Sphingomonas fennica sp. nov. and *Sphingomonas haloaromaticamans* sp. nov., outliers of the genus *Sphingomonas*

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Bacterial isolates obtained from polychlorophenol-contaminated sites in Finland (strain K101^T) and from a Dutch drinking water well (strain A175^T) were characterized taxonomically. 16S rRNA gene sequence analysis, determination of DNA G + C content, physiological characterization, estimation of the ubiquinone and polar lipid patterns and fatty acid content revealed that strains K101^T and A175^T were similar to *Sphingomonas wittichii* RW1^T but also showed pronounced differences. The DNA G + C contents of the two novel strains were 63.6 and 66.1 mol%, respectively. On the basis of these results, two novel species of the genus *Sphingomonas* are described, for which the names *Sphingomonas haloaromaticamans* sp. nov. [type strain A175^T (=DSM 13477^T=CCUG 53463^T)] and *Sphingomonas fennica* sp. nov. [type strain K101^T (=DSM 13665^T=CCUG 53462^T)] are proposed.

Several bacterial strains have been isolated from different habitats that are able to grow at the expense of chlorinated aromatic compounds, many of which are considered to be xenobiotics. Several studies have been conducted to elucidate the environmental behaviour of these novel isolates (White *et al.*, 1996) and many of them were originally assigned to the genus *Sphingomonas* (Balkwill *et al.*, 1997; Karlson *et al.*, 1996; Moore *et al.*, 1993; Nohynek *et al.*, 1996a, b; Stolz *et al.*, 2000; Zipper *et al.*, 1996). However, with the exception of *Sphingomonas wittichii*, all of these isolates have since been reclassified as species of the genera *Sphingobium* or *Novosphingobium* (Pal *et al.*, 2006;

Takeuchi *et al.*, 2001). There are some indications that the intermediary halocatechols, especially 4-fluorocatechol and 4-chlorocatechol, originating from the bacterial breakdown of some haloaromatics, can be mineralized through a proposed novel pathway involving protoanemonin (Blasco *et al.*, 1995; Nikodem *et al.*, 2003; Wittich *et al.*, 1999). This mechanism is possible in several strains that express only the genes encoding the ortho-pathway for the breakdown of catechol. Inducing extradiolically-cleaving catechol dioxygenase activity would lead to the misrouting of halogenated intermediates in those strains lacking a functional chlorocatechol pathway. In sphingomonads, a chlorocatechol pathway (in *sensu stricto*) has been detected only in the 1,4-dichlorobenzene-mineralizing strain A175^T and may also be found in some chlorophenoxy herbicide-degrading isolates (Ka *et al.*, 1994) and in Gram-positive bacteria (Konig *et al.*, 2004).

Strain A175^T was isolated from a mixture of various soil and water samples that used 1,4-dichlorobenzene as the sole carbon and energy source (Schraa *et al.*, 1986). The

Abbreviations: pNA, *p*-nitroanilide; pNP, *p*-nitrophenyl.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains A175^T and K101^T are X94101 and AJ009706, respectively.

An additional phylogenetic tree and tables detailing polar lipid distribution, FAB of phospholipids and polyamine patterns for the novel species are available with the online version of this paper.

novel strain was initially identified as a member of the genus *Alcaligenes* and only later was it discovered that the strain actually belongs to the family *Sphingomonadaceae* (Kosako *et al.*, 2000). Strain K101^T was isolated from 2,4,6-trichlorophenol-, 2,3,4,6-tetrachlorophenol- and pentachlorophenol-contaminated boreal groundwater in Finland and this novel strain was able to degrade all of these compounds (Männistö *et al.*, 1999). Here, we report the results of a polyphasic study of these two novel isolates which are currently assigned to the genus *Sphingomonas* (Takeuchi *et al.*, 2001; Yabuuchi *et al.*, 1990).

