

Thermaerobacter subterraneus* sp. nov., a novel aerobic bacterium from the Great Artesian Basin of Australia, and emendation of the genus *Thermaerobacter

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A strictly aerobic, thermophilic, Gram-positive, spore-producing, rod-shaped bacterium (2.0–10.0 × 0.3 µm), designated isolate C21^T, was isolated from a sample collected from an open drain run-off channel of a bore in the Great Artesian Basin of Australia (New Lorne Bore, registered number 17263). Isolate C21^T grew optimally at 70 °C (temperature range for growth was 55–80 °C) and pH 8.5 (pH range for growth was 6.0–10.5), with a generation time of 90 min. The isolate was strictly heterotrophic and grew on yeast extract and/or tryptone as carbon and energy sources. An increase in growth was not observed with carbohydrates (sucrose, cellobiose, glucose, dextrin, amylopectin, chitin, carboxymethylcellulose, xylan, inositol, arabinose, mannose, fructose, gelatin, starch, amylose, galactose, dextrose, xylose, maltose, L-sorbose or raffinose), organic acids (lactic acid, pyruvic acid or benzoic acid) or Casamino acids as sole carbon sources or in the presence of yeast extract and/or tryptone. The G+C content of the chromosomal DNA, as measured by the thermal denaturation method, was 71 mol%. Phylogenetic analysis of the 16S rRNA gene of isolate C21^T placed it as a member of the phylum *Firmicutes*, with *Thermaerobacter marianensis* as the closest relative (similarity value of 98%). However, isolate C21^T and *T. marianensis* differed in a number of key physiological and phenotypic properties and also had a DNA–DNA hybridization value of less than 5%. Based on this evidence, it is proposed that strain C21^T be designated *Thermaerobacter subterraneus* sp. nov. (type strain C21^T = ATCC BAA-137^T = DSM 13965^T).

Keywords: *Thermaerobacter subterraneus* sp. nov., Great Artesian Basin of Australia, thermophile, phylogeny, 16S rRNA

INTRODUCTION

Though the deeper phyla of domain *Bacteria*, such as *Aquificae* (Huber *et al.*, 1992) and *Thermotogae* (Fardeau *et al.*, 1997), consist exclusively of thermophiles, other, more recently evolved phyla such as *Firmicutes* are also represented by thermophilic members (Boone *et al.*, 1995; Collins *et al.*, 1994). Most thermophilic bacteria have been isolated from

volcanic hot springs or hydrothermal vents (Brock & Freeze, 1969; Harmsen *et al.*, 1997; Ward *et al.*, 1998), but within the past few years an increasing number of thermophilic microbes have also been isolated from subsurface non-volcanic thermal environments such as oilfields and aquifers (Denman *et al.*, 1991; Andrews & Patel, 1996; Magot *et al.*, 1997). In this report, isolation of a novel spore-forming, thermophilic strict aerobe from the Great Artesian Basin (GAB) of Australia is described. The GAB is a deep, multi-layered, subsurface, geothermal aquifer that underlies approximately 20% of Australia's landmass, mainly the arid and semi-arid regions (Habermahl, 1980). The water is brought to the surface by some 5000 free-

Abbreviations: GAB, Great Artesian Basin; RDP, Ribosomal Database Project.

The GenBank accession number for the 16S rDNA sequence of *Thermaerobacter subterraneus* strain C21^T is AF343566.

flowing bores and is distributed through open-drain run-off channels for use as drinking water for domestic animals. The temperature at the sources of these bores can be as high as 99 °C, with temperatures in run-off channels cooling to ambient, thereby producing unique temperature gradients in which distinct microbial communities and mats develop. A wide variety of physiological groups of bacteria, including sulfate reducers, carbohydrate fermenters, strict aerobes and strict anaerobes, has been isolated from this ecosystem.

METHODS

Source of cultures. Sediment samples were collected from the run-off channel of the New Lorne Bore (registered bore number 17263) situated near Blackall, some 1000 km north-west of Brisbane, Queensland, Australia (24° 54' 48" S and 145° 08' 18" E). The bore was drilled in 1966 and has a depth of 1613 m and a flow rate of 7.6 l s⁻¹. The temperature of the free-flowing water at the bore head was 88 °C and the pH was 8.5. A typical smell of H₂S was evident. Samples were collected to represent as many micro-niches as possible. A water sample was collected at the bore well head (source sample, 88 °C), sediment slurries were collected at various temperatures of the run-off channel and different coloured microbial mats developing at specific temperatures in the run-off channel, with grey (75 °C), red (66 °C), green (57 °C) and brown (52 °C) filaments, were also collected. Sterile glass vessels were filled to the brim, capped, transported to the laboratory and stored at room temperature until used. *Thermaerobacter marianensis* (JCM 10246^T = DSM 12885^T) was purchased from the DSMZ and cultured as described previously (Takai *et al.*, 1999).

