Phenylobacterium lituiforme sp. nov., a moderately thermophilic bacterium from a subsurface aquifer, and emended description of the genus *Phenylobacterium*

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A facultative anaerobic bacterium, strain Fail3^T, was isolated from samples collected from the free-flowing waters of a bore well (Fairlea Bore, registration number 3768) which taps into the Australian Great Artesian Basin subsurface thermal aquifer. Strain Fail3^T developed yellow to pale-yellow colonies (0.5–1.5 mm) after 48 h. The non-spore forming rods (0.5 × 1–3 µm) were slightly curved, occurred singly and as pairs and were motile with a single polar flagellum. Cells tended to form clumps in liquid medium and rosettes were commonly observed. The cells stained Gram-negative and electron micrographs of thin sections revealed a multi-layered complex Gram-negative cell wall structure. Strain Fail3^T grew optimally at 40–41 °C, with growth observed at 45 °C but not at 50 °C. The pH growth range was between pH 6 and 9 and optimal growth occurred between pH 6 and 6.5. Strain Fail3^T grew best with yeast extract as the sole carbon and energy source. Peptone, yeast extract, acetate, xylose, sucrose, glucose, glycerol, succinate, butyrate, lactate, fumarate, citrate, L-phenylalanine, cellobiose and gelatin supported growth but maltose, fructose, glycine, ethanol, benzoate and oxalate did not. Tyrosine was produced from L-phenylalanine. Strain Fail3^T was catalase-positive and oxidase-negative and did not hydrolyse starch. Growth was inhibited by neomycin, tetracycline, streptomycin, chloramphenicol, ampicillin, vancomycin and spectinomycin. The G + C content was determined to be 66.5 ± 0.5 mol%. On the basis of the 16S rRNA gene sequence analysis, strain Fail3^T was assigned as a novel species of the genus *Phenylobacterium*, *Phenylobacterium lituiforme* sp. nov. in the order *Caulobacterales*, subclass *α-Proteobacteria*, class *Proteobacteria*. The type strain is Fail3^T (= ATCC BAA-294^T = DSM 14363^T).

The Great Artesian Basin (GAB) of Australia, discovered in 1880, spans 1.7 million square kilometres across four Australian states, and is one of the largest underground water reservoirs in the world (8.7 × 10¹² m³). It has been estimated that the underground natural flow of water from its recharge zones in the east coast around the Great Dividing mountain ranges to its natural discharge in the Australian central region as natural mound springs is between 1 and 5 metres per year. The water has been isotopically dated to nearly 2 million years. Though natural discharge of water occurs through mound springs, as many as 5000 man-made free-flowing bores have been drilled to tap the basin's water resource. The aquifer water is geothermally heated at around 1 °C per 30 metres depth and

temperatures of up to 98 °C have been recorded for some bores. Though the aquifer water is predominantly freshwater around the rain recharged areas of the basin and suitable for drinking and domestic use, the quality deteriorates quickly, mainly due to the underlying geological chemistry of the basin and chemical reactions that occur due to temperature fluctuations. The hot water brought to the surface via the bores cools to ambient temperature in open-drain channel systems of up to 150 km long and is used as drinking water for farm animals. The deep subsurface GAB aquifer and its open-drain surface systems where temperature gradients form is an ideal environment for microbial diversity studies. A large number of diverse groups associated with the GAB environment have been isolated and studied over the past decade (Spanevello *et al.*, 2002; Kanso & Patel, 2003; Kanso *et al.*, 2002). Here we report on the characterization of a moderate thermophile, *Phenylobacterium lituiforme* sp. nov. strain Fail3^T, isolated from a 42 °C water sample collected at the source of a 295 m deep bore (Fairlea Bore, registration number 3768).

Abbreviation: GAB, Great Artesian Basin.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Phenylobacterium lituiforme* strain Fail3^T (= ATCC BAA-294^T = DSM 14363^T) is AY534887.