Biomass for the extraction of quinones, polar lipids and polyamines was grown on PYE medium (Busse *et al.*, 2005). For the extraction of fatty acids, biomass was grown on trypticase soy agar. Liquid and solid Luria–Bertani (LB) or R2A media were used for culturing the strains for the other taxonomic tests described in this study. Solid mineral salts medium plates were used to check the degradation of individual organic carbon sources at a concentration of organic carbon corresponding to about 25 mM single carbon atom. Cultures were grown aerobically at 28 °C. Cell morphology and dimensions were determined by phase-contrast microscopy. Cells were rod-shaped, 0.8–1.5 µm in length and 0.4–0.6 µm in diameter. Cells of both strains showed a tendency to grow in rosette-like formations. However, when grown on selective media, cells of both novel strains tended to grow as branched thread-like/hyphae-like aggregates.

The procedures used for physiological and biochemical characterizations were those described earlier (Kämpfer & Altwegg, 1992; Kämpfer *et al.*, 1991). The results obtained using API galleries (bioMérieux) and Biolog substrate utilization tests are given in detail in the species description. Strain A175^T has been reported to grow on 1,4-dichlorobenzene, but not on chlorophenols (Schraa *et al.*,

1986), and strain K101^T has been found to use tri-, tetra- and pentaphenols (Männistö *et al.*, 1999).

Interestingly, strain K101^T was capable of assimilating only two of the long list of carbon sources tested, namely acetate and DL-3-hydroxybutyrate, and is thus unique in the genus *Sphingomonas* (Denner *et al.*, 1999; Kämpfer *et al.*, 1997) (Table 1). With this highly specialized characteristic, strain K101^T resembles strains of another sphingomonad species, *Novosphingobium lentum*, which was isolated from a chlorophenol-degrading bioreactor purifying water from the same aquifer (Tirola *et al.*, 2005).

For the isolation of genomic DNA for the determination of G + C content, bacterial DNA was purified using proteinase K lysis, phenol/chloroform extractions and isopropanol precipitation according to Wilson (1994). The purity of the DNA samples was confirmed with caesium chloride gradient centrifugation. DNA G + C content was determined as described by Johnson (1994) using λ phage DNA as a standard. The separation was performed on a Purospher endcapped reverse-phase HPLC column (Merck) of 250 × 4 mm. The mobile phase was 20 mM triethylamine phosphate in 12 % aqueous methanol at a flow rate of 1 ml min⁻¹ at 22 °C. The DNA G + C contents were 66.1 mol% for strain A175^T and 63.6 mol% for strain K101^T. These values are within the range commonly found for members of the family *Sphingomonadaceae* but did not allow a clear affiliation to be made to a specific genus.

For the amplification of the 16S rRNA gene by PCR, single colonies were boiled in 100 µl of TE buffer for about 10 min at 95 °C to obtain DNA. A nearly complete 16S rRNA gene sequence was obtained as described previously (Abraham *et al.*, 1999). Reactions were evaluated on an Applied Biosystems 377 Genetic Analyzer and the final contig was assembled using the SEQUENCHER program version 4.0.5 (Gene Codes Corporation). The sequences

Table 1. Differentiating biochemical characteristics of strains A175^T, K101^T and *S. wittichii* RW1^T

Strains: 1, A175^T; 2, K101^T; 3, *S. wittichii* RW1^T. +, Positive; –, negative.

Characteristic	1	2	3
Assimilation of:			
N-Acetyl-D-glucosamine, L-arabinose, <i>p</i> -arbutin, azelate, D-cellobiose, D-galactose, D-glucose, D-maltose, α-D-melibiose, D-xylose	+	–	–
Phenylacetate, L-phenylalanine, salicin	–	–	+
L-Alanine, L-aspartate, L-histidine, L-leucine, L-tryptophan, propionate	+	–	+
Adipate, 4-aminobutyrate, fumarate, L-malate, L-ornithine	–	–	–
DL-Lactate, pyruvate	+	–	+
L-Proline	+	–	–
Hydrolysis of:			
Aesculin, pNP-α-D-glucopyranoside, pNP-β-D-glucopyranoside, pNP-phenylphosphonate, L-proline-pNA	+	–	–
pNP-β-D-glucuronide	–	–	+
bis-pNP-phosphate, L-glutamate-γ-3-carboxy-pNA	+	–	+
pNP-phosphorylcholine	–	+	–

