Journal of Industrial Microbiology & Biotechnology (1999) 23, 252–260

http://www.stockton-press.co.uk/jim

Detection of *Sphingomonas* spp in soil by PCR and sphingolipid biomarker analysis

KT Leung[1,2], YJ Chang[1], YD Gan[1], A Peacock[1], SJ Macnaughton[1], JR Stephen[1], RS Burkhalter[1], CA Flemming[1,3] and DC White[1,4]

[1]*Center for Environmental Biotechnology, University of Tennessee, Knoxville, TN 37932-2575;* [4]*Biological Science Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA*

***Sphingomonas* spp possess unique abilities to degrade refractory contaminants and are found ubiquitously in the environment. We developed *Sphingomonas* genus-specific PCR primers (SPf-190 and SPr1-852) which showed specific amplification of a 627-bp 16S rDNA fragment from *Sphingomonas* spp. A PCR assay using these *Sphingomonas* specific primers was developed to detect *Sphingomonas aromaticivorans* B0695R in three texturally distinct soil types, showing detection limits between 1.3–2.2 $\times 10^3$ CFU g^{-1} dry soil. A sphingolipid extraction protocol was also developed to monitor *Sphingomonas* populations in soil quantitatively. The detection limit of the assay was 20 pmol g^{-1} dry soil, equivalent to about 3×10^5 cells g^{-1} dry soil. Survival of *S. aromaticivorans* B0695R was monitored in the three different soils by antibiotic selective plate counting, PCR and sphingolipid analysis. All three approaches showed that the B0695R cells persisted in the low biomass Sequatchie sub-soil at about 3–5 $\times 10^7$ cells g^{-1} dry soil. In comparison to the plate counting assay, both the PCR and sphingolipid analysis detected a significantly higher level of B0695R cells in the clay soil and Sequatchie top-soil, indicating the possibility of the presence of viable but non-culturable B0695R cells in the soils. The combination of PCR and sphingolipid analysis may provide a more realistic estimation of *Sphingomonas* population in the environment.**

Keywords: *Sphingomonas*; sphingolipid analysis; PCR; soil

Introduction

The genus *Sphingomonas* has recently received a lot of attention because of its diverse metabolic capability [20]. Many members of the *Sphingomonas* genera possess unique abilities in degrading refractory organic pollutants [26] and antagonizing phytopathogenic fungi [3]. Some members of the genus are involved in metal corrosion [1] and some are considered as potential opportunistic human pathogens [28].

Based on 16S rDNA sequence comparison, *Sphingomonas* species are classified as a monophyletic group within the α-4 Proteobacteria. Members of the *Sphingomonas* genus are Gram-negative, aerobic, flagellated and mostly yellow-pigmented. One unique phenotypic feature, which distinguishes *Sphingomonas* from other members of the Eubacteria, is the possession of sphingolipids in their cellular membrane. These sphingolipids contain 18–21 carbon straight chain sphingosine bases, and amide-linked 2-hydroxy straight chain saturated fatty acids. Unlike other Gram-negative bacteria, members of the *Sphingomonas* genus do not contain lipopolysaccharide [12].

The genus *Sphingomonas* was proposed by Yabuuchi *et al* [28] to describe bacterial strains isolated from human clinical specimens and hospital environments. It was later found that this group of bacteria could also be isolated from natural sources, such as soil, rhizosphere, sediment, freshwater and marine environments [26]. Many *Sphingomonas* strains isolated from the environment possess broad catabolic capabilities towards recalcitrant organic pollutants, which include dibenzo-*p*-dioxin and dibenzofuran [27], carbofuran [6], hexachlorocyclohexane [9], chlorinated biphenyls [24], polychlorophenols [11,16], 2,4-dichlorophenoxyacetic acid [10], halogenated diphenyl ethers [21], and single-ring aromatic and polyaromatic hydrocarbons [7,13]. Despite the catabolic diversity of the genus *Sphingomonas*, its members are related phylogenetically. Balkwell *et al* [2] showed that the 16S rRNA gene sequence similarities of their subsurface *Sphingomonas* isolates were as high as 99.9%. Furthermore, the sequence similarities between these strains and other known *Sphingomonas* species, such as *S. capsulata* were about 98%.

Recently, *Sphingomonas* strains were isolated from deep subsurface sediments (108–407 meters below the surface) and found to be able to degrade aromatic hydrocarbons [2,7]. This revealed the wide environmental distribution of this group of bacteria, as well as its potential importance in recycling recalcitrant organic compounds. Despite the ecological importance of the *Sphingomonas* genus, information related to the ecology of this group of bacteria in soil environments is limited. Members of the genus *Sphingomonas* lack easily identified phenotypic features and, until now, no attempt to monitor the dynamics of these bacteria by molecular methods has been made.

The objectives of this study were threefold: (i) to design specific PCR primers to detect members of the genus *Sphin-*

Correspondence: DC White, Center for Environmental Biotechnology, University of Tennessee, 10515 Research Dr, Suite 300, Knoxville, TN 37932-2575, USA
[2]Present address: TM Bioscience Corporation, 439 University Ave, 11th floor, Toronto, Ontario, Canada M5G 1Y8
[3]Present address: Nalco Canada Inc, 1055 Truman St, Burlington, ON, Canhada, L7R 3Y9
Received 17 March 1999; accepted 7 April 1999

gomonas; (ii) to extract and quantify sphingolipids from *Sphingomonas* cells in soils; and (iii) to combine these novel methods, along with traditional plate-counting, to monitor the survival of an aromatic hydrocarbon-degrading *S. aromaticivorans* strain B0695R.

