

Fusibacter paucivorans gen. nov., sp. nov., an anaerobic, thiosulfate-reducing bacterium from an oil-producing well

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A strictly anaerobic, halotolerant, spindle-shaped rod, designated strain SEBR 4211^T, was isolated from an African saline oil-producing well. Cells stain Gram-positive, which was confirmed by electron microscopy observations. Strain SEBR 4211^T was motile by means of one to four peritrichous flagella, had a G+C content of 43 mol% and grew optimally at 37 °C, pH 7.3, with 0 to 3% (w/v) NaCl. It utilized a limited number of carbohydrates (cellobiose, glucose, fructose, mannitol and ribose) and produced acetate, butyrate, CO₂ and H₂ as end products from glucose fermentation. It reduced thiosulfate to sulfide. In the presence of thiosulfate, a decrease in butyrate and an increase in acetate production was observed. Phylogenetically, strain SEBR 4211^T was related to members of the low G+C *Clostridiales* order with *Clostridium halophilum* as the closest relative (16S rDNA sequence similarity of 90%). On the basis of phenotypic, genotypic and phylogenetic characteristics of the isolate, it is proposed to designate it as a new species of a new genus, *Fusibacter* gen. nov., as *Fusibacter paucivorans* sp. nov. The type strain is SEBR 4211^T (=DSM 12116^T).

Keywords: *Fusibacter paucivorans*, oil-producing well, thiosulfate reduction, taxonomy

INTRODUCTION

Though members of the domain *Archaea* are known to thrive in oilfield ecosystems (L'Haridon *et al.*, 1995; Stetter *et al.*, 1993), it now appears that fermentative bacteria could be the dominant microbial community in these ecosystems (Davydova-Charakhch'yan *et al.*, 1993; Fardeau *et al.*, 1993, 1996; Holloway *et al.*, 1980; Jeanthon *et al.*, 1995; L'Haridon *et al.*, 1995; Ravot *et al.*, 1995a; Stetter *et al.*, 1993). We recently isolated from oilfields fermentative bacteria that have the ability to reduce thiosulfate to sulfide during growth on organic compounds (Fardeau *et al.*, 1993; Magot *et al.*, 1997a, b; Ravot *et al.*, 1995a, b, 1997). The presence of thiosulfate is thought to increase biocorrosion risks in oilfield installations (Crolet & Magot, 1996; Magot *et al.*, 1994), and we provided recently the first experimental evidence of the in-

volvement of a non-carbohydrate-utilizing peptide-utilizing thiosulfate-reducing bacterium, *Dethiosulfovibrio peptidovorans*, in steel corrosion (Magot *et al.*, 1997b). These results provided a strong incentive to try to isolate other thiosulfate-utilizing micro-organisms from the oilfield environment.

We report here on the isolation of a new halotolerant thiosulfate-reducing bacterium having phenotypic and phylogenetic characteristics consistent with its placement in a new genus as a new species within the *Clostridiales* as *Fusibacter paucivorans* gen. nov., sp. nov.

METHODS

Sample source and sample collection. Strain SEBR 4211^T was isolated from a reservoir water sample from an offshore oil-producing well (Emeraude oilfield) in Congo, Central Africa. The *in situ* temperature was 35–40 °C and the concentration of sodium chloride was 40 g l⁻¹ (total salinity, 53 g l⁻¹). A 1 l sample was collected at the well head as

The GenBank accession number for the 16S rRNA-sequence of strain SEBR 4211^T is AF050099.



described by Bernard *et al.* (1992), and culture broths were immediately inoculated.

