Novosphingobium tardaugens sp. nov., an oestradiol-degrading bacterium isolated from activated sludge of a sewage treatment plant in Tokyo

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An oestradiol-degrading bacterium isolated at a sewage treatment plant in Tokyo was studied phenotypically, genotypically and phylogenetically. Analysis of its 16S rDNA sequence, DNA base composition, whole-cell fatty acid profile and isoprenoid quinone composition, as well as the presence of sphingoglycolipid, revealed that the isolate is a member of the genus Novosphingobium. However, the sequence similarity of its 16S rDNA to those of known Novosphingobium species was no higher than 97%, implying that the isolate is distinctive. The results of DNA–DNA hybridization experiments and physiological characterization also indicated that the isolate represents a novel Novosphingobium species, for which the name Novosphingobium tardaugens sp. nov. is proposed; strain ARI-1T (JCM 11434T = ATCC BAA-531T = IFO 16725T) is the type strain.

Abbreviations: E2, 17β-oestradiol; FAME, fatty acid methyl ester.

The DDBJ accession number for the 16S rDNA sequence of strain ARI-1T is AB070237.

There has been increasing concern recently over the potential of sewage treatment plant effluent to cause oestrogenic effects in aquatic fauna. Widespread sexual disruption, or so-called 'feminization', has been observed in riverine fish in several developed nations, including the UK, the USA and Japan (Folmar et al., 1996; Harries et al., 1996, 1997; Desbrow et al., 1998; Jobling et al., 1998; Nakada et al., 2000), and is thought to be due to environmental contaminants in the effluent. While there still remains some room for discussion as to the chemicals that cause this phenomenon, natural oestrogens entering the environment through the excretions of humans, domestic and farm animals and wildlife are thought to be the most likely suspects (Shore et al., 1993; Routledge et al., 1998; Metcalfe et al., 2001). Among the natural oestrogens, 17β-oestradiol (E2) is the most potent and is found ubiquitously in many water systems (Williams et al., 1999; Baronti et al., 2000; Ishii et al., 2000; Matsui et al., 2000; Tajima et al., 2000; Nasu et al., 2001). Therefore, it is thought that E2 is responsible for the majority of the oestrogenic effects found in sewage treatment plant effluent. These facts led us to search for micro-organisms with strong E2-degrading activity.

Recently, we found a significant E2-degrading activity in the activated sludge of a sewage treatment plant in Tokyo and isolated an E2-degrading bacterium, which was designated strain ARI-1T (Fujii et al., 2002). Analysis of 16S rDNA sequences strongly suggested that the strain represents a novel species of the genus Novosphingobium. In order to identify strain ARI-1T and assess its precise phylogenetic placement, analyses of DNA base composition, fatty acid composition, isoprenoid quinone composition, polar lipid pattern, phenotypic traits and DNA–DNA hybridization values were carried out in addition to 16S rDNA sequencing. On the basis of our results, we propose that strain ARI-1T should be placed in a novel species of the
genus *Novosphingobium*, *Novosphingobium tardaugens* sp. nov.

**Isolation of strain ARI-1<sup>T</sup>**

Strain ARI-1<sup>T</sup> was isolated from the activated sludge of a sewage treatment plant in Tokyo on a yeast nitrogen base without amino acids (YNB; Difco) on agar plates (pH 7.0 at 25 °C) containing 0.1% (w/v) E2 (E2/YNB agar). YNB consists of (NH₄)₂SO₄ as the nitrogen source, other salts (KH₂PO₄, MgSO₄, NaCl and CaCl₂), trace metals and very small amounts of vitamins, as described in the *Difco Manual* (Difco Laboratories, 1998). Therefore, E2 is considered to be effectively the sole carbon source in E2/YNB. The bacterial strains used in this study were obtained from the Institute for Fermentation (IFO), Osaka, Japan, and the Japan Collection of Microorganisms (JCM), Saitama, Japan. Recommended growth media listed in the institutes’ catalogues were used to grow these organisms.

