INTRODUCTION

Nonylphenol (NP) polyethoxylates and octylphenol (OP) polyethoxylates have been used worldwide and in large quantities as non-ionic detergents mainly for industrial use. After they are used and discarded, they flow into sewage treatment plants where they are partially biodegraded into NP and OP (Soares et al., 2008; Ying et al., 2002). These compounds possess the ability to mimic the female hormone oestrogen by interacting with its receptor (Renner, 1997; White et al., 1994). Moreover, it has been reported that they are concentrated in living organisms where they can be detected at quantities as non-ionic detergents mainly for industrial use. After they are used and discarded, they flow into sewage treatment plants where they are partially biodegraded into NP and OP (Soares et al., 2008; Ying et al., 2002). These compounds possess the ability to mimic the female hormone oestrogen by interacting with its receptor (Renner, 1997; White et al., 1994). Moreover, it has been reported that they are concentrated in living organisms where they can be detected at concentrations of 411-nmol litre⁻¹ (Ahel et al., 1993; Ekelund et al., 1990). As a result, increasing attention has been paid to the environmental fates and biodegradability of NP and OP.

Since the late 1990s, biodegradation of NP and OP has been studied extensively using soils, sludges and bioreactors (Fujii et al., 2000; Hesselsøe et al., 2001; Mortensen & Kure, 2003; Soares et al., 2003; Tanghe et al., 1998; Yuan et al., 2004). NP degradation by pure cultures has also been studied to understand its biodegradation mechanisms. An NP-degrading yeast, Candida aquaealexoris (formerly Candida maltosa) LMAR 1 (Corti et al., 1995; Vallini et al., 1997), NP-degrading aquatic fungi with laccases (Junghanns et al., 2005) and several NP-degrading bacterial strains belonging to the sphingomonads (de Vries et al., 2001; Fujii et al., 2000, 2001; Gabriel et al., 2005a; Tanghe et al., 1999; Ushiba et al., 2003) have been isolated and characterized.

Two identical nonylphenol monooxygenase genes linked to IS6100 and some putative insertion sequence elements in Sphingomonas sp. NP5

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Two identical nonylphenol monooxygenase genes linked to IS6100 and some putative insertion sequence elements in Sphingomonas sp. NP5 can degrade a wide range of nonylphenol (NP) isomers that have widely contaminated aquatic environments as major endocrine-disrupting chemicals. To understand the biochemical and genetic backgrounds of NP degradation, a gene library of strain NP5 was constructed using a broad-host-range vector pBBR1MCS-2 and introduced into Sphingobium japonicum UT26. Several transformants accumulated reddish brown metabolites on agar plates dispersed with a mixture of NP isomers. Two different DNA fragments (7.6 and 9.3 kb) involved in the phenotype were isolated from the transformants. Sequence analysis revealed that both fragments contained an identical 1593 bp monooxygenase gene (nmoA), the predicted protein sequence of which showed 83% identity to the octylphenol-4-monooxygenase of Sphingomonas sp. PWE1. The nmoA gene in the 7.6 kb fragment was surrounded by an IS21-type insertion sequence (IS) and IS6100, while another in the 9.3 kb fragment was adjacent to an IS66-type IS, suggesting that they have been acquired through multiple transposition events. A fast-growing recombinant Pseudomonas putida strain harbouring nmoA was constructed and used for degradation of a chemically synthesized NP isomer, 4-(1-ethyl-1-methylhexyl)phenol. This strain converted the isomer into hydroquinone stoichiometrically. 3-Methyl-3-octanol, probably originating from the alkyl side chain, was also detected as the metabolite. These results indicate that these two nmoA genes are involved in the NP degradation ability of strain NP5.
intermediate, the aromatic moiety and the side chain are separated as hydroquinone and a carboxylation with nine carbon atoms (finally forming an alcohol or several aliphatic compounds), respectively. Alternatively, 4-alkoxyphenols and alkylhydroquinones are also formed by 1,2-CO shift and NIH shift subsequent to the ipso-hydroxylation, depending on the structure of the alkyl group (particularly branching at the α-carbon atom) (Corvini et al., 2006a, b; Gabriel et al., 2005a, b, 2007). Furthermore, it was found that this degradation mechanism could be expanded for degradation of a structural homologue, bisphenol A, as well as most NP isomers in technical-grade NP (Gabriel et al., 2008; Kolvenbach et al., 2007). Porter & Hay (2007) reported isolation of an OP-degrading bacterium, Sphingomonas sp. PWE1, and its OP 4-monoxygenase gene (opdA). A recombinant Escherichia coli strain harbouring opdA converted an OP isomer, 4-(2,4,4-trimethylpentyl)phenol, to hydroquinone and 2,4,4-trimethylpentene (or 2,4,4-trimethylpentanol). This indicated that the opdA-coded monoxygenase caused a very similar reaction to those reported in the NP degradation by strains TTNP3 and Bayram. Very recently, from strains TTNP3 and Bayram, each opdA homologue was cloned and OP degradation was confirmed using recombinant E. coli strains harbouring the opdA homologue (Porter et al., 2012). Therefore, these opdA homologues were designated opdABayram and opdATTNP3 (opdA from PWE1 was designated opdAPWE1). Sequence comparison revealed that the nucleotide sequence difference among these opdAs was within 7 bp in the 1605 bp coding regions. Although only the opdA sequences had been reported and registered within DNA databases, little genetic information around these opdAs has so far been provided. In addition, opdA-dependent NP degradation has not yet been shown.