Media, enrichment and isolation. Medium D was prepared as described previously (Brock & Freeze, 1969). Samples (0.5 ml) and 10-fold serial dilutions of the sample to 10⁻¹⁰ were inoculated into 10 ml sterile medium D and incubated at 65 and 75 °C for up to 72 h. Growth was determined microscopically and positive enrichment cultures were sub-cultured again under identical growth conditions. Pure cultures were isolated by streaking onto medium D plates amended with 2% agar, followed by incubation at the enrichment temperatures. Single well-separated distinct colonies were picked and grown. This procedure was repeated at least twice before the culture was considered pure and characterized further. The pure culture, designated strain C21^T, was stored in a 50:50 medium D/glycerol mixture at -20 °C.

Light and electron microscopy. Light and electron microscopy were performed as described previously (Andrews & Patel, 1996). Gram reaction and oxidase and catalase tests were performed as described by Collee *et al.* (1996).

Growth characterization. All growth experiments were carried out in duplicate in liquid medium D at 70 °C unless otherwise stated. Growth was determined by inserting culture tubes directly into a Novaspec LKB spectrophotometer (Pharmacia-Biotech) and measuring optical density at 660 nm.

The ability of isolate C21^T to grow under anaerobic conditions was tested in a glucose-containing trypticase/peptone/yeast extract/glucose (TYEG) medium (Andrews & Patel, 1996) and in a medium used for culturing sulfate-reducing bacteria (Love *et al.*, 1993). The effect of different

concentrations of yeast extract and/or tryptone (up to 0.2%) on the growth of isolate C21^T was determined in medium D. NaCl tolerance was determined by adding NaCl to medium D to concentrations of up to 3%.

The nutritional spectrum of isolate C21^T was tested in medium D containing Casamino acids, sucrose, cellobiose, glucose, dextrin, amylopectin, inositol, arabinose, mannose, fructose, gelatin, amylose, galactose, dextrose, xylose, lactic acid, pyruvic acid, maltose, L-sorbose, raffinose, benzoic acid, carboxymethylcellulose, cellulose, chitin, xylan or starch at a final concentration of 0.1 and/or 0.5%.

The effect of antibiotics (ampicillin, neomycin, penicillin, phosphomycin, polymyxin B, streptomycin and tetracycline) on the growth of isolate C21^T was tested. The antibiotics were added from filter-sterilized stock solutions to medium D to give final concentrations of 10 and 100 µg ml⁻¹. Sodium azide was added to final concentrations of 250 and 500 µg ml⁻¹.

The temperature range for growth (55–80 °C) of isolate C21^T was determined in medium D. The pH range for growth (pH 4.9–11.5) was determined in the same medium by adjusting the pH with HCl or NaOH at room temperature prior to sterilization. The generation time was determined at optimum growth parameters (temperature of 70 °C and pH 8.5).

DNA extraction and G+C content of the DNA. Thirty millilitres of a 48-h-old culture was pelleted in a Sigma 4K15 (Quantum Scientific) by centrifuging at 5400 r.p.m. for 5 min. The pellet was resuspended in 487 µl TE buffer (10 mM Tris/HCl, pH 7.4, 1 mM EDTA, pH 8.0), 8 µl lysozyme (50 mg ml⁻¹) and 40 µl achromopeptidase (10 mg ml⁻¹) and incubated for 1 h at 37 °C. Thirty microlitres 10% SDS and 3 µl proteinase K (20 mg ml⁻¹) were added and the mixture was incubated at 50 °C for 1 h. Cell lysis was checked by phase-contrast microscopy. NaCl (5 M, 100 µl) and 80 µl 10% CTAB/0.7 M NaCl were added. The solution was vortexed and incubated at 65 °C for 10 min. DNA was purified from the suspension by first extracting with equal volumes of chloroform/isoamyl alcohol (24:1) and then with phenol/chloroform/isoamyl alcohol (25:24:1). Chromosomal DNA was recovered by adding 450 µl 2-propanol and centrifuging at 14000 r.p.m. for 15 min in a Sigma (1-15) microcentrifuge. The chromosomal DNA pellet was then washed with 250 µl 70% ethanol, dried and resuspended in 100 µl TE buffer. RNase A was added to a final concentration of 200 µg ml⁻¹. Chromosomal DNA integrity was checked by agarose gel electrophoresis and ethidium bromide staining and visualized by UV fluorescence. The G+C (mol%) content of the chromosomal DNA was determined at the DSMZ.