Materials and methods

Bacterial strains

The subsurface *Sphingomonas* strains B0478, B0522, B0712, B0695 and F199 were obtained from the DOE Subsurface Microbial Culture Collection (SMCC) at Florida State University [2]. *S. aromaticivorans* B0695 was isolated from a deep subsurface sediment (259 m beneath the surface) at the Southeast Coastal Plain of South Carolina and is able to metabolize aromatic hydrocarbons as sole carbon sources [7]. *S. aromaticivorans* B0695R is a spontaneous rifampicin-resistant mutant of strain B0695. The growth rate and aromatic hydrocarbon-degrading capability of *S. aromaticivorans* B0695R were unaffected by the mutation. The rifampicin resistance phenotype was stable after four transfers in Nutrient Broth (Difco, Detroit, MI, USA; data not shown). *S. aromaticivorans* B0695R was routinely grown in and maintained on Nutrient Broth and Nutrient Agar containing 50 μg ml^{-1} rifampicin, respectively. *Sphingomonas* sp strain UG30 and *S. yanoikuyae* B1 were obtained from Dr JT Trevors at the University of Guelph, Ontario, Canada and Dr DT Gibson at the University of Iowa, Iowa, USA, respectively. *Shewanella putrifaciens* strain 200 was an oil pipeline isolate [17]. *Pseudomonas aeruginosa* strain FRD-1 was isolated from a cystic fibrosis patient [18]. The *Bacillus* strain was isolated from a fresh water sample at the Center for Environmental Biotechnology, University of Tennessee. The rest of the bacterial strains in Table 1 were obtained from the American Type Culture Collection (ATCC). The *S. chlorophenolicum* strains and *Escherichia coli* were grown in a minimal salt-glutamate [14] and Luria-Bertani (LB broth, Difco) medium, respectively. *Desulfovibrio vulgaris* ATCC 29579 was cultured anaerobically in an acetate-yeast extract medium [19]. The rest of the strains were grown in Nutrient Broth at 30°C.

Soils

Three soils were used, two sandy loam soils, an agricultural top soil (0–15 cm) and a subsurface soil (135–150 cm) from the University of Tennessee Agriculture Experiment Station in Alcoa (Sequatchie series). A subsurface clay soil (135–150 cm) was collected from an uncultivated field next to the Center of Environmental Biotechnology, University of Tennessee, Knoxville. The physical, chemical and biological characteristics of the soils are summarized in Table 2.

DNA extraction, purification and amplification

DNA, from soil or bacteria, was extracted by a bead-beating system adapted from Borneman *et al* [4] with modifications. Five hundred milligrams of soil, 400 μl sodium phosphate buffer (0.12 M, pH 8.0), 200 μl chaotropic reagent (CRSR, BIO 101, Vista, CA, USA) and 500 mg glass beads (0.17 mm in diameter) were agitated in a 1.5-ml microcentrifuge tube using a high speed Crescent WIG-L-BUG™ bead beater (Crescent Dental MFG Co, Lyons, IL, USA) for 1.5 min. The sample was centrifuged at 13 000 $\times$ *g* for 5 min. The supernatant was collected and 300 μl chloroform was added to the soil pellet, mixed thoroughly, and centrifuged at 13 000 $\times$ *g* for 5 min. The aqueous supernatant was collected, pooled with the first supernatant fraction and DNA was precipitated with an equal volume of isopropanol in an ice bath for 30 min. The DNA extract was washed twice with 80% ethanol, re-dissolved in 200 μl TE buffer (pH 8.0), extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v v^{-1} v^{-1}) and followed by a glass milk DNA purification protocol using a Gene Clean Kit (BIO 101) as described by the manufacturer. The concentrations of the DNA extracts were estimated by densiometry and confirmed by UV spectrophotometry at 260 nm. DNA extraction efficiency was measured by comparing the amount of DNA recovered from uninoculated soil, soil inoculated with 5×10^8 B0695R cells, and the same number of cells in pure culture. Experiments were performed in triplicate. Purified DNA samples were used directly in a PCR assay with primer pair SPf-190/SPr1-852 or SPf-190/SPr2-852. The reaction was performed in a Labline Thermal Block II Thermocycler (Lab Line Instruments, Inc, Melrose Park, IL, USA) in a 25-μl volume of reaction mixture, which

Table 1 Specificity of the *Sphingomonas*-specific PCR primers[a]

Bacterial strains	PCR amplification by primers	
	SPf-190 and SPr1-852	SPf-190 and SPr2-852
Sphingomonas aromaticivorans		
F199	+	–
B0695	+	–
B0695R	+	–
Sphingomonas subterranea		
B0478	+	–
B0522	+	–
Sphingomonas stygia		
B0712	+	–
Sphingomonas capsulata	+	–
Sphingomonas paucimobilis	+	–
Sphingomonas parapaucimobilis	+	–
Sphingomonas yanoikuyae B1	+	–
Sphingomonas terrae	+	–
Sphingomonas chlorophenolicum		
ATCC 39723	–	+
ATCC 33790	–	+
UG30	–	+
Rhizomonas suberifaciens	–	–
Zymomonas mobilis	–	–
E. coli K-12	–	–
Pseudomonas aeruginosa FRD-1	–	–
Pseudomonas oleovorans ATCC 19347	–	–
Pseudomonas putida ATCC 33015	–	–
Alcaligenes eutropus CH34	–	–
Bacillus sp	–	–
Shewenella putrefaciens 200	–	–
Desulfovibrio vulgaris strain Hildenborough	–	–

[a]+ and – represent positive and negative amplification by the *Sphingomonas*-specific 16S rDNA PCR primers, respectively.

Table 2 Characteristics of the Sequatchie top-soil, Sequatchie sub-soil and clay soil

Property[a]	Sequatchie top-soil	Sequatchie sub-soil	Clay soil
Textural class	Sandy loam	Sandy loam	Clay
Sand/silt/clay (%)	71.5/24.0/4.5	55.9/34.1/10.0	32.9/21.6/45.5
WHC[b] (%)	23.1	31.4	42.1
Soil pH[c]	5.9	5.7	4.5
Organic carbon content (%)	1.6	1.2	1.3
Total nitrogen content (ppm)	394	167	174
Total sulphur content (ppm)	74	96	64
CFU g^{-1} dry soil	2.1×10^7	2.2×10^6	5.8×10^4
PLFA[d] (pmol g^{-1} dry soil)	16 685	400	255
Soil DNA (μg g^{-1} dry soil)	6.1	0.8	<0.1

[a] Measurements shown in this table are means of duplicate measurements.
[b] Water-holding capacity.
[c] Soil pH was measured in a 1:1 ratio of soil:deionized water mixture.
[d] Phospholipid fatty acids (PLFA) were extracted by the Bligh and Dyer procedure and analyzed by gas chromatography [25].

contained 1× Expand High Fidelity (HF) PCR buffer, 1.2 U of the Expand HF DNA polymerase (Boehringer Mannheim, IN, USA), 200 μM each of dNTPs, 0.2 μM each of primers, 1% formamide and 1 μl of template DNA, and a layer of 50 μl overlaying mineral oil. The mixture was subjected to a 5-min denaturation at 94°C followed by a 2-min annealing at 50°C and extension at 72°C. Parameters for the subsequent 34 cycles were 1 min denaturation, annealing and extension, followed by a final 10-min extension. The PCR product was analyzed by electrophoresis in a 1.2% (wt/vol) agarose gel containing ethidium bromide (0.2 μg ml^{-1}).