were matched against the EMBL database (Kanz *et al.*, 2005) using BLAST 2.2.9 (Altschul *et al.*, 1990). The 16S rRNA gene sequences of strains A175^T and K101^T have already been deposited in the EMBL database under the accession numbers X94101 (Nohynek *et al.*, 1996a) and AJ009706 (Männistö *et al.*, 1999), respectively. The sequences were aligned using CLUSTAL_X software (Thompson *et al.*, 1997). Tree topologies were reconstructed with the neighbour-joining algorithm with 1000 bootstrap replications, according to the method of Junca and Pieper (2004) (Fig. 1) and the UPMGA algorithm with the Kimura two-parameter model was calculated using MEGA 3.1 software (Kumar *et al.*, 2004) (see Supplementary Fig. S1 in IJSEM Online) using the EMBL database (Kanz *et al.*, 2005). The 16S rRNA gene sequence of strain A175^T showed 93.3 % similarity to that of *Sphingomonas paucimobilis* GIFU 2395^T, 96.0 % to strain K101^T and 96.7 % to the closest

recognized species, *S. wittichii* RW1^T (Yabuuchi *et al.*, 2001). These relatively low gene sequence similarities indicate that strain A175^T represents a novel species. Strain K101^T showed a 16S rRNA gene sequence similarity of 93.1 % to *S. paucimobilis* GIFU 2395^T and 95.8 % to *S. wittichii* RW1^T.

The procedures used for extraction and HPLC analysis of bacterial polyamines were performed as described by Busse & Auling (1988) and Busse *et al.* (1997). Strains A175^T (Busse *et al.*, 1999) and K101^T represent species containing *sym*-homospermidine as the major polyamine and they share this characteristic with the other species of the genus *Sphingomonas sensu stricto* (Busse *et al.*, 1999; Takeuchi *et al.*, 2001) and the genus *Sphingosinicella* (Geueke *et al.*, 2007). Strain A175^T appeared to be unique in the entire genus with respect to its unusually high content of

Fig. 1. Unrooted neighbour-joining dendrogram of the phylogenetic relationships between *Sphingomonas haloaromaticamans* sp. nov. A175^T, *Sphingomonas fennica* sp. nov. K101^T, all recognized species of the genus *Sphingomonas* and the type species of the genera *Sphingobium*, *Sphingopyxis*, *Novosphingobium* and *Sphingosinicella* based on a distance matrix analysis of the 16S rRNA gene sequences. GenBank accession numbers are given in parentheses. Bootstrap percentages are indicated at tree branching points. Bar, 0.005 substitutions per nucleotide.

cadaverine (see Supplementary Table S2 in IJSEM Online). Respiratory quinones were extracted as reported by Ventosa *et al.* (1993) and Altenburger *et al.* (1996). While strain A175^T formed only ubiquinone Q-10, strain K101^T also contained small amounts of Q-9 (2 %).

GC was used to analyse the fatty acid profiles of the novel strains, as described previously (Kämpfer *et al.*, 1992). The fatty acid C_{16:1}ω9*c* was shared by *S. wittichii* RW1^T and strain K101^T only. These two strains also contained fatty acid C_{16:1}ω11*c*, which has not been found previously within members of the genus *Sphingomonas* (Busse *et al.*, 1999). Fatty acid C_{18:0} was found in *S. wittichii* RW1^T, strain A175^T and strain K101^T. In contrast to strain A175^T, strain K101^T did not contain fatty acids C_{16:1}ω5*c*, C_{17:1}ω6*c*, C_{17:1}ω8*c*, C_{18:1}ω5*c* or those fatty acids that comprise summed feature 4 (Table 2).