DNA-DNA hybridization. DNA was extracted from 11 cultures of *Thermaerobacter marianensis* JCM 10246^T and isolate C21^T. Cells were lysed in lysozyme (final concentration of 1 mg ml⁻¹) and the DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and precipitated with ethanol. The DNA was dissolved in TE buffer. RNase A (10 µg ml⁻¹) was added and the mixture was incubated for 30 min at 35 °C, after which proteinase K (10 µg ml⁻¹) and 0.3% SDS were added and incubation was continued at 50 °C for 60 min. DNA purification was repeated using the phenol mixture and ethanol precipitation steps described above. The purified DNA was finally dissolved in TE buffer and stored at -20 °C until used.

DNA–DNA hybridization was performed at 55 °C using a colorimetric microplate hybridization method (Ezaki *et al.*, 1989) as modified by Kusunoki *et al.* (1991) and Maruyama *et al.* (2000). The DNA of *Escherichia coli* (SMUM 344^T = JCM 1649^T) was used as a negative reference.

16S rRNA gene sequencing and phylogenetic analysis. The 16S rRNA gene of isolate C21^T was amplified by PCR prepared in sterile 0.2 ml thin-wall tubes. Each reaction consisted of: 10 × PCR buffer (50 mM Tris/HCl, pH 8.3, 20 mM MgCl₂, 2.5 mg BSA ml⁻¹), 5 μl; 20 mM dNTPs, 0.5 μl; 50 μM forward primer and reverse primers (Redburn & Patel, 1993), 1 μl; chromosomal DNA, 200 ng; 1 U *Taq* polymerase; and sterile double-distilled water, 40.3 μl. The PCR thermal cycling was carried out in a RapidCycler (Idaho Technology) using the following parameters: 94 °C for 2 min followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min and 74 °C for 1.3 min with a slope of 9.9. PCR products were purified using QiaQuick PCR Purification spin columns according to the manufacturer's instructions (Qiagen).

Sequencing of the 16S rRNA gene was carried out using primers described previously (Redburn & Patel, 1993) and the products were analysed on a 96-lane ABI 377 DNA sequencer (Applied Biosystems). Sequence data were corrected using the sequence editor BIOEDIT and a contiguous consensus sequence was generated (Hall, 1999). The sequence was aligned using SEQUENCE ALIGNER version 8.0 of the Ribosomal Database Project (RDP) (Maidak *et al.*, 1999) and subsequently adjusted manually to conform to the *E. coli* 16S rRNA secondary structure model (Winker & Woese, 1991). The most homologous sequences to the 16S rRNA gene sequence of isolate C21^T were identified in the GenBank database, release 121, using BLAST (Altschul *et al.*, 1997). These were extracted and aligned with other related sequences extracted from the RDP (Maidak *et al.*, 1999). Unambiguous nucleotides in the alignment were used in the pairwise evolutionary distance estimation (Jukes & Cantor, 1969). Dendrograms were constructed using the neighbour-joining method (Saitou & Nei, 1987) and confidence in the tree topology was determined using 100 bootstrapped trees (Felsenstein, 1985).

RESULTS

Isolation and colony morphology

Dilutions of 10⁰, 10⁻¹ and 10⁻² of the enrichment cultures initiated from water-sediment slurries taken at 66 °C showed growth after incubation at 68 and 75 °C for 72 h in medium D. Microscopic examination revealed rod-shaped cells (2.0–10.0 × 0.3 μm) and similar colony morphology (small, translucent and circular colonies) from all three enrichment cultures on medium D agar plates after 48 h incubation at 65 °C. Several pure cultures were obtained by picking single well-isolated colonies and one of the cultures, isolate C21^T, was characterized further.