PCR primers

PCR primers were designed by an alignment of 16S rRNA gene sequences from 14 *Sphingomonas* strains and other Proteobacteria. A *Sphingomonas* genus-specific forward PCR primer, SPf-190, was synthesized based on a conserved region (nucleotide positions 190–126, based on the *E. coli* 16S rDNA sequence) of the *Sphingomonas* 16S rDNA sequences. Another *Sphingomonas* genus-specific reverse PCR primer, SPr1-852, was also designed based on a stretch of the 16S rDNA sequence between nucleotide positions 833–852. However, sequence alignment revealed that the 16S rDNA of the *S. chlorophenolicum* has a different sequence at the position of SPr1-852 primer. Consequently, primer SPr2-852 was designed to amplify the 16S rDNA of the *S. chlorophenolicum* strains (Figure 1).

Sphingolipid analysis

All solvents were of GC grade and were obtained from Fisher Scientific (Pittsburgh, PA, USA). *S. aromaticivorans* B0695R (50 mg fresh weight) or 10 g soil sample was used for sphingolipid analysis. Total lipid of the sample was extracted by the modified Bligh and Dyer procedure [25]. The lipid extract was dried under a stream of nitrogen gas at room temperature, re-dissolved in 1 ml of 3 M hydrochloric acid and heated in a capped 15-ml glass tube at 100°C for 3 h. The acid digest was cooled to room temperature and adjusted to pH 12 by KOH. The digest was extracted three times with 2 ml chloroform. The chloroform fraction was pooled, dried under nitrogen and derivatized by in 100 μl bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 60°C for 30 min. After evaporation under a stream of nitrogen, samples were redissolved in hexane and analyzed by GC-MS. The sample was analyzed using a Hewlett-Packard (HP) 5890 series II GC equipped with a HP 5973 series mass selective detector and a HP-5 capillary column (Hewlett-Packard Co, Palo Alto, CA, USA). The carrier gas (helium) was set at an inlet pressure of 30 p.s.i. and the temperature program of the column was: starting at 100°C, 100–200°C at 10°C min^{-1}, 200°C for 1 min, 200–300°C at 4.5°C min^{-1}, and 300°C for 3 min. Sphingosine (C18:1, Sigma, Chemical Co, St Louis, MO, USA) was used as an internal standard for the assay.

Microcosm design

S. aromaticivorans B0695R cells were grown in Nutrient Broth (Difco) containing 50 μg ml^{-1} rifampicin at 30°C, harvested at late log phase, washed twice with 0.85% sterile NaCl solution, and suspended in sterile deionized water at a density of 1×10^{10} cells ml^{-1}. Hundred-gram portions of the samples (sandy loam top-soil, fine sandy loam sub-soil and clay sub-soil) were added to sterile 250-ml polypropylene beakers. *S. aromaticivorans* B0695R cells were inoculated at a cell density of about 5×10^8 cells g^{-1} dry soil. Control microcosms received an equal volume of sterile deionized water instead of the cell suspension. Sterile deionized water was added to the soil to achieve a final moisture content of 75% of its water-holding capacity. The soil microcosms were covered with aluminum foil and incubated in the dark at 22°C and 100% humidity. Soil samples were collected at days 0, 3, 7, 14 and 21. Dilution series of the soil samples were prepared and 100 μl of each dilution was spread-plated on Nutrient Agar with or without rifampicin. Portions of the soil samples were used for the PCR assay and sphingolipid analysis as described earlier. All treatments were conducted in triplicate.

Results and discussion

Sphingomonas-specific PCR primers

Genus- and species-specific PCR primers for 16S rDNA of various groups of eubacteria have been used to detect spe-

Primers SPf-190	**5′MRGWCCAAAGATTTATCG**
Sphingomonas aromaticivorans B0695	CGGACCAAAGATTTATCG
Sphingomonas aromaticivorans F199	.....
Sphingomonas subterranea B0478	.....
Sphingomonas subterranea B0522	.....
Sphingomonas stygia B0712	.....
Sphingomonas capsulata	...T.....
Sphingomonas adhaesiva	AA.....
Sphingomonas paucimobilis	AA..T.....
Sphingomonas yanoikuyae B1	AA.T.....
Sphingomonas sanguis	AA.T.....
Sphingomonas chlorophenolicum ATCC 33790	AA.T.....
Sphingomonas chlorophenolicum ATCC 39723	AA.....
Sphingomonas chlorophenolicum RA2	AA.....
Sphingomonas chlorophenolicum SR3	AA.....
Rhizomonas suberifaciens	AA.T.....
Escherichia coli	AA.....GGGGGAC
Burkholderia cepacia	....TG....CGGGNGAC
Pseudomonas aeruginosa	A..GAG....GGGGGGAT

Primer SPr1-852	**5′CMAADCACCAWGTGMCCKGA**
Primer SPr2-852	**5′TGAAATGCCATGCACCCCAG**
Sphingomonas aromaticivorans B0695	CCAATCACCAAGTGACCGGA
Sphingomonas aromaticivorans F199	.....
Sphingomonas subterranea B0478	....A.....C.....
Sphingomonas subterranea B0522	.....
Sphingomonas stygia B0712	....A.....C.....
Sphingomonas capsulata	....A.....C.....
Sphingomonas adhaesiva	..N.A..N.NN...N.....
Sphingomonas paucimobilis	....G.....C.....
Sphingomonas yanoikuyae B1	.A..A.....T...C..T..
Sphingomonas sanguis	....G.....C.....
Sphingomonas chlorophenolicum ATCC 33790	TG..ATG...T.CAC..CAG
Sphingomonas chlorophenolicum ATCC 39723	TG..ATG...T.CAC..CAG
Sphingomonas chlorophenolicum RA2	TG..ATG...T.CAC..CAG
Sphingomonas chlorophenolicum SR3	TG..ATG...T.CAC..CAG
Rhizomonas suberifaciens	.T..GTT.T...AAC..T..
Escherichia coli	...CG.CT....G.CA.AAC
Burkholderia cepacia	TA.GGA.ATG.A.-C..CA.
Pseudomonas aeruginosa	TA.GATCT....GAT..CA.