Medium, enrichment and isolation. Enrichments were performed at 30 °C without agitation by inoculating directly a 2 ml sample from the reservoir water into a basal medium containing (per litre of distilled water) 1 g NH₄Cl, 0.3 g K₂HPO₄, 0.3 g KH₂PO₄, 3 g MgCl₂·6H₂O, 0.1 g CaCl₂·2H₂O, 40 g NaCl, 1 g KCl, 0.5 g cysteine-HCl, 0.5 g CH₃COONa, 1 g yeast extract (Difco Laboratories), 1 g bio-Trypticase (bioMérieux), 3.6 g glucose, 10 ml of the trace element solution of Balch *et al.* (1979) and 0.1 mg resazurin. The pH was adjusted to pH 7.3 with 10 M KOH. The medium was boiled under a stream of O₂-free N₂ gas and cooled to room temperature. Five or 20 ml aliquots were dispensed into Hungate tubes or serum bottles, respectively, under a stream of N₂/CO₂ (4:1, v/v), and the vessels were sealed and autoclaved for 45 min at 110 °C. Prior to inoculation, Na₂S·9H₂O, NaHCO₃ and sodium thiosulfate were injected from sterile stock solutions, to final concentrations of 0.04% (w/v), 0.2% and 20 mM, respectively. The final pH was pH 7.2. Unless otherwise indicated the basal medium was used throughout these studies. The strain was purified by repeated isolation on Petri dishes containing the same medium solidified with 1.6% (w/v) agar as already reported (Magot *et al.*, 1997a, b; Ravot *et al.*, 1997). Incubation was performed in an anaerobic glove box at 30 °C.

Growth conditions. All growth experiments were performed in duplicate, using Hungate tubes (Hungate, 1969) containing the basal medium unless otherwise indicated. For pH growth experiments, the medium was adjusted to different pH values by injecting NaHCO₃ or Na₂CO₃ into Hungate tubes from 10% (w/v) sterile anaerobic stock solutions. The temperature range for growth was determined using the culture medium adjusted to the pH optimum for growth. For studies on sodium chloride requirements, sodium chloride was weighed directly into Hungate tubes, and the basal medium dispensed into the tubes as described above. The strain was subcultured at least once under the same experimental conditions prior to inoculation for each growth experiment.

Tests for sporulation. The presence of spores was determined by microscopic examination of the culture at different phases of growth. The presence of heat-resistant spores was determined by heating at 80 °C for 10–20 min, at 90 °C for 5–10 min and at 100 °C for 10 min a culture previously grown in a rich medium containing 20 mM glucose, and 5 g yeast extract l⁻¹ and 5 g bio-Trypticase l⁻¹ in distilled water.

Substrate-utilization tests. For substrate-utilization tests, the basal medium without glucose was used. The substrates (D-arabinose, cellobiose, dulcitol, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, D-mannose, D-melibiose, D-raffinose, L-rhamnose, D-ribose, D-sorbitol, L-sorbose, sucrose, D-trehalose, DL-xylose, cellulose, starch, formate, acetate, butyrate, lactate, propionate) were injected from autoclaved stock solutions into Hungate tubes to a final concentration of 20 mM for sugars and 10 mM for organic and fatty acids. Formate and methanol were added to obtain a final concentration of 40 mM, whereas peptides, Casamino acids and gelatin were added at a final concentration of 5 g l⁻¹. The substrates were tested in the presence or absence of sodium thiosulfate (final concentration, 20 mM). To test for electron acceptors, 20 mM sodium thiosulfate, 20 mM sodium sulfate and 2% (w/v) elemental sulfur were added to the medium.

Light and electron microscopy. Light microscopy was performed as described by Cayol *et al.* (1994). For electron microscopy, exponentially-grown cells were negatively stained with 1% (w/v) sodium phosphotungstate (pH 7.2). For preparation of thin sections, exponentially-grown cells were centrifuged and fixed for 45 min in 4% (w/v) glutaraldehyde prepared in 25 mM cacodylate/HCl buffer (pH 6.0) containing 2% (w/v) NaCl. Then the cells were washed in the same buffer containing 0.3 M sucrose and 2% (w/v) NaCl and postfixated for 1 h in 1% (w/v) osmium tetroxide prepared in the same buffer with 0.25 M sucrose and 2% NaCl. Then the cells were diluted and washed with water, embedded in 2% (w/v) agarose and stained with 4% (w/v) uranyl acetate. The agarose was cut into small cubes, dehydrated in acetone and embedded in Araldite. Thin sections were stained with 5% (w/v) uranyl acetate for 20 min and with 2% (w/v) lead citrate for 10 min. Micrographs were taken with a Hitachi model H600 electron microscope at an accelerating voltage of 75 kV.