**Morphological characteristics**

To observe strain ARI-1<sup>T</sup> by transmission electron microscopy, a cell suspension of the strain was put on Formvar-coated grids and fixed with osmium vapour for several minutes. The grids were then washed with distilled water, negatively stained with uranyl acetate solution and observed with a Hitachi H-300 transmission electron microscope. The Gram reaction was tested as described by Yokota (1999). Strain ARI-1<sup>T</sup> (Fig. 1) was found to be an aerobic, Gram-negative, rod-shaped (approximately 1.6–7 days at 25 °C for strain ARI-1<sup>T</sup> to form tiny colonies on YNB agar). YNB consists of (NH₄)₂SO₄ as the nitrogen source, other salts (KH₂PO₄, MgSO₄, NaCl and CaCl₂), trace metals and very small amounts of vitamins, as described in the *Difco Manual* (Difco Laboratories, 1998). Therefore, E2 is considered to be effectively the sole carbon source in E2/YNB. The bacterial strains used in this study were obtained from the Institute for Fermentation (IFO), Osaka, Japan, and the Japan Collection of Microorganisms (JCM), Saitama, Japan. Recommended growth media listed in the institutes’ catalogues were used to grow these organisms.

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**Analysis of 16S rDNA and DNA base composition**

Chromosomal DNA was purified by standard procedures (Sambrook *et al.*, 1989) from bacterial cells harvested from nutrient broth after growth for 48 h at 25 °C. Subsequently, the bacterial cells were suspended in Tris/EDTA buffer (pH 8.0) and lysed with lysozyme (final concentration, 2 mg ml⁻¹) and SDS (final concentration, 0.5%). DNA was then recovered from the lysate by phenol/chloroform extraction, followed by RNase treatment, cetyltrimethylammonium bromide treatment and ethanol precipitation. Each procedure was performed twice to obtain highly pure DNA. Nearly complete 16S rDNA (1418 bases) was amplified by a PCR, using *Taq* DNA polymerase and universal primers corresponding to positions 8–27 (forward primer) and 1492–1510 (reverse primer) of the *Escherichia coli* numbering system (Weisburg *et al.*, 1991). The thermal profile involved 25 cycles of 94 °C for 60 s, 58 °C for 60 s and 72 °C for 90 s, with a final polymerization at 72 °C for 7 min. Direct sequencing of the amplified DNA fragments was carried out as described by Satomi *et al.* (1997). The 16S rDNA sequence for ARI-1<sup>T</sup> was compared with all known sequence data in the GenBank, EMBL and DDBJ databases using the BLAST algorithm (Altschul *et al.*, 1990). Multiple alignment, calculation of nucleotide substitution rates (K<sub>nuc</sub> values; Kimura, 1980) and the construction of phylogenetic trees by the neighbour-joining method (Saitou & Nei, 1987) were performed by using the program CLUSTAL W (Thompson *et al.*, 1994). The robustness of topology on phylogenetic trees was evaluated by a bootstrap analysis through 1000 replications. Alignment gaps, primer regions for PCR amplification and unidentified base positions were not taken into consideration for the calculations. The phylogenetic tree of the novel isolate and related organisms is shown in Fig. 2. Strain ARI-1<sup>T</sup> was positioned in the genus *Novosphingobium* and had the signature pattern of this genus (Takeuchi *et al.*, 2001). We have confirmed, using the program PHYLIP (Felsenstein, 1995), that other clustering methods (maximum-likelihood methods) also lead to the same proposed grouping. The species closest to strain ARI-1<sup>T</sup> was *Novosphingobium subterraneum* (97.0% sequence similarity).

The DNA base composition was determined by HPLC, as described by Kumagai *et al.* (1988). The G+C content of genomic DNA from strain ARI-1<sup>T</sup> was 61 mol% (Table 1), which is consistent with the values reported for other members of *Novosphingobium* (Nicholson *et al.*, 1994; Takeuchi *et al.*, 2001).

