**Microvirga subterranea gen. nov., sp. nov., a moderate thermophile from a deep subsurface Australian thermal aquifer**

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A strictly aerobic bacterium, strain Fail4^T^, was isolated from free-flowing geothermal waters of a bore (bore register no. 3768) tapping the Great Artesian Basin of Australia. The non-sporulating, Gram-negative cells of strain Fail4^T^ produced light-pink colonies, were rod-shaped (1 × 1.5–4 μm) and were motile by a single polar flagellum. Strain Fail4^T^ grew optimally at 41 °C at a pH of 7.0 and had an absolute requirement for yeast extract. The strain grew on casein hydrolysate, tryptone, gelatin, xylose and acetate in a medium supplemented with 0.06 or 0.006 % yeast extract. Weak acid production was detected from glucose and arabinose. Catalase was produced. Nitrite was produced from nitrate. Strain Fail4^T^ was sensitive to antibiotics that inhibit growth of bacteria. The G+C content was 63.5 ± 0.5 mol%. Strain Fail4^T^ was a member of the class 'Alphaproteobacteria', phylum Proteobacteria, placed almost equidistantly between *Methylobacterium* species, *Chelatococcus asaccharovorans* and *Bosea thiioxoidans* (similarity value of 93 %) as its nearest phylogenetic relatives. Phylogenetic and phenotypic evidence suggest that strain Fail4^T^ (= ATCC BAA-295^T^ = DSM 14364^T^) should be placed as the type strain of a species in a newly created genus, for which the name *Microvirga subterranea* gen. nov., sp. nov. is proposed.

**INTRODUCTION**

The Great Artesian Basin (GAB) is a subsurface geothermal aquifer that covers 1.7 × 10^9 km^2^ of the Australian continent and is considered to be the most extensive water-containing body (8.7 × 10^12 m^3^) of this type in the world (Habermahl, 1980). The aquifer is interbedded with up to four sequential layers of artesian water and confining Mesozoic sediments (70–200 million years old). The sequence varies from less than 100 m to over 3000 m and some of the sequences contain oil and gas. The aquifer water has a natural flow rate between 40 and 98 °C. The water from the bores cools to ambient in the hundreds of kilometres long, open-ditch runoff channels, and is used as drinking water by cattle and sheep. The chemical composition of the bore water is complex and is influenced by the chemistry of the underlying geological formation, the temperature and the various chemical interactions that occur at different temperatures. Consequently, the GAB has been recognized as a unique and exceedingly interesting environment for studies on microbial diversity. A variety of novel microbial physiologies have been isolated from this environment and include *Desulfotomaculum australicum* (Love et al., 1993), *Desulfovibrio longreachensis* (Redburn & Patel, 1994), *Fervidobacterium gondwanense* (Andrews & Patel, 1996), *Thermaerobacter subterraneus* (Spanevello et al., 2002) and *Bacillus subterraneus* (Kanso et al., 2002). In addition, extensive studies on the production of industrially important thermostable enzymes such as amylases and dextranase has been undertaken (Wynter et al., 1996), but the vast majority of these strains have remained taxonomically uncharacterized. Microbial communities observed to thrive along the temperature gradient sometimes as visible mat communities have also been under investigation in our laboratory (Spanevello, 2001). As part of ongoing efforts to unravel the microbial diversity of this unique pristine ecosystem, we report here on the isolation and characterization of a novel bacterium, strain Fail4^T^.
METHODS

Sample collection. Samples were collected at the bore outflow source of the 295-m-deep metal-encased Failea bore (bore no. 3768), situated in the district of Longreach, Queensland, Australia, by filling sterile glass containers completely to the brim and sealing with air-tight enclosures. The flow rate of the bore water was 9.1 l min⁻¹ and the source water temperature was 42 °C.

Culture media. Rouf’s agar medium (Mulder & Deinema, 1992) was used for enrichment and isolation. The medium contained (l⁻¹): 1 g yeast extract, 5 g peptone, 0.2 g MgSO₄.7H₂O, 0.05 g CaCl₂, 0.15 g ferric ammonium citrate, 0.05 g MnSO₄.4H₂O, 0.01 g FeCl₃.4H₂O, 17 g agar, 10 ml vitamin solution (Wolin et al., 1963) and 1 ml trace-element solution (Zeikus et al., 1979). Routine cultivation was performed in the same medium but without agar. Rouf’s medium containing 0.06% or 0.006% (w/v) yeast extract instead of 1 g l⁻¹ and without peptone and agar (referred to as Rouf’s minimal medium) was used to test the physiological properties of the strain. Rouf’s medium lacking agar but containing 5 g instead of 1 g yeast extract l⁻¹ (referred to as Rouf’s maximal medium) was used to determine the optimal environmental growth parameters. The pH of the medium was adjusted to 7.1 with NaOH when necessary.

