Frondihabitans cladoniiphilus sp. nov., an actinobacterium of the family *Microbacteriaceae* isolated from lichen, and emended description of the genus *Frondihabitans*

Massimiliano Cardinale,[1] Martin Grube[2] and Gabriele Berg[1]

Correspondence
Massimiliano Cardinale
m.cardinale@tugraz.at

[1]Institute of Environmental Biotechnology, Graz University of Technology, Petersgasse 12, A-8010 Graz, Austria

[2]Institute of Plant Science, Karl-Franzens University Graz, Holteigasse 6, A-8010 Graz, Austria

A novel actinobacterium, designated strain CafT13^T, was isolated from the thallus of the reindeer lichen *Cladonia arbuscula* sampled in the Austrian Alps (Koralpe). The organism was aerobic, with rod- to irregular-shaped cells (often forming dense clusters of cells when grown in liquid medium), Gram-stain-positive, oxidase-negative, catalase-positive and non-motile. It was able to grow at 1 °C and at low to neutral pH, but not above 30 °C or at high pH. The peptidoglycan type was B2β with ornithine as the diagnostic diamino acid. The menaquinones were MK-7 and MK-8. The polar lipid profile comprised diphosphatidylglycerol, phosphatidylglycerol, three unidentified phospholipids, three unidentified glycolipids and one unidentified aminolipid. The predominant fatty acids were $C_{18:1}$, $C_{14:0}$ 2-OH, $C_{17:1}\omega9c$, $C_{16:0}$ and anteiso-$C_{15:0}$. The mean DNA G+C content of strain CafT13^T was 69.0±0.17 mol%. 16S rRNA gene sequence analysis showed that strain CafT13^T belongs to the family *Microbacteriaceae*, within the genus *Frondihabitans*. The mean level of DNA–DNA relatedness between strain CafT13^T and the type strain of *Frondihabitans australicus* was 35.2±5.23%. The enzyme spectrum of strain CafT13^T differentiated it from recognized species of the genus *Frondihabitans*. Based on molecular, chemotaxonomic and physiological data, strain CafT13^T is considered to represent a novel species of the genus *Frondihabitans*, for which the name *Frondihabitans cladoniiphilus* sp. nov. is proposed; the type strain is CafT13^T (=DSM 23273^T=LMG 25550^T).

Bacterial communities associated with lichens were recently characterized based on cultivation-dependent and cultivation-independent methods (Grube & Berg, 2009). These communities are species-specific and include members of many bacterial phyla (Grube *et al.*, 2009; Schneider *et al.*, 2011). *Alphaproteobacteria* constitute the most abundant group, as detected by specific *in situ* hybridization, followed by *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Actinobacteria* and *Firmicutes* (Cardinale *et al.*, 2008; Hodkinson & Lutzoni, 2009). The occurrence of other bacterial groups and *Archaea* was also shown (Bjelland *et al.*, 2011; Selbmann *et al.*, 2010; Bates *et al.*, 2011; J. Vieira de Castro and others, unpublished). During a comparative study of bacterial diversity in different thallus parts of the reindeer lichen *Cladonia arbuscula* (Wallr.) Rabenh., we isolated two actinobacteria (strains CafT13^T and CafM5) from the juvenile part of a healthy thallus that was growing in the understorey of a natural spruce forest. The 16S rRNA gene sequences of the two strains were identical (100% similarity) and indicated placement in the family *Microbacteriaceae*; CafT13^T was chosen as a representative strain and characterized further. Highest levels of 16S rRNA gene sequence similarity were found between strain CafT13^T and the type strains of *Frondihabitans peucedani* and *Frondihabitans australicus* (97.2 and 97.1%, respectively). Strain CafT13^T was also shown to be closely related to members of the genera *Subtercola*, *Frigoribacterium* and *Curtobacterium* (highest levels of 16S rRNA gene sequence similarity of 96.2, 96.2 and 95.5%, respectively).