Analytical techniques. Growth was measured by inserting Hungate tubes directly into a model UV-160A spectrophotometer (Shimadzu) and measuring the optical density at 580 nm. Sulfide was determined photometrically as colloidal CuS by using the method of Cord-Ruwisch (1985). Hydrogen and fermentation products (alcohols, and volatile and non-volatile fatty acids) were measured as described by Fardeau *et al.* (1993).

Determination of G + C content of DNA. The G + C content of DNA was determined by the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). The DNA was isolated and purified by chromatography on hydroxyapatite and the G + C content was determined by using HPLC as described by Mesbah *et al.* (1989). Non-methylated lambda DNA (Sigma) was used as the standard.

16S rDNA sequence studies. The methods for the purification and extraction of DNA and the amplification and sequencing of the 16S rRNA gene have been described previously (Andrews & Patel, 1996; Love *et al.*, 1993; Redburn & Patel, 1993). The 16S rRNA gene sequence, which we determined, was manually aligned with reference sequences of various members of the domain *Bacteria* by using the editor ae2 (Maidak *et al.*, 1996). Reference sequences were obtained from the Ribosomal Database Project (Maidak *et al.*, 1996), EMBL and GenBank databases. Positions of sequence and alignment uncertainty were omitted from the analysis. A phylogenetic analysis was performed by using the various programs that are part of the PHYLIP package (Felsenstein, 1993) as described below. The pairwise evolutionary distances based on 1229 unambiguous nucleotides were computed by using the method of Jukes & Cantor (1969) and dendrograms were constructed from these distances by using the neighbour-joining method.

RESULTS

Enrichment and isolation

One enrichment culture on medium containing glucose as the energy source was positive and H₂S was detected after incubation at 30 °C for 7 d. Microscopic examination showed the presence of different morphological types of micro-organisms including spindle-shaped rods. The latter formed colonies on Petri dishes after 5 d incubation at 30 °C. Single colonies were picked

FICHE DESCRIPTIVE

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Mots-clés matières : *Fusibacter paucivorans*, phylogénie, taxonomie, anaérobiose, thiosulfato-réduction, pétrole
(10 au plus)

Résumé en Français :
(150 mots maximum)

