Note

Novosphingobium tardaugens sp. nov., an oestradiol-degrading bacterium isolated from activated sludge of a sewage treatment plant in TokyoKatsuhiko Fujii,¹ Masataka Satomi,² Naoki Morita,³ Taizo Motomura,⁴ Tadaharu Tanaka⁵ and Shintaro Kikuchi¹

Correspondence
Katsuhiko Fujii
kfu@mmm.muroran-it.ac.jp

¹Department of Applied Chemistry, Muroran Institute of Technology, Mizumoto, Muroran 050-8585, Japan

²Food Processing Division, National Research Institute of Fisheries Science, Fukuura, Kanazawa, Yokohama 236-8648, Japan

³Research Institute of Biological Resources, National Institute of Advanced Industrial Science and Technology, Toyohira, Sapporo 062-8517, Japan

⁴Muroran Marine Station, Field Science Centre for Northern Biosphere, Hokkaido University, Bokoi-minamimachi, Muroran 051-0003, Japan

⁵Technical Research Laboratory, Kurabo Industries, Shimokida-cho, Neyagawa, Osaka 572-0823, Japan

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-1^T (=JCM 11434^T =ATCC BAA-531^T =IFO 16725^T) is the type strain.

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, 17 β -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-1^T (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-1^T 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-1^T should be placed in a novel species of the

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

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

genus *Novosphingobium*, *Novosphingobium tardaugens* sp. nov.

Isolation of strain ARI-1^T

Strain ARI-1^T 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^T 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^T (Fig. 1) was found to be an aerobic, Gram-negative, rod-shaped (approximately 1.2 µm in length and 0.8 µm in diameter) and non-motile bacterium. It took 6–7 days at 25 °C for strain ARI-1^T to form tiny colonies on nutrient agar, brain/heart infusion agar or tryptic soy agar. We also tried each of the above agar media at half nutritional strength, but the growth rate of strain ARI-1^T was not improved. Compared with all other known *Novosphingobium* species, which can form colonies within 1 or 2 days under the same culture conditions, strain ARI-1^T seems to have the weakest growth activity. Colony growth of strain ARI-1^T was not observed at temperatures of 4 or 42 °C,

Fig. 1. Transmission electron micrograph of ARI-1^T cells. Bar, 1 µm.

which was consistent with the characteristics of other *Novosphingobium* species.

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^T 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_{nuc} 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^T was positioned in the genus *Novosphingobium* and had the signature sequences 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^T 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^T 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)

Fig. 2. Phylogenetic tree constructed by using the neighbour-joining method based on 16S rDNA sequences of strain ARI-1^T and related bacteria. Numbers at nodes indicate levels of bootstrap support based on 1000 resampled datasets. *Agrobacterium rhizogenes* was used as the outgroup. Bar, evolutionary distance (K_{nuc}) of 0.01.

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

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

Values are percentages of total fatty acids. The following fatty acids were not detected in either taxon: 16:0 2-OH, 18:0, 19:0, 22:0, 16:1 2-OH, 3-OH fatty acids. tr, Trace.

Fatty acid	ARI-1 ^T	<i>N. capsulatum</i>
Saturated fatty acids		
14:0	0.22	0.60
14:0 2-OH	12	1.9
16:0	3.1	21
17:0	tr	tr
Unsaturated fatty acids*		
16:1 ^b	15.6	5.1
17:1 ^a	2.6	0.52
18:1 ^b	55	66

*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-1^T 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

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

Strain	G+C content (mol%)*	Similarity (%) to labelled DNA from:				
		1	2	3	5	7
1. Strain ARI-1 ^T (=JCM 11434 ^T)	61	100	10	9	7	9
2. <i>N. aromaticivorans</i> ATCC 700278 ^T	64 ^a	14	100	22	36	22
3. <i>N. capsulatum</i> ATCC 14666 ^T	63 ^b	12	16	100	24	22
4. <i>N. rosa</i> ATCC 51837 ^T	65 ^c	12	12	12	12	12
5. <i>N. subarcticum</i> JCM 10398 ^T	66 ^d	12	34	25	100	18
6. <i>N. stygium</i> ATCC 700280 ^T	65 ^a	14	28	22	21	19
7. <i>N. subterraneum</i> ATCC 700279 ^T	60 ^a	11	14	16	16	100

*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).