Isolation and cellular characterization. Agar plates were spread directly with 100 µl of the bore-water sample and incubated at 37, 40 and 50 °C for up to 3 days. Several colonies that developed at 40 °C were picked and restreaked onto new plates and the procedure was repeated twice. A colony with a light-pink colour was picked as being representative of several similar colonies, designated strain Fail47 and studied further. Phase-contrast and electron microscopy of cells were performed as described previously (Andrews & Patel, 1996). The presence of poly-β-hydroxybutyrate (PHB) was demonstrated by staining lightly heat-fixed cells with Sudan black B [0.3% (w/v) in 70% (v/v) ethanol] for 10 min followed by decolorizing with xylene for 10 s, drying and counterstaining with Safranin [0.5% (w/v) in water] for 10 s. Phase-contrast microscopy and spore staining (Smibert & Krieg, 1994) of early, exponential and late growth phase and 1-week-old cultures was used to determine the presence of spores. Mid-exponential and mid-stationary phase cultures exposed to heat at 70, 80, 90 and 100 °C for 10 and 20 min were used to inoculate fresh medium and incubated at the 41 °C in order to determine heat resistance.

Substrate utilization and physiological studies. Rouf’s minimal medium was used to test for growth on different substrates. Unless otherwise stated, substrates were tested by adding aliquots from 2 or 3 M sterile stock solutions to 5 ml medium to a final concentration of 21 mM. Yeast extract, peptone and casein hydrolysate (Amyl Media) were tested at 0.05 and 0.1% (w/v), ethanol at 10 and 21 mM, methanol at 0.1, 0.5 and 1-0% (w/v), N,N-dimethylformamide (DMF) and formamide at 0.15 and 0.5% (w/v) and nitritoltriacetate (NTA) at concentrations from 0-007 to 0-5% (w/v). Methane was injected into tubes of autoclaved medium capped with butyl septa, using a syringe with a single-use filter unit (Sartorius) attached at the end to filter-sterilize the gas at the same time. The ratio of methane to air in the tube was roughly 1:4. Thiosulfate (5 and 10 mM) was tested as an alternate electron acceptor with 0-02 and 0-003% yeast extract. The pH was adjusted to 7.1 prior to inoculation when necessary and all tests were carried out in duplicate. Growth was measured as OD₆₀₀ after incubation at 41 °C for up to 24 h.

API 20E (bioMérieux) and BBL Crystal E/NF (Becton Dickinson) identification kits were used to determine acid production from sugars, nitrate reduction, enzymic hydrolysis and utilization of various substrates using the manufacturers’ recommended protocols. Catalase activity was determined on freshly growing colonies using 3% (v/v) hydrogen peroxide solution. Hydrolysis of starch and casein and determination of oxidative or fermentative catabolism were carried out according to methods described by Smibert & Krieg (1994).

Rouf’s maximal medium was used to determine pH (range pH 5–10), temperature (range 25–60 °C) and NaCl (range 0–11% (w/v)) optima.

Anaerobic growth was tested in TYEG medium (Wynter et al., 1996). The presence of photosynthetic pigments and carotenoids was determined by scanning acetone extracts of 10 and 50 ml cultures that had been incubated in the dark and in the light, in a Cintra20 spectrophotometer (GBC Scientific Equipment) at wavelengths between 250 and 900 nm.

Antibiotic sensitivity. Sensitivity to antibiotics (penicillin, ampicillin, neomycin, tetracycline, streptomycin, vancomycin, chloramphenicol and spectinomycin) was determined by adding the antibiotic to 10 ml aliquots of Rouf’s maximal medium to final concentrations of 10, 50 and 100 µg ml⁻¹. The cultures were incubated under optimal conditions for 24 h and growth was recorded by measuring the OD₆₀₀. Percentage growth inhibition was calculated from cultures that had been grown under similar conditions in antibiotic-free medium.