Plan de classement : Monde végétal et Animal - Fermentations

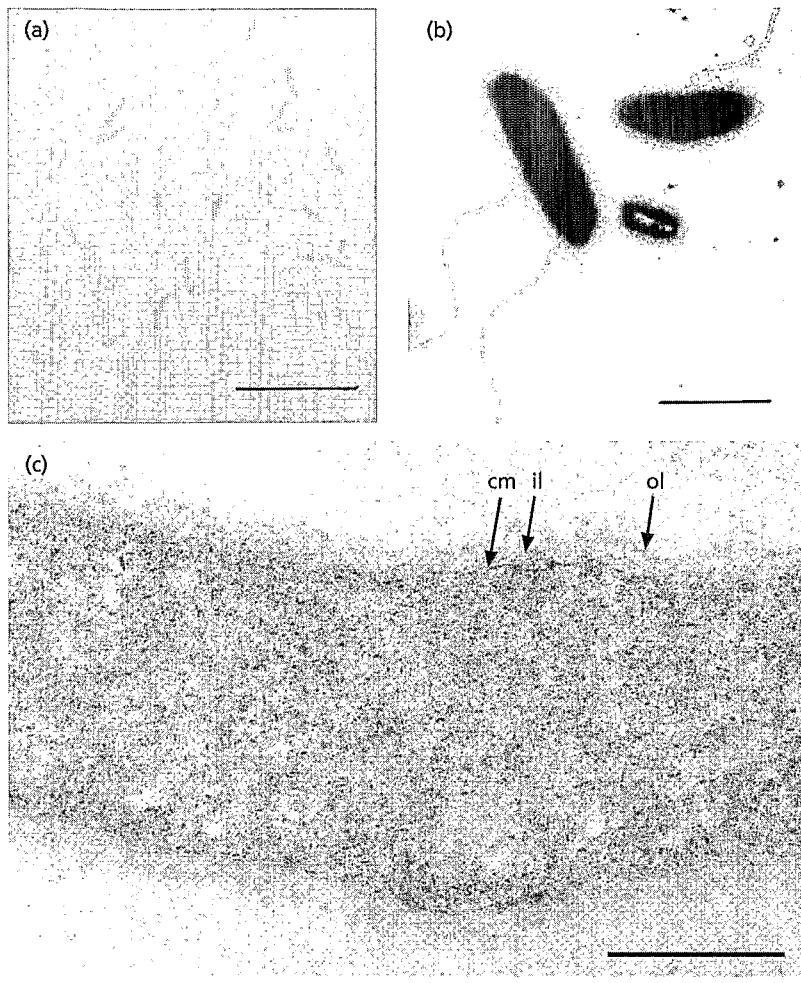


Fig. 1. (a) Phase-contrast micrograph of strain SEBR 4211^T (bar, 10 μm); (b) electron micrograph of a negatively stained culture of strain SEBR 4211^T showing one or two peritrichous flagella (bar, 2 μm); (c) electron micrograph of an ultrathin section of strain SEBR 4211^T showing the cytoplasmic membrane (cm), the inner layer (il) and the outer layer (ol) (bar, 0.2 μm).

and reinoculated twice before the culture was tested for purity. A spindle-shaped bacterium, designated SEBR 4211^T was used for further studies.

Morphology

White regular colonies (1 mm in diameter) were obtained after 8 d incubation at 30 °C. Strain SEBR 4211^T was a spindle-shaped rod. The cells were 1 \times 2–5 μm and occurred singly or in pairs (Fig. 1a). Each cell possessed one to four peritrichous flagella (Fig. 1b). Cells stained Gram-positive. Electron microscopy of sections of strain SEBR 4211^T revealed an atypical Gram-positive cell wall with a thin inner layer and a loose undulating outer layer (Fig. 1c). Spores were never observed. In addition, no growth was observed after the culture was heated at 100 °C for 10 min.

Optimum growth conditions

Strain SEBR 4211^T was anaerobic since it did not grow in oxidized medium (oxidation was indicated by the pink colour of the resazurin). It grew at temperatures

ranging from 20 to 45 °C, with an optimum at 37 °C (Fig. 2a). The isolate grew at sodium chloride concentrations ranging from 0 to 100 g NaCl l⁻¹, with an optimum between 0 and 30 g NaCl l⁻¹ (Fig. 2b). The optimum pH for growth was 7.3, and growth occurred between pH 5.7 and 8.0 (Fig. 2c).

Substrates for growth

No growth was obtained in the basal medium in the absence of glucose. Yeast extract was required for growth on carbohydrates and could not be replaced by bio-Trypticase. The following sugars were used as energy sources: D-cellobiose, fructose, glucose, D-mannitol and D-ribose. Strain SEBR 4211^T could not utilize arabinose, dulcitol, galactose, lactose, maltose, mannose, melibiose, raffinose, rhamnose, sorbitol, sorbose, sucrose, trehalose, xylose, cellulose, starch, formate, acetate, butyrate, lactate, propionate, methanol, bio-Trypticase, Casamino acids or gelatin. Acetate, butyrate, CO₂ and H₂ were produced during glucose fermentation (Table 1). The presence of thiosulfate did not affect the substrate utilization pattern.

