NOTE

Characterization and reclassification of an aromatic- and chloroaromatic-degrading *Pseudomonas* sp., strain HV3, as *Sphingomonas* sp. HV3

Kim Yrjälä,¹ Sini Suomalainen,² Eeva L. Suhonen,³ Sisko Kilpi,¹
Lars Paulin² and Martin Romantschuk^{1,4}

Author for correspondence: Kim Yrjälä. Tel: +358 0 708 59220. Fax: +358 9 59262.
e-mail: kim.yrjala@helsinki.fi

Department of Biosciences,
Division of General
Microbiology, PO Box 56
(Biocenter 1C)¹, Institute of
Biotechnology²,
Department of Applied
Chemistry and
Microbiology³,
Department of
Environmental and
Ecological⁴, FIN-00014
University of Helsinki,
Finland

Phylogenetic analyses of 16S rRNA gene sequences showed that the Gram-negative aromatic- and chloroaromatic-degrading *Pseudomonas* sp. strain HV3 carrying the mega-plasmid pSKY4 belongs to the genus *Sphingomonas*. The 16S rRNA sequence is most related to *Sphingomonas chlorophenolica* strains ATCC 33790^T (98.5%) and SR3 (98.4%) and *Sphingomonas* sp. SS86 (98.4%). The G+C content was 64 mol%, and the DNA–DNA-hybridization-based relative homology of strain HV3 to the *S. chlorophenolica* ATCC 33790^T and *S. chlorophenolica* RA2 was 59.6% and 35.9%, respectively. The results showed that although strain HV3 is related to *S. chlorophenolica* it differs in certain characteristics. It is therefore proposed to reclassify *Pseudomonas* sp. strain HV3 as *Sphingomonas* sp. HV3.

Keywords: taxonomy, 16S rRNA, DNA–DNA hybridization, sphingolipids, whole-cell fatty acids

Pseudomonas spp. constitute a very large and diverse group of bacteria. The genus has been a repository for incompletely described, aerobic, polarly flagellated, Gram-negative, rod-shaped bacteria (De Vos *et al.*, 1989). A reorganization of the genus has been taking place (Kerstens *et al.*, 1996). Some bacteria have been transferred to the genus *Sphingomonas* emended by Takeuchi *et al.* (1993). They are members of the α -4 subclass of *Proteobacteria* (Takeuchi *et al.*, 1994). The organisms are Gram-negative, non-spore-forming, straight rods that have a single polar flagellum when motile. *Sphingomonas* species seem to be widespread in water, soil and sediments (Fredrickson *et al.*, 1995; Ka *et al.*, 1994; Puhakka *et al.*, 1995; Wittich *et al.*, 1992) showing the importance of this genus in biodegradation.

Pseudomonas sp. strain HV3 was isolated as a 2-methyl-4-chlorophenoxyacetic acid (MCPA)- and 2,4-dichlorophenoxyacetic acid (2,4-D)-metabolizing strain from Finnish field soil (Kilpi *et al.*, 1980). In mixed cultures with a *Nocardia* sp. it also degrades

polychlorobiphenols (PCBs) (Kilpi *et al.*, 1988). Strain HV3 degrades aromatic and polyaromatic compounds (Kilpi *et al.*, 1983, 1988), probably through plasmid-encoded pathways (Yrjälä *et al.*, 1994, 1997). Catabolic *meta*-pathway genes from the mega plasmid pSKY4 of strain HV3 were found to show a unique gene organization (Yrjälä *et al.*, 1997). Here we present the data of a chemotaxonomic, physiological and phylogenetic study of strain HV3. The results point to a need for reclassification of strain HV3.

All strains used in this study are described in Table 1. *Pseudomonas* sp. strain HV3 (HV3) was maintained on mineral salts solution agar (Kilpi *et al.*, 1980) and solid naphthalene crystals were added to the lid of inverted plates. Strain HV3 forms yellow colonies in 2–4 d. *Sphingomonas* reference strains were maintained on half strength Trypticase soy agar (TSA) and grown at 28 °C.

Chemotaxonomy

Whole-cell fatty acids were analysed as methyl esters by GC according to the instructions of the MIDI Microbial Identification System.

