Alkanibacter difficilis gen. nov., sp. nov. and *Singularimonas variicoloris* gen. nov., sp. nov., hexane-degrading bacteria isolated from a hexane-treated biofilter

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Two Gram-negative, rod-shaped, non-spore-forming bacterial strains were isolated from a hexane-treated, full-scale biofilter from an oil mill. The strains were cultivated with hexane as the sole carbon source. One strain, MN154.3^T, showed a fatty acid profile that contained 16:0, 18:1*cis*11 and 19:0 cyclo11–12 as major compounds, while the second strain, isolate MN28^T, contained 14:0 3-OH, 16:0 and 18:1*cis*11 as the predominant fatty acids. On the basis of almost-complete 16S rRNA gene sequences, both strains could be allocated to the *Nevskia* branch of the class *Gammaproteobacteria*. The sequence similarities for strains MN154.3^T and MN28^T with respect to the most closely related type strains of this branch were 90.5 and 94.1 %, respectively. The sequence similarity between strains MN154.3^T and MN28^T was 90.6 %. The DNA G+C content of strain MN154.3^T was 62.8 mol% and that for strain MN28^T was 64.9 mol%. Both strains possessed ubiquinone-8 as the major quinone. On the basis of the 16S rRNA gene sequences of these two new isolates and several phenotypic differences exhibited with respect to known species of the *Nevskia* branch, strains MN154.3^T and MN28^T represent two novel genera and species, for which the names *Alkanibacter difficilis* gen. nov., sp. nov. and *Singularimonas variicoloris* gen. nov., sp. nov. are proposed. The type strain of *Alkanibacter difficilis* gen. nov., sp. nov. is MN154.3^T (=DSM 14804^T=LMG 22842^T) and that of *Singularimonas variicoloris* gen. nov., sp. nov. is MN28^T (=DSM 15731^T=LMG 22844^T).

During characterization of the hexane-degrading microbial community in a hexane-treated, full-scale biofilter for waste gas treatment, several groups of bacteria were isolated. Because hexane is a hydrophobic compound with low water solubility but high cytotoxic potential, micro-organisms that can utilize it are of great interest. Two strains, designated MN154.3^T and MN28^T, showed good growth on hexane and on several other short-chain alkanes. Analysis of almost-complete 16S rRNA gene sequences from strains MN154.3^T and MN28^T resulted in the placement of these strains in the *Nevskia* branch of the class *Gammaproteobacteria*. Sequence differences of more than 5 % between these strains and type strains known to belong to this branch prompted us to characterize strains MN154.3^T and MN28^T in more detail.

The strains were enriched from 1 g filter material and then cultivated in defined medium consisting of 0.8 g K₂HPO₄, 0.2 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.01 g FeSO₄·7H₂O, 1.0 g (NH₄)₂SO₄, 1 ml trace element solution and 5 ml vitamin solution. The medium solution was then made up to 1 l with double-distilled water. Strain MN154.3^T was cultivated in medium adjusted to pH 5.0 and strain MN28^T was grown in medium adjusted to pH 7.0. The trace element solution contained the following (l⁻¹): 3.0 g Na₂-EDTA, 0.05 g MnCl₂·2H₂O, 0.19 g CoCl₂·6H₂O, 0.041 g ZnCl₂, 0.006 g H₃BO₃, 0.024 g NiCl₂·6H₂O, 0.002 g CuCl₂ and 0.018 g NaMoO₄·2H₂O. The pH was adjusted to 6.0. The vitamin solution contained the following (l⁻¹): 0.01 g thiamine, 0.02 g nicotinic acid, 0.02 g pyridoxine hydrochloride, 0.01 g *p*-aminobenzoic acid, 0.02 g riboflavin, 0.02 g pantothenic acid, 0.001 g biotin and 0.001 g cyanocobalamin and was adjusted to a pH of 7.0. Liquid cultures 50 ml in volume were grown in screw-capped 100 ml flasks supplemented with 10 µl hexane. For solid media, 15 g agar l⁻¹ was included in the defined medium. Agar plates were incubated in 5 l desiccators, 1 ml hexane was placed in a separate beaker in the desiccator to supply hexane to the desiccator

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain MN154.3^T and strain MN28^T are AJ313020 and AJ555478, respectively.