In this study, we isolated an NP-degrading Sphingomonas strain from activated sludge and cloned two different DNA fragments from the isolate, which conferred NP degradation activity to a Sphingobium host strain. Both fragments encoded a 1593 bp identical monoxygenase gene (named nmoA), which existed between or beside some putative insertion sequence (IS) elements. In addition, by using a Pseudomonas clone harbouring nmoA, we achieved stoichiometric conversion of a chemically synthesized NP isomer to hydroquinone. Herein, we show the nature of bacterial NP monoxygenase genes with the surrounding sequences for the first time to our knowledge.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** Sphingomonas sp. NP5 was isolated in this study and used for NP degradation tests. Sphingobium japonicum UT26 (Nagata et al., 2007), Pseudomonas putida KT2440 (Bagdasarian et al., 1981) and E. coli JM109 (TaKaRa Bio) were used as hosts for gene cloning and expression. Strain JM109 was grown at 37 °C and other strains were grown at 30 °C. MSB medium consisting of (per litre) 1.0 g K2HPO4, 1.0 g (NH4)2SO4, 0.2 g MgSO4·7H2O, 0.02 g FeCl3, 0.1 g NaCl and 0.1 g CaCl2 (pH 7.6) was used as a mineral-salt medium. Luria–Bertani (LB) medium (Sambrook et al., 1989) and threefold-diluted LB medium (1/3 LB) were used as rich media. Ampicillin and kanamycin (Km) were added to the media at 100 and 50 mg l−1, respectively, when necessary.

**Isolation and identification of NP-degrading bacteria.** As a bacterial source, an activated sludge sample was taken from the Wakagawa final treatment facility (Wakayama city, Japan). One millilitre of the activated sludge sample was added to 100 ml MSB medium supplemented with 300 mg l−1 of a commercial NP reagent (‘p-Nonylphenol; Kanto Chemical), which is a mixture of NP isomers (hereafter abbreviated as NPmix), in a 300 ml flask. The culture was incubated at 30 °C and at 120 r.p.m. on a rotary shaker. Every 2 weeks, 1 ml of the culture was transferred to 100 ml of the same fresh medium and the culture was incubated in the same way. After 6 weeks, aliquots of the culture were spread on MSB agar plates, in which 1 g NPmix l−1 was dispersed, and incubated at 30 °C for several days. For preliminary bacterial identification, the 16S rRNA gene sequence of the isolate was analysed as described previously (Takeo et al., 2003).

**Chemical synthesis of 4-(1-ethyl-1-methylhexyl)phenol.** A mixture of 3-methyl-3-octanol (27.7 mmol) and 48% HBr (5 ml) was refluxed for 1 h, and then chilled in an ice bath. The mixture was extracted with ethanol, washed with ice-cold water, ice-cold 40% methanol, ice-cold 10% NaHCO3 and ice-cold water in turn, and then dried over Na2SO4. After evaporation of the solvent, distillation under reduced pressure (22 mmHg, boiling point 89–93 °C) yielded a colourless oil, which was presumed to be 3-bromo-3-methylheptane (8.88 mmol) due to the similarity of the boiling point to that of 3-bromo-3,6-dimethylheptane (83–104°C at 45 mmHg) (Lalah et al., 2001). AlCl3 (8.14 mmol) was added to anisole (46.2 mmol) and the mixture was stirred at room temperature until the AlCl3 was dissolved. After cooling in an ice bath, the colourless oil (putative 3-bromo-3-methylheptane, 7.96 mmol) in CH2Cl2 (5 ml) was added to the mixture. The mixture was stirred and kept below 20 °C for 22.5 h. Crushed ice was then added to the reaction mixture to hydrolyse the components. The organic layer was separated and the aqueous layer was extracted with CHCl3. The combined organic layer was washed with saturated NaCl solution, dilute K2CO3 solution and water, and was then dried over Na2SO4. The solvents were removed under vacuum. The residue was observed by chromatography on silica gel with CHCl3/n-hexane (1:3) and the structure of the product, 4-(1-ethyl-1-methylhexyl)anisolole (5.93 mmol), was confirmed by NMR analysis (Table 1). BBr3 (12.6 mmol) was added dropwise to an ice-cold solution of the anisole (5.93 mmol) in CH2Cl2 (20 ml). The mixture was stirred and kept at 0 °C for 1.5 h, and then hydrolysed with water by adding it dropwise. The organic layer was separated, washed with saturated NaCl solution and dried over Na2SO4. After evaporation of the solvent, the residue was observed via chromatography on silica gel with CHCl3/ethyl acetate (10:1), and the structure of the final product, 4-(1-ethyl-1-methylhexyl)phenol (411-NP) (3.83 mmol), was confirmed by chemical analyses (Table 1). The reagents used in this synthesis were purchased from the following companies: 3-methyl-3-octanol (Sigma-Aldrich Japan); 48% HBr and silica gel (Wako Pure Chemical Industries); NaHCO3, K2CO3, CH3Cl2, CHCl3 and n-hexane (San‘ei Kako); Na2SO4 (Shikoku Chemicals); AlCl3 (Kanto Chemical); anisole (Tokyo Chemical Industry); NaCl (Naikai Salt Industries); and BBr3 (Merck).