Samples were collected by completely filling sterile glass containers with water emitted from the mouth of the outflow of Fairlea Bore (temperature 42 °C and pH 8.2), which is located in Longreach district, Queensland. The bottles were then capped and transported to the laboratory at Griffith University and stored at room temperature until used. For strain isolation, modified RouF's medium agar (pH 7.1) plates were spread with 10, 50 and 100 µl each of the bore water sample and the plates incubated at 37, 40 and 50 °C for 24 h. Modified RouF's medium (Mulder & Deinema, 1992) contained (per 1000 ml distilled water) 1 g yeast extract, 5 g peptone, 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g CaCl_2 , 0.15 g ammonium iron (III) citrate, 0.05 g $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.01 g $\text{FeCl}_3 \cdot 4\text{H}_2\text{O}$, 17 g agar, 10 ml Wolin vitamin solution (Wolin *et al.*, 1963) and 1 ml Zeikus's trace element solution (Zeikus *et al.*, 1979). Based on the colony morphology and pigmentation, a representative colony from each of the five different colony types that developed was picked and streaked onto new plates. This procedure was repeated at least twice before the isolates were deemed to be pure. The isolates were finally subcultured in liquid RouF's medium which lacked agar, sterile glycerol added to a final concentration of 50 %, and the isolates frozen as stock cultures at -20 °C. Strain FaiI3^T, which produced 0.5–0.15 mm yellow colonies after 48 h incubation at 37 °C, was selected for further characterization. Carotenoids with absorbance peaks at A_{460} – A_{468} were detected from acetone-extracted cell-free supernatants using a Cintra20 Spectrophotometer (GBC Scientific Equipment). The colonies of strain FaiI3^T were circular and convex with entire edges, had a smooth surface and possessed a sticky texture, which emulsified in water easily. An odour was present. Cellular characterization and sporulation tests performed as described previously (Kanso & Patel, 2003) showed that the cells of strain FaiI3^T stained Gram-negative, occurred singly or in pairs and short chains of three cells were rarely observed. The cells were usually short to slightly curved rods (0.5 × 1–3 µm), but filamentous cells (4–7 µm) were also present. Cell rosettes were frequently observed. Strain FaiI3^T was motile with a single polar flagellum (Fig. 1a). Electron microscopic examination of thin sections revealed a Gram-negative type cell wall ultrastructure (Fig. 1b). Spores were never observed and cells were heat sensitive.

Strain FaiI3^T was a facultative anaerobe as it also grew in anaerobic liquid RouF's medium (pH 7.1), which had been prepared by boiling, cooling and dispensing 10 ml aliquots into Hungate tubes under a stream of oxygen-free N_2 . Further proof of the strain's facultative nature comes from our observation that the strain grew along the stab in RouF's deep agar slants.

Maximal RouF's medium lacking agar and containing 5 g yeast extract l^{-1} instead of 1 g l^{-1} was used to determine optimum growth conditions and inhibitory effects of antibiotics and NaCl. Strain FaiI3^T grew optimally at 40–41 °C with growth occurring at 45 °C but not at 50 °C. The pH growth range was between pH 6 and 9 with an optimum

Fig. 1. (a) Electron micrograph of negatively stained cells showing a single polar flagellum (arrow). Bar, 0.2 µm. (b) Transmission electron micrograph of a thin section of strain FaiI3^T showing a Gram-negative type cell wall. Bar, 0.2 µm.

between pH 6 and 6.5. Strain FaiI3^T had a generation time of 4 h under optimal growth conditions. Strain FaiI3^T grew best without NaCl and as little as 0.5 % NaCl inhibited 75 % of its growth. Neomycin, tetracycline, streptomycin and chloramphenicol at 10 µg ml^{-1} and ampicillin, vancomycin and spectinomycin at 50 µg ml^{-1} completely inhibited growth of strain FaiI3^T.

Characterization studies performed by adding appropriate substrates from 2 or 3 M sterile stock solutions to a final concentration of 20 mM to RouF's minimal medium (10 ml) containing 0.006 % (w/v) yeast extract and lacking peptone and agar indicated that strain FaiI3^T grew on a wide range of substrates (Table 1). Inoculation of API 20E (bioMérieux) and BBL Crystal E/NF identification kits (Becton Dickinson) with strain FaiI3^T, following the manufacturer's recommended protocols, showed weak acid production from glucose and arabinose but not from mannose, sucrose, melibiose, rhamnose, sorbitol, mannitol, adonitol, galactose, amygdalin or inositol. In addition, nitroanilidase, glucosidase and catalase were produced but not oxidase, urease, tryptophan deaminase, ornithine decarboxylase, arginine dihydrolase, H_2S , lysine

Table 1. Differentiating features of *Phenylobacterium lituiforme* strain FaiI3^T and *Phenylobacterium immobile* strain E^T, the type species of the genus

Both *P. lituiforme* and *P. immobile* stain Gram-negative, do not form spores, prostheca or grow on maltose, fructose or hydrolyse starch. Both species are catalase-positive and urease-negative and do not produce indole.