**Cellular fatty acid composition and polar lipid pattern**

Whole-cell lipids were extracted according to the methods of Bligh & Dyer (1959). Fatty acid methyl esters (FAMEs)
were prepared by reacting the extracted lipids with 2 M HCl in methanol at 100 °C for 24 h in test tubes with Teflon-lined caps. Subsequently, the FAMEs were extracted three times with hexane and the hexane layer was then evaporated completely by vacuum centrifugation. The FAME residue was then redissolved in an appropriate volume of hexane. Analysis of FAMEs was performed with a GC1700 gas chromatograph (Shimadzu) equipped with a BPX70 capillary column (50 m × 0.22 mm i.d.; SGE). Helium was used as the carrier gas (column head pressure, 130 kPa). The injector and detector were maintained at 260 °C and the column oven was programmed to increase from 155 to 235 °C at a rate of 4 °C min⁻¹ and then maintained at 235 °C for 10 min. Peak areas were quantified with a C-R8A chromatography recorder (Shimadzu). Compounds were identified by comparison of their retention times with those of known standards and were confirmed by GC/MS using a Saturn 2000 ion-trap mass spectrometer (Varian) connected to a Varian 3800 gas chromatograph equipped with a BPX70 capillary column (25 m × 0.22 mm i.d.). The oven was programmed to increase from 80 to 240 °C at a rate of 4 °C min⁻¹. Helium was used as the carrier gas (1 ml min⁻¹). GC/MS was performed at an ionization voltage of 70 eV and a trap temperature of 230 °C, with a mass range of 40–400 Da. The whole-cell fatty acid profile of strain ARI-1T is shown in Table 2. The major non-polar fatty acid was 18 : 1, while 2-hydroxymyristic acid (14 : 0 2-OH) was present as the dominant hydroxylated fatty acid. However, 3-hydroxylated fatty acids were not detected. These results are consistent with the description of the genus Novosphingobium (Yabuuchi et al., 1990; Takeuchi et al., 1993, 1994, 2001; Kämpfer et al., 1997).

The polar lipid profile was examined with two-dimensional TLC. The crude lipids were separated twice on silica gel 60 TLC plates (20 × 20 cm; Merck) with two solvent systems, chloroform/methanol/water (65:25:4, v/v) and methanol/chloroform (30:70, v/v). The polar lipids were separated twice on silica gel 60 TLC plates (20 × 20 cm; Merck) with two solvent systems, chloroform/methanol/water (65:25:4, v/v) and methanol/chloroform (30:70, v/v).

**Table 1.** DNA–DNA hybridization of various *Novosphingobium* species

<table>
<thead>
<tr>
<th>Strain</th>
<th>G+C content (mol%)*</th>
<th>Similarity (%) to labelled DNA from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1. Strain ARI-1T (=JCM 11434T)</td>
<td>61</td>
<td>100</td>
</tr>
<tr>
<td>2. <em>N. aromaticivorans</em> ATCC 700278T</td>
<td>64a</td>
<td>14</td>
</tr>
<tr>
<td>3. <em>N. capsulatum</em> ATCC 14666T</td>
<td>63b</td>
<td>12</td>
</tr>
<tr>
<td>4. <em>N. rosa</em> ATCC 51837T</td>
<td>65c</td>
<td>12</td>
</tr>
<tr>
<td>5. <em>N. subarcticum</em> JCM 10398T</td>
<td>66d</td>
<td>12</td>
</tr>
<tr>
<td>6. <em>N. stygium</em> ATCC 700280T</td>
<td>65a</td>
<td>14</td>
</tr>
<tr>
<td>7. <em>N. subterraneum</em> ATCC 700279T</td>
<td>60a</td>
<td>11</td>
</tr>
</tbody>
</table>

*Data for reference species were taken from: a, Balkwill et al. (1997); b, Yabuuchi et al. (1990); c, Takeuchi et al. (1995); d, Nohynek et al. (1996).*

**Table 2.** Whole-cell fatty acid profiles of strain ARI-1T and *N. capsulatum*

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>ARI-1T</th>
<th>N. capsulatum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated fatty acids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 : 0</td>
<td>0-22</td>
<td>0-60</td>
</tr>
<tr>
<td>14 : 0 2-OH</td>
<td>12</td>
<td>1-9</td>
</tr>
<tr>
<td>16 : 0</td>
<td>3-1</td>
<td>21</td>
</tr>
<tr>
<td>17 : 0</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>Unsaturated fatty acids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 : 1b</td>
<td>15-6</td>
<td>5-1</td>
</tr>
<tr>
<td>17 : 1a</td>
<td>2-6</td>
<td>0-52</td>
</tr>
<tr>
<td>18 : 1b</td>
<td>55</td>
<td>66</td>
</tr>
</tbody>
</table>

*Including at least two (a) or three (b) isomers.*

Saturn 2000 ion-trap mass spectrometer (Varian) connected to a Varian 3800 gas chromatograph equipped with a BPX70 capillary column (25 m × 0.22 mm i.d.). The oven was programmed to increase from 80 to 240 °C at a rate of 4 °C min⁻¹. Helium was used as the carrier gas (1 ml min⁻¹). GC/MS was performed at an ionization voltage of 70 eV and a trap temperature of 230 °C, with a mass range of 40–400 Da. The whole-cell fatty acid profile of strain ARI-1T is shown in Table 2. The major non-polar fatty acid was 18 : 1, while 2-hydroxy-13-myrystic acid (14 : 0 2-ΟΗ) was present as the dominant hydroxylated fatty acid. However, 3-hydroxylated fatty acids were not detected. These results are consistent with the description of the genus *Novosphingobium* (Yabuuchi et al., 1990; Takeuchi et al., 1993, 1994, 2001; Kämpfer et al., 1997).