**NP degradation tests and chemical analyses.** NP degradation tests were carried out using strain NP5 or recombinant strains. Bacterial cells from an agar plate were inoculated into LB medium (containing Km for recombinant strains) and incubated for 5 days for strain NP5, or for 3 days for recombinant Sphingobium strains or overnight for recombinant Pseudomonas strains. Cells were collected...
Table 1. Analytical data on chemical compounds used and metabolites detected

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>RT (min) in HPLC analysis</th>
<th>RT (min) in GC/MS analysis</th>
<th>IR (cm⁻¹, in KBr)</th>
<th>MS (m/z, relative intensity)</th>
<th>¹H-NMR (p.p.m. in CDCl₃ at 500 MHz)</th>
<th>¹³C-NMR (p.p.m. in CDCl₃ at 126 MHz)</th>
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<tr>
<td>411-NP</td>
<td><img src="image" alt="Structural formula" /></td>
<td>20.0</td>
<td>10.4</td>
<td>3344.3, 2931.6, 2929.7, 1612.4, 1514.0, 1458.1, 1228.6, 1182.3, 827.4</td>
<td>41 (22), 77 (10), 91 (9), 107 (100), 135 (47), 191 (8), 220 (M⁺, 4)</td>
<td>0.65 (t, J=6.8 Hz, 3H, 3CH₃), 0.82 (t, J=6.3 Hz, 3H, CH₃), 0.92-1.24 (m, 9H, 3CH₂+CH₃), 1.43-1.51 (m, 2H, CH₂), 1.60-1.69 (m, 2H, CH₂), 5.27 (brs, 1H, OH), 6.77 (d, J=8.5 Hz, 2H, ArH), 7.12 (d, J=8.5 Hz, 2H, ArH)</td>
<td>8.59, 14.07, 22.56, 23.53, 23.83, 32.64, 35.63, 40.33, 42.98, 114.69, 127.60, 140.33, 152.70</td>
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<tr>
<td>4-(1-Ethyl-1-methyl-hexyl)anisole</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.64-0.69 (m, 3H, CH₃), 0.80-0.85 (m, 3H, CH₃), 0.93-1.27 (m, 9H, 3CH₂+CH₃), 1.47-1.56 (m, 2H, CH₂), 1.62-1.72 (m, 2H, CH₂), 3.76 (s, 3H, OCH₃), 6.81-6.86 (m, AA’BB’, 2H, ArH), 7.16-7.21 (m, AA’BB’, 2H, ArH)</td>
<td>8.60, 14.06, 22.58, 23.54, 23.88, 32.69, 35.68, 40.29, 43.03, 54.94, 113.13, 127.32, 139.91, 157.07</td>
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Table 1. cont.

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<th>Compound</th>
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<th>MS (m/z, relative intensity)</th>
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<th>¹³C-NMR (p.p.m. in CDCl₃ at 270 MHz)</th>
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<td>Hydroquinone</td>
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<td>4.7</td>
<td>7.0</td>
<td>30 m</td>
<td>254 nm (or 277 nm for hydroquinone)</td>
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<tr>
<td>Metabolite IV</td>
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<td>7.0</td>
<td>7.0</td>
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</table>

Cloning of NP monooxygenase genes. Total DNA of strain NP5 extracted using phenol treatment (Saito & Miura, 1963) was partially digested with Sau3AI (TaKaRa Bio) and resolved on a 0.8 % (w/v) SeaPlaque GTG agarose gel (TaKaRa Bio). DNA fragments ranging from 6 to 23 kb were isolated from the gel according to the protocol for the use of the agarose. They were ligated at 16 °C for 1 h using a TaKaRa DNA ligation kit ver. 2.0 (TaKaRa Bio) to a broad-host-range vector, pBBR1MCS-2 (5.1 kb, Km-resistant, mob) (Kovach et al., 1995), which had been digested with BamHI and dephosphorylated with a bacterial alkaline phosphatase (TaKaRa Bio) according to the supplier’s instructions. Then, the mixture was used to transform E.
coli JM109 and transformants were screened on LB agar plates containing Km. Aliquots of 100 mM IPTG aqueous solution and 40 mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside 1 M in DMSO were spread on the surface for blue/white colony selection. Colonies of the transformants that appeared were suspended with small amounts of sterilized water on the plates. They were then recovered from the plates and subjected to plasmid extraction by the alkaline lysis method (Sambrook et al., 1989). The extracted plasmid DNA was introduced into Sphingobium japonicum UT26 by electroporation as described previously (Takeo et al., 2003). For electroporation, UT26 cells were prepared by suspending the colonies grown overnight on 1/3 LB agar plates with ice-cold sterilized water at OD600 4.5. After electroporation, the cell suspension was 10-fold diluted with 1/3 LB medium and incubated at 30 °C and at 160 r.p.m. on a rotary shaker for 1.5 h. As screening plates, 1/3 LB plates containing Km and NPmix (1 g l⁻¹), which were further overlaid with the same agar without NPmix to minimize the toxicity of NPmix, were prepared and used.

