Frondicola australicus gen. nov., sp. nov., isolated from decaying leaf litter from a pine forest

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An aerobic bacterium, designated strain E1HC-02^T, was isolated from the decaying leaf litter of a slash pine forest located in southeast Queensland, Australia. Cells of strain E1HC-02^T were short irregular rods (0.5–1.0 × 0.2–0.4 µm) which stained Gram-positive and possessed a cell-wall ultrastructure which appeared to be made of protein subunits. The novel strain grew optimally in 1 % trypticase soy broth (TSB) at 25 °C and at a pH of 9.1. Strain E1HC-02^T metabolized a range of carbohydrates, organic acids and amino acids. The G + C content of the DNA was 71 ± 1 mol% as determined by the thermal denaturation method. 16S rRNA gene sequence analysis of strain E1HC-02^T showed that it was a member of the family *Microbacteriaceae*, phylum *Actinobacteria*. The cell wall contained a type B2β peptidoglycan, the dominant cellular fatty acid was 18 : 1ω7c and the major hydroxy fatty acid was 2-OH 14 : 0. The major menaquinones were MK-8 (76 %) and MK-7 (24 %) and the glycolipids present were disphosphatidylglycerol, phosphatidylglycerol and three unidentified phospholipids. The chemotaxonomic properties of strain E1HC-02^T were distinctly different to all of the 17 genera of the family *Microbacteriaceae* and hence strain E1HC-02^T is designated as representing a novel species of a new genus, *Frondicola australicus* gen. nov., sp. nov. The type strain of the type species is E1HC-02^T (=JCM 13598^T = DSM 17894^T).

Nutrients released from the microbial decomposition of plant residues, such as leaf litter, in both natural and agricultural ecosystems, are important for plant growth. It is well known that the quantity of leaf litter decreases during the process of decomposition due to the loss of readily available carbon with a concomitant increase in microbial numbers and an accumulation of refractory compounds (Wardle *et al.*, 2006). Extensive studies on the culture-dependent and culture-independent microbial diversity of soils and the plant rhizosphere and, to a lesser extent, the leaf litter biosphere, have suggested that members of the phyla *Actinobacteria* and *Proteobacteria* dominate these environments (Sharma *et al.*, 2005; Xu *et al.*, 1996). During our culture-dependent and culture-independent bacterial diversity studies of the leaf litter of a slash pine forest located in southeast Queensland, Australia, we frequently isolated members of the phylum *Actinobacteria*. Here, we report on

the characteristics of an irregular rod-shaped bacterium, designated strain E1HC-02^T, which represents a novel species of a new genus.

For the isolation of strain E1HC-02^T, 10 g of moist leaf litter was mixed with 50 ml of sterile distilled water and vortexed for 5 min. The suspension was then rocked horizontally for 5 min, serially diluted ten-fold and 100 µl of the mixture was spread onto trypticase soy broth (TSB) Gelrite plates, pH 7.2. The Gelrite plates contained (g l⁻¹) 3 g TSB, 2.033 g MgCl₂·6H₂O, 0.882 g CaCl₂·2H₂O and 20 g Gelrite. The plates were incubated at room temperature (approx. 25 °C) for up to 6 days. Several morphologically distinct single colonies that developed were picked and individually restreaked onto fresh TSB Gelrite plates at least twice before being considered pure. Colonies from pure cultures were resuspended in sterile preservation medium (0.3 g TSB, 15 ml glycerol and 85 ml distilled water) and stored at –80 °C. One of the isolates that produced cream, round, smooth colonies within 3 days of incubation was designated strain E1HC-02^T and selected for further characterization.

Cell morphology was determined by phase-contrast and electron microscopy (Kanso & Patel, 2003). Cells of strain E1HC-02^T were non-flagellated and non-sporulating irregular short rods (0.5–1.0 × 0.2–0.4 µm). Electron

Abbreviation: DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Frondicola australicus* sp. nov. strain E1HC-02^T is DQ52589.