Cell morphology and cell wall ultrastructure

Isolate C21^T cells were non-motile rods (2.0–10.0 × 0.3 μm), occurring singly or in pairs, and stained Gram-negative. However, EM examination of thin

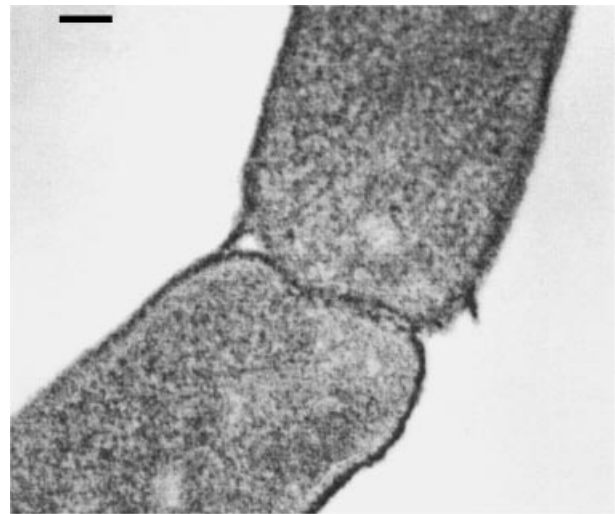


Fig. 1. EM of a thin section of a dividing cell of strain C21^T showing a Gram-positive type cell wall. Bar, 50 nm.

sections revealed a cell wall lacking an outer cell membrane that is typical of Gram-negative species (Fig. 1). Isolate C21^T produced terminal ellipsoidal spores that distended the cell.

Growth characteristics and substrate utilization

Isolate C21^T was a strict aerobe and grew better with agitation than as a stationary culture. It grew in medium D with either yeast extract or tryptone. The presence of both of these substrates at concentrations equal to or less than 0.2% increased biomass and improved generation time.

Carbohydrates (sucrose, cellobiose, glucose, dextrin, amylopectin, chitin, carboxymethylcellulose, xylan, inositol, arabinose, mannose, fructose, gelatin, starch, amylose, galactose, dextrose, xylose, maltose, L-sorbose and raffinose), organic acids (lactic acid, pyruvic acid and benzoic acid) and Casamino acids could not be used as sole carbon sources or when supplemented with yeast extract and tryptone (0.2%). An increase in growth was not detected in medium D containing either a lower concentration (0.1%) of substrate (glucose, lactose, sucrose, maltose, acetate and fructose) or lower concentrations of yeast extract and tryptone (0.025 and 0.05%, respectively).

The optimal temperature and pH for growth in medium D containing 0.1% yeast extract and 0.1% tryptone were 70 °C (temperature range of 55–80 °C) and pH 8.5 (pH range of 6.0–10.5), during which time a generation time of 90 min was obtained. Isolate C21^T did not require NaCl for growth; no growth was evident in NaCl concentrations greater than 1%. The growth of isolate C21^T was slower on medium D agar plates than in medium D broth.

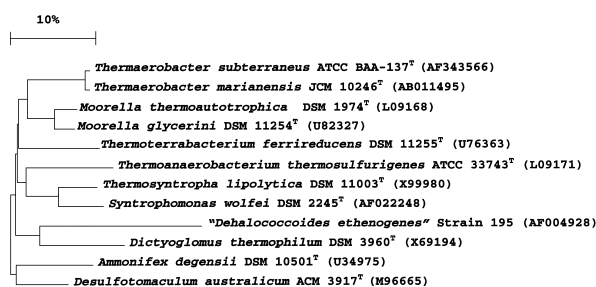


Fig. 2. Dendrogram showing the position of isolate C21^T within the radiation of members of the phylum *Firmicutes*. The dendrogram was constructed using the neighbour-joining method and Jukes & Cantor evolutionary distance matrix data generated from the pairwise of 1318 unambiguous nucleotides. Sequences used in the analysis were extracted from RDP version 8.0 and GenBank release 121. Sequence accession numbers are shown in parentheses. Bar, 10 substitutions per 100 nt.

Antibiotic susceptibility

Isolate C21^T did not grow in the presence of ampicillin, neomycin, penicillin, phosphomycin, polymyxin B, streptomycin or tetracycline at concentrations of 10 µg ml⁻¹ but growth was not affected in the presence of sodium azide at a concentration of 500 µg ml⁻¹.