Figure 1 Alignment of *Sphingomonas*-specific 16S rDNA PCR primer sequences. The dots represent nucleotides identical to the *S. aromaticivorans* B0695 sequences. The dash represents a gap in the sample sequence when aligned with the *S. aromaticivorans* B0695 sequence. Sequence differences are indicated by the replacement nucleotides shown below the *S. aromaticivorans* B0695 sequences. IUPAC abbreviations: D = (A, G or T), K = (G or T), M = (A or C), R = (A or G), and W = (A or T).

cific groups of bacteria [5,8,15]. However, no attempt was made to detect members of the genus *Sphingomonas* by PCR amplification based on the genus-specific DNA sequences located within the 16S rDNA. 16S rRNA gene sequences of nine *Sphingomonas* spp and some common soil bacterial strains were compared (Figure 1). A *Sphingomonas* genus-specific sequence was located between nucleotide positions 190–207 of the 16S rRNA gene (based on the *E. coli* 16S rDNA). This sequence was chosen to be the *Sphingomonas*-specific forward PCR primer (SPf-190). Its 3′ end sequence has a complete match between the 14 *Sphingomonas* strains and has seven to eight 3′ end mismatch bases with other bacterial strains. A second *Sphingomonas* genus-specific sequence was located at the nucleotide positions 852–871 of the 16S rRNA gene. This sequence is conserved between eight of the nine *Sphingo-*

Table 3 DNA extraction efficiency of *S. aromaticivorans* B0695R from soils

	Top soil (sandy loam)	Sub soil (fine sandy loam)	Clay soil
Inoculum size (CFU)	5.1×10^8	4.3×10^8	8.0×10^8
Cells only (ηg DNA)	816.4 (± 234.9)[a]	646.9 (± 338.1)	1203.8 (± 118.7)
Soil + cells (ηg g^{-1} dry soil)	895.6 (± 184.5)	424.3 (± 85.5)	992.9 (± 308.2)
Soil only (ηg g^{-1} dry soil)	245.5 (± 32.8)	u.d.[b]	u.d.
Extraction efficiency (%)	79.6 (± 7.6)	65.6 (± 36.7)	82.5 (± 26.9)
PCR detection limit (CFU g^{-1} dry soil)	1.3×10^3	2.2×10^3	1.5×10^3

[a] Mean of triplicate samples ± standard deviation ($n = 3$).
[b] Undetectable.

monas species tested (Figure 1), showing two to five mismatches in the center region of the sequence. A degeneracy primer (SPr1-852) was designed to correct for the mismatches of the sequences. 16S rDNA sequence of *S. chlorophenolicum* at the position of the SPr1-852 primer is distinct from other *Sphingomonas* species, showing 12 mismatches to the SPr1-852 primer. Therefore, a different reversed PCR primer, SPr2-825, was designed for the detection of the *S. chlorophenolicum* strains. The two *Sphingomonas*-specific PCR primer sets (SPf-190/SPr1-852 and SPf-190/SPr2-852) were tested on 13 different *Sphingomonas* strains and 10 other bacterial strains which can commonly be found in the environment. Primer set SPf-190/SPr1-852 showed positive amplification only to the *Sphingomonas* strains (except *S. chlorophenolicum* strains) giving a 627-bp DNA fragment. Primer set SPf-190/SPr2-852 was specific only to the *S. chlorophenolicum* strains (Table 1). Both the *Sphingomonas*-specific PCR primer sets were negative in amplifying DNA extracts of *Rhizomonas suberifaciens* and *Zymomonas mobilis*, which are two of the very few eubacteria that possess sphingolipids.

PCR detection of Sphingomonas aromaticivorans B0695R in soil

S. aromaticivorans B0695R is a well-characterized subsurface isolate capable of utilizing benzoate, *p*-cresol, *o*- and *m*-xylene and salicylate, as sole carbon and energy sources [7]. Because of its potential importance in breaking down recalcitrant aromatic compounds in the environment, it was used in this study as a model bacterium to study *Sphingomonas* detection in soil. Two approaches are commonly used to extract DNA from soil. The indirect approach involves the separation of cells from soil prior to DNA extraction. This is problematic in recovering microbial cells protected by clay particles [22], causing inconsistency in DNA extraction from soils with different physical and chemical characteristics. In this study, a direct soil DNA extraction protocol was tested on three soils, two sandy loam soils and a clay soil, each of which had different biomass contents (Table 2). DNA extraction efficiencies for the red clay and the Sequatchie top- and sub-soils were 83, 80 and 66%, respectively, suggesting that the extraction efficiency of the bead-beating protocol was consistent regardless of the texture of the soils (Table 3). Following spiking a serial dilution of *S. aromaticivorans* B0695R cells into the soils, detection limits of the PCR assay for *S. aromaticivorans* B0695R using the *Sphingomonas* genus-specific primers (SPf-190/SPr1-852) were between 1.3–2.2×10^3 cells g^{-1} dry soil (Table 3). Indigenous *Sphingomonas* in the Sequatchie top-soil and clay soil were below the detection limits of the assay. However, PCR analysis on the Sequatchie sub-soil showed a weak 627-bp amplified fragment indicating the presence of a low level of indigenous *Sphingomonas*. This PCR assay can only follow the changes of *Sphingomonas* population in soil qualitatively. To monitor the dynamics of *Sphingomonas* populations, a protocol for the quantitative measurement of sphingolipids was developed in this study.

Sphingolipid analysis

S. aromaticivorans B0695R was used as a model bacterium to develop our protocol to extract and analyze sphingolipids from soil samples. The four major long-chain sphinganine bases of *S. aromaticivorans* B0695R were C18:0, C20:1, and two C21:1 isomers. They constituted about 83% of the total bacterial cellular sphinganine bases (Table 4). The TMS-derivatized long-chain bases were fragmented to generate signature fragments with m/z of 73 (TMS^+), 103 ($CH_2O\text{-}TMS^+$), 116 (CH- $CH_2O\text{-}TMS^+$), and 132 ($CHNH_2\text{-}CH_2O\text{-}TMS^+$) (Figure 2). The bacterium contained about 7×10^{-5} pmoles sphingolipids $cell^{-1}$ (ie about 4×10^7 sphingolipid molecules $cell^{-1}$, Table 5), which was comparable to the amount of lipopolysaccharide found on the surface of one Gram-negative bacterium (about 9×10^6 molecules $cell^{-1}$). This observation agreed with the findings of Kawasaki *et al* [12] that *Sphingomonas* cells contain sphingolipids instead of LPS.