atmosphere. All incubations were performed at 30 °C. Both strains grew well on agar plates with hexane as the sole carbon source. In liquid minimal medium, strain MN28^T showed weak growth or no growth at all. Growth with other carbon sources was tested in 20 ml tubes with 5 ml liquid medium. Amino acids, carbohydrates and carbonic acids were tested at a concentration equivalent to 60 mM carbon. Alkanes were used at a concentration of 0.2 % (v/v). Growth experiments with alkanes were performed in screw-capped tubes. In liquid minimal medium, strain MN154.3^T showed good growth on β -hydroxybutyric acid, weak growth on succinate and no growth on L-arabinose, D-fructose, D-alanine, D-threonine, D-glucose, L-aspartic acid, L-lysine or citrate. Strain MN154.3^T grew well with pentane, hexane and decane, but not with heptane or octane. Growth on nonane was weak with this strain. The optimal growth temperature for strain MN154.3^T was 30 °C; growth was slightly slower at 23 °C and considerably slower at 37 °C. No growth was observed at 13 or 45 °C. Strain MN28^T did not grow with any of these alkanes or with β -hydroxybutyric acid in liquid minimal medium. An expanded collection of carbon sources was tested for strains MN154.3^T and MN28^T using the API 20NE system and API 50 CH strips with API 50 CHB/E medium (bioMérieux) and using Biolog GN microplates. All tests were performed according to the manufacturers' instructions, but the incubation time was extended to 10 days. Strain MN154.3^T did not show growth or positive reactions in any of these test systems. However, strain MN28^T gave positive results for gelatinase activity in the API 20NE system and also for acid production from glycerol and sucrose and hydrolysis of aesculin (with the API 50 CH system). In the Biolog GN system, strain MN28^T was positive for the oxidation of Tween 40, D-glucose, methyl pyruvate, monomethyl succinate, *cis*-aconitic acid, β -hydroxybutyric acid, α -ketobutyric acid, succinic acid, bromosuccinic acid, succinamic acid, alaninamide, L-alanyl glycine, L-glutamic acid, glycyl L-glutamic acid, L-threonine and urocanic acid.

For fatty acid analyses, the isolates and two reference strains were grown, according to their specific requirements, in different media. Strain MN28^T was grown on trypticase soy agar plates (Becton Dickinson) for 7 days; strain MN154.3^T failed to grow on trypticase soy agar and so was grown on defined medium with hydroxybutyrate at 30 °C for 9 days. The reference strains *Nevskia ramosa* DSM 11499^T and *Hydrocarboniphaga effusa* DSM 16095^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). For the fatty acid analyses, *N. ramosa* DSM 11499^T was grown for 7 days at 25 °C on DSM medium 828 as recommended by the culture collection. *H. effusa* DSM 16095^T was grown on trypticase soy agar for 6 days at 30 °C. Saponification, methylation and extraction of the fatty acids from isolates and reference strains were performed as described by Sasser (1990). The fatty acid methyl ester extracts were analysed using a GC with a flame

ionization detector (GC-FID; model 6890, Hewlett Packard) equipped with a 5 % phenyl-methyl-silicone capillary column. The identities of the fatty acids were verified by GC-MS with a gas chromatograph (model 5890, series II; Hewlett Packard) equipped with a 5 % phenyl-methyl-silicone capillary column and a model 5972 mass selective detector. The chromatographic conditions used were as described previously (Lipski & Altendorf, 1997). The positions of double bonds and of cyclopropane, hydroxy and methyl groups were determined from the carboxyl group of the fatty acid molecule, according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (1977). Both isolates had a straight-chain fatty acid pattern dominated by vaccenic acid and palmitic acid (18:1*cis*11 and 16:0). Isolate MN154.3^T could be differentiated from isolate MN28^T by the presence of the cyclopropane fatty acids 17:0 cyclo9–10 and 19:0 cyclo11–12 in the former organism. Conversely, isolate MN28^T was characterized by the presence of the branched-chain fatty acids 12:0 iso, 14:0 iso and 16:0 iso and the monounsaturated fatty acid 16:1*cis*11 (Table 1). Both strains showed 14:0 3-OH as the

Table 1. Fatty acid compositions (%) of strain MN154.3^T, strain MN28^T and two reference strains

Strains: 1, MN154.3^T; 2, MN28^T; 3, *N. ramosa* DSM 11499^T; 4, *H. effusa* DSM 16095^T.