DNA base composition and DNA–DNA hybridization

The DNA G+C composition of isolate C21^T was 71 mol% (thermal denaturation method). Quantitative DNA–DNA hybridization experiments showed less than 5% genomic relatedness between C21^T and *Thermaerobacter marianensis*.

16S rRNA gene sequence analysis

A sequence of 1552 nt of the 16S rRNA gene of isolate C21^T, corresponding to positions 7–1541 of the 16S rRNA gene of *E. coli* (Winker & Woese, 1991), was generated using seven primers. The G+C content of the 16S rRNA gene sequence was 64 mol%. Phylogenetic analysis of this sequence with representative members of the domain *Bacteria* revealed a relationship with members of the *Firmicutes*. A more detailed analysis within this phylum showed that isolate C21^T had a high similarity to *Thermaerobacter marianensis* (value of 98%) and bootstrap analysis gave a 100% confidence level for this relationship (Fig. 2).

DISCUSSION

A variety of different physiological groups of bacteria, including sulfate reducers, carbohydrate fermenters, strict aerobes and strict anaerobes, has been isolated from the GAB environment, including *Desulfovibrio longreachensis* (Redburn & Patel, 1994), *Desulfotomaculum australicum* (Love *et al.*, 1993), *Fervidobacterium gondwanense*, a phylogenetic deep member of the order *Thermotogales* (Andrews & Patel, 1996), members of the genera *Thermus* (Denman *et al.*, 1991),

Bacillus (Love *et al.*, 1992), *Caloramator* and *Thermoanaerobacter* (Wynter *et al.*, 1996) and as yet unnamed taxa. The isolation of strain C21^T from this unique non-volcanically heated subsurface aquifer extends the known microbial diversity of this environment. Isolate C21^T is a strictly aerobic, spore-forming, thermophilic bacterium that has a typical Gram-positive type cell wall and, based on these properties, resembles members of the genera *Bacillus* (Blanc *et al.*, 1997) and *Saccharococcus* (Ahmad *et al.*, 2000), to the exclusion of non-spore-formers such as *Thermus* (Brock & Freeze, 1969; Denman *et al.*, 1991). The DNA G+C content of isolate C21^T is 71 mol% and is also much higher than that reported for any members of the family *Bacillaceae* (De Bartolomeo *et al.*, 1991).

Thermaerobacter marianensis was isolated from the world's deepest sea-floor (10897 m), the Mariana Trench Challenger Deep (Takai *et al.*, 1999). In the same paper, the authors cited a personal communication from their colleague on a phylogenetically similar thermophilic bacterium (similarity value of 98%) isolated from a shallow hydrothermal vent. The increased hydrostatic pressure sensitivity of *Thermaerobacter marianensis* points to the possibility that it is not perhaps a normal inhabitant of the Trench environment and that it could have been deposited as a result of subduction activities, which greatly perturb the upper mantle. However, the isolation of isolate C21^T from a habitat very different from the other two, very different, ecosystems cannot be explained by this hypothesis, as the GAB is a relatively recent, rainwater-recharged, closed environment (Habermahl, 1980).

Phylogenetic analysis of the 16S rRNA gene of isolate C21^T indicates that it is a member of the phylum *Firmicutes*, within the family *Syntrophomonadaceae* of the order *Clostridiales*, with *Thermaerobacter marianensis* being the closest relative (similarity value of 98%). Interestingly, all members of the order *Clostridiales* possess an anaerobic metabolism (Collins *et al.*, 1994), with the exception of *Thermaerobacter marianensis* (Takai *et al.*, 1999) and isolate C21^T described in this study. This lack of congruence of physiology with phylogeny is notable, but not unusual. For example, all members of the family *Bacillaceae* are strict aerobes or facultative anaerobes with the exception of *Bacillus infernus*, which is an obligate anaerobe (Boone *et al.*, 1995), necessitating an amendment to the genus description. Our results therefore confirm that physiologies do not have phylogenetic boundaries and that different metabolic variants can co-exist within a cohesive phylogenetic cluster.

Thermaerobacter marianensis and isolate C21^T share a number of characteristics, including the high G+C content of the DNA, an aerobic metabolism and a Gram-positive cell wall ultrastructure. However, a number of differences also exist. Isolate C21^T is an alkaliphile that does not require NaCl, does not use substrates other than yeast extract and tryptone for growth and produces spores, whereas *Thermaero-*

Table 1. Characteristics of isolate C21^T and *Thermaerobacter marianensis*

Data for isolate C21^T were taken from this study and those for *Thermaerobacter marianensis* from Takai *et al.* (1999). Both species grow on yeast extract and peptone.