We used a polyphasic approach to further characterize strain CafT13^T in accordance with the standard guidelines for the suborder *Micrococcineae* (Schumann *et al.*, 2009).

Phylogenetic analysis demonstrated that strain CafT13^T is separate from recognized species of the genera *Frondihabitans*, *Frigoribacterium*, *Curtobacterium* and *Subtercola*. Based on phenotypic, physiological and chemotaxonomic differences as well as phylogenetic distance from its closest

Abbreviation: ISP, International *Streptomyces* Project.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CafT13^T is FN666417.

relatives, we propose that strain CafT13^T represents a novel species of the genus *Frondihabitans* in the family *Microbacteriaceae* (following standard nomenclature rules; Tindall *et al.*, 2006).

Strain CafT13^T has been conserved at −70 °C in the Strain Collection of Antagonistic Micro-organisms (SCAM) at the Institute of Environmental Biotechnology, Graz University of Technology (TU-Graz), and in the DSMZ and BCCM/LMG public culture collections.

A thallus of the lichen *Cladonia arbuscula* was collected, by wearing gloves and by using sterilized, ethanol-cleaned forceps, under a natural spruce forest at Koralpe, in the Austrian Alps at the border between Styria and Carinthia (Handalm: 46° 50′ 35″ N 15° 01′ 20″ E, about 1780 m above sea-level). The sample was placed in a sterile 50 ml screw-capped tube and stored in a refrigerated bag. After about 6 h, the sample was processed for bacterial isolation. The thallus was crushed in 0.8 % NaCl solution and serial diluents of the suspension were plated onto tryptone-yeast extract medium (Carl Roth). After 6 days of incubation at 20 °C, two yellowish-pigmented colonies were picked, streaked onto fresh plates and designated CafT13^T and CafM5. Growth was tested on several media and the best results were achieved with International *Streptomyces* Project (ISP) medium 2 (Shirling & Gottlieb, 1966). A considerable amount of extracellular matrix was produced by strain CafT13^T on this medium, especially when grown below 10 °C; the colonies appeared yellowish, turning to bright yellow after 4−5 days of growth. Growth in liquid culture was performed at 24 °C with shaking at 220 r.p.m., and was characterized by the formation of a mucous and filamentous cell aggregate sticking to the bottom of the tube, this aggregate being relatively difficult to disaggregate or resuspend. The pH range for growth was established in liquid cultures; a pre-inoculum was grown in ISP 2 at 24 °C for 30 h, and 350 µl (about 5×10^6 cells) was then added to 50 ml filter-sterilized ISP 2 with pH values adjusted to 3–12 (single pH unit intervals). The flasks were incubated with shaking at 150 r.p.m. for 70 h and the OD_{600} was then measured with a spectrophotometer. Growth occurred at (initial) pH 3–8, optimally at pH 4–7. Growth was very poor at pH 3 and was absent at pH 9–12. pH was also measured after bacterial growth; acidification of the medium at initial pH 6, 7 and 8 was detected (pH 4.58, 4.91 and 5.02, respectively, after growth), whereas at initial pH 4 and 5 there was only a slight acidification (pH 3.73 and 4.76, respectively). The experiments were repeated at pH 3–9 by using a buffering system consisting of 0.05 M NaH_2PO_4/Na_2HPO_4 (Miller *et al.*, 2005) to define exactly the optimal pH range for growth. Growth was confirmed to occur at the initial pH values given above; after bacterial growth, acidification of the medium was again noted (values of pH before/after bacterial growth: 4/3.72, 5/4.36, 6/5.31 and 7/5.34). We concluded that strain CafT13^T is an acidophilic bacterium, showing optimal growth at pH 3.7−5.4.

The temperature range for growth was established on the basis of growth on solid medium. ISP 2 plates were streaked with strain CafT13^T, incubated at 1, 4, 15, 20, 22, 24, 26, 30 and 37 °C and monitored for 14 days. Growth was observed at 1–30 °C, with optimum growth at 22–26 °C.