Fatty acid	1	2	3*	4
12:0 iso		2.2		
12:0	4.3	11.4	3.2	16.3
12:0 2-OH		1.5		
12:0 3-OH		0.7		3.4
14:0 iso		0.4		
14:0	1.2	2.5	20.5	2.2
14:1 <i>trans</i> 2		1.1		
15:0		0.5		0.8
ECL 15.082†		0.9		
14:0 2-OH	2.1			
14:0 3-OH	6.6	11.5		2.8
16:0 iso		4.6		
16:1 <i>cis</i> 9	3.8	2.2	16.1	16.4
16:1 <i>cis</i> 11		3.0		
16:0	30.1	15.6	42.2	18.9
17:0 cyclo9–10	2.4			
17:1 <i>cis</i> 11		0.5		
16:0 2-OH		0.8		1.6
16:0 3-OH	1.3	0.3		
18:1 <i>cis</i> 9	2.3			
18:1 <i>cis</i> 11	15.8	37.3	17.9	37.6
18:0	14.9	0.4		
19:0 cyclo11–12	15.3	2.1		

**N. ramosa* DSM 11499^T contained four more lipids (equivalent chain lengths: 13.528, ECL 15.355, ECL 15.553 and ECL 17.379) whose mass spectra indicated long-chain monounsaturated alcohols.

†ECL, Equivalent chain length.

predominant hydroxy fatty acid. The presence of a straight-chain fatty acid pattern was in accord with the profiles of the two reference strains but was different from the patterns of most other genera of the family *Xanthomonadaceae*, the fatty acid profiles of which are dominated by iso/anteiso fatty acids (Finkmann *et al.*, 2000; Kim *et al.*, 2006; Lee *et al.*, 2005; An *et al.*, 2005). To date, the only species of the family *Xanthomonadaceae* known to possess a straight-chain fatty acid pattern is *Xylella fastidiosa*. This species comprises a group of nutritionally fastidious and plant-pathogenic strains, which were isolated exclusively from the xylem of plant tissue (Wells *et al.*, 1987). The specific fatty acids, the ecology and the 16S rRNA gene sequence of *X. fastidiosa* serve to differentiate this species clearly from the two novel isolates and two reference strains analysed in this study. The allocation of the genera *Nevskia* and *Hydrocarboniphaga* to the family *Xanthomonadaceae* is currently based exclusively on 16S rRNA gene sequence data (Palleroni *et al.*, 2004; Saddler & Bradbury, 2005) but both the deep branching position within this family and the chemotaxonomic differences suggest separation of the *Nevskia* branch from the family *Xanthomonadaceae* and the definition of a separate family for this particular group of species.

Isoprenoid quinones and polar lipids were extracted and analysed using the small-scale integrated procedure of Minnikin *et al.* (1984). Quinones were separated into menaquinones and ubiquinones by using TLC. Separation of the ubiquinones according to the different isoprenyl chain lengths was performed on HPTLC-RP 18 F₂₅₄ plates (Merck) according to Kroppenstedt (1982). The two novel isolates and the two reference strains all possessed a quinone of the ubiquinone-8 type. The polar lipid patterns of the two novel isolates were different from each other and also from the patterns of the two reference strains (Fig. 1).

For 16S rRNA gene sequencing, bacterial DNA was extracted from pure cultures by using the DNeasy Tissue kit (Qiagen). Almost-complete 16S rRNA gene sequence PCR products were obtained as described previously (Friedrich *et al.*, 2002). Full-length sequences of the PCR products were obtained by GAG BioScience (Bremen, Germany). Sequences were checked manually using the CHROMAS programme (Technelysium) and assembled using the DNAMAN programme (Lynnon BioSoft). The sequences were compared with sequences in the National Centre for Biotechnology Information nucleotide sequence database, using the BLAST program (Altschul *et al.*, 1997). The BLAST analyses of the 16S rRNA gene sequence for strain MN154.3^T revealed *N. ramosa* Soe1^T as the type strain with the most similar sequence (94.1 % similarity). For strain MN28^T, the next most closely related type strain was *H. effusa* AP103^T (90.5 % sequence similarity). The sequence similarity between strains MN154.3^T and MN28^T was 90.6 %. A phylogenetic analysis with the MEGA program, version 3.1 (Kumar *et al.*, 2004), showed that both the reference species and isolates MN154.3^T and MN28^T are members of a deep branch within the family

