Petrobacter succinatimandens gen. nov., sp. nov., a moderately thermophilic, nitrate-reducing bacterium isolated from an Australian oil well

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A novel Gram-negative, aerobic and moderately thermophilic bacterium, strain 4BON^T, was isolated from a non-water-flooded Australian terrestrial oil reservoir. Cells were non-spore-forming straight rods, which were motile by means of a polar flagellum. The optimum growth conditions were 55 °C, pH 6.9 and 0.5 % NaCl. Strain 4BON^T was oxidase- and catalase-positive; it grew on fumarate, pyruvate, succinate, formate, ethanol and yeast extract in the presence of oxygen or nitrate as terminal electron acceptor. Nitrate was reduced to nitrous oxide. The DNA G + C content of the strain was 58.6 mol%. The closest phylogenetic relative of strain 4BON^T was *Hydrogenophilus thermoluteolus* (similarity of 91.8 %), of the β -*Proteobacteria*. As strain 4BON^T is physiologically and phylogenetically different from *H. thermoluteolus*, it is proposed that it be assigned to a novel species of a novel genus, *Petrobacter succinatimandens* gen. nov., sp. nov. The type strain is 4BON^T (= DSM 15512^T = CIP 107790^T).

Petroleum reservoirs harbour a rich and diverse community of micro-organisms including (i) fermentative, (ii) sulfate-, thiosulfate- and sulfur-reducing, (iii) nitrate-reducing, (iv) methanogenic and (v) metal-respiring micro-organisms (Magot *et al.*, 2000). Among these micro-organisms, heterotrophic together with hydrogenotrophic micro-organisms (e.g. methanogens and sulfate-reducing bacteria) are considered as common inhabitants of oilfield environments (Ivanov *et al.*, 1983; Fardeau *et al.*, 1993, 1997, 2000; Grassia *et al.*, 1996; Magot *et al.*, 2000; Mueller & Nielsen, 1996; Nilsen *et al.*, 1996; Ollivier *et al.*, 1998). Despite the fact that organic acids are known to occur in oil reservoirs (Rees *et al.*, 1997), a great deal of attention has been paid by microbiologists to heterotrophic micro-organisms using carbohydrates in these environments (Fardeau *et al.*, 1993, 1997, 2000; Magot *et al.*, 2000); therefore, little information is available on the isolation of heterotrophic non-sulfate-reducing bacteria that use organic acids (e.g. fumarate, succinate, etc.)

aerobically or anaerobically. The only organic-acid-using micro-organisms isolated from oilfield ecosystems, unable to use sulfate as terminal electron acceptor, include members of the genera *Marinobacter* (Huu *et al.*, 1999), *Deferribacter* (Greene *et al.*, 1997), *Denitrovibrio* (Myhr & Torsvik, 2000) and *Anaerobaculum* (Rees *et al.*, 1997).

Here, we describe a novel moderately thermophilic, organic-acid-using, nitrate-reducing bacterium (strain 4BON^T), belonging to the β -*Proteobacteria*, isolated from a non-water-flooded Australian terrestrial oil reservoir. It presented significant phenotypic, genotypic and phylogenetic differences when compared with members of the β -*Proteobacteria*, which led to the proposal of it being assigned to a novel genus, *Petrobacter*, as the type and sole species, *Petrobacter succinatimandens*.

The oil sample used in this study was collected from the Riverslea oilfield in the Bowen–Surat basin of Eastern Australia (Queensland). The sample was designated OCA5 and was stored at 4 °C until used. Enrichment was performed in a medium prepared anaerobically (Fardeau *et al.*, 2000) containing (l⁻¹ distilled water) 0.3 g K₂HPO₄, 0.3 g KH₂PO₄, 0.2 g MgCl₂·6H₂O, 0.1 g CaCl₂·2H₂O, 0.1 g KCl, 1 g NaCl, 1 g NH₄Cl, 10 mM CH₃COONa, 1 g yeast extract (Difco Laboratories) and 10 ml of the trace mineral solution of Balch *et al.* (1979). The pH was adjusted to 7.0 with 10 M KOH. The vessels were autoclaved for 45 min

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Petrobacter succinatimandens* 4BON^T is AY219713.