Details of the cellular fatty acid content of strain E1HC-02^T are available as a supplementary table with the online version of this paper.

Fig. 1. (a, b) Transmission electron micrographs of thin sections of cells of *Frondicola australicus* sp. nov. E1HC-02^T showing irregular rod-shaped cells with inclusion bodies (IB), a typical Gram-positive cell-wall ultrastructure with an S layer (SL), and septum (S) formation during cell division. Bars, 200 nm.

micrographs of thin sections revealed a Gram-positive cell-wall ultrastructure with an S-layer (Messner & Sleytr, 1992; Sleytr *et al.*, 1988) and electron-dense inclusion bodies (Fig. 1).

Growth of strain E1HC-02^T was tested in different concentrations of TSB (up to 3 %) with temperatures ranging from 4 to 50 °C and pH values ranging from 5.1 to 9.5. Growth was measured at 660 nm in a Novaspec LKB spectrophotometer (Pharmacia-Biotech Pty. Ltd.). Strain E1HC-02^T grew optimally in 1 % TSB at a pH of 9.1 (pH growth range, 6.0–9.5) and an incubation temperature of 25 °C (growth temperature range 15–37 °C) with a generation time of 5 h. Unless indicated otherwise, all subsequent growth experiments were conducted using these conditions.

The metabolic profile of strain E1HC-02^T was determined using GN2 Biolog plates (Hayward). For this, the novel strain was grown on Biolog Universal Growth medium agar plates. Colonies were resuspended in an inoculation fluid and this was used to inoculate the wells of GN2 Biolog plates as recommended by the manufacturer. After 24 h incubation at room temperature (approx. 25 °C), the absorbances in the wells of the microtitre plates were read and the results transformed into positive, borderline and negative scores by using Biolog MicroStation System software. The results of the analysis are provided in the species description.

Catalase activity was determined by dropping H₂O₂ on to colonies that developed on 1 % TSB agar plates and activity was determined to be positive. The oxidase test, determined

by using Oxidase detection strips (Medvet Science Pty. Ltd.), was negative.

The cell-wall peptidoglycan of strain E1HC-02^T was isolated and analysed at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany, using previously described procedures (MacKenzie, 1987; Schleifer, 1985; Schleifer & Kandler, 1972; Rhuland *et al.*, 1955). The total hydrolysate was found to contain the amino acids ornithine, alanine, glycine, homoserine and glutamic acid in the molar ratio of 1.0 : 0.9 : 1.7 : 0.5 : 1.0. The presence of these amino acids was confirmed by GC and MS (MacKenzie, 1984). Hydroxyglutamic acid was not detected. The partial hydrolysate of the cell-wall peptidoglycan showed that strain E1HC-02^T contained the peptides Gly-L-Glu and D-Orn-D-Ala. On the basis of amino acid and peptide analyses of cell-wall hydrolysates, strain E1HC-02^T was determined to contain a peptidoglycan of type B2β {Gly} [L-Hsr] D-Glu-D-Orn (Schleifer & Kandler, 1972). No glycolate was detected in the cell-wall acid hydrolysate, which suggests that muramic acid occurs in the *N*-acetyl form.

Fatty acid methyl ester (FAME) analysis of the whole-cell was determined at DSMZ using GC (MIDI, Microbial ID). Strain E1HC-02^T possessed a very large amount of fatty acid 18 : 1ω7c (77.9 %) and smaller amounts of other fatty acids (see Supplementary Table S1 in IJSEM Online).

Polar lipid analysis was carried out by the identification services of DSMZ and Dr B. J. Tindall, DSMZ, Germany. Strain E1HC-02^T possessed disphosphatidylglycerol, phosphatidylglycerol, at least 6 glycolipids, of which 3 were probably diglycosyl diglycerides (the nature of sugars and their linkages have not been identified), and 4 unidentified phospholipids.