Polar lipids were extracted according to the method of Tindall (1990) and analysed by two-dimensional TLC. Unique polar lipid profiles distinguished strains A175^T and K101^T from each other and from *S. wittichii* RW1^T. Lack of phosphatidylmonomethylethanolamine and phosphatidyl-dimethylethanolamine in extracts from strain A175^T enabled this strain to be differentiated from *S. wittichii* RW1^T (Busse *et al.*, 1999) and strain K101^T. The absence of detectable amounts of phosphatidylcholine and the unknown lipids PL3, GL1 and GL4 in extracts from strain K101^T was unique among these closely related strains (see Supplementary Table S1a in IJSEM Online).

Table 2. Cellular fatty acids of species of the genus *Sphingomonas*

Strains: 1, *S. wittichii* RW1^T; 2, strain A175^T; 3, strain K101^T. Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 4 consists of one or more of the following fatty acids: C_{16:1}ω7*c* and C_{15:0} iso 2-OH. Summed feature 7 consists of one or more of the following isomers: C_{18:1}ω7*c*, C_{18:1}ω9*t*, and/or C_{18:1}ω12*t* (*cis* and *trans* isomers are indicated by the suffixes *c* and *t*, respectively).

Fatty acid	1	2	3
C _{14:0}	1.7	0.7	0.5
C _{15:0}		0.8	
C _{16:0}	12.8	9.2	11.4
C _{18:0}	0.3	0.7	0.2
C _{16:1} ω5 <i>c</i>	6.0	5.2	
C _{16:1} ω9 <i>c</i>	11.0		18.5
C _{16:1} ω11 <i>c</i>	3.3		2.1
C _{17:1} ω6 <i>c</i>	0.7	7.1	
C _{17:1} ω8 <i>c</i>		0.9	
C _{18:1} ω5 <i>c</i>	1.4	1.6	
C _{14:0} 2-OH	7.4	8.3	8.6
C _{15:0} 2-OH	<0.1	0.6	0.4
C _{16:0} 2-OH	<0.1		1.0
Summed feature 4	9.3	11.0	
Summed feature 7	55.6	51.6	54.3

Lipids were extracted by a modified Bligh–Dyer method (Fredrickson *et al.*, 1986), followed by analysis in a mass spectrometer using fast atom bombardment (FAB-MS) ionization recorded in the negative mode of selected fractions as previously described by Abraham *et al.* (1997). Analysis of the individual phospholipids by MS revealed that C_{18:1}–C_{16:1}-phosphatidyl-*N,N*-dimethylethylamine (743 Da), the corresponding choline (756 Da), C_{18:1}–C_{18:1}-phosphatidic acid (700 Da), the corresponding -*N,N*-dimethylethylamine (771 Da) and corresponding choline (785 Da) were formed by nearly all members of the family *Sphingomonadaceae* investigated. Interestingly, for the separated group of strains in the *Sphingomonas* cluster, *S. wittichii* RW1^T and strains K101^T and A175^T, some lipids could be identified as occurring preferentially in these strains. These were C_{19:1}–C_{16:0}-phosphatidic acid (688 Da) and -glycerol (762 Da), C_{18:1}–C_{16:1}-*N*-methyl-ethylamine (729 Da) and C_{18:1}–C_{16:2}-phosphatidyl-*N,N*-dimethylethylamine (741 Da) (see Supplementary Table S1b in IJSEM Online).

On the basis of the 16S rRNA gene sequences, strains A175^T and K101^T were found to be only remotely related to other species of the genus *Sphingomonas*. Although strain A175^T is more closely related to *S. wittichii* RW1^T than to strain K101^T on the basis of 16S rRNA gene sequence comparisons, strain A175^T differs significantly from these sphingomonads in its polyamine pattern (see Supplementary Table S2 in IJSEM Online).