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chloroform/methanol/acetic acid/water (80:12:15:4, v/v). A 50 % sulfuric acid solution was used to detect spots of all kinds of lipids. 2-Naphthol/sulfuric acid, periodate-Schiff, Zinolide (Dittmer reagent) and ninhydron were respectively used for the detection of sugars, 2-glycols, phosphate and free amino groups (Stolz et al., 2000). Sphingoglycolipid, the unique lipid of the genus Novosphingobium, was detected, along with phospholipids, an unidentified lipid and a pigment (data not shown).

### Isoprenoid quinone analysis

The isoprenoid quinone composition of strain ARI-1T was determined by one-dimensional TLC and HPLC, using the methods described by Yamada & Kuraishi (1982). The total acetone-soluble extract of whole cells was separated by one-dimensional TLC with benzene as the eluant. Isoprenoid length was analysed by using HPLC, with a reverse-phase column (Wako handy ODS, 250 mm × 4.6 mm i.d.; Wako Pure Chemicals). The extract contained ubiquinone, which consisted mainly of Q-10 (data not shown). The presence of ubiquinone Q-10 as the predominant isoprenoid quinone is typical of members of the α-Proteobacteria, including the genus Novosphingobium (Yabuuchi et al., 1990; Yrjälä et al., 1998; Stolz et al., 2000; Takeuchi et al., 2001).

### DNA–DNA hybridization experiments

As described above, analyses of 16S rDNA sequences, DNA base composition, fatty acid composition, polar lipid pattern and isoprenoid quinone composition indicated that strain ARI-1T is a member of the genus Novosphingobium. However, the 16S rDNA sequence similarity to the most closely related Novosphingobium species was 97 % at most, implying that strain ARI-1T represents a distinct species. Therefore, to obtain more conclusive information on the relationship between strain ARI-1T and other Novosphingobium species, DNA–DNA hybridization experiments were carried out by the microplate hybridization method (Ezaki et al., 1989) using photobiotin labelling and colorimetric detection (Satomi et al., 1997), with 1,2-phenylenediamine (Sigma) as the substrate and streptavidin–peroxidase conjugate (Boehringer) as the colorimetric enzyme. Table 1 shows the levels of DNA–DNA reassociation among these species; all are relatively low (maximum 36%). It has been recommended that a phylogenetically defined species consists of strains that exhibit approximately 70 % or higher DNA–DNA hybridization (Wayne et al., 1987). Therefore, our findings strongly suggest that strain ARI-1T is distinct from other known Novosphingobium species.

### Other characteristics

The physiological characteristics of strain ARI-1T and known Novosphingobium species are summarized in Table 3. Oxidase and catalase tests were respectively carried out with the Poremedia oxidase test indicator (Eiken Chemical) and 3.0 % hydrogen peroxide. The API 20NE system (bioMérieux) was used to determine assimilation patterns and biochemical characteristics of the test organisms. Strain ARI-1T was positive for catalase activity and reduction of nitrate, which is consistent with other members of Novosphingobium. While each Novosphingobium species had unique assimilation patterns, ARI-1T did not assimilate any substrates tested in this study. Cultivation of strain ARI-1T with YNB (pH 7.0 at 25 °C) containing 1.0 % of the substrates was also carried out using a rotary shaker, but similar results were obtained. We suspected that the negative results for strain ARI-1T might be due to its adaptation to E2. It is known that highly adapted strains must be subcultured several times with a full medium before they recover their typical assimilation patterns. Thus, we subcultured ARI-1T repeatedly (10 times) with nutrient broth before the assimilation-pattern test. However, strain ARI-1T never showed any positive results in the test, while E2-degrading activity was retained as a stable characteristic.