For genomic DNA purification, a 50 ml sample of actively growing culture was centrifuged at 5400 r.p.m. at 4 °C for 20 min (4K15; Sigma) and the cell pellet was resuspended in 4 ml buffer A (5 mM Tris/HCl pH 8.0, 100 mM NaCl and 5 mg lysozyme). After incubation on ice for 5 min, 0.85 ml of buffer [300 mM EDTA, pH 7.5 and 5 % (w/v) SDS] and 100 µl of 20 mg ml⁻¹ proteinase K was added. Following overnight incubation at 55 °C, an equal volume of phenol : chloroform : isoamylalcohol (25 : 24 : 1) was added and the mixture was agitated gently for 30 min. Three volumes of cold 100 % ethanol were then carefully overlaid and the flocculent DNA that developed was spooled onto a sterile disposable plastic loop. The spooled DNA was resuspended in 5 ml TE buffer (10 mM Tris/HCl pH 7.4, 1 mM EDTA pH 8) and the quality assessed by agarose gel electrophoresis, ethidium bromide staining and visualization under a Gel Documentation Analysis System (GDAS 1200; UVP). The mol% G + C content of the genomic DNA was determined by the thermal denaturation method (Marmur & Doty, 1961) using a spectrophotometer (Cintra20; GBC Scientific Equipment) as described by Spanevello *et al.*, 2002 and was calculated to be 71 ± 1 mol%.

For studies on the 16S rRNA gene, 10 ml of an overnight culture of strain E1HC-02^T was centrifuged at 5400 r.p.m. at 4 °C for 20 min (4K15; Sigma). The pellet was resuspended in 100 µl TE buffer and the cells were lysed by boiling for 15 min. The lysate was centrifuged at 14 000 r.p.m. for 15 min in a microcentrifuge (model 1-15; Sigma) and 2 µl aliquots of the supernatant were used as a template for the amplification of the 16S rRNA gene (Andrews & Patel, 1996). The sequencing of the gene was performed essentially as described previously (Andrews & Patel, 1996). The partial sequences that were generated were assembled using BioEdit v5.0.1 (Hall, 1999) and the consensus sequence of 1433 nucleotides was manually corrected for errors. The most closely related sequences in GenBank (version 152) and the Ribosomal Database Project II (release 9.37) identified using BLAST (Altschul *et al.*, 1997) and the Sequence Match program (Cole *et al.*, 2005) were extracted, aligned and manually adjusted according to the 16S rRNA secondary structure using BioEdit. Nucleotide ambiguities were omitted and evolutionary distances were calculated by using the Jukes and Cantor option (Jukes & Cantor, 1969) in TreeCon (Van de Peer *et al.*, 1997). Phylogenetic trees were constructed from evolutionary distances using the neighbour-joining method (Saitou & Nei, 1987). Tree topology was re-examined by the bootstrap method (1000 replications) of resampling (Felsenstein, 1985).

16S rRNA gene sequence analysis indicates that strain E1HC-02^T is a member of the phylum *Actinobacteria*, class *Actinobacteria*, subclass *Actinobacteridae*, order