Fig. 1. Polar lipid patterns of strain MN154.3^T (a), strain MN28^T (b), *N. ramosa* DSM 11499^T (c) and *H. effusa* DSM 16095^T (d). AL, unknown aminolipid; APL, unknown aminophospholipid; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

Xanthomonadaceae (Fig. 2). Remarkably, one species from this branch, *H. effusa*, is able to grow on aliphatic hydrocarbons from C₆ to C₁₉ (Palleroni *et al.*, 2004). At least one strain of this species, *H. effusa* LX1, was isolated from a toluene-treated biofilter (Juteau *et al.*, 1999).

The G+C content of the novel isolates was determined after degradation of isolated genomic DNA by P1 nuclease and alkaline phosphatase, followed by separation and quantification of the resulting nucleosides by using HPLC (Mesbah *et al.*, 1989). Lambda-DNA was used as the reference. The DNA G+C content of strain MN154.3^T was 62.8 mol% (SD, 0.19) and that for strain MN28^T was 64.9 mol% (SD, 0.07).

The 5 % differences in gene sequence similarity between the novel isolates and their most closely related type strains and between the novel isolates themselves, as well as the presence of several phenotypic differences (Table 2), led us to the proposal of two novel genera and species to accommodate strains MN154.3^T and MN28^T. Phenotypically, strain MN154.3^T can be differentiated from the most closely related species, *N. ramosa*, on the basis of the DNA G+C content, which is 67.8 % for *N. ramosa* Soe1^T but only 62.8 % for MN154.3^T. Rosette-like microcolonies at the surfaces of liquid cultures, typical of *N. ramosa*, were never observed for strain MN154.3^T. In contrast to *N. ramosa* Soe1^T, strain MN154.3^T exhibited the cyclopropane fatty acids 17:0 cyclo9–10 and 19:0 cyclo11–12. Both strains showed different polar lipid profiles. Both species from this

Fig. 2. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of the isolates within the family *Xanthomonadaceae*. Bootstrap percentages (based on 1000 replications) >70 % are shown at nodes. *Escherichia coli* was used as the outgroup. Bar, 0.02 substitutions per nucleotide position.

branch, *N. ramosa* and *H. effusa*, can grow on pyruvate and lactate, unlike our novel isolates.

During the revision of this manuscript, the species *Solimonas soli* was described (Kim *et al.*, 2007). The type strain of this species, *Solimonas soli* DCY12^T, shows a 16S rRNA gene sequence similarity of 98.2 % with respect to strain MN28^T, which suggests a strong phylogenetic relationship between these taxa. However, for *Solimonas soli* DCY12^T, a DNA G+C content of 40.5 mol% was described; this is considerably lower than the value (64.9 mol%) found for strain MN28^T. In contrast to the high level of sequence similarity, the difference in the DNA G+C contents (>20 mol%) supports the differentiation of these two taxa at the genus level. The taxonomic relationship of these two

taxa should be clarified by means of a direct experimental comparison between the two strains.

Description of *Alkanibacter* gen. nov.

Alkanibacter (Al.ka.ni.bac'ter. N.L. n. *alkanum* saturated hydrocarbon; N.L. masc. n. *bacter* from Gr. n. *bakterion* rod; N.L. masc. n. *Alkanibacter* rod assimilating alkanes).

Cells are aerobic, non-spore-forming rods. Gram-negative and oxidase- and catalase-positive. The major fatty acids are 16:0 and 18:1*cis*11. The quinone type is ubiquinone-8. The type species is *Alkanibacter difficilis*.

Description of *Alkanibacter difficilis* sp. nov.

Alkanibacter difficilis (diff'i.cil.is. L. masc. adj. *difficilis* difficult, because it is difficult to cultivate).