Motility and oxygen relationships were tested in ISP 2 plus 0.3 % agar-agar (Roth) by using transparent inoculation tubes. A few identical colonies were harvested from a plate with a sterile needle and inoculated through the centre of the medium to approximately one-half the depth of the medium (Tittsler & Sandholzer, 1936). Growth behaviour was observed during 3 weeks of incubation at both 22 and 30 °C in the dark and in vertical inoculation tubes, demonstrating that cells of strain CafT13^T are strictly aerobic and non-motile.

For API tests, strain CafT13^T and the type strains of *F. australicus* and *F. peucedani* were grown on ISP 2 at 24 °C for 3 days, and all tests were performed as recommended by the manufacturer (bioMérieux). API ZYM tests were performed after incubation at 26 °C for 20 h. Strain CafT13^T was positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase and α-mannosidase, but negative for valine arylamidase, β-glucuronidase and β-fucosidase. API Coryne tests were performed after incubation at 26 °C for 45 h. Strain CafT13^T was positive for pyrrolidonyl arylamidase and gelatin hydrolysis, but negative for nitrate reduction, pyrazinamidase, urease, and fermentation of glucose, ribose, xylose, mannitol, maltose, lactose, sucrose and glycogen. Catalase activity (21st test of the API Coryne strip) was positive. API 20NE tests were performed after incubation at 26 °C for 45 h. Strain CafT13^T was positive for β-glucosidase (aesculin degradation) and assimilation of *N*-acetyl-D-glucosamine, but negative for indole production, arginine dihydrolase, and assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. The cytochrome oxidase test, determined with oxidase detection strips (bioMérieux), was negative. The API tests results showed remarkable differences between the enzyme spectrum of strain CafT13^T and those of the type strains of *F. australicus* and *F. peucedani*, with 17 differences out of 49 tested reactions (Table 1).

Morphology and colour of the colonies were checked with a stereomicroscope after 5 days of growth on ISP 2 at 24 °C. Single colonies were ~1 mm in diameter, bright yellow, slightly translucent, circular, raised-to-convex and with entire margins. When grown at <10 °C, single colonies appeared punctiform.

Cell morphology was determined by bright-field and differential interference contrast microscopy. Cells of strain CafT13^T were non-flagellated, non-sporulating short rods,

Table 1. Differential characteristics of strain CafT13^T and the type strains of recognized *Frondihabitans* species

Strains: 1, CafT13^T; 2, *F. australicus* E1HC-02^T; 3, *F. peucedani* RS-15^T. ND, Not determined.

Characteristic	1	2	3
Colony colour	Yellow	White	Yellow
Cellular arrangement	Aggregates	Single cells	Single cells
Growth temperature (°C)			
Range	1–30	15−37	4−37
Optimum	22−26	30	20−30
Growth pH			
Range	3.0−8.0	6.0−9.5	5.1−12.1
Optimum	3.7−5.4	9.1	8.1−11.1
DNA G+C content (mol%; mean ± SD)	69.0 ± 0.17	71 ± 1	68.3
Major fatty acids (%)	$C_{18:1}$ (79.3), $C_{14:0}$ 2-OH (7.22), $C_{17:1}\omega9c$ (3.2), $C_{16:0}$ (2.7), anteiso-$C_{15:0}$ (2.4)	$C_{18:1}\omega7c$ (70.0−71.1), $C_{14:0}$ 2-OH (6.5−9.7)	$C_{18:1}\omega7c$ (31.5−35.0), anteiso-$C_{15:0}$ (28.4−30.9), $C_{14:0}$ 2-OH (16.7−18.4)
Major menaquinones (%)	MK-8 (67), MK-7 (33)	MK-8 (76), MK-7 (24)	MK-8 (75), MK-9 (15), MK-7 (10)
Alkaline phosphatase	+	+	−
Esterase (C4)	+	−	−
Lipase (C14)	+	+	−
Valine arylamidase	−	+	−
Cystine arylamidase	+	+	−
Trypsin	+	+	−
α-Chymotrypsin	+	+	−
α-Galactosidase	+	+	−
α-Mannosidase	+	+	−
α-Fucosidase	−	+	−
Gelatin hydrolysis	+	−	−
Assimilation of:			
D-Glucose	−	+	−
D-Mannose	−	+	−
D-Mannitol	−	+	−
N-Acetyl-D-glucosamine	+	+	−
Pyrazinamidase	−	+	ND
Pyrrolidonyl arylamidase	+	−	ND