Characteristic	<i>P. lituiforme</i> strain FaiI3 ^T * (=ATCC BAA-294 ^T =DSM 14363 ^T)	<i>P. immobile</i> strain E ^T † (=DSM 1986 ^T)
Ecology	Water from subsurface aquifer	Soil
Pigmentation, cell shape and size (µm)	Yellow to pale-yellow, rods, 0.5 × 1–3	Colourless, rods to coccoid rods, 0.7–1 × 1–2
Motility	Motile	Non-motile
Flagella	Single, polar	None
O ₂ requirement	Facultative anaerobe	Obligate aerobes
Growth temperature (°C)	Range 25–45, no growth at 50, optimum 40–41	Optimum 28–30, no growth at 37
pH growth range	6–9, optimal at 6–6.5	6.5–8, optimal at 6.8–7
NaCl growth range	Optimal at 0%, no growth at 1%	Optimal at 0%, no growth at 1%
Oxidase activity	–	+
Gelatin hydrolysis	+	–
Growth on substrates:		
Glucose	+	–
Sucrose	+	–
Xylose	+	–
Glycerol	+	–
Acetate	+	–
Succinate	+	+
Citrate	+	–
G + C (mol%)	66.5 ± 0.5	65.5

*RouF's medium containing 0.006% yeast extract did not support growth of strain FaiI3^T and was therefore used for substrate utilization tests. Growth was measured after incubation for up to 48 h at 41 °C at OD₆₀₀. Strain FaiI3^T utilized yeast extract (the best growth substrate), peptone, acetate, pyruvate, succinate, butyrate, lactate, citrate, fumarate, L-phenylalanine, xylose, sucrose, glucose, glycerol and cellobiose but not maltose, fructose, glycine, ethanol, benzoate, oxalate, aesculin or nitrilotriacetate.

†Data from Lingens *et al.* (1985).

decarboxylase, β-galactosidase, arabinosidase, glucuronidase, glucosaminidase, xylosidase, indole from tryptophan or acetoin. Nitrate was reduced to nitrogen. Casein but not starch was hydrolysed as determined by the method of Smbert & Krieg (1994).

Growth in RouF's minimal medium containing either 0.06% or 0.006% yeast extract and L-phenylalanine (0.5 g l⁻¹) was determined over a 76 h incubation period at 41 °C by measuring growth at OD₆₀₀ and the formation of tyrosine using the modified method of Lowry *et al.* (1951). An uninoculated culture medium served as control. An increase in the growth of strain FaiI3^T with the concomitant increase in tyrosine was observed in minimal RouF's media (Fig. 2).

The fact that strain FaiI3^T has an optimum growth temperature of 41 °C reflects closely its environmental habitat of the moderately thermal waters (42 °C) of the GAB of Australia, a deep subsurface aquifer. Strain FaiI3^T was isolated by plating aquifer samples that had been collected directly from the bore source without surface contamination. Both these observations suggest that the primary habitat of the strain is the aquifer. Strain FaiI3^T uses a wide range

of organic substrates including the aromatic amino acid L-phenylalanine for growth. The latter is a rare characteristic not commonly reported in bacteria. The ability of strain

Fig. 2. Growth of strain FaiI3^T in RouF's minimal medium containing 0.5 g L-phenylalanine l⁻¹ with 0.006% yeast extract [growth (○) and tyrosine production (●)] and with 0.06% yeast extract [growth (□) and tyrosine production (■)].

FaiI3^T to grow on such a wide range of organic compounds may reflect its survival and adaptation on organic matter released from decomposing dead cells in the otherwise pristine GAB waters.

Genomic DNA was prepared using a modified method (Marmur, 1961) in which achromopeptidase (final concentration 1 mg ml⁻¹) was used for cell lysis and RNase (20 µg ml⁻¹) used to digest RNA. The DNA was dissolved overnight at 4 °C in 0.1 × SSC to a concentration of 20 µg ml⁻¹. Its thermal denaturation temperature (T_m) was determined to be 66.5 ± 0.5 mol% using a Cintra20 spectrophotometer (GBC Scientific Equipment). *Escherichia coli* genomic DNA prepared in the same manner was used as reference DNA.