Displays the following characteristics in addition to those given in the genus description. Cells are small rods of $0.6 \pm 0.1 \times 0.9 \pm 0.3$ µm. Colonies are transparent, yellow, circular and 1 mm in diameter on defined-medium agar with added hexane. KOH and aminopeptidase tests give positive results and Gram-staining is negative. Grows on hydroxybutyric acid and succinate (weakly) as carbon sources. Optimal growth is at 30 °C; no growth occurs at 13 or 45 °C. The type strain, MN154.3^T, grows on pentane, hexane and decane. No growth occurs in nutrient broth or on trypticase soy agar. The major fatty acids are 16:0, 18:0, 18:1*cis*11 and 19:0 cyclo11-12; 14:0 3-OH is present. The DNA G+C content is 62.8 mol%.

The type strain, MN154.3^T (=DSM 14804^T=LMG 22842^T), was isolated from an industrial biofilter.

Description of *Singularimonas* gen. nov.

Singularimonas (Sin.gu.la.ri.mo'nas. L. adj. *singularis* extraordinary; Gr. fem n. *monas* unit, cell; N.L. fem. n. *Singularimonas* extraordinary unit).

Table 2. Phenotypic characteristics of strains MN154.3^T, MN28^T and related taxa

Strains: 1, MN154.3^T; 2, MN28^T; 3, *N. ramosa* DSM 11499^T; 4, *H. effusa* DSM 16095^T; data for 3 and 4 are from Stürmeyer *et al.* (1998), Palleroni *et al.* (2004) or this study. +, Positive; −, negative; ND, no data available.

Characteristic	1	2	3	4
DNA G+C content (mol%)	62.8	64.9	67.8	60
Presence of iso-branched fatty acids	−	+	−	−
Presence of cyclopropane fatty acids (>10 %)	+	−	−	−
Swarming on agar surface	−	−	ND	+
Growth on complex media	−	+	ND	+
Rosette-like colonies at air–water interface	−	−	+	−
Growth on:				
Lactate and pyruvate	−	−	+	+
Malate	−	−	−	+
Succinate	−	+	−	+
Glutamate	−	+	+	+
Acetate	−	−	+	−

Cells are aerobic, non-spore-forming rods. Gram-negative and oxidase- and catalase-positive. The major fatty acids are 16:0 and 18:1*cis*11; iso-branched fatty acids are also present. The quinone type is ubiquinone-8. The type species is *Singularimonas variicoloris*.

Description of *Singularimonas variicoloris* sp. nov.

Singularimonas variicoloris (va.ri.i.co.lo'ris. L. adj. *varius* varying; L. gen. n. *coloris* of colour; N.L. gen n. *variicoloris* of varying colour).

Displays the following characteristics in addition to those given in the genus description. Catalase-positive (weak reaction). Colonies vary in colour between yellow, orange and brown. Colonies on trypticase soy agar are 1–3 mm in diameter, umbonate and are each surrounded by a flat, pale white rim. The colour is more intense in the centre of the colony. Gram-staining is negative and the KOH and aminopeptidase tests give positive results. The DNA G+C content is 64.9 mol% (SD, 0.07). Growth is positive with hexane on minimal medium agar but is negative in liquid medium. Positive for growth on the following: Tween 40, α -D-glucose, methyl pyruvate, monomethyl succinate, *cis*-aconitic acid, β -hydroxybutyric acid, α -ketobutyric acid, succinic acid, bromosuccinic acid, succinamic acid, alaninamide, L-alanylglycine, L-glutamic acid, glycyl L-glutamic acid, L-threonine and urocanic acid. The gelatinase reaction is positive. No growth occurs on the following: α -cyclodextrin, dextrin, glycogen, Tween 80, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, cellobiose, L-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, glucose, *myo*-inositol, α -D-lactose, lactulose, maltose, D-mannitol, D-mannose, melibiose, methyl β -D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, xylitol, acetic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α -hydroxybutyric acid, γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α -ketoglutaric acid, α -ketovaleric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, caprate, adipate, malate, glucuronamide, D-alanine, L-alanine, L-asparagine, L-aspartic acid, glycyl L-aspartic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, DL-carnitine, γ -aminobutyric acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, DL- α -glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate. Negative for reduction of nitrate, indole production, arginine dihydrolase, urease, β -galactosidase and β -glucosidase. The major fatty acids are 14:0 3-OH, 16:0 and 18:1*cis*11; iso-branched fatty acids are present. The DNA G+C content is 64.9 mol% (SD, 0.07).

The type strain, MN28^T (=DSM 15731^T=LMG 22844^T), was isolated from an industrial biofilter.

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