typically curved in the middle and with clubbed ends (Fig. 1a). Cell size was 1.2–2.0 × 0.5–0.9 μm. The rod–coccoid life cycle was not detected. Clusters of a few to about a thousand cells were often observed in samples collected from both liquid and solid cultures (Fig. 1b). This tendency to form aggregates might explain the occurrence of floccules in liquid culture. Interestingly, Bright & Bulgheresi (2010) showed that auto-aggregation is often a prerequisite of host attachment and therefore it is a characteristic related to a symbiotic lifestyle (e.g. for *Sinorhizobium meliloti*).

For DNA extraction, cell pellets collected from the plate were treated with 10 mg lysozyme ml^{-1}, 20 mg proteinase K ml^{-1} and 10 % SDS; phenol/chloroform/isoamylalcohol at 25 : 24 : 1 was used to separate DNA from proteins and finally the DNA was precipitated with ice-cold ethanol, dried, resuspended in 10 mM Tris/HCl, pH 7.5, and stored at −20 °C. About 150−200 ng DNA was used as a template for amplification of the 16S rRNA gene by PCR. Primers Eub1/1492r (5′-AGAGTTTGATCCTGGCTCAG-3′/5′-GGYTACCTTGTTACGACTT-3′) were added to the *Taq*-and-go kit (MP Biomedicals) and the following program was run: initial denaturation (95 °C for 10 min) followed by 30 cycles of denaturation (94 °C, 30 s), annealing (52 °C, 30 s) and extension (72 °C, 60 s), and a final extension (72 °C, 5 min). Five microlitres of PCR product was checked by 1 % agarose gel electrophoresis. The PCR product was purified, both strands were sequenced and the sequences were assembled with the software MEGA4 (Tamura *et al.*, 2007). A final 16S rRNA gene sequence of 1390 nt of strain CafT13^T was obtained and used for phylogenetic analysis.

The 16SrRNA gene sequence of strain CafT13^T was aligned with those of the type strains of the type species of each genus in the family *Microbacteriaceae* and with that of *F. peucedani* RS-15^T (Lee, 2010), by using the program Clustal X2 (Larkin *et al.*, 2007). The resulting multi-alignment was improved with the software MEGA4 by removing

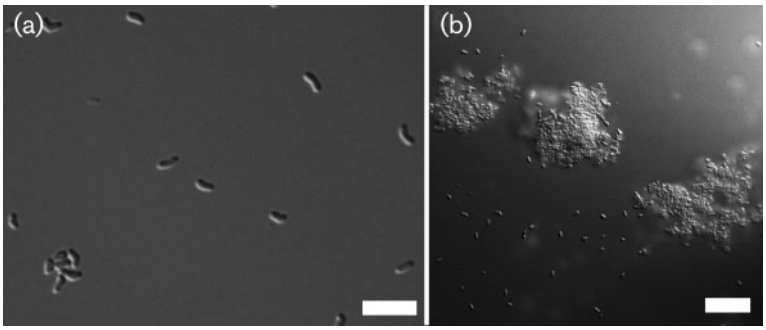

Fig. 1. Differential interference contrast micrographs showing the cell morphology of strain CafT13^T. Cells were grown in liquid ISP 2 at 24 °C and 150 r.p.m. and were observed with a 63× oil-immersion objective (numerical aperture 1.30; Leica Microsystems). (a) The typical coryneform shape, the clubbed ends and a small cluster of six cells are visible; bar, 5 μm. (b) Large auto-aggregated cell clusters were detected after 48 h of growth in liquid ISP 2; bar, 15 μm.