Characteristic	Isolate C21 ^T	<i>Thermaerobacter marianensis</i>
Habitat	Great Artesian Basin of Australia bore outflow	Challenger Deep sediment, Mariana Trench
Cell size (µm)	2.0–10.0 × 0.3	2.0–7.0 × 0.3–0.6
Presence of spores	Ellipsoidal, terminal and larger than the cell	–
Growth conditions:		
Temperature growth range (°C)	55–80 (optimum 70)	50–80 (optimum 74–76)
pH growth range	6.0–10.5 (optimum 8.5)	5.4–9.5 (optimum 7.0–7.5)
NaCl requirement	–	0.5–5.0% (optimum 2.0%)
Growth on:		
Amino acids	–	+
Carbohydrates	–	+
Carboxylic acids	–	+
Requirement for yeast extract or peptone for growth on carbohydrates	NA	–
G+C content (mol%)	71 (thermal denaturation)	72.5 (HPLC)

bacter marianensis is an obligate halophile and is more nutritionally versatile and spores have not been detected (Table 1). In addition, isolate C21^T and *Thermaerobacter marianensis* have a DNA homology of less than 5%.

Based on the results presented above, it is proposed that isolate C21^T be designated a novel member of the genus *Thermaerobacter*, *Thermaerobacter subterraneus* sp. nov. It is widely accepted that spore-forming and non-spore-forming species can be included in the same genus, as exemplified by the case of genus *Thermoanaerobacter* (Cayol *et al.*, 1995).

Emended description of *Thermaerobacter* (Takai *et al.* 1999)

Thermaerobacter (Therm.ae.ro.bac'ter. Gr. adj. *thermos* hot; Gr. n. *aer* air; N.L. *bacter* masc. equivalent of Gr. neut. n. *baktron* rod or staff; N.L. masc. n. *Thermaerobacter* rod that grows at high temperatures in the presence of air).

Rod-shaped, may or may not form spores. Gram-variable cells are non-motile and flagella are absent. Aerobic and thermophilic. Heterotrophic. Grows at neutral to alkaline pH. NaCl may or may not be required for growth. May utilize organic substrates such as yeast extract, peptone, cellulose, starch, chitin, casein, Casamino acids, a variety of sugars, carboxylic acids and amino acids. The G+C content of genomic DNA is 71–73 mol%. Major cellular fatty acids are iso-C_{17:0}, C_{14:1}, anteiso-C_{17:0}, iso-C_{15:0}, anteiso-C_{15:0}, C_{16:0} and C_{18:0}. On the basis of 16S rRNA gene analysis, the genus *Thermaerobacter* is most closely related to the genus *Moorella*. *Thermaerobacter* species habitats known so far include deep sea-floor environments, hydrothermal vents and subterranean thermal

environments such as the Great Artesian Basin of Australia. The type species is *Thermaerobacter marianensis*.

Description of *Thermaerobacter subterraneus* sp. nov.

Thermaerobacter subterraneus (sub.ter.ran'è.us. L. prep. *sub* under, beneath; L. n. *terra* earth, ground; L. masc. adj. *subterraneus* under the earth).

Cells are rod-shaped (2.0–10.0 × 0.3 µm) with rounded ends and occur singly or in pairs. Cells are non-motile and do not possess flagella. Stains Gram-negative but possesses a Gram-positive cell wall ultrastructure. Forms terminal ellipsoidal spores that distend the cells. Strictly aerobic. Temperature range for growth is 55–80 °C (optimum at 70 °C). pH range for growth is 6.0–10.5 (optimum at pH 8.5). Does not require NaCl and growth is inhibited by NaCl concentrations higher than 1%. Grows on yeast extract and/or tryptone, but not on any other carbon source as sole source of carbon/energy. Ampicillin, neomycin, penicillin, phosphomycin, polymyxin B, streptomycin and tetracycline, but not sodium azide, inhibit growth. Genomic DNA G+C composition is 71 mol%. Phylogenetically related to *Thermaerobacter marianensis* (16S rRNA gene similarity value of 98%). Obtained from a sediment sample from the outflow of a Great Artesian Basin bore (the New Lorne Bore) in Queensland, Australia. Type strain is C21^T (= ATCC BAA-137^T = DSM 13965^T).

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