Extraction efficiencies of the sphingolipids from the red clay and the Sequatchie top- and sub-soil were assessed.

Table 4 Characteristics of major sphinganine bases of *Sphingomonas aromaticivorans* B0695R

Sphinganine base	Retention time (min)	Major MS fragments (m/z)	Percentage
C18:0	24.78	73, 103, 116, 132, 313, 342	16.2 (± 2.9)
C20:1	27.63	73, 103, 116, 132, 339, 368	24.3 (± 1.9)
C21:1 (isomer 1)	28.01	73, 103, 116, 132, 353, 382	15.3 (± 2.9)
C21:1 (isomer 2)	29.36	73, 103, 116, 132, 353, 382	27.0 (± 4.4)

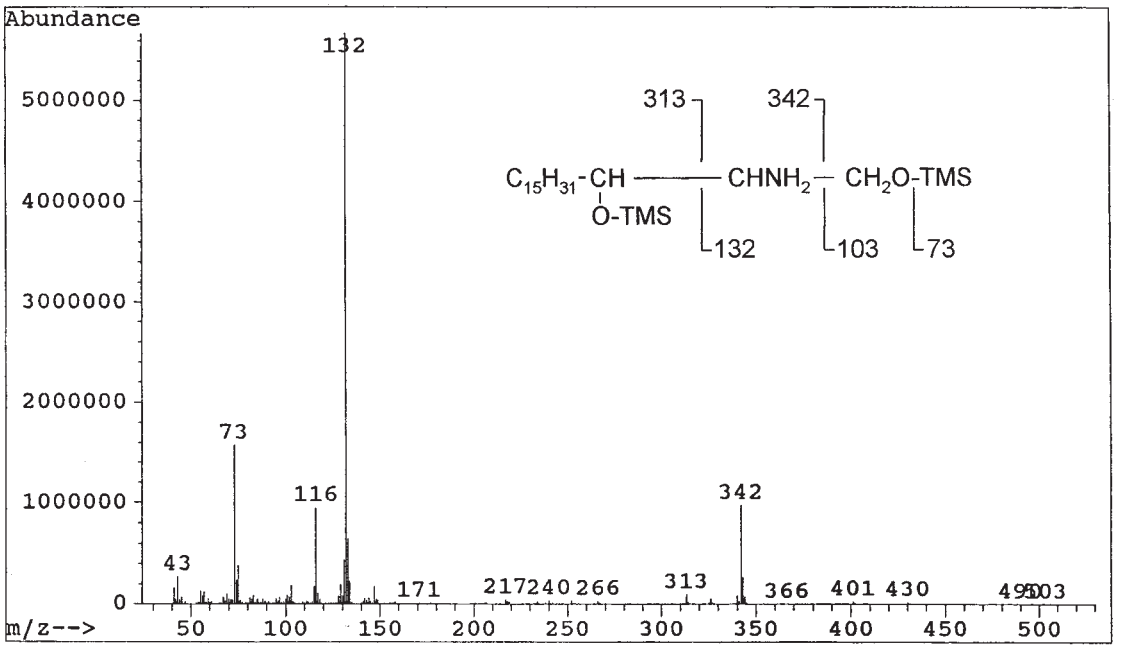

Figure 2 Mass spectrum of C18:0 sphinganine base of *S. aromaticivorans* B0695R.

Table 5 Extraction efficiency of *S. aromaticivorans* B0695R sphinganine bases from soils

	Sphinganine bases recovered from 7×10^9 B0695R cells[a]	Extraction efficiency of B0695R sphinganine bases
Cell culture	4.7×10^5 ($\pm 5.6 \times 10^4$) pmol	100
Top soil	2.3×10^2 ($\pm 4.6 \times 10^1$) pmol g^{-1} dry soil	–
Sub soil	Undetected	–
Clay soil	Undetected	–
Top soil + B0695R cells	3.2×10^5 ($\pm 7.3 \times 10^3$) pmol g^{-1} dry soil	67.9 (± 8.2)
Sub soil + B0695R cells	3.6×10^5 ($\pm 3.1 \times 10^4$) pmol g^{-1} dry soil	75.7 (± 11.2)
Clay soil + B0695R cells	3.3×10^5 ($\pm 3.4 \times 10^4$) pmol g^{-1} dry soil	70.1 (± 11.1)

[a] Mean of triplicate samples ± standard deviation ($n = 3$).

Despite differences in the soils' clay content, 68–76% of the sphingolipids were recovered (Table 5). The detection limit of the sphinganine bases was about 20 pmol g^{-1} dry soil, which was equivalent to about 3×10^5 cells g^{-1} dry soil (data not shown). Sphingolipids were not detected in the clay soil and Sequatchie sub-soil (Table 5). Although the sub-soil tested positive for containing a low level of *Sphingomonas* by the PCR assay, it could be below the detection limit of the sphingolipid assay. About 230 pmol g^{-1} dry soil of sphinganine bases, C18:0 (28.9%) and C20:1 (71.9%), were detected in the Sequatchie top-soil (Table 5). The background C18:0 was identical to the C18:0 of *S. aromaticivorans* B0695R but the background C20:1 had a retention time of 27.95 min which was distinct from the C20:1 of the *S. aromaticivorans* B0695R (Table 4). This observation seems to disagree with the PCR assay that the top-soil contained less than 1.3×10^3 cells g^{-1} dry soil. One possibility is that other sphingolipid-positive bacterial genera, such as *Rhizomonas* and *Zymomonas*, are present in the soil. However, the *Sphingomonas*-specific primers (SPf-190/SPr1-852) did not amplify the ATCC type strains of *Rhizomonas suberifaciens* and *Zymomonas mobilis* (Table 1). Another possibility is the presence of novel *Sphingomonas* species which cannot be detected by primers SPf-190 and SPr1-852. The latter explanation agrees with the findings of Stephen *et al* [23] that rDNA closely related to *Sphingomonas* spp was detected in the Sequatchie top-soil by DGGE analysis. Therefore, the combined approach of using both PCR and sphingolipid analysis improved our understanding of the complexity of *Sphingomonas* populations in the environment.