Actinomycetales, suborder *Micrococccineae*, family *Microbacteriaceae* (Fig. 2). The inclusion of strain E1HC-02^T as a member of the phylum to the suborder level is supported by the presence of the defining nucleotide signatures and, with the exception of an A:U at the positions 771–808 (*Escherichia coli* numbering) instead of a G:C, to the family level (Stackebrandt *et al.*, 1997). Phylogenetic analysis undertaken with representative members of the family *Microbacteriaceae* placed strain E1HC-02^T almost equidistantly between the cluster represented by the strains of the psychrophile *Frigoribacterium faeni* (Miteva *et al.*, 2004; Kämpfer *et al.*, 2000) and an isolate from alpine subnival plants (H. M. Sheng, L. Z. Ang, S. J. Xu, T. Chen & X. L. Zheng, unpublished, GenBank accession no. DQ339615) and a 16S rRNA clone from DNA extracted from volcanic deposits (J. Gomez-Alvarez, J. Baniak, G. Harrison, J. G. Medrano & K. Nusslein, unpublished, GenBank accession no. DQ490454). This relationship was not robust when the sequences of the 16S rRNA clone and the alpine subnival plant isolate sequences were excluded from the analysis, suggesting that strain E1HC-02^T is only distantly related to *Frigoribacterium faeni*. The phylogenetic analysis, taken together with the information that strain E1HC-02^T possesses a protein subunit cell-wall ultrastructure, is unable to grow at psychrophilic temperatures, has a higher optimum pH for growth and has a number of significant chemotaxonomic differences in its cell wall and cell membranes and in its metabolizable substrates (Table 1), clearly differentiates the novel strain from members of the genus *Frigoribacterium*.

Fig. 2. Neighbour-joining phylogenetic tree showing the position of *Frondicola australicus* sp. nov. E1HC-02^T within the radiation of members of the family *Microbacteriaceae*. GenBank accession numbers are given in parentheses. Clusters of members of the same genus are represented as triangles and include *Mycetocola saprophilus* DSM 15178^T (GenBank accession no. AB012647) and *Mycetocola tolaasinivorans* DSM 15179^T (AB012646) for the genus *Mycetocola*, *Microbacterium imperiale* DSM 20530^T (X77442), *Microbacterium phyllosphaerae* DSM 13468^T (AJ277840), *Microbacterium liquefaciens* DSM 20638^T (X77444) and *Microbacterium thalassium* DSM 12511^T (AM181507) for the genus *Microbacterium* and *Curtobacterium citreum* DSM 20528^T (X77436) and *Curtobacterium flaccumfaciens* LMG 3645^T (AJ312209) for the genus *Curtobacterium*. Bootstrap values above 95% from 1000 replicates are shown. Bar, 10% change per nucleotide.

Table 1. Key characteristics that differentiate genera of the family *Microbacteriaceae*

Taxa: 1, *Frondicola australicus* gen. nov., sp. nov.; 2, *Frigoribacterium*; 3, *Clavibacter*; 4, *Rathayibacter*; 5, *Rhodoglobus*; 6, *Leifsonia*; 7, *Agromyces*; 8, *Agrococcus*; 9, *Subtercola*; 10, *Agreia*; 11, *Cryobacterium*; 12, *Leucobacter*; 13, *Curtobacterium*; 14, *Microbacterium*; 15, *Mycetocola*; 16, *Okibacterium*; 17, *Plantibacter*; 18, *Zimmermannella*. R, Red; W, white; Y, yellow; O, orange; P, pink; BL, blue; B, brown; YW, yellowish-white; PG, phosphatidylglycerol; DPG, disphosphatidylglycerol; GL, glycolipids; PL, unidentified phospholipids; Orn, ornithine; Lys, lysine; DAB, diaminobutyric acid; NR, not reported.