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. α -Naphthol/sulfuric acid, periodate-Schiff, Zinzadze (Dittmer reagent) and ninhydrin were respectively used for the detection of sugars, α -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-1^T 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 $\times$ 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-1^T 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-1^T represents a distinct species. Therefore, to obtain more conclusive information on the relationship between strain ARI-1^T 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-1^T is distinct from other known *Novosphingobium* species.

Other characteristics

The physiological characteristics of strain ARI-1^T 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

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

Strains: 1, ARI-1^T; 2, *N. subarcticum* JCM 10398^T; 3, *N. subterraneum* ATCC 700279^T; 4, *N. stygium* ATCC 700280^T; 5, *N. capsulatum* ATCC 14666^T; 6, *N. rosa* ATCC 51837^T; 7, *N. aromaticivorans* ATCC 700278^T. All strains were positive for catalase and nitrate oxidase activity. All strains were negative for oxidase, arginine dihydrolase and urease activity, production of indole, acid production from glucose and assimilation of *n*-capric acid, citrate and phenyl acetate.

Characteristic	1	2	3	4	5	6	7
Assimilation of:							
Glucose	–	+	+	+	+	+	+
L-Arabinose	–	+	+	+	+	+	+
D-Mannose	–	–	+	+	+	+	+
D-Mannitol	–	+	–	+	+	–	+
N-Acetyl-D-glucosamine	–	+	–	–	+	–	–
Maltose	–	+	+	+	+	+	+
Glucanate	–	–	–	–	+	+	–
Adipic acid	–	–	–	–	–	–	+
DL-Malate	–	+	–	–	+	–	–
Enzyme activity:							
β -Glucosidase	–	+	+	+	+	+	+
Gelatinase	–	–	–	–	+	–	–
β -Galactosidase	–	+	+	+	+	+	+

and biochemical characteristics of the test organisms. Strain ARI-1^T 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-1^T did not assimilate any substrates tested in this study. Cultivation of strain ARI-1^T 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-1^T 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-1^T repeatedly (10 times) with nutrient broth before the assimilation-pattern test. However, strain ARI-1^T 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-1^T belongs to the genus *Novosphingobium*. Moreover, the similarity values for the 16S rDNA of ARI-1^T and its closest phylogenetic neighbours suggested that ARI-1^T is distinct from them. Finally, the DNA–DNA hybridization experiment clearly showed that strain ARI-1^T represents a novel species of the genus *Novosphingobium*. An additional *Novosphingobium* species (*Novosphingobium hassiacum*) was recently isolated from wastewater (Kämpfer

et al., 2002). Thus, we obtained the whole 16S rDNA sequence of *N. hassiacum* (AJ416411) and subjected it to the BLAST similarity search. The 16S rDNA similarity between strain ARI-1^T and *N. hassiacum* 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^T should be classified as a novel species of the genus *Novosphingobium*, *Novosphingobium tardaugens* sp. nov.

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

Description of *Novosphingobium tardaugens* sp. nov.

Novosphingobium tardaugens (L. adj. *tardus* slow; L. adj. *augens* growing; N.L. adj. *tardaugens* slowly growing).

Cells are Gram-negative, aerobic, rod-shaped (approx. 1.2 µm long and 0.8 µm 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 °C, but not at 4 or 42 °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. Sphingoglycolipid is present. The major isoprenoid quinone is ubiquinone Q-10. The type strain, strain ARI-1^T (=JCM 11434^T =IFO 16725^T =ATCC BAA-531^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

