

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).

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 ($9600 \times g$ 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.

37 Concentrations of tNP, 4-*tert*-OP, and other APs were determined by high-performance liquid
38 chromatography (HPLC). The metabolites produced by tNP and 4-*tert*-OP degradation were analyzed by
39 gas chromatography-mass spectrometry (GC-MS). In the experiments using tNP-spiked sediments and
40 4-*tert*-OP-spiked sediments, three vials from each treatment were sampled on each sampling day. The
41 upper parts of the plants (shoots and leaves) were separated from the roots, which were left in the
42 sediment, before extraction of tNP or 4-*tert*-OP. Both types of sediment (i.e., rhizosphere sediment with
43 roots and unvegetated sediment without roots) were acidified with 5 mL of salting-out solution (1 N HCl,
44 30% [w:v]), shaken with 20 mL of an ethyl acetate-*n*-hexane mixture (2:1, vol/vol) at 300 rpm for 20
45 min, sonicated in an ultrasonic bath (20 kHz, 200 W, 5-s intervals, 4 °C) for 20 min, and shaken again
46 for 20 min. Then, the organic layer was collected. The extract was dried under flowing nitrogen,
47 dissolved in an equal volume of acetonitrile, and analyzed by HPLC. In addition, the extract was directly
48 analyzed by GC-MS to determine the decrease in levels of NP isomers in the tNP mixture. We
49 confirmed that almost all of the tNP (more than 94%) and 4-*tert*-OP (more than 97%) was recovered
50 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.