The whole-cell fatty acid composition of strain HV3

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; MCPA, 2-methyl-4-chlorophenoxyacetic acid.

The EMBL accession number for the sequence reported in this paper is Y12803.

Table 1. Bacterial strains used in the study

Strain/species	Source/reference
<i>Pseudomonas</i> sp. HV3	This study
<i>S. chlorophenolica</i> ATCC 33790 ^{T*}	R. L. Crawford (Ederer <i>et al.</i> , 1997)
<i>S. chlorophenolica</i> RA2 [†]	R. L. Crawford (Ederer <i>et al.</i> , 1997)
<i>S. chlorophenolica</i> ATCC 39723 [‡]	Ederer <i>et al.</i> (1997)
<i>S. yanoikuyae</i> DSM7462 ^T	HAMBI (Yabuuchi <i>et al.</i> , 1990)
<i>S. paucimobilis</i> ATCC 29837 ^T	HAMBI (Yabuuchi <i>et al.</i> , 1990)
<i>S. terrae</i> IFO15098 ^T	HAMBI (Takeuchi <i>et al.</i> , 1993)
<i>S. macrogoltabidus</i> IFO 15033 ^T	HAMBI (Takeuchi <i>et al.</i> , 1993)
<i>S. parapaucimobilis</i> DSM 7463 ^T	HAMBI (Yabuuchi <i>et al.</i> , 1990)
<i>P. saccharophila</i> KF1	J. A. Puhakka (Puhakka <i>et al.</i> , 1995)
<i>P. aeruginosa</i> ATCC 27853	H. Jousimies-Somer, National Public Health Institute (NPHI)
<i>E. coli</i> ATCC 35401	NPHI
<i>Hafnia alvei</i> ATCC 29927	NPHI
<i>E. coli</i> DH5 α	BRL
<i>P. fluorescens</i> A506	S. E. Lindow (Wilson & Lindow, 1993)

* Received as *Arthrobacter* sp. ATCC 33790.

† Received as *Sphingomonas* RA2.

‡ Received as *Flavobacterium* ATCC 39723.

Table 2. Whole-cell fatty acid profile of *Pseudomonas* sp. HV3 and reference strains

Strain	Percentage fatty acid composition (w/w, $\pm 2\%$)						
	14:0 2-OH	16:1 $\omega 7c$	16:0	17:1 $\omega 6c$	Summed feature 7*	18:1 $\omega 5c$	Other
<i>Pseudomonas</i> sp. strain HV3	7	10	12	3	61	2	5
<i>S. paucimobilis</i> ATCC 29837 ^T	5	4	9	2	76	2	2
<i>S. chlorophenolica</i> SR3	7	11	11	2	64	1	4
<i>S. chlorophenolica</i> RA2	7	14	14	2	60	1	2
<i>S. chlorophenolica</i> ATCC 39723	7	12	15	2	60	2	2
<i>S. subarctica</i> KF1 ^T	6	9	7	1	69	2	6
<i>S. yanoikuyae</i> DSM 7462 ^T	8	18	11	1	54	2	6
<i>S. parapaucimobilis</i> DSM 7463 ^T	6	10	9	2	66	3	4
<i>S. terrae</i> IFO 15098 ^T	1	11	6	42	17	1	22
<i>S. macrogoltabidus</i> ATCC 15033 ^T	4	32	14	1	41	ND	8
<i>P. aeruginosa</i> ATCC 27853 [†]	ND	15	26	ND	39	ND	20
<i>P. fluorescens</i> A506 [‡]	ND	26	33	ND	20	ND	21
<i>H. alvei</i> ATCC 29927	ND	17	32	ND	7	ND	44

ND, Not detected.

* Summed feature 7 consisted of one or more of the following isomers, which could not be separated by the Microbial Identification System: 18:1 $\omega 7cis$, 18:1 $\omega 9trans$, 18:1 $\omega 12trans$.

† *P. aeruginosa* also contained 3% 10:0 3-OH, 3% 12:0, 4% 12:0 2-OH, and 4% 12:0 3-OH.