incomplete parts at both the beginning and the end of the sequence, and by manually changing the position of a few nucleotides, where necessary. The optimized multi-alignment comprised 1396 unambiguously aligned positions (including alignment gaps) and was used for phylogenetic analyses performed with the PHYLIP package version 6.69 (Felsenstein, 1989, 2005). The maximum-parsimony tree (Eck & Dayhoff, 1966; Kluge & Farris, 1969) was inferred directly from the aligned sequences. A distance matrix was obtained with the Jukes–Cantor model (Jukes & Cantor, 1969) and used as input for both the neighbour-joining (Saitou & Nei, 1987) and the minimum-evolution tree (Kidd & Sgaramella-Zonta, 1971; Rzhetsky & Nei, 1993). Bootstrap analysis based on 1000 replicates was applied to test for the confidence of tree topologies. The results showed that strain CafT13^T formed a monophyletic branch together with recognized species of the genus *Frondihabitans* (Fig. 2). The position of strain CafT13^T did not vary with the method of tree reconstruction used and it was supported by high bootstrap values (Fig. 2).

Cells of strain CafT13^T were disrupted with a French pressure cell and the DNA was purified by chromatography on hydroxyapatite according to Cashion *et al.* (1977).

For calculation of the G+C content, DNA was hydrolysed with P1 nuclease and the nucleotides were dephosphorylated with bovine alkaline phosphatase (Mesbah *et al.*, 1989). The resulting deoxyribonucleosides were analysed by HPLC. The HPLC system (Shimadzu) consisted of the following: an LC-20AD solvent delivery module, DGU-3A online degasser, CTO-10AC column oven, SIL-20A automatic sample injector and SPD-6A UV spectrophotometric detector. Chromatograms were analysed by using the CLARITY software package (DataApex). The analytical column was a VYDAC 201SP54,