General molecular protocols, such as restriction digestion and transformation, were carried out according to Sambrook et al. (1989). Sequencing and sequence analyses were carried out as described previously (Takeo et al., 2006). A custom-sequencing service (Macrogen) was also used to confirm the result of the sequencing.

Subcloning of the NP monoxygenase gene. The NP monoxygenase gene (nmoA) of strain NP5 was amplified by PCR using a pair of primers, NMO-F-BAM-A (5′-CGTGGATCCAGAAGAGCACAAGCAGAATAGCC-3′) and NMO-R-BAM-A (5′-CGCTAGCTTATCCAAGCGGTGTGCAAG-3′), in the following reaction mixture: 10 ng template DNA, 10 μmol each primer, 2.5 mM each dNTP, 5 μl 10× ExTaq buffer and 0.5 U ExTaq polymerase (TaKaRa Bio) in a total volume of 50 μl. The PCR conditions employed were as follows: 95 °C for 3 min; 25 cycles of 95 °C for 1 min, 50 °C for 45 s and 72 °C for 2 min; and 72 °C for 5 min. The amplified PCR product was digested with BamHI and electrophoretically purified using a 0.8 % (w/v) agarose gel and a Suprec-01 spin column (TaKaRa Bio). Cloning of the recovered DNA fragments into pBBR1MCS-2 was performed as described above and the resultant plasmid was introduced into P. putida KT2440 by electroporation.

Nucleotide sequence accession numbers. The nucleotide sequences of the partial 16S rRNA gene and the insert fragments of pNAP2 and pNAP5 including nmoA were deposited in the DDBJ/GenBank/EMBL databases under accession nos AB518068, AB519148 and AB519680, respectively.

RESULTS AND DISCUSSION

Isolation of NP-degrading bacterium

Enrichment cultivation for 6 weeks and selection using NPmix-dispersed plates resulted in the appearance of small white colonies on the plates and formation of clear zones around the colonies. This suggests that the colonies degraded NPmix around them and used it as a sole carbon source. We then purified a single strain from the colonies by repeatedly streaking them on the same plate and designated it strain NP5. In the MSB liquid medium including NPmix (0.2 mM), this strain grew from OD600 0.05 to 0.20 in 1 week, but growth then stopped. An additional spike of NPmix (0.2 mM) did not support further growth. Such low growth on NP or OP has been observed in the degradation by other degraders (Porter & Hay, 2007; Tanghe et al., 1999). This might be caused by the toxicity of the metabolites from NPmix. To confirm NP degradation by this strain, NPmix degradation was carried out using the cell suspension of strain NP5. GC/MS analysis of the culture extracts showed that many peaks of m/z = 220 (the molecular ion of NP), corresponding to various NP isomers in NPmix were detected in the 5-day culture at a retention time (RT) of 8–10 min (Fig. 1a). In the control culture without cells, Metabolite I and Metabolite II detected in Fig. 1(a) showed almost identical mass fragment patterns to those of authentic C9 alcohols, 3-nonanol and 2,3-dimethyl-3-heptanol, respectively (Table 1). These results suggest that strain NP5 degraded a wide range of NP isomers in NPmix with the release of their side chains as C9 alcohols.

For preliminary identification of strain NP5, the partial sequence of the 16S rRNA gene was amplified by PCR, cloned and sequenced. The 1409 bp sequence determined showed significant identity to those of γ-hexachlorocyclohexane-degrading Sphingomonas strains Alpha4-5 (accession no. AT771798, nucleotide sequence identity 99 %), Alpha1-2 (AT771794, 99 %) and Alpha4-2 (AY771797, 98 %), and an oestrogen-degrading bacterium, Sphingomonas sp. JEM-2.
(AB219360, 98 %). It also showed considerable identity to that of an NP-degrading bacterium Sphingomonas sp. TTNP3 (EF514925, 97 %), but lower identity to those of other NP- or OP-degrading bacteria, Sphingobium cloacae S-3 (NR_027233, 94 %), Sphingobium amiense Y (NR_028622, 95 %) and Sphingomonas sp. PWE1 (EU004850, 94 %). From this analysis, we tentatively identified strain NP5 as representing a Sphingomonas sp.