Phylogenetic characterizations of isolated strains

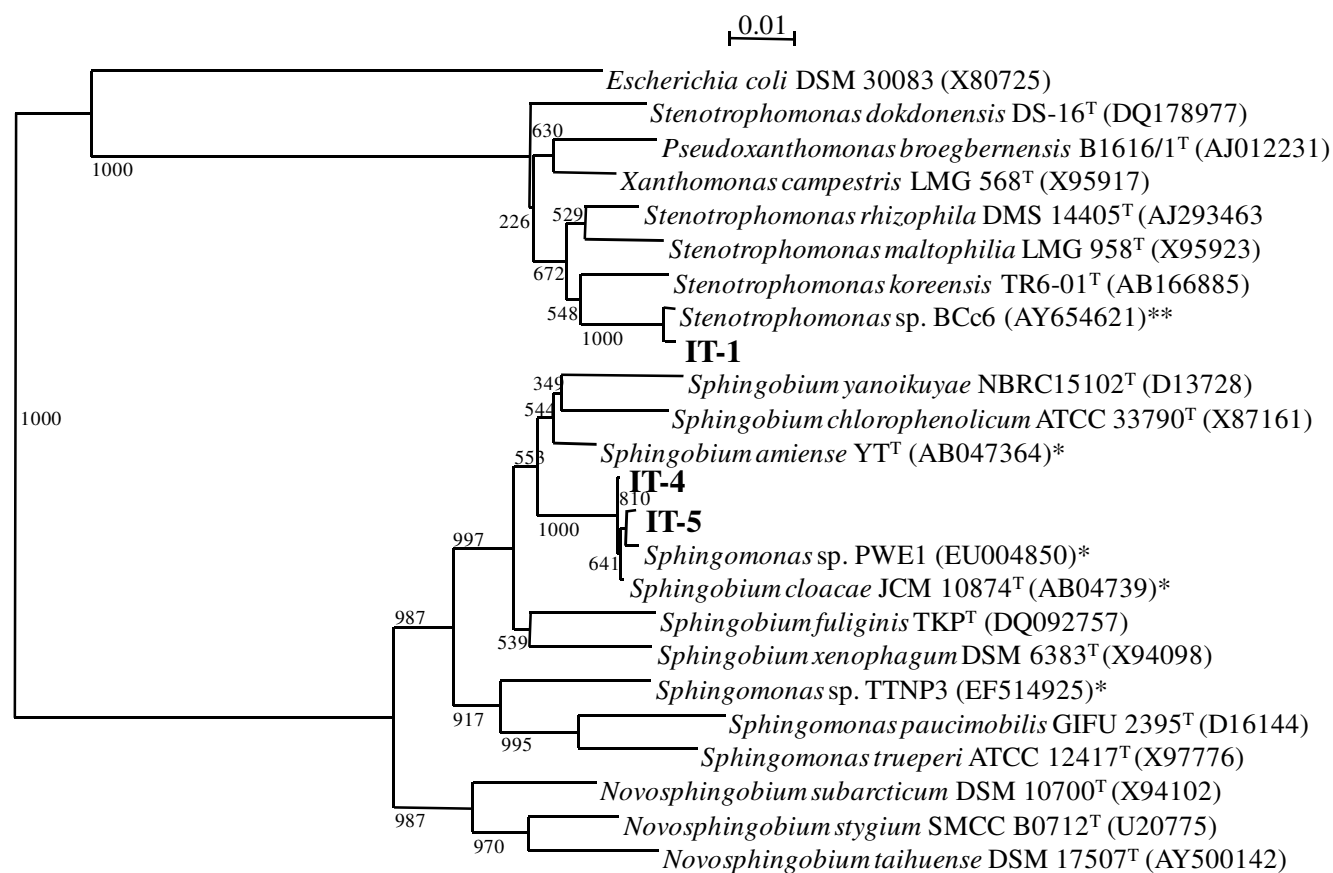


FIGURE S1. Phylogenetic relationships among strains IT-1, IT-4, and IT-5, type strains of *Sphingomonadaceae* and *Stenotrophomonas* sp., and previously isolated 4-NP-degrading (*), 4-*tert*-OP-degrading (*), and NPEO-degrading (**) bacterial strains, established by the neighbor-joining method using 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.