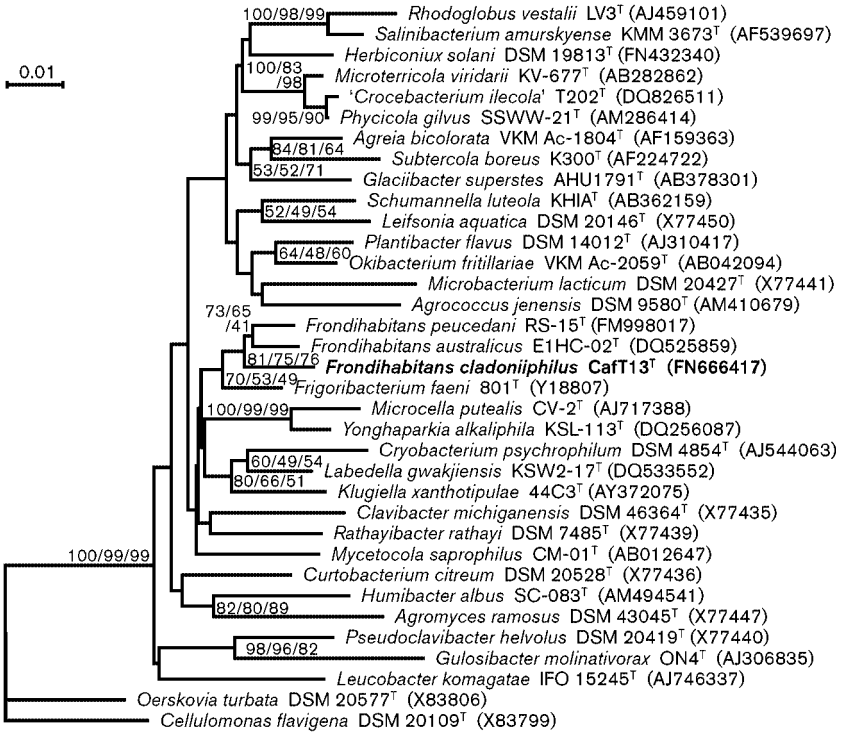

Fig. 2. Phylogenetic tree based on a multi-alignment of 1396 nt of the 16S rRNA gene sequence showing the relationships between strain CafT13^T and the type strains of recognized *Frondihabitans* species and of the type species of all other genera in the family *Microbacteriaceae*. The tree topology was inferred by the neighbour-joining method. Numbers at nodes are bootstrap values as percentages of 1000 data resamplings, obtained by the neighbour-joining/minimum-evolution/maximum-parsimony methods, respectively; only values ⩾50 % (obtained by at least one method) are shown. Sequences of members of the *Cellulomonadaceae* were used as the outgroup. Bar, 0.01 substitutions per site.

C_{18}, 5 μm (250 × 4.6 mm) equipped with a 201GD54H guard column (Vydac). The chromatography conditions were: 45 °C, 10 μl sample, 0.3 M $(NH_4)H_2PO_4$/acetonitrile (40 : 1, v/v) as solvent, pH 4.4, 1.3 ml min^{-1} (Tamaoka & Komagata, 1984). As reference DNA we used: non-methylated Lambda-DNA (G+C content 49.858 mol%), *Bacillus subtilis* DSM 402 (43.518 mol%), *Xanthomonas campestris* pv. *campestris* DSM 3586^T (65.069 mol%) and *Streptomyces violaceoruber* DSM 40783 (72.119 mol%). The DNA G+C content was calculated from the ratio of deoxyguanosine (dG) to thymidine (dT) according to the method of Mesbah *et al.* (1989). The DNA G+C content of strain CafT13^T was 69.0 ± 0.17 mol% (mean ± SD of 2 determinations).

DNA–DNA hybridization experiments with strain CafT13^T and *F. australicus* DSM 17894^T (Zhang *et al.*, 2007; Greene *et al.*, 2009) were carried out as described by De Ley *et al.* (1970) considering the modifications described by Huß *et al.*, (1983) by using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in situ* temperature probe (Varian). Hybridization was performed twice in 2× SSC at 69 °C and with 10 % formamide. The level of DNA–DNA relatedness between strain CafT13^T and *Frondihabitans australicus* DSM 17894^T was 35.20 ± 5.23 % (mean ± SD).

Strain CafT13^T was cultivated on ISP 2. Analyses of respiratory quinones and polar lipids were carried out by the Identification Service of the DSMZ (Braunschweig, Germany). The major menaquinones were MK-8 (67 %) and MK-7 (33 %). Analysis of the polar lipids revealed the presence of diphosphatidylglycerol, phosphatidylglycerol, three unidentified phospholipids, three unidentified glycolipids and one unidentified aminolipid. This polar lipid profile does not match those of any of the close relatives of strain CafT13^T and, in particular, the aminolipids were not found in any related genera of *Microbacteriaceae* (Table 1).

The major cellular fatty acids of strain CafT13^T were $C_{18:1}$ (79.3 %), $C_{14:0}$ 2-OH (7.22 %), $C_{17:1}\omega9c$ (3.20 %), $C_{16:0}$ (2.70 %) and anteiso-$C_{15:0}$ (2.40 %).

The total hydrolysate (4 M HCl, 100 °C, 16 h) of the peptidoglycan of strain CafT13^T contained ornithine, alanine, glycine, homoserine and glutamic acid in an approximate molar ratio of 1.0 : 0.5 : 1.0 : 0.5 : 1.0. Their presence was confirmed by GC/MS (320 Singlequad; Varian) performed according to MacKenzie (1984). Hydroxyglutamic acid could not be detected. The partial hydrolysate (4 M HCl, 100 °C, 0.75 h) contained the peptides Gly–L-Glu and D-Orn–D-Ala. These data indicated that strain CafT13^T has a peptidoglycan of type B2β {Gly} [L-Hsr] D-Glu–D-Orn (Schleifer & Kandler, 1972).

The molecular, chemotaxonomic and physiological data presented thus indicate that strain CafT13^T represents a novel species of the genus *Frondihabitans*, for which the name *Frondihabitans cladoniiphilus* sp. nov. is proposed.