DNA G+C content and 16S rRNA sequencing. Genomic DNA was prepared using a modification of the method of Marmur (1961) in which a chromopeptidase (final concentration 0.8 µg ml⁻¹) was used to improve cell lysis following lysozyme treatment. The genomic DNA was dissolved overnight in 0.1× SSC at a concentration of 20 µg ml⁻¹ and the Tm was determined in 0.1× SSC using Escherichia coli DNA as a reference in a Cintra20 spectrophotometer.

The methods used for 16S rRNA gene amplification and sequencing have been reported previously (Andrews & Patel, 1996). Sequences generated during this work were assembled into one complete sequence using Bioedit (Hall, 1999). The consensus sequence was corrected manually for errors and the most homologous sequences, determined against the GenBank database using BLAST (Altschul et al., 1997) and using the ‘Sequence Match’ option against the Ribosomal Database Project (Maidak et al., 2001), were extracted and aligned manually. Sequence uncertainties were omitted from the analysis and phylogeny from 1108 unambiguous nucleotides was determined as described previously (Andrews & Patel, 1996).

RESULTS

Morphology and growth characteristics

Colonies (0.5–2 mm) of strain Fail47 were light pink, circular and convex with entire smooth edges and shiny surfaces, thin in texture and easily emulsifiable. The rod-shaped cells (1×1.5–3.4 µm) with round ends occurred singly and as pairs and were motile with a single polar flagellum (Fig. 1a). Pleomorphic L-, T-, V- and Y-shaped cells could be observed in cultures that were a few days old (Fig. 1b). Large intracellular granules were observed under phase-contrast microscopy from cells from ageing cultures (Fig. 1c). Staining revealed that the darkly stained granules against pink-coloured cells were PHB. The cells stained Gram-negative and electron micrographs of thin sections revealed a Gram-negative type cell wall (Fig. 1d). Speros were not observed from cultures grown under different conditions. Cultures were sensitive to heat (70 °C for 10 min), supporting the notion that strain Fail47 was a non-spor-former.
Strain FaiI4T grew optimally at 41°C (growth range 25–45°C; no growth at 50°C) and pH 7.0 (pH range 6–9). Strain FaiI4T grew best without NaCl and tolerated 1% NaCl (w/v), with no growth in the presence of 2% (w/v) NaCl. The doubling time of strain FaiI4T under optimal growth conditions was 270 min.

Strain FaiI4T did not grow anaerobically in TYEG medium and did not contain bacteriochlorophyll, indicating that it was not a photosynthetic bacterium. However, carotenoids were present in light- and dark-grown cultures.

Substrate utilization and physiological characteristics

Strain FaiI4T grew on yeast extract, which was absolutely required for growth on other substrates. Strain FaiI4T grew well on acetate, xylose, peptone and casein hydrolysate (Amyl Media) but not on glucose, sucrose, fructose, maltose, cellobiose, glycerol, ethanol, methanol, pyruvate, succinate, methanol, benzoate, butyrate, oxalate, citrate, lactate, fumarate, glycine, aesculin, NTA, DMF or formamide as sole sources of carbon and energy in a medium containing 0.06 or 0.006% yeast extract. Gelatin, but not starch or casein, was hydrolysed. Very weak acid production was observed from glucose and arabinose. Oxidase, urease, tryptophan deaminase, ornithine decarboxylase, arginine dihydrolase, H₂S, lysine decarboxylase, β-galactosidase, arabinosidase, glucuronidase, glucosaminidase, xylosidase, indole from tryptophan and acetoin were not produced. Nitroanilidase, glucosidase and catalase were positive. Nitrate was reduced to nitrite. Thiosulfate did not stimulate growth.

Antibiotic susceptibility

Strain FaiI4T did not grow in the presence of 10 μg penicillin, ampicillin, neomycin, tetracycline, streptomycin and chloramphenicol ml⁻¹ or 100 μg spectinomycin ml⁻¹ but growth was inhibited only partially in medium containing 100 μg vancomycin ml⁻¹.

G+C content and 16S rRNA sequence analysis

The G+C content of strain FaiI4T determined by the Tₘ method was found to be 63.5 ± 0.5 mol%.