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Balkwill, D. L., Drake, G. R., Reeves, R. H. & 7 other authors (1997). Taxonomic study of aromatic-degrading bacteria from deep-terrestrial-subsurface sediments and description of *Sphingomonas aromaticivorans* sp. nov., *Sphingomonas subterranea* sp. nov., and *Sphingomonas stygia* sp. nov. *Int J Syst Bacteriol* **47**, 191–201.
- Baronti, C., Curini, R., D'Ascenzo, G., Di Corcia, A., Gentili, A. & Samperi, R. (2000). Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. *Environ Sci Technol* **34**, 5059–5066.
- Bligh, E. J. & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**, 911–917.
- Desbrow, C., Routledge, E. J., Brighty, G. C., Sumpter, J. P. & Waldock, M. (1998). Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. *Environ Sci Technol* **32**, 1549–1558.
- Difco Laboratories (1998). Yeast media. In *Difco Manual*, 11th edn, pp. 576–580. Detroit: Difco Laboratories.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Felsenstein, J. (1995). PHYLIP – Phylogeny Inference Package (version 3.57c). Distributed by the author. Department of Genetics, University of Washington, Seattle, USA.
- Folmar, L. C., Denslow, N. D., Rao, V., Chow, M., Crain, D. A., Enblom, J., Marcino, J. & Guillelte, L. J., Jr (1996). Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. *Environ Health Perspect* **104**, 1096–1101.
- Fujii, K., Kikuchi, S., Satomi, M., Ushio-Sata, N. & Morita, N. (2002). Degradation of 17β-estradiol by a gram-negative bacterium isolated from activated sludge in a sewage treatment plant in Tokyo, Japan. *Appl Environ Microbiol* **68**, 2057–2060.
- Harries, J. E., Sheahan, D. A., Jobling, S., Matthiessen, P., Neall, P., Routledge, E. J., Rycroft, R., Sumpter, J. P. & Tylor, T. (1996). A survey of estrogenic activity in United Kingdom inland waters. *Environ Toxicol Chem* **15**, 1993–2002.
- Harries, J. E., Sheahan, D. A., Jobling, S., Matthiessen, P., Neall, P., Sumpter, J. P., Tylor, T. & Zaman, N. (1997). Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environ Toxicol Chem* **16**, 534–542.
- Ishii, Y., Okita, S., Torigai, M. & Yun, S. (2000). Determination of oestrogens in environmental water samples by LC/MS/MS. *Bunseki Kagaku* **49**, 753–758 (in Japanese).
- Jobling, S., Nolan, M., Tyler, C. R., Brighty, G. & Sumpter, J. P. (1998). Widespread sexual disruption in wild fish. *Environ Sci Technol* **32**, 2498–2506.
- Kämpfer, P., Denner, E. B. M., Meyer, S., Moore, E. R. B. & Busse, H.-J. (1997). Classification of “*Pseudomonas azotocolligans*” Anderson 1955, 132, in the genus *Sphingomonas* as *Sphingomonas trueperi* sp. nov. *Int J Syst Bacteriol* **47**, 577–583.
- Kämpfer, P., Witzemberger, R., Denner, E. B. M., Busse, H.-J. & Neef, A. (2002). *Novosphingobium hassiacum* sp. nov., a new species isolated from an aerated sewage pond. *Syst Appl Microbiol* **25**, 37–45.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kumagai, M., Fujimoto, M. & Kuninaka, A. (1988). Determination of base composition of DNA by high performance liquid chromatography of its nuclease P1 hydrolysate. *Nucleic Acids Symp Ser* **19**, 65–68.