‡ *P. fluorescens* also contained 3% 10:0 3-OH, 2% 12:0, 5% 12:0 2-OH, and 4% 12:0 3-OH.

and selected reference strains are shown in Table 2. The strains contained octadecenoic acid as the dominant fatty acid. Other significant fatty acids were 2-hydroxytetradecanoic acid (2-hydroxymyristic acid),

cis-9-hexadecenoic acid and hexadecanoic acid. The closest match to strain HV3 in the whole-cell TSBA fatty acid library (MIDI) was *Flavobacterium resinovorum*, and in the clinical library (MIDI) was

Fig. 1. Dendrogram based on the whole-cell fatty acid composition of strain HV3 and reference strains. The dendrogram was constructed using the MIDI Microbial Identification System software.

Sphingomonas paucimobilis. Fig. 1 shows a dendrogram based on the whole-cell fatty acid analysis where strain HV3 clusters with *S. chlorophenolica* RA2 (Ederer *et al.*, 1997; Nohynek *et al.*, 1995), *S. chlorophenolica* ATCC 39723 (Ederer *et al.*, 1997; Nohynek *et al.*, 1995) and *S. yanoikuyae* DSM 7462^T (Yabuuchi *et al.*, 1990). The occurrence of 2-hydroxymyristic acid, which was found in strain HV3, is typical for the genus *Sphingomonas* (Takeuchi *et al.*, 1993; Takeuchi *et al.*, 1995; Yabuuchi *et al.*, 1990).

Sphingolipids were extracted from 0.5–0.7 g wet cells and analysed using a method of Yabuuchi *et al.* (1990) modified as described by Nohynek *et al.* (1996). *S. paucimobilis* ATCC 29837^T was used as a positive control. TMS derivatives of dihydrosphingosines analysed by gas chromatography-mass spectrometry (Fig. 2) showed that strain HV3 contained the major dihydrosphingosines d-18:0, d-20:1 and d-21:1 (no. carbon atoms:no. double bonds), which were the same as in *S. paucimobilis* ATCC 29837^T used as reference (not shown). The main dihydrosphingosines of strain HV3 were also similar to those of *S. capsulata*, *S. yanoikuyae*, *S. adhaesiva* and the proposed *S. subarctica* strains (Nohynek *et al.*, 1996; Takeuchi *et al.*, 1993; Yabuuchi *et al.*, 1990). The proposed *S. chlorophenolica* strains were also reported to have closely similar major sphingosines to *S. paucimobilis* (Nohynek *et al.*, 1995). The most abundant sphingosine in the genus *Sphingobacterium* is branched-chain methyl-hexadecasphinganine, d-17:0 (Dees *et al.*, 1985; Yabuuchi *et al.*, 1983), which was not among the main sphingosines in strain HV3. Thus the sphingosine data place strain HV3 among the *Sphingomonas* strains, apart from the *Sphingobacterium* strains, *S. sanguis*, *S. macroglutabidus* and *S. terrae* which contain d-18:0, d-19:1 and d-20:1 as the main dihydrosphingosines (Takeuchi *et al.*, 1993).

Respiratory quinones were analysed by HPLC of extracts of 100–200 mg lyophilized cells (Tindall,

Fig. 2. Mass fragmentogram of the d-18:0 sphingosine peak of strain HV3 and structural formulae of trimethylsilyl (TMS) derivatives of the most abundant sphingosines of strain HV3.

1990). Samples were further purified on preparative TLC (Silica Gel F254) and developed in hexane/diethylether (85:15, v/v). The spots were scraped off and dissolved in methanol using ultrasound. The samples were chromatographed with Lichrospher 100 RP-18 column (5 µm, 125 × 4 mm) using isocratic elution with methanol at 25 °C. Chromatograms were compared with commercially available ubiquinone Q-10 and ubiquinones from *S. paucimobilis* ATCC 29837^T, *P. fluorescens* A506 and *E. coli* DH5α reference strains. The absorption at 273 nm was used for measuring ubiquinones. The major ubiquinone of strain HV3 had a retention time (21.5 min) identical to that of the reference strain *S. paucimobilis* ATCC 29837^T and the model compound, ubiquinone Q-10. Quinones of other reference strains such as *E. coli* DH5α (Yrjälä *et al.*, 1994) and *P. fluorescens* A506 (Wilson & Lindow, 1993), had clearly different retention times. The main respiratory quinone in strain HV3, ubiquinone Q-10, is typical for the genus *Sphingomonas* (Yabuuchi *et al.*, 1990).