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

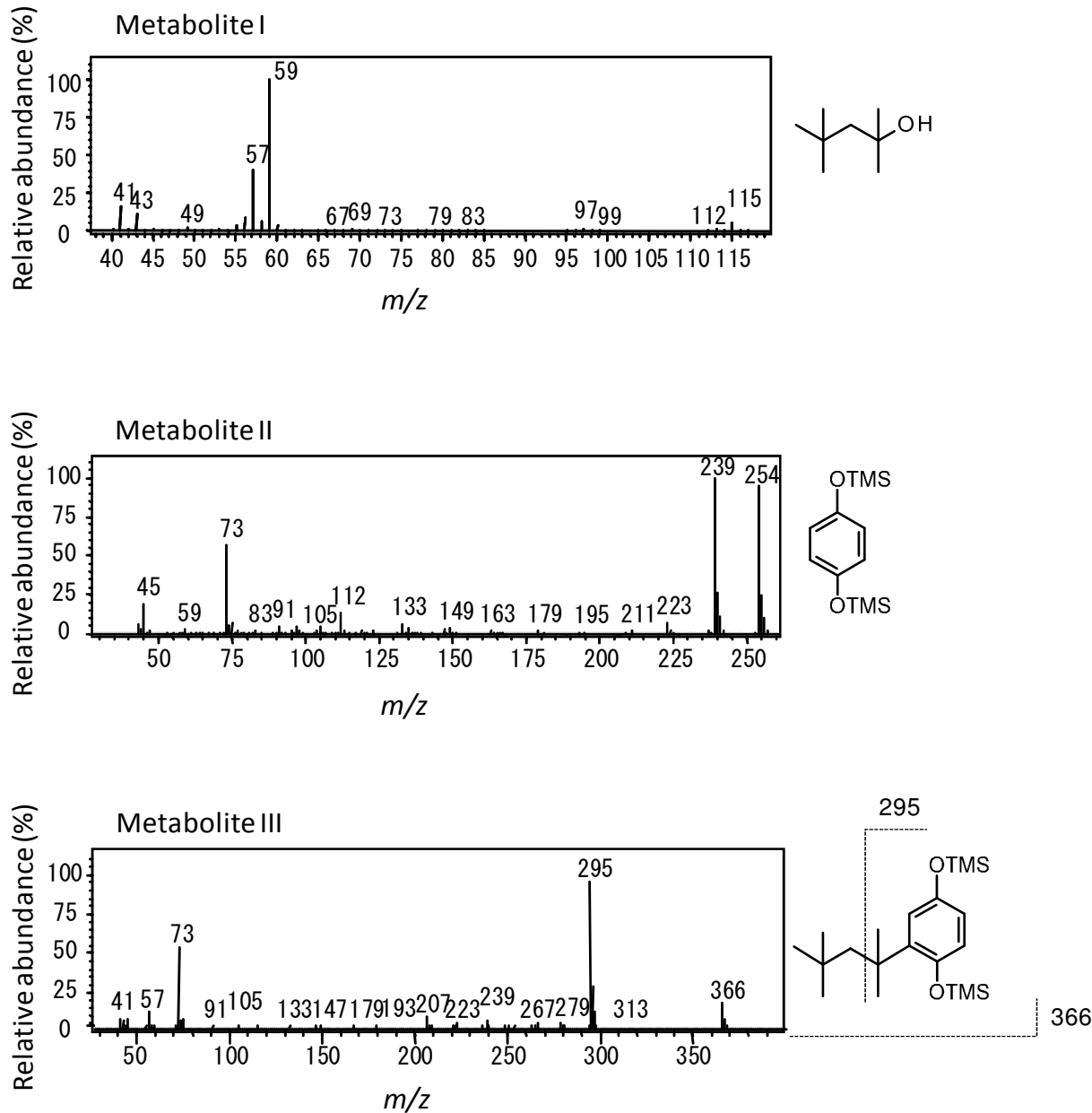


FIGURE S2. EIMS spectra of metabolites I, II, and III formed by 4-*tert*-OP degradation by isolated strains. (A) EIMS spectrum of metabolite I, identified as 2,2,4-trimethyl-1-pentanol, and its structure; (B) EIMS spectrum of trimethylsilyl (TMS) derivative metabolite II, identified as hydroquinone,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

Utilization and degradability of APs by isolated three strains

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

Substrate	Strain IT-1		Strain IT-4		Strain IT-5	
	Growth ^a	Transformation	Growth ^a	Transformation	Growth ^a	Transformation
		ratio (%) ^b		ratio (%) ^b		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

^a “+” indicates a substantial increase in cell density (OD₆₀₀) from the initial 0.02 to OD₆₀₀ > 0.05; “–” indicates no substantial increase in cell density.

^b Transformation ratios (TR) were calculated from HPLC chromatograms obtained from 24-h cultures of each strain whole cells and autoclaved sterile controls as follows: TR (%) = [1 – (substrate peak area from whole-cell culture)/(substrate peak area from sterile control)] × 100.

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

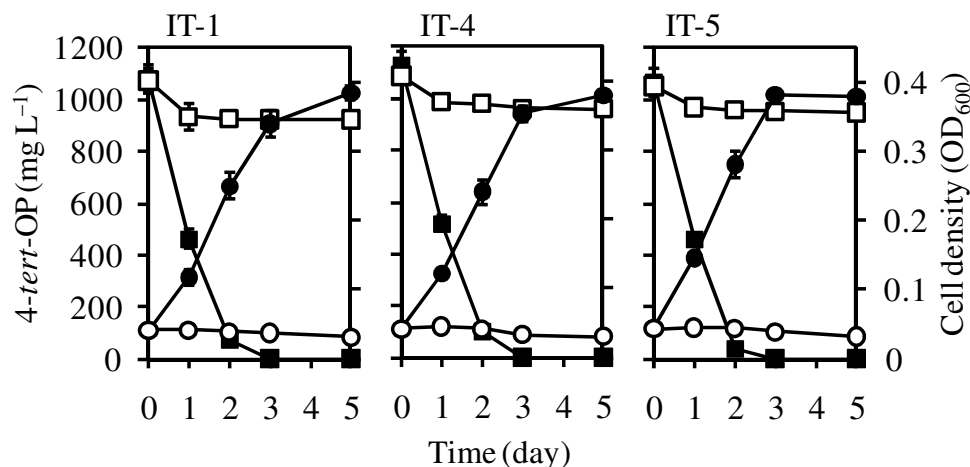


FIGURE S3. Degradation of 4-*tert*-OP (squares) and cell growth of the isolated strains (circles) under aerobic (closed symbols) and anaerobic (open symbols) conditions. Data are the means of triplicate experiments, and the error bars indicate 95% confidence intervals.