- Matsui, S., Takigami, H., Matsuda, T., Taniguchi, N., Adachi, J., Kawami, H. & Shimizu, Y. (2000). Estrogen and estrogen mimics contamination in water and the role of sewage treatment. *Water Sci Technol* **42**(12), 173–179.
- Metcalfe, C. D., Metcalfe, T. L., Kiparissis, Y., Koenig, B. G., Khan, C., Hughes, R. J., Croley, T. R., March, R. E. & Potter, T. (2001). Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by in vivo assays with Japanese medaka (*Oryzias latipes*). *Environ Toxicol Chem* **20**, 297–308.
- Nakada, N. H., Takada, H., Nyuunoya, H., Nakamura, M. & Iguchi, T. (2000). Identification of chemicals disrupting endocrine systems of aquatic biota in sewage effluent using GC-MS determination, chemical fractionation and recombinant yeast assay. In *Proceedings of the 9th Symposium on Environmental Chemistry*, pp. 122–123. Sapporo, Japan (in Japanese).
- Nasu, M., Goto, M., Kato, H., Oshima, Y. & Tanaka, H. (2001). Study on endocrine disrupting chemicals in wastewater treatment plants. *Water Sci Technol* **43**(2), 101–108.
- Nicholson, L. A., Morrow, C. J., Corner, L. A. & Hodgson, A. L. M. (1994). Phylogenic relationship of *Fusobacterium necrophorum* A, AB, and B biotypes based upon 16S rRNA gene sequence analysis. *Int J Syst Bacteriol* **44**, 315–319.
- Nohynek, L. J., Nurmiäho-Lassila, E.-L., Suhonen, E. L., Busse, H.-J., Mohammadi, M., Hantula, J., Rainey, F. & Salkinoja-Salonen, M. S. (1996). Description of chlorophenol-degrading *Pseudomonas* sp. strains KF1^T, KF3, and NK1 as a new species of the genus *Sphingomonas*, *Sphingomonas subarctica* sp. nov. *Int J Syst Bacteriol* **46**, 1042–1055.
- Routledge, E. J., Sheahan, D., Desbrow, C., Brighty, G. C., Waldock, M. & Sumpter, J. P. (1998). Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach. *Environ Sci Technol* **32**, 1559–1565.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Satomi, M., Kimura, B., Mizoi, M., Sato, T. & Fujii, T. (1997). *Tetragenococcus muriaticus* sp. nov., a new moderately halophilic lactic acid bacterium isolated from fermented squid liver sauce. *Int J Syst Bacteriol* **47**, 832–836.
- Shore, L. S., Gurevitz, M. & Shemesh, M. (1993). Estrogen as an environmental pollutant. *Bull Environ Contam Toxicol* **51**, 361–366.
- Stolz, A., Schmidt-Maag, C., Denner, E. B. M., Busse, H.-J., Egli, T. & Kämpfer, P. (2000). Description of *Sphingomonas xenophaga* sp. nov. for strains BN6^T and N,N which degrade xenobiotic aromatic compounds. *Int J Syst Evol Microbiol* **50**, 35–41.
- Tajima, H., Tsujimura, K. & Yamaguchi, M. (2000). Development of an analytical method of 17 β -estradiol in river water by liquid chromatography/tandem mass spectrometry. *Bunseki Kagaku* **49**, 843–848 (in Japanese).
- Takeuchi, M., Kawai, F., Shimada, Y. & Yokota, A. (1993). Taxonomic study of polyethylene glycol-utilizing bacteria: emended descriptions of the genus *Sphingomonas* and new description of *Sphingomonas macrogoltabidus* sp. nov., *Sphingomonas sanguis* sp. nov., and *Sphingomonas terrae* sp. nov. *Syst Appl Microbiol* **16**, 227–238.
- Takeuchi, M., Sawada, H., Oyaizu, H. & Yokota, A. (1994). Phylogenetic evidence for *Sphingomonas* and *Rhizomonas* as nonphoto-synthetic members of the alpha-4 subclass of the *Proteobacteria*. *Int J Syst Bacteriol* **44**, 308–314.
- Takeuchi, M., Sakane, T., Yanagi, M., Yamasato, K., Hamana, K. & Yokota, A. (1995). Taxonomic study of bacteria isolated from plants: proposal of *Sphingomonas rosa* sp. nov., *Sphingomonas pruni* sp. nov., *Sphingomonas asaccharolytica* sp. nov., and *Sphingomonas mali* sp. nov. *Int J Syst Bacteriol* **45**, 334–341.
- Takeuchi, M., Hamana, K. & Hiraishi, A. (2001). Proposal of the genus *Sphingomonas sensu stricto* and three new genera, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*, on the basis of phylogenetic and chemotaxonomic analyses. *Int J Syst Evol Microbiol* **51**, 1405–1417.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.
- Williams, R. J., Jürgens, M. D. & Johnson, A. C. (1999). Initial predictions of the concentrations and distribution of 17 β -oestradiol, oestrone and ethinyl oestradiol in 3 English rivers. *Water Res* **33**, 1663–1671.
- Yabuuchi, E., Yano, I., Oyaizu, H., Hashimoto, Y., Ezaki, T. & Yamamoto, H. (1990). Proposals of *Sphingomonas paucimobilis* gen. nov. and comb. nov., *Sphingomonas parapaucimobilis* sp. nov., *Sphingomonas yanoikuyae* sp. nov., *Sphingomonas adhaesiva* sp. nov., *Sphingomonas capsulata* comb. nov., and two genospecies of the genus *Sphingomonas*. *Microbiol Immunol* **34**, 99–119.
- Yamada, Y. & Kuraishi, I. (1982). Ubiquinone and menaquinone. In *Biseibutsu no Kagakubunrui Jikkenhou*, pp. 143–155. Edited by K. Komagata. Tokyo: Gakkai Shuppan Centre (in Japanese).
- Yokota, A. (1999). Gram staining. In *Biseibutsugaku-jikkenhou*, p. 199. Edited by J. Sugiyama, S. Watanabe, K. Oowada, T. Kuroiwa, H. Takahashi & G. Tokuda. Tokyo: Kodansha Scientific (in Japanese).
- Yrjölä, K., Suomalainen, S., Suhonen, E. L., Kilpi, S., Paulin, L. & Romantschuk, M. (1998). Characterization and reclassification of an aromatic- and chloroaromatic-degrading *Pseudomonas* sp., strain HV3, as *Sphingomonas* sp. HV3. *Int J Syst Bacteriol* **48**, 1057–1062.