BLAST analysis indicated that strain FaiI4T was most closely related to the environmental clone WCHB1-55 (Djoja et al., 1998) and the taxonomically undescribed strain SJA-9 (Von Wintzingerode et al., 1999) in the class ‘Alphaproteobacteria’, phylum Proteobacteria (as proposed in the 2nd edition of Bergey’s Manual of Systematic Bacteriology; Garrity & Holt, 2001). Methylobacterium species (Patt et al., 1976; Urakami et al., 1993; Urakami & Komagata, 1984) and Bosea thiooxidans (Das et al., 1996) were the mostly closely related of the taxonomically valid taxa, with a mean similarity value of 93% (Fig. 2). Bootstrap analysis gave a high degree of confidence to this relationship (100%). Strain FaiI4T was slightly less closely related to Chelatococcus asaccharovorans (Auling et al., 1993; Egli et al., 1988).

DISCUSSION

Strain FaiI4T was isolated from the waters of the subsurface aquifer of the GAB. There is a plethora of evidence that microbial communities exist in the deep biosphere such as the deep sea (Pedersen, 2000). A limited number of other studies have reported the presence of microbial communities in the deep-bore-well biosphere but, as water samples were collected with the assistance of pumps (Stevens & McKinley, 1995) and soil samples by coring (Phelps et al., 1989), extraneous contamination could not be ruled out. Collecting uncontaminated samples is a major problem associated with studying deep-subsurface microbial communities. However, we believe that the primary habitat for strain FaiI4T is the deep aquifer biosphere rather than the surface environment, as the strain was cultured from samples collected from the fast, freely flowing pristine waters of a 295 m metal-encased deep bore at the point of surface emission without surface contamination and it grows optimally at 41°C, the
temperature of the source water. It is highly likely that there is continual, slow growth of the isolate in the deep-subsurface environment, with subsequent seeding to the surface via the bore. On the other hand, colonization of the bore environment, which contains the rapidly flowing waters, by extraneous microbial contaminants and their subsequent release by the maintenance of extraordinarily high growth rates is improbable.

Penicillin and a number of other antibiotics that inhibit members of domain Bacteria also inhibited the growth of strain Fail4T, suggesting an affiliation to this group of microbes. This was confirmed by analysis of the 16S rRNA gene which, in addition, indicated that the closest relatives of the isolate were C. asaccharovorans, B. thiooxidans and Methylobacterium species, respectively members of the families ‘Beijerinckiaceae’, ‘Bradyrhizobiaceae’ and ‘Methylobacteriaceae’ of the class ‘Alphaproteobacteria’, phylum Proteobacteria. The dendrogram was constructed by using the neighbour-joining method and Jukes & Cantor evolutionary distance matrix data obtained from 1108 unambiguous nucleotides. Sequences were extracted from the RDP version 8.0 (including A. spiritensis, deposited by C. R. Woese) and GenBank release 121. GenBank accession numbers are shown in parentheses. The members of the family ‘Methylobacteriaceae’ (indicated by the triangle) included Methylobacterium rhodinum NCIMB 9421T (L20849) and Methylobacterium radiotolerans JCM 2831T (D32227), which were the most closely related to strain Fail4T. Numbers represent bootstrap values of the branching (100 replicates). Bar, 2 substitutions per 100 nt.

Fig. 2. Dendrogram showing the position of strain Fail4T within the radiation of the members of the families ‘Beijerinckiaceae’, ‘Bradyrhizobiaceae’, Hyphomicrobiaceae, ‘Methylocystaceae’ and ‘Methylobacteriaceae’ of the class ‘Alphaproteobacteria’, phylum Proteobacteria. The dendrogram was constructed by using the neighbour-joining method and Jukes & Cantor evolutionary distance matrix data obtained from 1108 unambiguous nucleotides. Sequences were extracted from the RDP version 8.0 (including A. spiritensis, deposited by C. R. Woese) and GenBank release 121. GenBank accession numbers are shown in parentheses. The members of the family ‘Methylobacteriaceae’ (indicated by the triangle) included Methylobacterium rhodinum NCIMB 9421T (L20849) and Methylobacterium radiotolerans JCM 2831T (D32227), which were the most closely related to strain Fail4T. Numbers represent bootstrap values of the branching (100 replicates). Bar, 2 substitutions per 100 nt.

