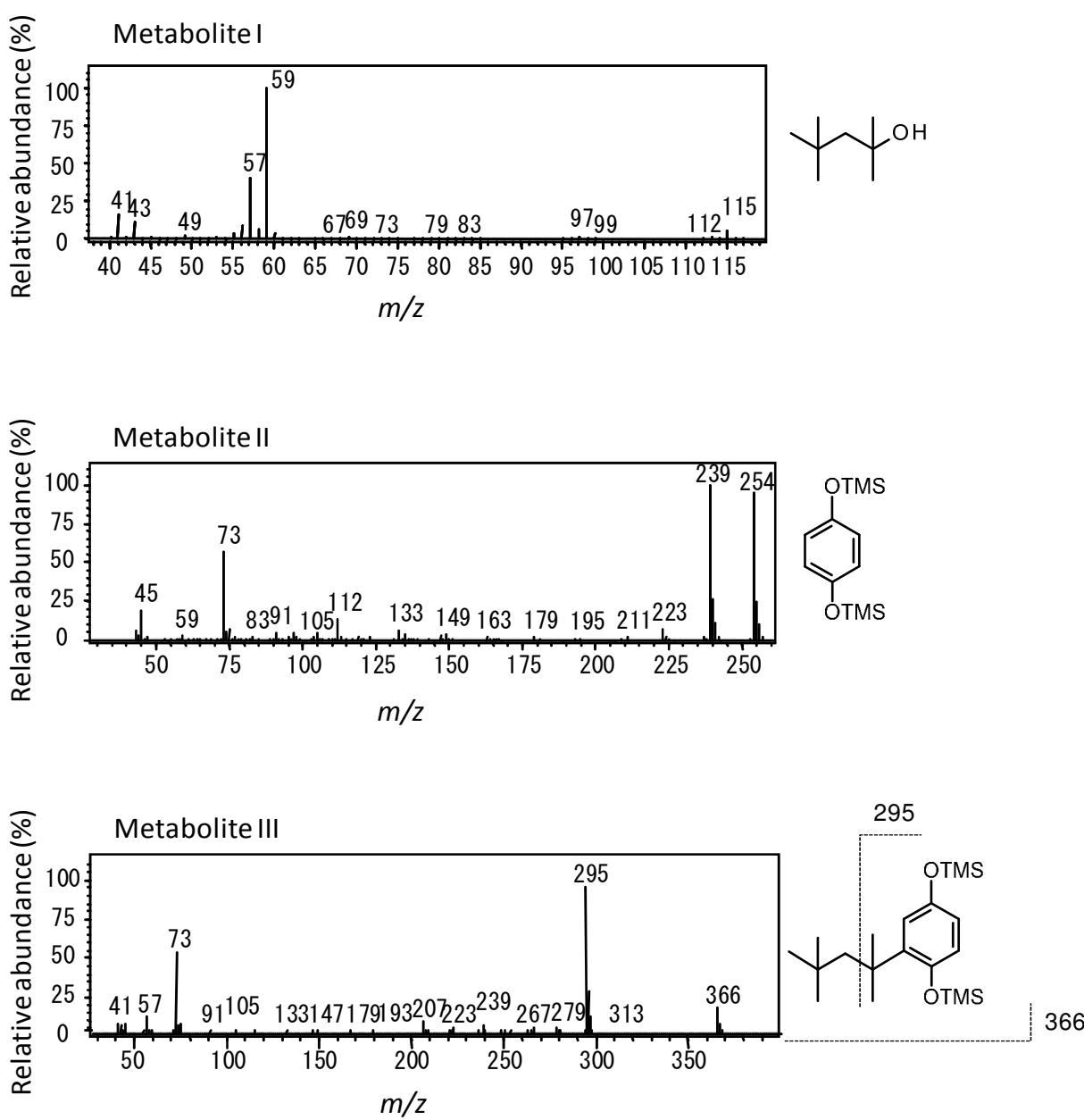


FIGURE S2. EIMS spectra of metabolites I, II, and III formed by 4-*tert*-OP degradation by isolated strains. (A) EIMS spectrum of metabolite I, identified as 2,2,4-trimethyl-1-pentanol, and its structure; (B) EIMS spectrum of trimethylsilyl (TMS) derivative metabolite II, identified as hydroquinone, and its structure; (C) EIMS spectrum of TMS derivative metabolite III, tentatively identified as 2-octylhydroquinone.