**Cloning of genes involved in NP degradation**

To understand the genetic background of NP degradation by this strain, a gene library of NP5 was constructed using a broad-host-range vector pBBR1MCS-2, and introduced into E. coli JM109 by transformation. More than 2000 transformants appeared on LB agar plates including Km. Plasmid DNA was extracted from 20 randomly chosen transformants and the sizes of the insert fragments were checked by restriction analysis. All the plasmids had insert fragments whose average size was 7.0 kb. Then, all the colonies that had appeared on the plates were collected together from the plates and plasmid DNA was extracted from the cells to obtain a plasmid library. As the chromosome size of a taxonomically close Sphingomonas strain, Sphingomonas wittichii RW1, is 5382 kb (accession no. NC_009511), this plasmid library (>14,000 kb in total) can be considered to cover most of the NP5 chromosome. Subsequently, for the efficient expression of Sphingomonas genes, the plasmid library was introduced into Sphingobium japonicum UT26 (Nagata et al., 2007) by electroporation, which shows high electroporation efficiency. After electroporation, aliquots of the culture were spread on 1/3 LB agar plates containing Km. Many transformants appeared, layer-like, on the plates [>2 x 10^4 transformants (µg DNA)^-1]. However, when the same plates supplemented with a high concentration of NP_mix (1 g l^-1) were used for screening, no transformants were observed due to the toxicity of NP_mix. To alleviate the toxicity, we employed 1/3 LB plates containing Km and NP_mix (1 g l^-1) overlaid with the same agar without NP_mix. Consequently, 19 colonies appeared on two screening plates. All of the colonies accumulated reddish brown compounds, suggesting that these colonies might reduce the toxicity of NP_mix by degrading NP_mix around them and survive on the plates. Plasmids were extracted from the transformants and their insert fragments were analysed by restriction enzymes. As a result, it was found that they converged on five plasmids, named pNAP1 to pNAP5 (Fig. S1, available with the online version of this paper). pNAP1 and pNAP2 shared a common 5 kb DNA region, which conferred the colour phenotype to the host strain again. pNAP4 and pNAP5 also shared a common 7 kb DNA region, which enabled the host strain to accumulate the reddish brown compounds from NP_mix, but was different from the former 5 kb region. pNAP3 had a unique DNA fragment different from the other pNAP plasmids, but we were unable to reproduce the colour phenotype using pNAP3, suggesting that the essential region for the phenotype had been lost. These results indicate that strain NP5 has at least two copies of a key gene(s) involved in NP degradation at different loci on the genome (or plasmids). Thus, the UT26 transformants harbouring pNAP2 or pNAP5 were selected and used for further studies.

**Degradability of 411-NP**

To confirm the NP degradability of the transformants, one of the abundant NP isomers found in technical-grade NPs from commercial companies (Tokyo Chemical Industry, Aldrich, Fluka and Acros) (Katase et al., 2008), 411-NP, was chemically synthesized and its structure was confirmed by chemical analyses (Table 1). 411-NP degradation tests were carried out using the cell suspensions of strain NP5, strain UT26 and the UT26 transformants. As we had not detected any great decrease (>2 %) in 411-NP concentration in the culture supernatants of the UT26 transformants by HPLC analysis, we checked metabolite formation from 411-NP by GC/MS analysis. In the culture extract of the host strain UT26, 411-NP was detected but no metabolites were detected by GC/MS analysis (Fig. 2b). In contrast, in the culture extract of strain NP5, one metabolite (Metabolite III-1) was detected at an RT of 4.7 mins in the GC/MS chromatogram (Fig. 2a), although 411-NP was not detected due to complete degradation. The mass spectrum of Metabolite III-1 was in good agreement with that of authentic 3-methyl-3-octanol (Table 1), which is an alcoholic form of the alkyl side chain of 411-NP. Metabolites with an identical RT to that of Metabolite III-1 were detected in the culture extracts of the UT26 transformants harbouning pNAP2 or pNAP5 (Fig. 2c, d). The mass fragment patterns were also almost identical.
to those of authentic 3-methyl-3-octanol (for example, Metabolite III-2 in Fig. 2c and Table 1). Thus, we concluded that genes encoding the side-chain-releasing activity from 411-NP resided on both insert fragments of pNAP2 and pNAP5.