A transmission electron micrograph of strain 4BON^T and a graph showing the effect of temperature on the growth of strain 4BON^T cultivated on succinate are available from IJSEM Online.

at 110 °C and, prior to inoculation, 10 mM KNO₃ and 0.5 % (v/v) of the vitamin solution of Balch *et al.* (1979) were added from sterile stock solutions. A H₂/CO₂ mixture (2 bars) was added in the gas phase. For enrichment, a 2 ml oil-well water sample was inoculated into 20 ml of medium and incubated at 50 °C without agitation. Three enrichment series were performed in the same medium before isolation. Strains were isolated by repeated use of the Hungate roll-tube technique (Hungate, 1969), with medium solidified with 2 % agar noble (Difco). The basal medium, used for characterization of pH, temperature and NaCl ranges for growth of the isolates, was similar to the enrichment medium supplemented with 20 mM sodium succinate. The culture medium was adjusted to different pH values by injecting NaHCO₃ from 10 % (w/v) sterile anaerobic stock solutions. For studies on NaCl requirements, NaCl was weighed directly in the tubes prior to dispensing the medium. Substrates were tested in aerobiosis (nitrogen was replaced by air in the gas phase) and anaerobiosis (in the presence and in the absence of nitrate as terminal electron acceptor) in basal medium at a final concentration of 20 mM. As a test for electron acceptors, sodium thiosulfate (20 mM), sodium sulfate (20 mM), sodium sulfite (2 mM), elemental sulfur (1 %, w/v), potassium nitrate (10 mM) and potassium nitrite (2 mM) were added to the medium. The use of electron acceptors was evaluated by measuring the optical density at 580 nm and by measuring H₂S, ammonium, nitrite or nitrous oxide production.

Sulfide was determined photometrically as colloidal CuS by using the method of Cord-Ruwisch (1985). Nitrite was detected by the colorimetric method of Griess–Illosvay (Knapp & Clark, 1984) and by the Quantofix test (Macherey–Nagel). Nitrous oxide was measured by gas chromatography (Fardeau *et al.*, 1993). Organic compounds were determined as described previously (Fardeau *et al.*, 1997). Morphological characteristics of isolates were observed with an optiphot (Nikon) phase microscope. Electron microscopy studies were performed as described by Koussémon *et al.* (2001).

The G+C content of the DNA of strain 4BON^T was determined at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) by using HPLC as described by Mesbah *et al.* (1989). Non-methylated lambda DNA (Sigma) was used as the standard. The 16S rRNA gene of the isolate was amplified as described previously (Miranda-Tello *et al.*, 2003). The PCR products were purified by using a NucleoSpin Extract kit (Macherey Nagel) and were cloned into the vector PGEM-T-easy (Promega). Plasmids containing the insert were purified using the Wizard Plus SV Minipreps DNA Purification System (Promega), according to the manufacturer's protocol. Sequencing was carried out by Genome Express (Grenoble, France). The new sequence was aligned to a full-length 16S rDNA consensus sequence, assembled and checked manually for accuracy using the

alignment editor BIOEDIT v5.0.9 (Hall, 1999). The 16S rRNA gene sequence of strain 4BON^T was compared with other sequences in the GenBank database (Benson *et al.*, 1999) and RDP (Maidak *et al.*, 2001), using BLAST (Altschul *et al.*, 1997) to identify its closest relatives. The sequences of the closest relatives were retrieved and, together with the 16S rRNA gene sequence of strain 4BON^T, were subjected to a phylogenetic analysis. Positions of sequence and alignment ambiguity were omitted from the analysis and the pair-wise evolutionary distances based on 1374 unambiguous nucleotides were computed by using the method of Jukes & Cantor (1969). A dendrogram was constructed using the neighbour-joining method (Saitou & Nei, 1987). Confidence in the tree topology was determined by using 100 bootstrapped trees (Felsenstein, 1993). The accession numbers for the 16S rDNA sequences of the reference organisms are included in Fig. 1.