Emended description of the genus *Frondihabitans*

The genus description follows Zhang *et al.* (2007), Greene *et al.* (2009) and the present work. Cells are aerobic, Gram-positive, non-endospore-forming, irregular-shaped rods. No mycelium is produced. A range of carbohydrates and organic compounds are metabolized. 16S rRNA gene sequence analysis indicates that the genus is a member of the family *Microbacteriaceae*. The cell-wall peptidoglycan type is B2β, the major cellular fatty acids are $C_{18:1}$, anteiso-$C_{15:0}$ and $C_{14:0}$ 2-OH, and menaquinones MK-7, MK-8 and MK-9 are present. The polar lipid profile contains unknown aminolipids, glycolipids, phospholipids, diphosphatidylglycerol and phosphatidylglycerol.

Description of *Frondihabitans cladoniiphilus* sp. nov.

Frondihabitans cladoniiphilus (cla.do.ni.i.phi′lus. N.L. fem. n. *Cladonia* referring to the host organism and isolation source; N.L. masc. adj. *philus* from Gr. masc. adj. *philos* loving; N.L. masc. adj. *cladoniiphilus Cladonia*-loving).

Cells are non-motile, non-spore-forming, aerobic, irregular, short rods (1.2–2.0 μm) that stain Gram-positive. Growth occurs at 1–30 °C (optimum, 22−26 °C) and at pH 3–8 (optimum, pH 3.7−5.4). Catalase-positive and oxidase-negative. The type B2β peptidoglycan contains the amino acids ornithine, alanine, glycine, homoserine and glutamic acid in an approximate molar ratio of 1.0 : 0.5 : 1.0 : 0.5 : 1.0. Ornithine is the diagnostic diamino acid. Hydroxyglutamic acid is absent. The major cellular fatty acids are $C_{18:1}$, $C_{14:0}$ 2-OH, $C_{17:1}\omega9c$, $C_{16:0}$ and anteiso-$C_{15:0}$; the major menaquinones are MK-8 and MK-7. Other physiological and biochemical properties are given in Table 1. The polar lipid profile comprises diphosphatidylglycerol, phosphatidylglycerol, three unidentified phospholipids, three unidentified glycolipids and one unidentified aminolipid.

The type strain is CafT13^T (=DSM 23273^T=LMG 25550^T), isolated from the thallus of the reindeer lichen *Cladonia arbuscula* (Wallr.) Rabenh. sampled from the understorey of a natural alpine spruce forest in the region of Styria, Austria. The mean DNA G+C content of the type strain is 69.0 ± 0.17 mol%.

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References

Bates, S. T., Cropsey, G. W. G., Caporaso, J. G., Knight, R. & Fierer, N. (2011). Bacterial communities associated with the lichen symbiosis. *Appl Environ Microbiol* **77**, 1309–1314.

Bjelland, T., Grube, M., Hoem, S., Jorgensen, S. L., Daae, F. L., Thorseth, I. H. & Øvreås, L. (2011). Microbial metacommunities in the lichen–rock habitat. *Environ Microbiol Rep* **3**, 434–442.

Bright, M. & Bulgheresi, S. (2010). A complex journey: transmission of microbial symbionts. *Nat Rev Microbiol* **8**, 218–230.

Cardinale, M., Vieira de Castro, J., Jr, Müller, H., Berg, G. & Grube, M. (2008). *In situ* analysis of the bacterial community associated with the reindeer lichen *Cladonia arbuscula* reveals predominance of *Alphaproteobacteria*. *FEMS Microbiol Ecol* **66**, 63–71.

Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.

De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.

Eck, R. V. & Dayhoff, M. O. (1966). *Atlas of Protein Sequence and Structure.* Silver Springs, MD: National Biomedical Research Foundation.

Felsenstein, J. (1989). PHYLIP – phylogeny inference package (version 3.2). *Cladistics* **5**, 164–166.

Felsenstein, J. (2005). PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.