The methods used for 16S rRNA gene amplification and sequencing have been reported previously (Andrews & Patel, 1996). Partial sequences generated in this investigation were assembled and the consensus sequence corrected manually for errors using BioEdit v5.0.1 (Hall, 1999). The most closely related sequences against GenBank and Ribosomal Database Project II were identified using BLAST (Altschul *et al.*, 1997) and the Sequence Match program (Maidak *et al.*, 2001); sequences were then extracted, aligned and manually adjusted according to the 16S rRNA secondary structure using BioEdit. Sequence uncertainties were omitted and phylogenetic reconstruction achieved using TreeCon (Van de Peer & De Wachter, 1994) in which pairwise evolutionary distances were computed from percentage similarities (Jukes & Cantor, 1969) and phylogenetic trees constructed from the evolutionary distances using the neighbour-joining method (Saitou & Nei, 1987). FastDNAm1 was also used in phylogenetic reconstruction (Olsen *et al.*, 1994). Tree topology was re-examined by using the bootstrap method of resampling (Felsenstein, 1985) using 1000 bootstraps.

16S rRNA gene sequence of strain FaiI3^T showed the greatest similarity to members of the order *Caulobacterales*, subclass α -*Proteobacteria*, class *Proteobacteria*. The closest relatives were *Phenylobacterium immobile* (similarity value of 96 %) and members of the genera *Caulobacter* (Abraham *et al.*, 1999) and *Brevundimonas* (mean similarity value of 94 %) (Fig. 3). The low level of similarity between strain FaiI3^T and *Caulobacter* and *Brevundimonas* is in itself indicative that it is a distinct species. However, the ability of strain FaiI3^T to grow optimally at 41 °C in the absence of NaCl differentiates it from members of the genera *Caulobacter* and *Brevundimonas*, which grow optimally at 20–25 °C but not above 35 °C and require NaCl for optimal growth (Abraham *et al.*, 1999; Lingens *et al.*, 1985).

Both strain FaiI3^T and *P. immobile*, the sole member of the genus *Phenylobacterium*, share the ability to metabolize the aromatic amino acid L-phenylalanine for growth, have a G + C content of 65–66 mol%, are sensitive to growth in the presence of 0.5 % NaCl, have a similar antibiotic sensitivity profile, possess a similar cell wall ultra-structure

and are close phylogenetic relatives. However, there are numerous phenotypic differences between them, which provide evidence of distinctness (Table 1). Strain FaiI3^T is a facultative anaerobe which grows optimally at 41 °C (maximum growth temperature of 45 °C), is actively motile with a single flagellum and its colonies are yellow, whereas *P. immobile* is an obligate aerobe which grows optimally between 28 and 30 °C with no growth occurring at 37 °C, is non-motile and the colonies are non-pigmented. In addition, *P. immobile* grows well only on restricted and specialized substrates, which include, in addition to L-phenylalanine, the herbicides chloridazon, antipyrin and pyrimidon. In contrast, strain FaiI3^T grows on a much wider range of substrates including carbohydrates such as glucose, sucrose and xylose, glycine and gelatin as well as fatty acids, which include acetate, succinate and butyrate. Furthermore, *P. immobile* is a slow-growing bacterium and requires 2–3 weeks incubation for colony development (Lingens *et al.*, 1985) whereas the colonies of strain FaiI3^T develop within 36–48 h incubation at 41 °C. However, the 16S rRNA gene sequence similarity of 96 % in itself is clearly sufficient to justify the inclusion of strain FaiI3^T as a new species of the genus *Phenylobacterium*. Based on the evidence presented above, we propose to emend the description of the genus *Phenylobacterium* and assign strain FaiI3^T as a new species, *Phenylobacterium lituiforme* sp. nov.