Phylogeny

The sequence of 16S rDNA from *Pseudomonas* sp. strain HV3 was determined using PCR and solid-phase DNA sequencing (Ridell *et al.*, 1995). DNA

Fig. 3. Phylogenetic dendrograms of 16S rRNA sequences from strain HV3, *Sphingomonas* and related strains. A 1.36 kb sequence of the strains was used in constructing the tree based on neighbour joining. Bootstrap analyses were made with 100 cycles and the values are indicated. Nucleotide sequence accession numbers of sequences of the reference strains obtained from the EMBL nucleotide sequence database: *S. chlorophenolica* strains ATCC 33790^T, SR3, ATCC 39723, RA2 are X87161, X87162, X87163 and X87164 respectively; *Sphingomonas* sp. SS86, D16148 (Maidak *et al.*, 1994); *S. paucimobilis* EPA 505, X94100; *Sphingomonas* sp. CF06, U52146; *S. yanoikuyae*, X85023; *Sphingomonas* sp. Q1, X87167; *Sphingomonas* sp. SS3, X87165; *Sphingomonas* sp. UN1F1, U37345; strain C7, L22759; *Sphingomonas* sp. BN6, X94098; *Sphingomonas* sp. SYK, D16149 (Maidak *et al.*, 1994); *S. terrae* IFO 15098^T, D13727; *S. macrogoltabidus* ATCC 51380^T, D13723; *Blastomonas natatorius*, X73043; *R. suberifaciens* IFO 15211^T, D13737; *S. rosa* IFO 15208^T, D13945; *Sphingomonas* sp. UN1P1, U37347; *S. subarctica*, X94102; *S. capsulata* ATCC 14666^T, D16147; *S. aromaticivorans* SMCC F199^T, U20756; *S. subterranea* SMCC B0478^T, U20773; *Sphingomonas* sp. RW1, X72723; *Sphingomonas* sp. A175, X94101; *Sphingomonas* sp. BF14, Z23157; *Caulobacter subvibrioides*, strain CB81, M83797; *S. trueperi* ATCC 12417^T, X97776; *S. paucimobilis* ATCC 29837^T, D16144; *S. sanguis* IFO 13937^T, D13726; *S. parapaucimobilis* IFO 15100^T, D13724; *Porphyrobacter neustonensis*, M96745; *Erythrobacter longus* ATCC 33941^T, M59062; *Agrobacterium tumefaciens*, D14504 and *Brevundimonas diminuta* ATCC 11568^T, M59064.

fragments were amplified and sequenced using primers described by Edwards *et al.* (1989). The search for most homologous sequences was done with the FASTA program and the alignment of the 16S rRNA sequences was done with the PILEUP program of Genetics Computer Group (GCG) software package, version 8. The pairwise evolutionary distances were estimated (Galtier & Gouy, 1994; Jukes & Cantor, 1969) and the phylogenetic tree was inferred by the neighbour-joining method (Saitou & Nei, 1987) using the software package TREECON for Windows (Van der Peer & Wachter, 1994).

A nearly complete 16S rRNA sequence of the strain HV3 (1.44 kb) was determined and deposited in the EMBL database with the accession number Y12803. The 16S rRNA sequence placed strain HV3 phylogenetically among several described *Sphingomonas* species (Karlson *et al.*, 1995). The inferred phylogenetic relationships derived from neighbour joining analysis (Saitou & Nei, 1987) of the pairwise comparisons among the rRNA gene sequences (1.36 kb) of 34 *Sphingomonas* and related species are presented in Fig. 3. They showed a sequence similarity to strain