Survival of Sphingomonas aromaticivorans B0695R in soils

Survival of *S. aromaticivorans* B0695R in the three soils was monitored by spread-plating it on rifampicin-supplemented nutrient agar, PCR and sphingolipid analysis. In the Sequatchie top-soil, the cell density of the inoculum decreased steadily from 4.8×10^8 to 4.9×10^5 CFU g^{-1} dry soil over 15 days incubation and dropped below the detection limit of plate counting (3.3×10^5 CFU g^{-1} dry soil) after 15 days (Figure 3a). The plate-count assay has two major disadvantages in this study. First, the high native population of rifampicin-resistant bacteria in the top soil

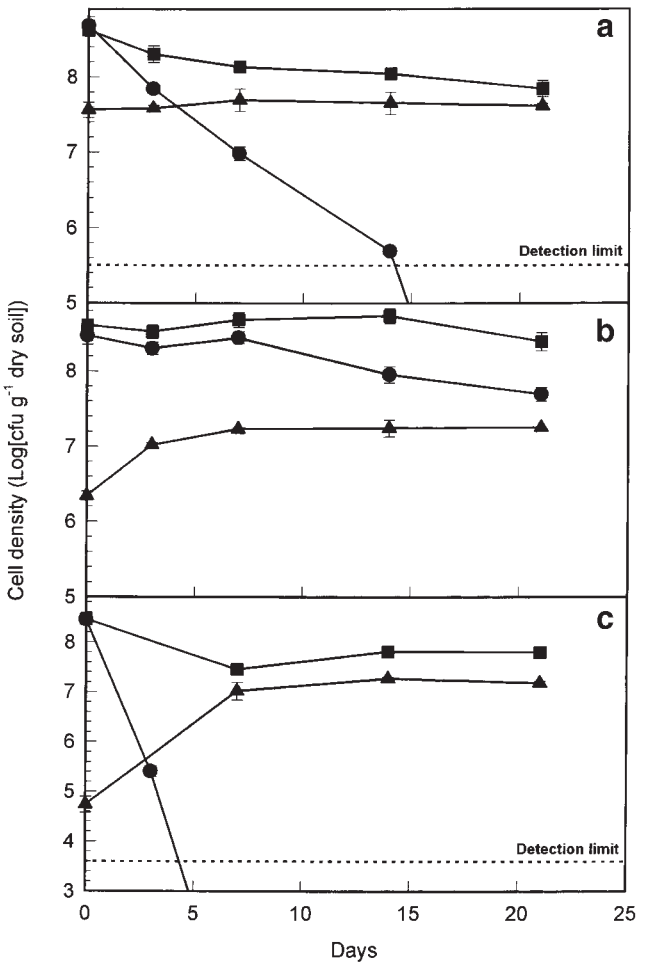

Figure 3 Survival of *S. aromaticivorans* B0695R in (a) Sequatchie top-soil; (b) Sequatchie sub-soil; and (c) clay soil as determined by antibiotic selective plate counting. ●, Cell density of *S. aromaticivorans* B0695R; ■, total bacterial counts of the inoculated soils; ▲, total bacterial counts of the uninoculated soils. Error bars represent ± one standard deviation ($n = 3$).

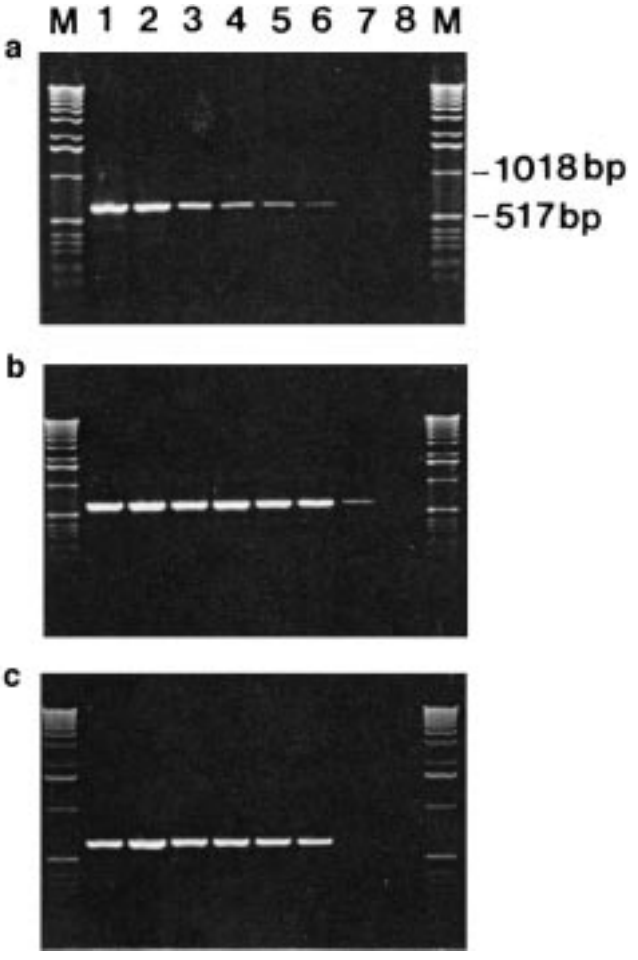

Figure 4 Agarose gel illustrating PCR products of DNA extracts from (a) Sequatchie top-soil; (b) Sequatchie sub-soil; and (c) clay soil seeded with *S. aromaticivorans* B0695R cells. Lanes 2 and 8 are positive and negative (sterile deionized water) controls, respectively. Lanes 2, 3, 4, 5 and 6 represent PCR products of soil extracts sampled at days 0, 3, 7, 14 and 21, respectively; lane 7 is uninoculated control; lane M is the 1-kb DNA ladder marker.