Characteristic	1	2	3	4	5	6	7	8	9
Colony colour	W	Y	W, Y, O, P, BL	NR	R	Y, W	Y	W, Y, O	Y
Growth temperature range (°C)	15–37	0–28	Up to 35	<37	–2–21	7–37	Up to 37	Up to 37	–2–28
Growth optimum (°C)	25	4–10	21–26	24–28	18	24–28	26–30	28	15–17
DNA G+C (mol%)	71 ± 1	72	67–78	63–72	62	66–73	70–72	74	64–68
Diamino acid	Orn	Lys	DAB	DAB	Orn	DAB	DAB	DAB	DAB
Major fatty acids (≥ 5%)*	C _{18:1} ω7c, C _{14:0} 2-OH	a-C _{15:0} , i-C _{16:0} , C _{16:0} , a-C _{15:1}	a-C _{15:0} , i-C _{16:0} , a-C _{17:0}	a-C _{15:0} , i-C _{16:0} , a-C _{17:0}	a-C _{15:0} , i-C _{16:0} , a-C _{17:0} , a-C _{15:1}	a-C _{15:0} , i-C _{16:0} , a-C _{17:0}	a, i	a-C _{15:0} , i-C _{16:0} , a-C _{17:0} , a-C _{15:1}	a-C _{15:0} , i-C _{16:0} , a-C _{17:0} , i-C _{14:0} , a-C _{15:1}
Polar lipids	PG, DPG, GL, PL	PG, DPG	PG, GL	PG, GL	NR	PG, DPG	PG, GL	PG, GL	PG, GL, DPG
Major menaquinone	MK-7, MK-8	MK-9	MK-9, MK-10	MK-10	MK-11, MK-12	MK-10, MK-11	MK-12, MK-13	MK-11, MK-12	MK-9, MK-10

Characteristic	10	11	12	13	14	15	16	17	18
Colony colour	Y, O	NR	B	Y, O, W, P	W, O	YW	Y	Y	W
Growth temperature range (°C)	Up to 37	<18	Up to 37	Up to 35	10–40	>4–33	7–37	NR	NR
Growth optimum (°C)	24–26	9–12	28	24–27	30	25	24–27	25	30
DNA G+C (mol%)	67	65	66	68–75	65–75	64–65	67	68–70	62–68
Diamino acid	DAB/Orn	DAB	DAB	Orn	Lys/Orn	Lys	Lys	DAB	DAB
Major fatty acids (≥ 5%)*	a-C _{15:0} , i-C _{16:0} , a-C _{17:0}	a-C _{15:0} , i-C _{16:0} , a-C _{17:0} , i-C _{15:0} , a-C _{15:1}	a-C _{15:0} , i-C _{16:0} , a-C _{17:0}	a-C _{15:0} , i-C _{16:0} , a-C _{17:0} , a-C _{15:1} , ch _{17:0}	a-C _{15:0} , i-C _{16:0} , a-C _{17:0} , i-C _{15:0}	a-C _{15:0} , i-C _{16:0} , a-C _{17:0}	a-C _{15:0} , i-C _{16:0} , a-C _{17:0} , i-C _{15:0}	a-C _{15:0} , i-C _{16:0} , a-C _{17:0} , i-C _{15:0}	a-C _{15:0} , i-C _{16:0} , C _{16:0} , a-C _{17:0}
Polar lipids	PG, DPG	PG, GL	PG, GL, DPG	PG, GL	PG, GL	NR	PG, DPG	NR	NR
Major menaquinone	MK-10	MK-10	MK-11	MK-9	MK-11, MK-12, MK-13, MK-14	MK-9, MK-10	MK-11, MK-12	MK-10, MK-11	MK-8, MK-9, MK-10

*a, Anteiso-branched acids; i, iso-branched acids; ch, ω-cyclohexyl acid. Except for *Agrococcus jenensis* ST54, monounsaturated 12-methyl-tetradecenoic acid (a-C_{15:1}) represents 5% or more of the total only when the strains were grown at lower temperatures (10 °C).

The members of the family *Microbacteriaceae* form a cohesive phylogenetic cluster and the 17 genera of the family are further defined and differentiated by key chemotaxonomic characteristics, as indicated in Table 1. Strain E1HC-02^T, however, possesses traits that are atypical and have so far not been reported for any of the genera of the family *Microbacteriaceae*, including the genus *Frigoribacterium* (Table 1). Strain E1HC-02^T contains a high concentration of the monounsaturated fatty acid 18:1 ω 7c (78 %), a fatty acid reported so far only in a few members of the class *Alphaproteobacteria* (Kim *et al.*, 2006; Martínez-Cánovas *et al.*, 2004), possesses a type B2 β cell-wall peptidoglycan, has MK-8 and MK-7 as the major menaquinones and contains the glycolipids disphosphatidylglycerol, phosphatidylglycerol and three unidentified phospholipids.