### Conclusion

The 16S rDNA sequence, G+C content of the genomic DNA, whole-cell fatty acid profile, isoprenoid quinone composition and the presence of sphingoglycolipid indicated that strain ARI-1T belongs to the genus Novosphingobium. Moreover, the similarity values for the 16S rDNA of ARI-1T and its closest phylogenetic neighbours suggested that ARI-1T is distinct from them. Finally, the DNA–DNA hybridization experiment clearly showed that strain ARI-1T represents a novel species of the genus Novosphingobium. An additional Novosphingobium species (Novosphingobium hassiacum) was recently isolated from wastewater (Kämpfer et al., 2000).

### Table 3. Characteristics that differentiate strain ARI-1T from other Novosphingobium species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<td>Assimilation of:</td>
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<td>Glucose</td>
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<tr>
<td>L-Arabinose</td>
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<tr>
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<td>Enzyme activity:</td>
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<tr>
<td>β-Glucosidase</td>
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</tr>
</tbody>
</table>

Strains: 1, ARI-1T; 2, N. subterraneum ATCC 700279T; 4, N. stygium ATCC 700280T; 5, N. capsulatum ATCC 14666; 6, N. rosa ATCC 51837; 7, N. aromatics ATCC 700278. All strains were positive for catalase and nitrate oxidase activity. All strains were negative for oxidase, arginine dihydrodrolase and urease activity, production of indole, acid production from glucose and assimilation of n-capric acid, citrate and phenyl acetate.
et al., 2002). Thus, we obtained the whole 16S rDNA sequence of \textit{N. hasiacum} (AJ416411) and subjected it to the BLAST similarity search. The 16S rDNA similarity between strain ARI-1\textsuperscript{T} and \textit{N. hasiacum} was found to be 96\%, indicating that they are distinct species. On the basis of the phenotypic, genotypic and phylogenetic data obtained in our study, we conclude that strain ARI-1\textsuperscript{T} should be classified as a novel species of the genus \textit{Novosphingobium}, \textit{Novosphingobium tardaugens} sp. nov.

Recently, Takeuchi et al. (2001) divided the genus \textit{Sphingomonas} into four new genera, \textit{Sphingomonas}, \textit{Sphingobium}, \textit{Sphingopyxis} and \textit{Novosphingobium}. \textit{Novosphingobium} consisted initially of six members: \textit{Novosphingobium aromativorans}, \textit{Novosphingobium capsulatum}, \textit{Novosphingobium rosa}, \textit{Novosphingobium stygium}, \textit{Novosphingobium subarcticum} and \textit{N. subterraneum}. Several members of this genus are known to degrade aromatic compounds. For instance, \textit{N. aromativorans}, \textit{N. stygium} and \textit{N. subterraneum} were reported to degrade benzoate, cresol, naphthalene or xylene (Balkwill et al., 1997) and \textit{N. subarcticum} can degrade chlorophenol (Nohynek et al., 1996). Strain ARI-1\textsuperscript{T} was isolated as a novel \textit{Novosphingobium} strain that can degrade E2, an aromatic compound.

**Description of Novosphingobium tardaugens sp. nov.**

\textit{Novosphingobium tardaugens} (L. adj. \textit{tardus} slow; L. adj. \textit{augens} growing; N.L. adj. \textit{tardaugens} slowly growing).

Cells are Gram-negative, aerobic, rod-shaped (approx. 1-2 \(\mu\)m long and 0.8 \(\mu\)m in diameter) and non-motile. Colonies are circular, entire, convex, opaque and whitish brown. Grows in nutrient broth, brain/heart infusion and tryptic soy broth at 25\(^\circ\)C, but not at 4 or 42\(^\circ\)C. Catalase-positive and oxidase-negative. Nitrate is reduced. The G+C content of the DNA is 61 mol\%. The major non-polar fatty acid is 18:1, and the major 2-hydroxy fatty acid is 14:0 2-OH. 3-Hydroxy fatty acids are not detected. Sphingolipid is present. The major isoprenoid quinone is ubiquinone Q-10. The type strain, strain ARI-1\textsuperscript{T} (=JCM 11434\textsuperscript{T} =IFO 16725\textsuperscript{T} =ATCC BAA-531\textsuperscript{T}), was isolated from activated sludge of a sewage treatment plant in Tokyo. The 16S rDNA sequence of the type strain has been deposited in the DDBJ database under accession number AB070237.

**Acknowledgements**

We would like to thank Mr Kenji Taii for his technical assistance. This work was supported by funding from the Showa Shell Sekiyu Foundation for Promotion of Environmental Research (to K. F.).

**References**