**Sequence analysis of the insert fragments of pNAP2 and pNAP5**

pNAP2 and pNAP5 had 7.6 and 9.3 kb DNA fragments, respectively. Sequence analysis revealed the presence of a 3121 bp segment that was completely identical in both fragments (Fig. 3). Homology searches indicated that a 1593 bp open reading frame (ORF) (named *nmoA*) with 74% nucleotide sequence identity to *opdAs* existed in the common segment. The DNA G+C content of *nmoA* was 51.3 mol%, considerably lower than those of the surrounding sequences (62.5 mol% for the insert of pNAP2 and 63.0 mol% for the insert of pNAP5 except *nmoA*). It was also lower than those of *opdAs* (56.5 mol%). The DNA G+C content of bacteria belonging to the genus *Sphingomonas* is in the range 59.0–67.2 mol% (Yabuuchi & Kosako, 2005). Therefore, this analysis suggests that *nmoA* might have come from other bacterial species of lower DNA G+C content. The *nmoA* gene was 5 bp preceded by a putative ribosome-binding sequence (5'–AGGAAG–3'), but

**Fig. 3.** Structures of recombinant plasmids pNAP2 and pNAP5, and putative genes and genetic elements including nonylphenol mono-oxygenase genes (*nmoAs*) on the inserts of the plasmids. Arrow-type boxes indicate complete or incomplete ORFs (orf's) and closed boxes show a broad-host-range vector pBBR1MCS-2. An asterisk indicates an incomplete ORF without a start codon, while delta marks indicate truncated ORFs. Arrowheads indicate the lac promoter (lacP) of the vector.

**Fig. 4.** Phylogenetic tree among flavoprotein mono-oxygenases including the predicted nonylphenol mono-oxygenase sequence (NmoA) of *Sphingomonas* sp. NP5 and the predicted octylphenol mono-oxygenase sequences (*opdAs*) of *Sphingomonas* sp. PWE1, *Sphingomonas* sp. TTN3 and *Sphingobium xenophagum* Bayram (a) and partial multiple alignment of the NmoA and OpdAs (b). The tree was constructed using the UPGMA method of GENETYX-MAC ver.16 (GENETYX) and the alignment was also done using the same software. Accession numbers and amino acid sequence identity with NmoA are shown in parentheses. Asterisks indicate conserved amino acid residues. Bold letters indicate the fingerprints for the binding of the ADP- and riboflavin-moieties of FAD reported by Wierenga et al. (1986) and Eppink et al. (1997). In the ADP-binding motif, Δ indicates K, R, H, S, T, Q or D, + indicates A, I, L, V, M, C or Y, and Δ indicates D or E. Also, a, b and TURN represent the secondary structures α-helix, β-sheet and turn, respectively. 3HPPH, 3-(3-hydroxyphenyl)propionate hydroxylase; FADM, FAD-binding (or FAD-dependent) mono-oxygenase; PKH, polyketide hydroxylase; C, Comamonas; R, Ralstonia; Myx, Myxococcus; St, Stigmatella; G, Gordonia; Myc, Mycobacterium; X, Xanthobacter; A, Amycolatopsis; T, Thermomonospora; N, Nocardioidea; Spb, Sphingobium.
no typical E. coli σ70-type promoter sequences were detected in the 152 bp flanking region common to pNAP2 and pNAP5. It was predicted to encode a protein (531 aa residues with a predicted molecular mass of 59.7 kDa) with 83% amino acid sequence identity to the OP 4-monoxygenase of Sphingomonas sp. PWE1 (opdA<sub>PWE1</sub> gene product, EU002557). Porter & Hay (2007) reported that a recombinant E. coli strain harbouring opdA<sub>PWE1</sub> was able to degrade an OP isomer, 4-(2',4',4'-trimethylpentyl)phenol. More recently, Porter et al. (2012) successfully cloned an opdA homologue (opdA<sub>Bayram</sub> and opdA<sub>TTNP3</sub>) from NP-degrading strains Bayram and TTNP3, respectively. The predicted protein sequences from opdA<sub>Bayram</sub> and opdA<sub>TTNP3</sub> showed 100 and 99% amino acid sequence identity to that from opdA<sub>PWE1</sub>, respectively. In contrast, the nmoA-coded protein showed lower identity to these opdA-coded proteins (83.0–83.4%). Fig. 4(a) shows a phylogenetic tree constructed based on the predicted protein sequences of nmoA and opdAs, and the amino acid sequences of their homologous flavoprotein monoxygenases. This figure clearly shows that these nmoA- and opdA-coded proteins are clustered and have been evolutionarily separated from other monoxygenases. However, nmoA seems to be somewhat different from these opdAs due to the difference in DNA G+C content and amino acid sequence identity mentioned above.

The nmoA-coded protein also showed around 30% amino acid sequence identity to bacterial 3-(3-hydroxyphenyl)-propionate hydroxylases and other FAD-binding monoxygenases (for example enzymes abbreviated as 3HPPH and FADM, respectively, in Fig. 4a). According to the classification of flavoprotein monoxygenases suggested by van Berkel et al. (2006), these monoxygenases can be classified within class A, members of which are encoded by a single gene and structurally composed of one dinucleotide-binding domain (Rossmann ββα fold) binding to FAD. As shown in Fig. 4(b), the ADP-binding and FAD-binding motifs (Ferrández et al., 1997; DiMarco et al., 1993) were also conserved in the N terminus and central regions of the nmoA-coded protein, respectively. The fingerprints for interacting directly with the ADP- and riboflavin-moieties of FAD (bold letters shown in Fig. 4b; Wierenga et al., 1986; Eppink et al., 1997) were also detected in the nmoA-coded protein.