122 Utilization and degradability of APs by isolated three strains

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125 TABLE S1. Utilization and degradability of APs (2-*tert*-BP, 4-*n*-BP, 4-*tert*-BP, 4-*n*-OP, 4-*tert*-OP, 4-*n*-
126 NP, and tNP) by isolated three strains.

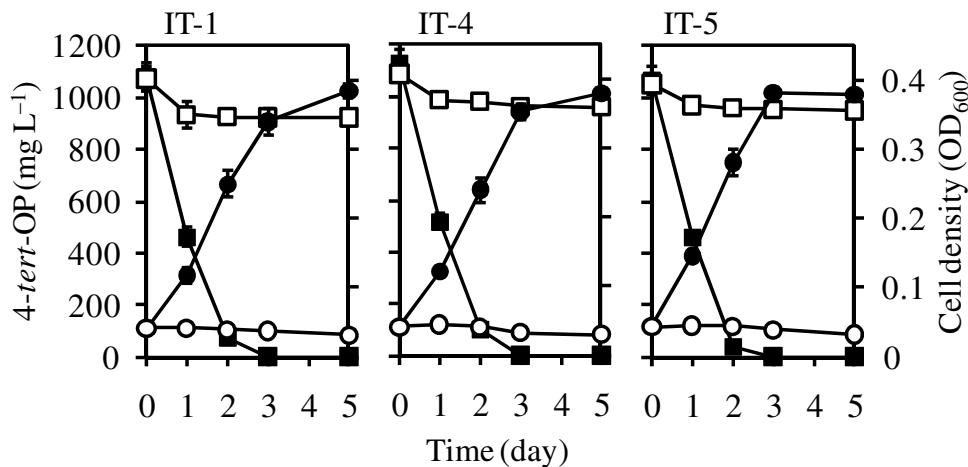
Substrate	Strain IT-1		Strain IT-4		Strain IT-5	
	Growth ^a	Transformation ratio (%) ^b	Growth ^a	Transformation ratio (%) ^b	Growth ^a	Transformation ratio (%) ^b
2- <i>tert</i> -BP	–	0	–	0	–	0
4- <i>n</i> -BP	–	8.1	–	70.2	–	0
4- <i>tert</i> -BP	–	16.5	–	98.9	–	40.3
4- <i>n</i> -OP	–	95.1	–	91.9	–	76.2
4- <i>tert</i> -OP	+	100	+	100	+	100
4- <i>n</i> -NP	–	25.6	–	44.7	–	46.5
tNP	+	87.8	+	92.6	+	82.1

127 ^a“+” indicates a substantial increase in cell density (OD₆₀₀) from the initial 0.02 to OD₆₀₀ > 0.05; “–”
128 indicates no substantial increase in cell density.129 ^bTransformation ratios (TR) were calculated from HPLC chromatograms obtained from 24-h cultures of
130 each strain whole cells and autoclaved sterile controls as follows: TR (%) = [1 – (substrate peak area
131 from whole-cell culture)/(substrate peak area from sterile control)] × 100.

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Degradation of 4-*tert*-OP and cell growth of the isolated strains under aerobic and

anaerobic conditions



147 FIGURE S3. Degradation of 4-*tert*-OP (squares) and cell growth of the isolated strains (circles) under
148 aerobic (closed symbols) and anaerobic (open symbols) conditions. Data are the means of triplicate
149 experiments, and the error bars indicate 95% confidence intervals.