decreased the sensitivity of detection of the rifampicin-resistant *S. aromaticivorans* B0695R cells in the soil. Secondly, the spread-plate assay could not account for the viable but non-culturable (VBNC) *S. aromaticivorans* B0695R cells in the soil samples. Since neither the PCR nor sphingolipid analysis require growth of the *S. aromaticivorans* B0695R cells, these assays represent a more realistic estimation of the bacterial population in soil. PCR analysis demonstrated steadily decreasing intensity of amplification products of *S. aromaticivorans* B0695R from day 0 to 21 (Figure 4a). Sphingolipid analysis provided a quantitative estimation of the population of *S. aromaticivorans* B0695R in the top-soil. The concentration of the sphingolipids decreased from 1.5×10^4 to 140 pmol g^{-1} dry soil, equivalent to about 2.1×10^8 to 1.7×10^6 cells g^{-1} dry soil, respectively, in 7 days and remained at a similar level from day 7 to 21 (Figure 5). The sphingolipid analysis agreed with the PCR amplification that a significant population of *S. aromaticivorans* B0695R was present in the soil sample even at 21 days. However, the steady decline of the *S. aromaticivorans* B0695R rDNA did not agree with the consistent level of sphingolipids from day 7 to 21. The discrepancy may be due to the reduction of the size of the *S. aromaticivorans* B0695R genome when the bacteria entered the VBNC phase in the top soil. It is also possible that sphingolipids of non-viable cells were protected by soil aggregates causing a reduction of sphingolipid degradation. These results were in general agreement with the findings of Stephen *et al* [23], who studied microcosms consisting of the same topsoil and inoculum containing *S. aromaticivorans* B0695 by kingdom-level PCR and denaturing gradient gel electrophoresis.

Antibiotic selective plate counts showed that survival of the *S. aromaticivorans* B0695R cells in the Sequatchie sub-soil was different from that in the top-soil. The inoculant density dropped from 3×10^8 to 4.9×10^7 CFU g^{-1} dry soil over 21 days incubation (Figure 3b). In agreement with the selective plate counting assay, sphingolipid analysis revealed that the sphingolipid concentration decreased from 1.0×10^4 to 2.0×10^3 pmol g^{-1}dry soil (ie about 1.4×10^8 to 2.9×10^7 cells g^{-1} dry soil, respectively) in 7 days and remained stable afterward (Figure 5). PCR analysis of the

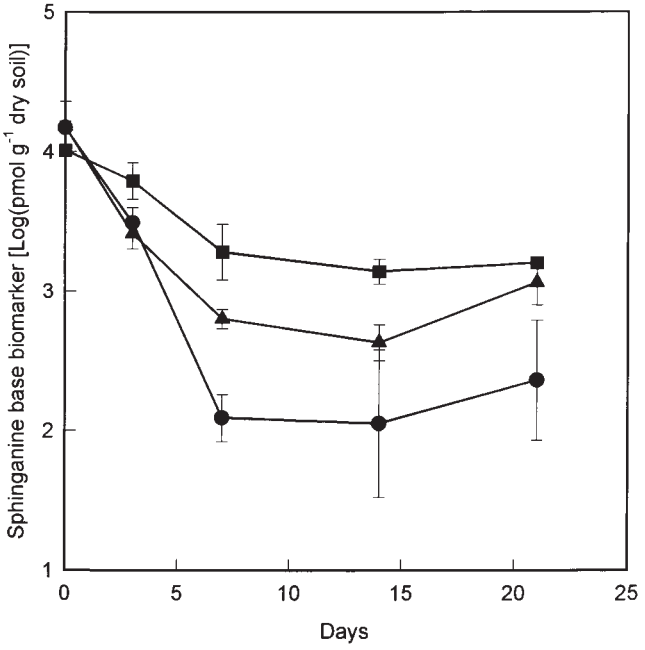

Figure 5 Dynamics of *S. aromaticivorans* B0695R sphinganine bases in soil seeded with the inoculum. ●, Sequatchie top-soil; ■, Sequatchie subsoil; and ▲, clay soil. Error bars represent ± one standard deviation ($n = 3$).

S. aromaticivorans B0695R rDNA showed that similar amounts of amplified product were observed throughout the 21 days incubation (Figure 4b) with the cell density constantly above the linear detection range (above 1×10^7 cells g^{-1} dry soil) of the PCR analysis. Despite the similar physical and chemical properties of the Sequatchie top- and subsoil, the native culturable bacterial population of the subsoil was 10-fold less than the top-soil (Table 2). The lower competitive pressure from the indigenous microbial population in the sub-soil could be a major factor which contributed to the better survival of the *Sphingomonas* inoculum.

The culturable *S. aromaticivorans* B0695R cell density in the clay soil decreased from 3.5×10^8 to 2.5×10^5 CFU g^{-1} dry soil in 3 days and dropped below 4×10^3 CFU g^{-1} dry soil (the detection limit of *S. aromaticivorans* B0695R in clay soil) after 7 days incubation (Figure 3c). A discrepancy was observed between antibiotic selective plating and the PCR and sphingolipid analysis. Despite the rapid decline of the culturable *S. aromaticivorans* B0695R population, PCR amplification showed that the amount of amplified rDNA was above the linear detection range of the bacteria (above 1×10^7 cells g^{-1} dry soil) (Figure 4c). Sphingolipid analysis revealed that the concentration of sphinganine bases decreased from 1.6×10^4 to 631 pmol g^{-1} dry soil (about 2.3×10^8 to 9.0×10^6 cells g^{-1} dry soil) in the first 7 days and remained between 398–1259 pmol g^{-1} dry soil afterward. The results of the PCR and sphingolipid analysis suggested that the decline of the culturable *S. aromaticivorans* B0695R population could be due to the loss of viability as well as to the culturability of the inoculum.

In conclusion, sphingolipid biomarker analysis provides a quantitative and non-culture-dependent means to monitor dynamics of a *Sphingomonas* population in the environment. In combination with PCR analysis using the *Sphingomonas* genus-specific primers, it can improve the specificity of the sphingolipid biomarker assay by reducing the possibility of false positive interpretations caused by other minor sphingolipid-containing bacteria.

Acknowledgements

This work was supported by a grant from the US Department of Energy, Natural and Accelerated Bioremediation Research Program (NABIR), award No. DE-FC02-96ER62278 to DCW. We thank Drs DL Balkwill, DT Gibson and JT Trevors for providing us some of the *Sphingomonas* strains.