Strain Fail4T is a strictly non-spore-forming aerobe, stains Gram-negative, is catalase-positive and does not produce indole or hydrolysis starch; these phenotypic properties are common to C. asaccharovorans, B. thiooxidans and Methylobacterium species. However, numerous other properties of strain Fail4T are different from those of these close phylogenetic relatives (Table 1). Strain Fail4T has the highest optimum growth temperature (41°C) and has a restricted substrate range for growth in comparison with these close relatives. In addition, the G+C content of the DNA of strain Fail4T (63 mol%) is very different from those of B. thiooxidans (68 mol%) and Methylobacterium species (66–71 mol%), but is similar to that of C. asaccharovorans. A number of other key metabolic differences also exist. Strain Fail4T is unable to utilize NTA as a carbon source, a key differentiating property of C. asaccharovorans, a property that is also unique amongst bacteria. All 14 Methylobacterium species are characterized by their pink-pigmented colonies, production of PHB and extreme nutritional versatility in their ability to grow on organic substrates as well as C1 compounds, such as methane and methanol. Therefore, they are commonly referred to as the pink-pigmented facultatively methylotrophic microbes. Strain Fail4T also produces pink colonies and PHB, but it has a number of key nutritional differences when compared with its four closest phylogenetic neighbours, namely Methylobacterium radiotolerans, Methylobacterium aminovorans, Methylobacterium extorquens and Methylobacterium organophilum, including its inability to grow on the C1 compounds tested (Table 1).

Growth of B. thiooxidans is stimulated in the presence of thiosulfate, but this is not the case with strain Fail4T.

The phylogenetic and phenotypic evidence presented above suggests that strain Fail4T cannot be assigned to any known taxon. We therefore propose to describe it as representative of a novel species of a new genus, Microvirga subterranea gen. nov., sp. nov.

Description of Microvirga gen. nov.

Microvirga (Mi.cro.vir’ga. Gr. adj. mikros small; L. fem. n. virga rod; N.L. fem. n. Microvirga a small rod).

Strictly aerobic, small, rod-shaped cells that stain Gram-negative and produce light-pink colonies. The optimum temperature for growth is 41°C, with a temperature range for growth between 25 and 45°C. Does not grow on glucose but uses xylose. Reduces nitrate to nitrite. Cells are susceptible to antibiotics that inhibit members of domain Bacteria. Bacteriochlorophyll is absent but carotenoids are present. PHB is produced. Thiosulfate does not stimulate growth. 16S rDNA sequence analysis indicates that it is a member of the ‘Alphaproteobacteria’, phylum Proteobacteria, placed almost equidistantly between C. asaccharovorans and B. thiooxidans, respectively members of the families ‘Beijerinckiaceae’ and ‘Bradyrhizobiaceae’, as its nearest phylogenetic relatives. The type species is Microvirga subterranea.

Description of Microvirga subterranea sp. nov.

Microvirga subterranea (sub.ter.ra’ne.a. L. fem. adj. subterranea underground, subterranean).

In addition to the properties described for the genus, the following properties apply. After 48 h, colonies (diameter...
0.5–2 mm) are light pink, convex, smooth and circular with entire margins. Cells are rod-shaped (1 × 1.5–4 μm), stain Gram-negative and possess a typical Gram-negative type cell wall ultrastructure, are motile by a single polar flagellum and do not form spores. Cells from old cultures are pleomorphic and contain large intracellular granules. Does not grow at 50°C; optimum pH is 7.0 (pH range for growth 6–9). Grows on yeast extract, which is absolutely required for growth. Grows on casein hydrolysate, tryptone, xylose and acetate as sole carbon and energy sources but not on other carbohydrates, organic acids, alcohols, methane, glycine, aesculin, NTA, DMF or formamide. Gelatin, but not starch or casein, is hydrolysed. Very weak acid production is detected from glucose and arabinose. Catalase is produced but not oxidase, urease, indole or acetoin. Nitrite is produced from nitrate. Sensitive to penicillin, ampicillin, chloramphenicol, tetracycline, streptomycin and neomycin but resistant to vancomycin and spectinomycin. The G + C content of the type strain is 63.5 ± 0.5 mol%.

The type strain, strain Fail4T (= ATCC BAA-295T = DSM 14364T), was isolated from the geothermal waters of a bore (bore register no. 3768) tapping the GAB of Australia. 

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REFERENCES