HV3 of 91.5%–98.5%. The 16S rRNA sequence of strain HV3 showed 98.5% homology to *S. chlorophenolica* ATCC 33790^T, 98.4% homology to *S. chlorophenolica* SR3 (Karlson *et al.*, 1995) and *Sphingomonas* sp. SS86, and 97.6% to *S. chlorophenolica* strains RA2 and ATCC 39723 (Fig. 3) (Karlson *et al.*, 1995). The 16S rRNA sequence of strain HV3 forms its own branch between the branches containing the proposed *S. chlorophenolica* strains SR3, ATCC 33790^T, and *Sphingomonas* sp. SS86, *S. chlorophenolica* RA2 and ATCC 39723 (Fig. 3). With the transformed distance method of Galtier & Gouy (1994) strain HV3 clusters with the *Sphingomonas* sp. SS86 (not shown). The results agree with the whole-cell fatty acid results showing that strain HV3 is similar to *S. chlorophenolica* RA2 and ATCC 39723 and a little less similar to *S. yanoikuyae* strains.

The 16S rRNA sequence similarity to *Rhizomonas suberifaciens* (Van Bruggen *et al.*, 1990) was relatively low, 93.6%. Although *R. suberifaciens* have characteristics shared by strain HV3, such as ubiquinone Q-10 and sphingolipids, they differ in mol % G + C contents. For base composition studies with strain HV3, DNA

was isolated (Visuvanathan *et al.*, 1989) and the DNA base composition determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, according to Mesbah & Whitman (1989) and Tamaoka & Komagata (1984). The G+C content of the DNA of strain HV3 was 64 ± 1 mol%, which is in the range found for *Sphingomonas* species (Yabuuchi *et al.*, 1990) but higher than for *Rhizomonas*, 59 mol%. Additionally strain HV3 does not contain 10-methyloctadecenoic acid that is found in *Rhizomonas* (Van Bruggen *et al.*, 1993) showing that it belongs to another genus.

DNA-DNA hybridization

For hybridization studies at DSMZ, DNA was isolated by chromatography on hydroxyapatite (Cashion *et al.*, 1977). Spectroscopic DNA-DNA hybridization (De Ley *et al.*, 1970; Huss *et al.*, 1983) was carried out and renaturation rates were computed with the TRANSFER.BAS program (Janke, 1992).

Total DNA of strain HV3 was hybridized to that of *S. chlorophenolica* ATCC 33790^T and *S. chlorophenolica* RA2. The homology to ATCC 33790 was 59.6% and to RA2 35.9%. The homology between *S. chlorophenolica* ATCC 33790^T and *S. chlorophenolica* RA2 was 71.8%. Wayne *et al.* (1987) recommend that a DNA-DNA homology of 70% would define a species, which places strain HV3 outside of *S. chlorophenolica*.

Metabolic properties

An assay for biodegradation of 2,4,6-trichlorophenol and pentachlorophenol was made in 2 ml mineral salts solution (8 ml vials) by shaking at room temperature. Cells grown on benzoic acid were added to 5 p.p.m. solutions of the substrate and the suspensions were extracted and analysed after seven days as described by Puhakka *et al.* (1995), except that the gas chromatograph was a Hewlett Packard 5890 equipped with an HP-5 (30 m, 0.25 mm i.d.) column. Killed cells in 2% formaldehyde were used as controls. *Pseudomonas* sp. strain HV3, initially isolated as a MCPA- and 2,4-D-degrader, did not degrade the chlorophenols tested and differs from the *S. chlorophenolica* and *S. subarctica* strains that are all isolated as chlorophenol-degraders. *S. chlorophenolica* meaning 'relating to chlorophenols', is thus not a suitable name for HV3, and it is therefore problematic to classify HV3 as *S. chlorophenolica* as currently described and designated.

Taken together, strain HV3 has in a detailed characterization been found to have characteristics typical for the genus *Sphingomonas*. The metabolic, phylogenetic and DNA-DNA hybridization data support the conclusion that strain HV3 belongs in the genus *Sphingomonas*. The strain is most related to *S. chlorophenolica*, but since the DNA hybridization with two of the *S. chlorophenolica* strains is lower (59.6% and 35.9% respectively), than found within the species

(*S. chlorophenolica*) (Nohynek *et al.*, 1996), the classification of this strain on species level requires further studies.

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