150 Cell growth of isolates strains with root extract

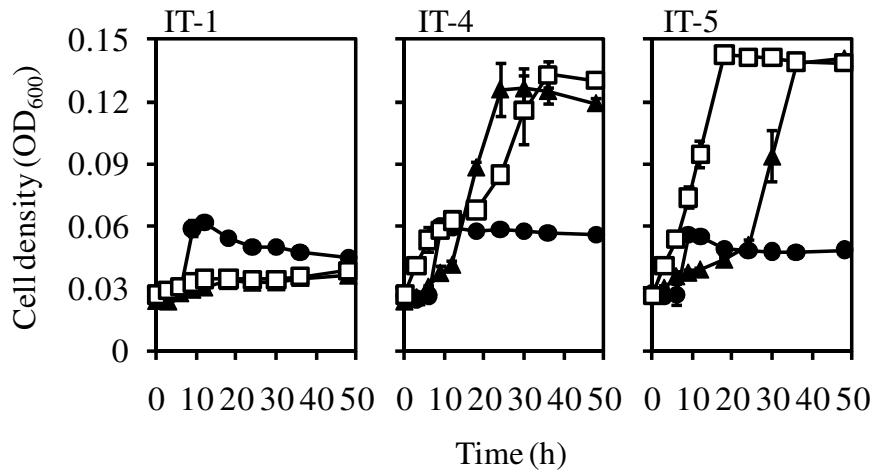


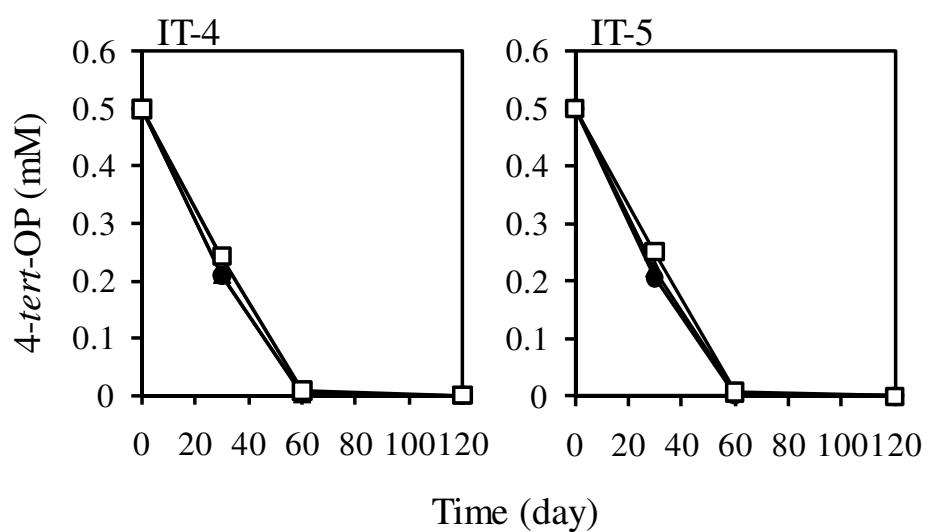
FIGURE S4. Cell growth of strains IT-1, IT-4, and IT-5. Circles represent cell densities in the presence of 4-*tert*-OP (1 mM); triangles represent cell densities in the presence of glucose (1 mM); and squares represent cell densities in the presence of root extract (140 mg-TOC L⁻¹). Data are the means of triplicate experiments, and the error bars indicate 95% confidence intervals.

166 Degradation of 4-*tert*-OP by the isolated strains grown with root extract

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178 FIGURE S5. Degradation of 4-*tert*-OP by the isolated strains grown on 4-*tert*-OP (circles), glucose

179 (triangles), or root extract (squares).

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Proposed pathway for the degradation of 4-*tert*-OP by the isolated strains

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194 FIGURE S6. Proposed pathway for the degradation of 4-*tert*-OP by the isolated strains. (A) 4-*tert*-OP;
 195 (B) putative *ipso*-hydroxylation intermediate (Appl. Environ. Microbiol. **2007**, 73, 7373–7379.); (C)
 196 hydroquinone; (D) 2,2,4,-trimethyl-1-pentanol; (E) 2-octylhydroquinone (tentative identification).
 197 Because hydroquinone and 2,2,4-trimethyl-1-pentanol were detected as metabolites produced by 4-*tert*-
 198 OP degradation by our three strains, we propose that a type II *ipso*-substitution mechanism is involved in
 199 degradation of NP and 4-*tert*-OP by our three strains. As in the case of strain TTNP3 (Appl. Microbiol.
 200 Biotechnol. **2006**, 72, 223–243; Appl. Environ. Microbiol. **2004**, 70, 6897–6900.), a NIH shift product
 201 (i.e., 2-*tert*-octylhydroquinone) was also detected as metabolites produced by 4-*tert*-OP degradation by
 202 our three strains.

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