In the insert fragment of pNAP2, the 3121 bp common segment including nmoA was surrounded by two putative

### Table 2. Analysis of gene candidates detected in the inserts of pNAP2 and pNAP5

<table>
<thead>
<tr>
<th>Name in Fig. 3</th>
<th>No. of nucleotides (aa)</th>
<th>DNA G+C content (mol%)</th>
<th>Putative function</th>
<th>Homologous protein (amino acid sequence identity, accession no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δorf1</td>
<td>1239 (413)</td>
<td>63.9</td>
<td>Transposase</td>
<td>IS21 family transposase from Sphingomonas sp. KA1 (83.8%, BAFO3490). Transposase from Sphingomonas wittichii RW1 (83.3%, ABQ71600).</td>
</tr>
<tr>
<td>orf2</td>
<td>765 (255)</td>
<td>63.1</td>
<td>Transposase (helper protein)</td>
<td>IstB ATP-binding domain-containing protein from Sphingomonas wittichii RW1 (88.6%, ABQ71601). IS21 family transposase from Sphingomonas sp. KA1 (88.6%, BAFO3941).</td>
</tr>
<tr>
<td>orf3 (nmoA)</td>
<td>1593 (531)</td>
<td>51.3</td>
<td>NP monoxygenase</td>
<td>OP monoxygenase from Sphingomonas sp. PWE1 (83.4%, ABU98341). OP monoxygenase from Sphingobium xenophagum Bayram (83.4%, ACT12953). OP monoxygenase from Sphingomonas sp. TTNP3 (83.0%, ACII2952).</td>
</tr>
<tr>
<td>orf4</td>
<td>792 (264)</td>
<td>61.1</td>
<td>Transposase</td>
<td>Transposase for insertion sequence IS6100 from E. coli A (100%, ACQ42062). Transposase from Acinetobacter baumannii (100%, CAJ77056).</td>
</tr>
<tr>
<td>orf5</td>
<td>1221 (407)</td>
<td>63.9</td>
<td>Alcohol dehydrogenase</td>
<td>Alcohol dehydrogenase zinc-binding domain protein from Mycobacterium rhodesiae JS60 (32.6%, EHB45716). Alcohol dehydrogenase zinc-binding domain protein from Frankia sp. EAN1pec (ABW16175, 31.6%).</td>
</tr>
<tr>
<td>orf6 (nmoA)</td>
<td>1593 (531)</td>
<td>51.3</td>
<td>NP monoxygenase</td>
<td>OP monoxygenase from Sphingomonas sp. PWE1 (83.4%, ABU98341). OP monoxygenase from Sphingobium xenophagum Bayram (83.4%, ACT12953). OP monoxygenase from Sphingomonas sp. TTNP3 (83.0%, ACII2952).</td>
</tr>
<tr>
<td>orf7</td>
<td>1608 (536)</td>
<td>67.2</td>
<td>Transposase</td>
<td>IS66 family transposase from Methyllobacterium nodulans ORS 2060 (51.5%, ACL62505). Transposase from Azorhizobium caulinodans ORS 571 (50.0%, BAFO87283).</td>
</tr>
<tr>
<td>orf8</td>
<td>372 (124)</td>
<td>60.8</td>
<td>Transposase</td>
<td>IS66 family orf2 protein from Methyllobacterium nodulans ORS 2060 (55.9%, ACL62506). Transposase from Azorhizobium caulinodans ORS 571 (53.4%, BAFO87282).</td>
</tr>
</tbody>
</table>
ISs (Fig. 3). One of them, just upstream of nmoA, contained two ORFs (orf1 and orf2) encoding putative transposases similar to those of known IS21 family ISs (Berger & Haas, 2001) (Table 2), while another with orf4, downstream of nmoA, was identical to IS6100 originally found in Tn610 of Mycobacterium fortuitum FC1 (Martin et al., 1990). In contrast, in the insert fragment of pNAP5, a different putative IS was found downstream of nmoA (Fig. 3). This IS contained two putative transposase genes (orf7 and orf8), the protein sequences of which were homologous to those of known IS66 family ISs (Han et al., 2001) (Table 2). In pNAP2, this IS66-like IS was disrupted by the insertion of IS6100. In conclusion, this sequence analysis indicates that nmoA has been acquired through multiple transposition events. Stolz (2009) described that, in sphingomonads, IS6100 and other ISs (IS3, IS4, IS21 and IS66 families) are closely linked to degradation genes and contribute to development and distribution of new degradation pathways. The sequences around nmoA may be a typical example of this. A possible protein-coding region without an ATG start codon (orf5* in Fig. 3) was found upstream of nmoA in the insert of pNAP5. The predicted protein sequence showed approximately 30% identity to alcohol dehydrogenases (Table 2). Porter et al. (2012) reported the presence of a part of an alcohol dehydrogenase gene in the upstream region of opdA, which was identical in all three strains PWE1, Bayram and TTNP3. However, the detailed sequence has not yet been reported.