Based on the phylogenetic, chemotaxonomic and phenotypic evidence presented, strain E1HC-02^T cannot be placed in any of the 17 genera of the family *Microbacteriaceae* and hence we propose to create a new genus, *Frondicola* gen. nov., to accommodate this novel species. Strain E1HC-02^T represents the type strain of the type species, *Frondicola australicus* sp. nov.

Description of *Frondicola* gen. nov.

Frondicola [Fron.di'co.la. L. fem. n. *frons* (genitive, *frondis*) fallen leaves; L. masc. suffix n. *-cola* from *incola* inhabitant; N.L. masc. n. *Frondicola* inhabitant of leaves, leaf dweller].

Cells are aerobic, non-endospore-forming, irregular-shaped rods which stain Gram-positive. No mycelium is produced. A range of carbohydrates, organic compounds and amino acids are metabolized. 16S rRNA gene sequence analysis indicates that the genus is a member of the family *Microbacteriaceae* within the radiation of the genus *Frigoribacterium*. The cell-wall peptidoglycan type is B2 β , the major cellular fatty acid is 18:1 ω 7c and menaquinones MK-7 and MK-8 are present. The type species is *Frondicola australicus*.

Description of *Frondicola australicus* sp. nov.

Frondicola australicus (aus.tra'li.cus. N.L. masc. adj. *australicus* pertaining to Australia, from where the type strain was isolated).

Has the following properties in addition to those given in the description of the genus. Cells are non-motile, non-spore-forming, aerobic, irregular, short rods (0.5–1.0 $\times$ 0.2–0.4 μ m) that stain Gram-positive. Cell wall appears to be made of a protein subunit layer and inclusion bodies are present. Growth occurs at temperature between 15 and 37 °C (optimum temperature, 25 °C) and at pH 6.0–9.5 (optimum, pH 9.1). The type strain metabolizes dextrin, Tween 40, Tween 80, maltose, D-melibiose, α -D-glucose, sucrose, D-trehalose, turanose, succinic acid monomethyl ester, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, DL-lactic acid, L-glutamic acid, L-pyrogutamic acid and urocanic acid. Does not metabolize α -cyclodextrin,

glycogen, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, D-cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, myo-inositol, α -D-lactose, lactulose, D-mannitol, D-mannose, methyl β -D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, xylitol, pyruvic acid methyl ester, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α -hydroxybutyric acid, β -hydroxybutyric acid, γ -hydroxybutyric acid, p-hydroxyphenylacetic acid, itaconic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinamic acid, glucuronamide, L-alaninamide, L-alanine, D-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-serine, D-serine, L-threonine, DL-carnitine, γ -aminobutyric acid, inosine, uridine, thymidine, phenylethylamine, 2-aminoethanol, 2,3-butanediol, glycerol, DL- α -glycerol phosphate, α -D-glucose 1-phosphate or D-glucose 6-phosphate. Catalase-positive and oxidase-negative. The type B2 β [Gly] [L-Hsr] D-Glu-D-Orn peptidoglycan contains the amino acids ornithine, alanine, glycine, homoserine and glutamic acid in a molar ratio of 1.0:0.9:1.7:0.5:1.0, respectively. The major cellular fatty acid is 18:1 ω 7c (78 %) and the major menaquinones detected are MK-8 (76 %) and MK-7 (24 %). The glycolipids disphosphatidylglycerol, phosphatidylglycerol and three unidentified phospholipids are present. The DNA base content is 71 $\pm$ 1 mol% G + C.

The type strain, strain E1HC-02^T (= JCM 13598^T = DSM 17894^T), was isolated from the decaying leaf litter of a slash pine forest located in southeast Queensland, Australia.

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