Our data do not support the notion of Takeuchi *et al.* (2001) that members of the genus *Sphingomonas* and those of the genera *Sphingobium*, *Novosphingobium* and *Sphingopyxis* can be discerned by the presence of *sym*-homospermidine and the absence of spermidine in *Sphingomonas* species while species of the other three genera possess spermidine but not homospermidine (see Supplementary Table S2). Both polyamines were detected in *S. wittichii* RW1^T and in strains A175^T and K101^T, but were also detected in the type strains of *Sphingomonas asaccharolytica*, *Sphingomonas paucimobilis*, *Sphingomonas trueperi*, *Sphingomonas abaci*, *Sphingomonas panni*, *Sphingomonas echinoides* and *Sphingomonas pituitosa* (Busse *et al.*, 1999, 2005; Denner *et al.*, 1999, 2001). However, all species of the genus *Sphingomonas sensu stricto* are characterized by the predominant polyamine *sym*-homospermidine. These results show that species of the genus *Sphingomonas* have mainly *sym*-homospermidine and some of them also produce minor quantities of spermidine. Species of the genera *Sphingobium*, *Novosphingobium* and *Sphingopyxis* produce no *sym*-homospermidine. However, *sym*-homospermidine also characterizes species of the genus *Sphingosinicella* (Geueke *et al.*, 2007; Maruyama *et al.*, 2006). Although strains K101^T and A175^T share most of the signature nucleotides proposed by Takeuchi *et al.* (2001) for the differentiation of members of the genus *Sphingomonas sensu stricto* from related genera, these nucleotides are also found in species of the genus *Sphingosinicella* (Geueke *et al.*, 2007; Maruyama

et al., 2006). In conclusion, neither the polyamine patterns nor the signature nucleotides allow the assignment of strains K101^T and A175^T to either of the two genera. The phylogenetic distance of the two novel strains to representatives of the genus *Sphingosinicella* and their close relatedness to *S. wittichii*, as well as differences in the signatures identified for members of the genus *Sphingosinicella* by Geueke *et al.* (2007) support the assignment of strains K101^T and A175^T to the genus *Sphingomonas*. Therefore, the names *Sphingomonas fennica* sp. nov. and *Sphingomonas haloaromaticamans* sp. nov. are proposed for strains K101^T and A175^T, respectively.

Description of *Sphingomonas haloaromaticamans* sp. nov.

Sphingomonas haloaromaticamans (ha.lo.a.ro.ma.tic.a'mans. N.L. n. *haloaromaticum* haloaromatic, class of chemical compound; L. part. adj. *amans* loving; N.L. part. adj. *haloaromaticamans* loving haloaromatics).

Cells are rod-shaped, 0.8–1.5 µm in length and 0.4–0.6 µm in diameter. Colonies are yellow-coloured. *N*-Acetyl-D-glucosamine, L-arabinose, *p*-arbutin, D-cellobiose, D-galactose, D-glucose, D-maltose, α-D-melibiose, D-xylose, acetate, propionate, azelate, DL-3-hydroxybutyrate, DL-lactate, pyruvate, L-alanine, L-aspartate, L-histidine, L-leucine, L-proline and L-tryptophan are used as substrates, but not D-fructose, gluconate, D-mannose, L-rhamnose, sucrose, salicin, D-trehalose, maltitol, D-mannitol, D-sorbitol, *cis*-aconitate, adipate, 4-aminobutyrate, citrate, fumarate, L-malate, L-ornithine, L-phenylalanine, 3-hydroxybenzoate, 4-hydroxybenzoate or phenylacetate. Aesculin, pNP-α-D-glucopyranoside, pNP-β-D-glucopyranoside, *bis*-pNP-phosphate, pNP-phenylphosphonate, 2-deoxythymidine-5'-pNP-phosphate, L-alanine-pNA, L-glutamate-γ-3-carboxy-pNA and L-proline-pNA are hydrolysed, but pNP-β-D-galactopyranoside, pNP-β-D-glucuronide and pNP-phosphorylcholine are not hydrolysed. Nitrate is not reduced. The main polar lipids are phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and sphingoglycolipid. Phosphatidylcholine, unidentified phospholipid PL3 and unidentified glycolipid GL4 are minor constituents. Major fatty acids are C_{18:1} (summed feature 7) and C_{16:1} (summed feature 4). Minor fatty acids are C_{14:0}, C_{15:0}, C_{16:0}, C_{18:0}, C_{16:1ω5c}, C_{17:1ω6c}, C_{17:1ω8c} and C_{18:1ω5c}. The major hydroxy-fatty acid is C_{14:0} 2-OH together with minor amounts of C_{15:0} 2-OH. The major polyamine is homospermidine; minor polyamines are diaminopropane, putrescine, cadaverine, spermidine and spermine. Produces only ubiquinone Q-10. The DNA G + C content of the type strain is 66.1 mol%. Grows between 10 and 40 °C with optimal growth between 30 and 37 °C. The optimal pH range is 5–8.