References

1 Arens P, GJ Tuschewitzki, M Wollmann, H Follner and H Jacob. 1995. Indicators for microbiologically induced corrosion of copper pipes in cold-water plumbing system. Zbl Hyg 196: 444–456.
2 Balkwill DL, RH Reeves, GR Drake, JY Reeves, FH Crocker, MB King and DR Boone. 1997. Phyogenetic characterization of bacteria in the subsurface microbial culture collection. FEMS Microbiol Rev 20: 201–216.
3 Berg G and G Ballin. 1994. Bacterial antagonists to *Verticillium dahliae* Kleb. J Phytopathol 141: 99–110.
4 Borneman J, PW Skroch, KM O'Sullivan, JA Palus, NG Rumjanek, JL Jansen, J Nienhuis and EW Triplett. 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl Environ Microbiol 62: 1935–1943.
5 Degrange V and R Bardin. 1995. Detection and counting of *Nitrobacter* populations in soil by PCR. Appl Environ Microbiol 61: 2093–2098.
6 Feng X, L-T Ou and A Ogram. 1997. Plasmid-mediated mineralization of carbofuran by *Sphingomonas* sp strain CF06. Appl Environ Microbiol 63: 1332–1337.
7 Fredrickson JK, DL Balkwill, JM Zachara, SW Li, JF Brockman and MA Simmons. 1991. Physiological diversity and distributions of heterotrophic bacteria in deep Cretaceous sediments of the Atlantic Coastal Plain. Appl Environ Microbiol 57: 402–411.
8 Hastings RC, MT Ceccherini, N Miclaus, JR Saunders, M Bazzicalupo and AJ McCarty. 1997. Direct molecular biological analysis of ammonia oxidising bacteria populations in cultivated soil plots treated with swine manure. FEMS Microbiol Ecol 23: 45–54.
9 Imai R, Y Nagata, M Fukada, M Takagi and K Yano. 1991. Molecular cloning of a *Pseudomonas paucimobilis* gene encoding for a 17-kilodalton polypeptide that eliminates HCl molecules from hexachlorocyclohexane. J Bacteriol 173: 6811–6819.
10 Ka JO, WE Holben and JM Tiedje. 1994. Genetic and phenotype diversity of 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacteria isolated from 2,4-D treated filled soils. Appl Environ Microbiol 60: 1106–1115.
11 Karlson U, F Rojo, JD van Elsas and EB Moore. 1995. Genetic and serological evidence for the recognition of four pentachlorophenol-degrading bacterial strains as a species of the genus *Sphingomonas*. Appl Syst Microbiol 18: 539–548.
12 Kawasaki SR, R Moriguchi, K Sekiya, T Nakai, E Ono, K Kume and K Kawahara. 1994. The cell envelope structure of the lipopolysaccharide-lacking Gram-negative bacterium *Sphingomonas paucimobilis* J Bacteriol 176: 284–290.
13 Khan AA, R-F Wang, W-W Cao, W Franklin and CE Cerniglia. 1996. Reclassification of a polycyclic aromatic hydrocarbon-metabolizing bacterium, *Beijerinckia* sp strain B1, as *Sphingomonas yanoikuyae* by fatty acid analysis, protein pattern analysis, DNA–DNA hybridization, and 16S ribosomal DNA sequencing. Int J Syst Bacteriol 46: 466–469.
14 Leung KT, O Tresse, D Errampalli, H Lee and JT Trevors. 1997. Mineralization of *p*-nitrophenol by pentachlorophenol-degrading *Sphingomonas* spp. FEMS Microbiol Lett 155: 107–114.
15 Marchesi JR, T Sato, AJ Weightman, TA Martin, JC Fry, SJ Hiom and WG Wade. 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbiol 64: 795–799.

16 Nohynek LJ, E-L Nurmiaho-Lassila, EL Suhonen, H-J Busse, M Mohammadi, J Hantula, F Rainey and MS Salkinoja-Salonen. 1995. Description of chlorophenol-degrading *Pseudomonas* sp strain KF1T, KF3, and NKF1 as new species of the genus *Sphingomonas*, *Sphingomonas subarctica* sp nov. J Syst Bacteriol 46: 1042–1055.
17 Obuekwe CO. 1980. PhD thesis. University of Alberta, Edmonton, Alberta, Canada.
18 Ohman DE and AM Chakrabarty. 1981. Genetic mapping of chromosomal determinants for the production of exopolysaccharide alginate in a *Pseudomonas aeruginosa* cystic fibrosis isolate. Infect Immun 33: 124–148.
19 Pfennig N, F Widdel and HG Truper. 1981. The dissimilatory sulfate-reducing bacteria. In: The Prokaryotes, Vol 1 (Starr MP, H Stolp, HG Trupes, A Ballows and HE Schlegel, eds), pp 926–940, Springer-Verlag KG, Heidelberg, Germany.
20 Romine MF, LC Stillwell, K-K Wong, SJ Thurston, EC Sisk, C Sensen, T Gaasterland, JK Fredrickson and JD Saffer. 1999. Complete sequence of a 184-kilobase catabolic plasmid from *Sphingomonas aromaticivorans* F199. J Bacteriol 181: 1585–1602.
21 Schmidt S, RM Wittich, D Erdmann, H Wilkes, W Francke and P Fortnagel. 1992. Biodegradation of diphenyl ether and its monohalogenated derivatives by *Sphingomonas* sp strain SS3. Appl Environ Microbiol 58: 2744–2750.
22 Steffan RJ, J Goksoyr, AK Bej and RM Atlas. 1988. Recovery of DNA from soils and sediments. Appl Environ Microbiol 54: 2908–2915.
23 Stephen JR, Y-J Chang, SJ Macnaughton, SL Whittaker, C Hicks, KT Leung, CA Flemming and DC White. 1999. Fate of a mixture of five metal resistant bacteria introduced into pristine and heavy-metal-contaminated soil microcosms assessed by DGGE. Environ Toxicol Chem 18: 1118–1123.
24 Taira K, N Hayase, N Arimura, S Yamashita, T Miyazaki and K Furukawa. 1988. Cloning and nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase gene from the PCB-degrading strain of *Pseudomonas paucimobilis* Q1. Biochemistry 27: 3990–3996.
25 White DC, WM Davis, JS Nichols, JD King and RJ Bobbie. 1979. Determination of sedimentary microbial biomass by extractable lipid phosphate. Oecologia 40: 51–62.
26 White DC, SD Sutton and DB Ringelberg. 1996. The genus *Sphingomonas*: physiology and ecology. Curr Opin Biotechnol 7: 301–306.
27 Wittich R-M, H Wilkes, V Sinnwell, W Francke and P Fortnagel. 1992. Metabolism of dibenzo-*p*-dioxin by *Sphingomonas* sp strain RW1. Appl Environ Microbiol 58: 1005–1010.
28 Yabuuchi E, I Yano, H Oyaizu, Y Hashimoto, T Ezaki and H Yamamoto. 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 genus *Sphingomonas*. Microbiol Immunol 34: 99–119.