Enrichment cultures were positive for growth after 10 days incubation at 37 °C or 4 days incubation at 50 °C. Microscopic examination of the cultures revealed the presence of motile, rod-shaped bacteria. Round, yellowish, smooth colonies (1–2 mm in diameter) developed on roll tubes after 10 days incubation at both temperatures. Single colonies were picked and streaking was repeated three times before cultures were considered pure. Three strains, similar in morphology, were isolated. Strain 2BON was isolated at 37 °C, whereas strains 3BON and 4BON^T were isolated at 50 °C. As all these strains had identical 16S rDNA sequences, and since strain 2BON also grew at 50 °C, strain 4BON^T was used for further characterization. Cells of strain 4BON^T were straight rods that were 0.3–0.4 × 2 µm in size, motile by means of a polar flagellum (see image available in IJSEM Online) and occurred singly or in pairs. No spore formation was detected. Cell ultra-thin sections showed a thin cell wall (data not shown). Strain 4BON^T grew at temperatures ranging from 35 to 60 °C, and optimum growth was obtained at 55 °C (pH 7) (see graph available in IJSEM Online). Growth occurred at initial pH values of between 5.5 and 8.0 at 55 °C, and the optimum pH was 6.9. The strain grew in the presence of NaCl concentrations ranging from 0 to 3 %, and the optimum NaCl concentration was 0.5 % at pH 7 and 55 °C. Growth was weak in the absence of yeast extract. Strain 4BON^T grew with oxygen or nitrate as terminal electron acceptors, using fumarate, pyruvate, formate, ethanol, succinate and yeast extract as energy sources. The following compounds were not used in aerobiosis and in the presence of nitrate as terminal electron acceptor: acetate, butyrate, propionate, valerate, isovalerate, lactate, adipate, alanine, L-glutamate, proline, serine, valine, fructose, glucose, lactose, maltose, ribose, xylose, vanillate, benzoate, 4-hydroxybenzoate, 3-methylbenzoate, phenylacetate, 4-aminobenzoate, benzaldehyde. Nitrate was reduced to nitrous oxide. Nitrite was an intermediary product of nitrate reduction.

The *in situ* physicochemical conditions, together with the

Fig. 1. Phylogenetic tree based on 16S rDNA sequences, constructed by the neighbour-joining method, showing the position of strain 4BON^T among members of the β -Proteobacteria. Sequence accession numbers are given in parentheses. Numbers on the branches are confidence limits (expressed as percentages) estimated by a bootstrap analysis performed using 100 replications. Confidence limits less than 80 % are not shown. Bar, 5 nt substitutions per 100 nt.

presence of phenotypically and phylogenetically closely related anaerobes in oilfields, suggest that oil reservoirs are mainly anaerobic ecosystems (Magot *et al.*, 2000). However, the accidental introduction of oxygen into oil reservoirs cannot be excluded due to water-flooding during oil extraction. This might partly explain the occurrence of aerobic to microaerophilic bacteria in oil-field ecosystems (Gevertz *et al.*, 2000; Voordouw *et al.*, 1996; Telang *et al.*, 1997). Among these micro-organisms, hydrogen-oxidizers, using nitrate as their terminal electron acceptor, have been isolated and characterized (Gevertz *et al.*, 2000). Attempts to isolate phenotypically similar micro-organisms from the Australian oil reservoirs that we studied were unsuccessful. Despite the presence of hydrogen as energy source in the enrichment medium that we used, our microbiological studies only led to the isolation of heterotrophic bacteria that used organic acids, aerobically or anaerobically, but not hydrogen. Isolation of these bacteria was only allowed by the presence of yeast extract in the original enrichment medium. The subsequent isolation on other substrates of organic-acid-users originating from oilfield ecosystems has already been reported (Davydova-Charakhch'yan *et al.*, 1992; Rees *et al.*, 1997). Strain 4BON^T, isolated from an Australian oil reservoir in Queensland, was further characterized and was shown to oxidize a limited range of organic acids (pyruvate, fumarate, succinate) together with ethanol. Despite the fact that strain 4BON^T is an aerobic, nitrate-reducing bacterium, it differed phylogenetically from strains CVO and FWKO B, isolated from Canadian oilfields, as they both belong to the epsilon subdivision of the Proteobacteria. In contrast, strain 4BON^T belongs to the beta subdivision of