Emended description of *Phenylobacterium* Lingens *et al.* 1985

Cells stain Gram-negative, are non-spore forming straight to slightly curved rods, coccobacilli or cocci measuring 0.7–1.0 × 1.0–2.0 µm and occur singly, in pairs or short chains. Strains may form rosettes. Filamentous cells tend to form in old cultures. Species may be strict aerobes or facultative anaerobes and may be motile or non-motile. Cells do not form sheaths or prosthecae and are not acid-fast. The members of the genus grow on L-phenylalanine. Based on the 16S rRNA gene sequence analysis, members of this genus form a monophyletic group within the order *Caulobacterales*, subclass α -*Proteobacteria* of the class *Proteobacteria*. The DNA base ratio is 65 ± 1 mol% G + C. Isolated from soil and water.

The type species is *Phenylobacterium immobile* Lingens *et al.* 1985 (strain E^T = DSM 1986^T).

Description of *Phenylobacterium lituiforme* sp. nov.

Phenylobacterium lituiforme (li.tu.i.for' me. L. masc. n. *lituus* curved rod of the augurs; L. neut. adj. suffix *-forme* having the form of; N.L. neut. adj. *lituiforme* formed like a curved rod).

Strain FaiI3^T is a facultative anaerobe isolated from water collected from a free-flowing bore well, tapping the underground water of the Great Artesian Basin of Australia. Yellow to pale-yellow colonies (0.5–2 mm) develop on

Fig. 3. Dendrogram showing the phylogenetic position of *Phenylobacterium lituiforme* strain Fail3^T (=ATCC BAA-294^T = DSM 14363^T) as a member of the family *Caulobacteraceae*, order *Caulobacterales*, subclass α -*Proteobacteria* in class *Proteobacteria*. The dendrogram was constructed by using the neighbour-joining method and Jukes & Cantor evolutionary distance matrix data obtained from 1381 unambiguous aligned nucleotides. The sequences were extracted from the Ribosomal Database Project (RDP) version 8.0 and GenBank database, release 121. The clusters indicated as triangles for *Caulobacter* species represent *Caulobacter vibrioides* VKM-B1496^T (AJ009957), *Caulobacter fusiformis* ATCC 15257^T (AJ227759) and *Caulobacter henricii* ATCC 15253^T (AJ227758) and for *Brevundimonas* species represent *Brevundimonas vesicularis* IAM 12105^T (AB021414) and *Brevundimonas diminuta* IAM 12691^T (AB021415). The triangle representing sequences for the members of the order *Sphingomonadales* were used as outgroups and includes *Zymomonas mobilis* subsp. *mobilis* ATCC 10988^T (RDP, unpublished), *Blastomonas natatoria* ATCC 35951^T (X73043), and *Sandaracinobacter sibiricus* strain RB16-17^T (Y10678). Bootstrap values (from 100) are indicated at the nodes. GenBank/EMBL/DDBJ accession numbers for the sequences are shown in parentheses. Scale bar indicates 10 substitutions per 100 nucleotides.

RouF's agar plates after 48 h at 41 °C. Colonies are circular, convex, with an entire edge and smooth surface, are sticky in texture, fairly easily emulsified and odour is present. The cells are non-spore-forming straight to slightly curved rods (0.5 by 1–3 µm), motile with a single polar flagellum, occur mainly singly, some in pairs and short chains. Cells often tend to clump in liquid medium to form rosettes. The cells stain Gram-negative and electron micrographs of thin sections reveal a multi-layered complex Gram-negative cell wall. Sheaths and prosthecae are not produced. Strain FaiI3^T grows optimally at 40–41 °C and growth is observed at 45 °C but not at 50 °C. The pH range for growth is 6–9 and optimal growth occurs between pH 6 and 6.5. Strain FaiI3^T is very sensitive to NaCl and growth is inhibited by 0.5 % (w/v) NaCl. Strain FaiI3^T grows best with yeast extract as the sole carbon and energy source. Peptone, yeast extract, acetate, pyruvate, xylose, sucrose, glucose, glycerol, succinate, butyrate, lactate, citrate, fumarate, L-phenylalanine, cellobiose and gelatin support growth but maltose, fructose, glycine, ethanol, benzoate and oxalate do not. Tyrosine is produced from L-phenylalanine. Strain FaiI3^T is catalase-positive, oxidase-negative and does not hydrolyse starch. Growth is inhibited by neomycin, tetracycline, streptomycin, chloramphenicol, ampicillin, vancomycin and spectinomycin. The G+C content is 66.5 ± 0.5 mol%.

The type strain is FaiI3^T (=ATCC BAA-294^T = DSM 14363^T).

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