**Evidence for the presence of two nmoA genes in strain NP5**

Southern hybridization using NP5 total DNA digested with restriction enzymes and a gene probe prepared from
the \textit{nmoA} sequence detected 3.5 and 9.0 kb positive signals in the \textit{Hind}III digest, and 1.9 and 7.0 kb positive signals in the \textit{Sph}I digest (Fig. 5b). This result is in good agreement with the restriction map constructed from the sequences of the inserts of pNAP2 and pNAP5 (Fig. 5c) and confirmed that two \textit{nmoA} genes existed at different loci in strain NP5. Intriguingly, in the \textit{Hind}III digest, the 9.0 kb signal was much stronger than the 3.5 kb signal. Also, in the \textit{Sph}I digest, the 1.9 kb signal was much stronger than the 7.0 kb signal. The restriction map implies that the stronger signals originated from pNAP5, while the weaker signals came from pNAP2. In the \textit{Pst}I digest, one strong signal was detected and seems to be the 3.1 kb fragment from pNAP5 (Fig. 5b, c). This observation suggests that \textit{nmoA} in pNAP5 may reside on a multi-copy-number plasmid in strain NP5, while another in pNAP2 exists on the NP5 chromosome (or a low-copy-number plasmid). In a pulse field gel electrophoresis and hybridization study using an \textit{opdA} gene probe, Wise (2008) detected two faint hybridization signals in strain PWE1, one on a 360 kb plasmid and another on the chromosome, suggesting that strain PWE1 may have two \textit{opdA} genes. However, the result was obscure and further information has not yet been provided.

**Degradation of 411-NP by a \textit{Pseudomonas} strain harbouring \textit{nmoA}**

To confirm the function of \textit{nmoA}, the \textit{nmoA}-coding region, together with the 42 bp flanking region including the putative ribosome-binding sequence, was amplified by PCR and introduced into pBBR1MCS-2 to construct pBNMOA-F. In this construct, \textit{nmoA} was located downstream of the \textit{lac} promoter of the vector. It was then introduced into \textit{P. putida} KT2440 by electroporation, because strain KT2440 can grow much faster than strains UT26 and NP5. When the recombinant strain harbouring pBNMOA-F was streaked onto an LB agar plate including Km and NPmix (1 g l$^{-1}$), it grew well on the plate in a few days and accumulated the reddish brown metabolites (Fig. S2).

We carried out 411-NP degradation tests using the cell suspension of the recombinant KT2440 strain. HPLC analysis of the culture supernatant revealed that 70% of 411-NP initially added was degraded in only 5 h (Fig. 6) and one metabolite (Metabolite IV) was detected at an RT of 7.0 min in the HPLC chromatogram (data not shown). After separation of this metabolite by preparative HPLC, it was subjected to GC/MS analysis. The mass spectrum of Metabolite IV was in good agreement with that of authentic hydroquinone (Table 1). No decrease in 411-NP concentration was detected in the control culture (\textit{P. putida} KT2440 cells harbouring the vector). Thus, we concluded that 411-NP was converted to hydroquinone, which has not previously been detected in cultures of the wild-type strain NP5. This \textit{nmoA} expression in \textit{P. putida} KT2440 was dependent on the transcriptional direction of \textit{nmoA} to the \textit{lac} promoter but not on the addition of IPTG (data not shown). GC/MS analysis of the control extract also revealed the presence of another metabolite (Metabolite III-3), the mass spectrum of which was almost identical to those of Metabolite III-1, Metabolite III-2 and authentic 3-methyl-3-octanol (Table 1). Therefore, we expected the side chain of 411-NP to be released from the aromatic-ring as 3-methyl-3-octanol (Fig. 7). These results indicate that 411-NP was degraded by ipso-hydroxylation caused by the \textit{nmoA}-coded monooxygenase, as has been suggested in the NP degradation by strains TTNP3 and Bayram (Corvini \textit{et al.}, 2004, 2006a, b; Gabriel \textit{et al.}, 2005a, b, 2007).

In the OP degradation test using \textit{E. coli} cells expressing \textit{opdA$_{PWE1}$} and an OP isomer 4-(2,4,4-trimethylpentyl)phenol,
Porter & Hay (2007) detected a molar ratio of 25% hydroquinone and 57% 2,4,4-trimethyl-1-pentene as the metabolites of the isomer, after the parent compound (0.61 μmol) was completely degraded in 70 h. In contrast, in this 411-NP degradation, we were able to recover an almost stoichiometric amount of hydroquinone from 411-NP degradation (120 μM in 5 h, Fig. 6). This may be due to a difference in the host strains used (E. coli and P. putida). From these results, we concluded that nmoA encodes a monooxygenase that is essential for the initial ipso-hydroxylation of NP in strain NP5.

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