Cell growth of isolates strains with root extract

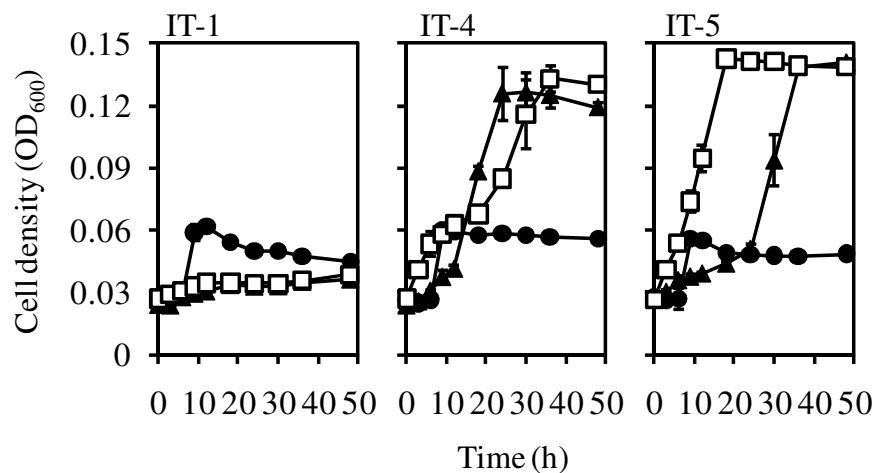


FIGURE S4. Cell growth of strains IT-1, IT-4, and IT-5. Circles represent cell densities in the presence of 4-tert-OP (1 mM); triangles represent cell densities in the presence of glucose (1 mM); and squares represent cell densities in the presence of root extract (140 mg-TOC L⁻¹). Data are the means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

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

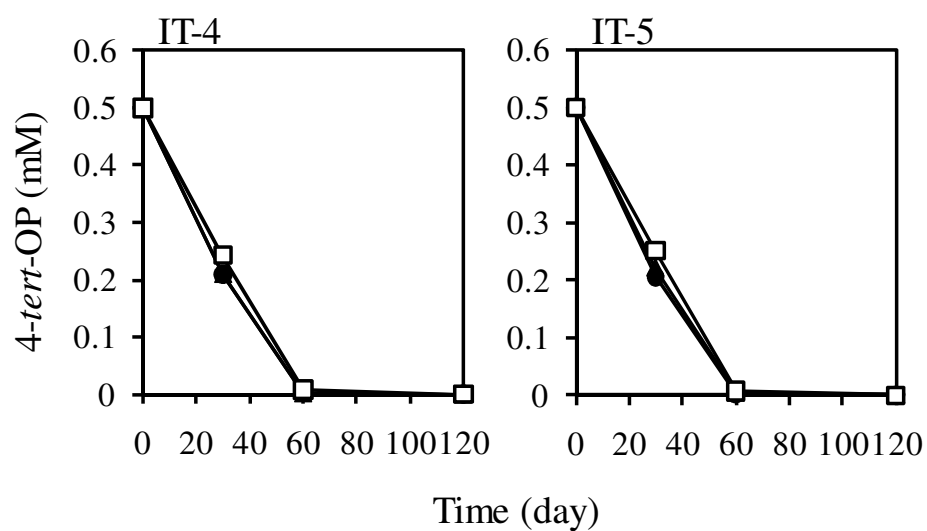


FIGURE S5. Degradation of 4-*tert*-OP by the isolated strains grown on 4-*tert*-OP (circles), glucose (triangles), or root extract (squares).

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

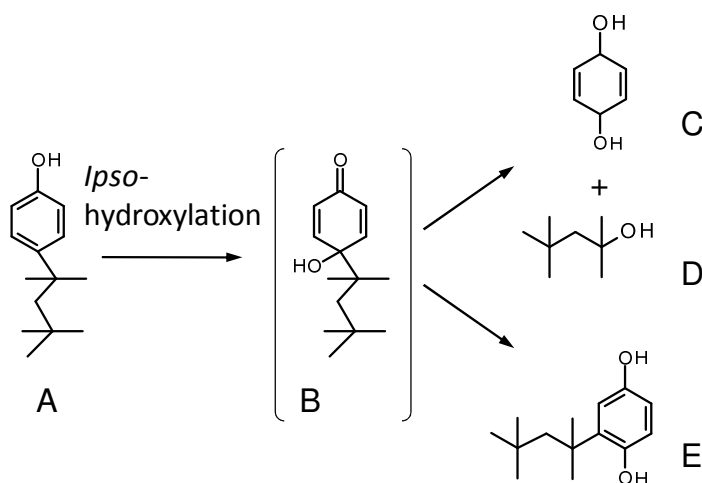


FIGURE S6. Proposed pathway for the degradation of 4-*tert*-OP by the isolated strains. (A) 4-*tert*-OP; (B) putative *ipso*-hydroxylation intermediate (Appl. Environ. Microbiol. **2007**, 73, 7373–7379.); (C) hydroquinone; (D) 2,2,4-trimethyl-1-pentanol; (E) 2-octylhydroquinone (tentative identification).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