The type strain, A175^T (=DSM 13477^T=CCUG 53463^T), was isolated from water and soil samples from the Netherlands as a degrader of benzene, catechol, chlorobenzene, 1,3-dichlorobenzene and 1,4-dichlorobenzene.

Description of *Sphingomonas fennica* sp. nov.

Sphingomonas fennica (fen.ni'ca. N.L. fem. adj. *fennica* pertaining to Finland, from where the type strain was isolated).

Colonies are coloured light yellow but the colour is not stable during subcultivation on rich medium. Short, plump, rod-shaped cells, 0.5–0.9 µm by 0.9–1.5 µm. Cells are motile. Reduces nitrate to nitrogen. Acetate and DL-3-hydroxybutyrate are used as substrate but not *N*-acetyl-D-glucosamine, L-arabinose, *p*-arbutin, D-cellobiose, D-fructose, D-galactose, gluconate, D-glucose, D-mannose, D-maltose, α-D-melibiose, L-rhamnose, sucrose, salicin, D-trehalose, D-xylose, maltitol, D-mannitol, D-sorbitol, propionate, *cis*-aconitate, adipate, 4-aminobutyrate, azelate, citrate, fumarate, DL-lactate, L-malate, pyruvate, L-alanine, L-aspartate, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-tryptophan, 3-hydroxybenzoate, 4-hydroxybenzoate or phenylacetate. pNP-phosphorylcholine, 2-deoxythymidine-5'-pNP-phosphate and L-alanine-pNA are hydrolysed, but aesculin, pNP-β-D-galactopyranoside, pNP-β-D-glucuronide, pNP-α-D-glucopyranoside, pNP-β-D-glucopyranoside, *bis*-pNP-phosphate, pNP-phenylphosphonate, L-glutamate-γ-3-carboxy-pNA and L-proline-pNA are not hydrolysed. Polar lipids are phosphatidylmonomethylethanolamine, phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylmethylethanolamine and sphingoglycolipid. Major fatty acids are C_{18:1} (summed feature 7), C_{16:1ω9c} and C_{16:0}. Minor fatty acids are C_{14:0}, C_{18:0}, and C_{16:1ω11c}. The major hydroxy-fatty acid is C_{14:0} 2-OH, with minor amounts of 2-C_{15:0} 2-OH and C_{16:0} 2-OH. Major polyamine is homospermidine; minor polyamines are putrescine, cadaverine, spermidine and spermine. Produces ubiquinone Q-10, but small amounts (2%) of Q-9 can also be detected. DNA G + C content of the type strain is 63.6 mol%. Grows between 10 and 37 °C with optimal growth between 20 and 30 °C. pH range for growth is 5–8, optimal pH is 7–8.

The type strain, K101^T (=DSM 13665^T=CCUG 53462^T), was isolated from polychlorophenol-contaminated groundwater adjacent to a sawmill, Southern Finland, as a degrader of 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol and pentachlorophenol. The environment from which the strain was originally isolated was cold (7–8 °C), oxygen-deficient, humic (4–23 mg dissolved organic carbon l⁻¹) and slightly acidic (pH 6–6.5).

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