the Proteobacteria, as indicated by the phylogenetic tree shown in Fig. 1, and it oxidizes neither elemental sulfur nor thiosulfate. Strain 4BON^T also differed significantly from other nitrate-reducing bacteria isolated from oil reservoirs: *Deferribacter thermophilus* is a strict anaerobe which is most closely related to *Flexistipes sinusarabici* (Greene *et al.*, 1997); *Denitrovibrio acetiphilus* is a strict anaerobe which oxidizes acetate and has *Geovibrio ferri-reducens* as its closest phylogenetic relative (Myhr & Torsvik, 2000); and *Marinobacter aquaeolei* is a facultative, mesophilic acetate-oxidizer (Huu *et al.*, 1999). Comparison of the almost-complete 16S rDNA sequence of strain 4BON^T (1529 nt) with sequences in the public databases indicated that the strain was related to a group of uncultured micro-organisms (LaPara *et al.*, 2000) (mean similarity of 98 %; data not shown). Nevertheless, the bacterial species phylogenetically most closely related to strain 4BON^T is *Hydrogenophilus thermoluteolus* (Hayashi *et al.*, 1999) (sequence similarity of 91.8 %). Strain 4BON^T differs from *H. thermoluteolus* by its inability to oxidize hydrogen and by having a lower DNA G + C content (58.6 mol% for strain 4BON^T vs 63–65 mol% for *H. thermoluteolus*). Because of its distinct phenotypic, genotypic and phylogenetic characteristics, it is proposed that strain 4BON^T represents a novel species, *Petrobacter succinatimandens*, within a novel genus, *Petrobacter*, within the β -Proteobacteria.

Description of *Petrobacter* gen. nov.

Petrobacter (Pe.tro.bac'ter. Gr. fem. n. *petra* rock, stone; M.L. masc. n. *bacter* equivalent of Gr. neut. n. *bakterion* rod, staff; N.L. masc. n. *Petrobacter* the stone bacterium).

Cells are straight rods. Gram-negative. Spores are not formed. Grow aerobically and anaerobically only with nitrate. Moderately thermophilic member of the domain *Bacteria*, class β -*Proteobacteria*. Organic acids serve as main substrates.

The type species is *Petrobacter succinatimandens*.

Description of *Petrobacter succinatimandens* sp. nov.

Petrobacter succinatimandens (suc.ci.na.ti.man'dens. N.L. n. *succinatum* succinate; L. part. adj. *mandens* eating, consuming; N.L. masc. adj. *succinatimandens* consuming succinate).

Cells are straight rods ($0.3\text{--}0.4 \times 2 \mu\text{m}$), which occur singly or in pairs and possess one polar flagellum. Spores are not formed. Stains Gram-negative. Round colonies (1–2 mm diameter) develop in roll tubes after 1 week incubation at 50°C . Chemo-organotroph. Obligate aerobe. Oxidase- and catalase-positive. Moderately thermophilic. The optimum temperature for growth is 55°C at pH 7; temperature range between 35 and 60°C . The optimum pH for growth is 6.9; growth occurs between pH 5.5 and 8.0. Slightly halo-tolerant, growing in the presence of up to 3% NaCl, with an optimum at 0.5%. Oxidizes fumarate, pyruvate, succinate, ethanol and yeast extract in the presence of oxygen or nitrate as terminal electron acceptor. The following compounds are not used in aerobiosis or in the presence of nitrate as terminal electron acceptor: acetate, butyrate, propionate, valerate, isovalerate, lactate, adipate, alanine, L-glutamate, proline, serine, valine, fructose, glucose, lactose, maltose, ribose, xylose, vanillate, benzoate, 4-hydroxybenzoate, 3-methylbenzoate, phenylacetate, 4-aminobenzoate, benzaldehyde. Nitrate is reduced to nitrous oxide. Elemental sulfur, sulfate, thiosulfate and nitrite are not used as electron acceptors. Isolated from an Australian oil well.

The type strain is 4BON^T (= DSM 15512^T = CIP 107790^T). The G+C content of the DNA is 58.6 mol% (HPLC).

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