Greene, A. C., Euzéby, J. P., Tindall, B. J. & Patel, B. K. C. (2009). Proposal of *Frondihabitans* gen. nov. to replace the illegitimate genus name *Frondicola* Zhang *et al.* 2007. *Int J Syst Evol Microbiol* **59**, 447–448.

Grube, M. & Berg, G. (2009). Microbial consortia of bacteria and fungi with focus on the lichen symbiosis. *Fungal Biol Rev* **23**, 72–85.

Grube, M., Cardinale, M., de Castro, J. V., Jr, Müller, H. & Berg, G. (2009). Species-specific structural and functional diversity of bacterial communities in lichen symbioses. *ISME J* **3**, 1105–1115.

Hodkinson, B. P. & Lutzoni, F. (2009). A microbiotic survey of lichen-associated bacteria reveals a new lineage from the Rhizobiales. *Symbiosis* **49**, 163–180.

Huß, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Kidd, K. K. & Sgaramella-Zonta, L. A. (1971). Phylogenetic analysis: concepts and methods. *Am J Hum Genet* **23**, 235–252.

Kluge & Farris, J. S. (1969). Quantitative phyletics and the evolution of anurans. *Syst Zool* **18**, 1–32.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A. & other authors (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947–2948.

Lee, S. D. (2010). *Frondihabitans peucedani* sp. nov., an actinobacterium isolated from rhizosphere soil, and emended description of the genus *Frondihabitans* Greene *et al.* 2009. *Int J Syst Evol Microbiol* **60**, 1740–1744.

MacKenzie, S. L. (1984). Amino acids and peptides. In *Gas Chromatography/Mass Spectrometry Applications in Microbiology*, pp. 157–204. Edited by G. Odham, L. Larsson & P. Mardh. New York: Plenum.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Miller, J. A., Kalyuzhnaya, M. G., Noyes, E., Lara, J. C., Lidstrom, M. E. & Chistoserdova, L. (2005). *Labrys methylaminiphilus* sp. nov., a novel facultatively methylotrophic bacterium from a freshwater lake sediment. *Int J Syst Evol Microbiol* **55**, 1247–1253.

Rzhetsky, A. & Nei, M. (1993). Theoretical foundation of the minimum-evolution method of phylogenetic inference. *Mol Biol Evol* **10**, 1073–1095.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.

Schneider, T., Schmid, E., de Castro, J. V., Jr, Cardinale, M., Eberl, L., Grube, M., Berg, G. & Riedel, K. (2011). Structure and function of the symbiosis partners of the lung lichen (*Lobaria pulmonaria* L. Hoffm.) analyzed by metaproteomics. *Proteomics* **11**, 2752–2756.

Schumann, P., Kämpfer, P., Busse, H. J., Evtushenko, L. I. & Subcommittee on the Taxonomy of the Suborder Micrococcineae of the International Committee on Systematics of Prokaryotes (2009). Proposed minimal standards for describing new genera and species of the suborder *Micrococcineae*. *Int J Syst Evol Microbiol* **59**, 1823–1849.

Selbmann, L., Zucconi, L., Ruisi, S., Grube, M., Cardinale, M. & Onofri, S. (2010). Culturable bacteria associated with Antarctic lichens: affiliation and psychrotolerance. *Polar Biol* **33**, 71–83.

Shirling, E. B. & Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* **16**, 313–340.

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Tindall, B. J., Kämpfer, P., Euzéby, J. P. & Oren, A. (2006). Valid publication of names of prokaryotes according to the rules of nomenclature: past history and current practice. *Int J Syst Evol Microbiol* **56**, 2715–2720.

Tittsler, R. P. & Sandholzer, L. A. (1936). The use of semi-solid agar for the detection of bacterial motility. *J Bacteriol* **31**, 575–580.

Zhang, L., Xu, Z. & Patel, B. K. C. (2007). *Frondicola australicus* gen. nov., sp. nov., isolated from decaying leaf litter from a pine forest. *Int J Syst Evol Microbiol* **57**, 1177–1182.