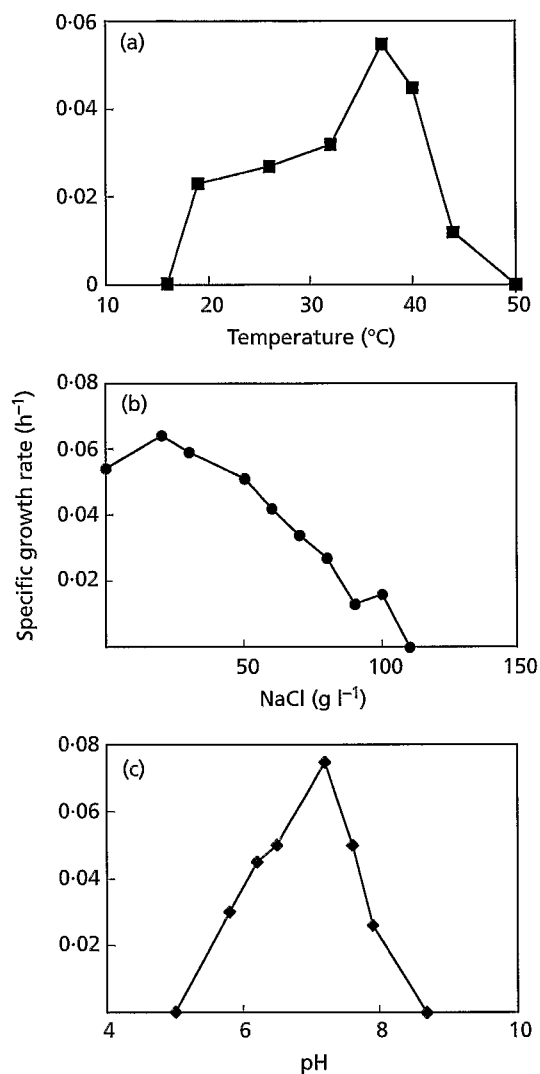


Fig. 2. Effect of (a) temperature (pH 7.3, no NaCl), (b) NaCl (37 °C, pH 7.3) and (c) pH (37 °C, no NaCl) on the growth of strain SEBR 4211^T cultivated in basal medium.

Effect of electron acceptors

Strain SEBR 4211^T used thiosulfate and sulfur but not sulfate as an electron acceptor. Hydrogen inhibited the growth of strain SEBR 4211^T but inhibition was

relieved in the presence of thiosulfate. Growth was not observed on hydrogen as the sole energy source in the presence of thiosulfate as the electron acceptor and acetate (1 mM) as the carbon source. A decrease in butyrate production and an increase in acetate production were observed in the presence of thiosulfate (Table 1).

G + C content of DNA and 16S rDNA sequence analysis

The G + C content of DNA of isolate SEBR 4211^T was 43 mol % (as determined by HPLC). Using 12 primers, we determined an almost complete sequence consisting of 1525 bases of the 16S rRNA gene of strain SEBR 4211^T (corresponding to *Escherichia coli* positions 8–1542). Phylogenetic analysis showed that strain SEBR 4211^T was a member of low G + C content Gram-positive branch and grouped with members of cluster XI. The closest relatives were *Clostridium halophilum* (similarity of 90%), '*Clostridium aminobutyricum*' and *Clostridium felsineum* (mean similarity of 89%) (Fig. 3).

DISCUSSION

Strain SEBR 4211^T isolated from oil reservoir water reduces thiosulfate to sulfide. Thiosulfate reduction is a common physiological trait exhibited by many fermentative mesophilic, thermophilic and hyperthermophilic micro-organisms of the domain *Bacteria* originating from oilfield ecosystems (Fardeau *et al.*, 1993, 1997; Jeanthon *et al.*, 1995; Magot *et al.*, 1994, 1997a, b; Ravot *et al.*, 1995a, b, 1997). Interestingly, thiosulfate has been detected in oilfield facilities where it is thought to increase biocorrosion (Crolet & Magot, 1996; Magot *et al.*, 1994), but the ecological significance of thiosulfate reduction in oil reservoirs is so far unknown.

Phylogenetic analysis of 16S rRNA indicates that strain SEBR 4211^T is a member of the *Clostridium* phylum (Cato *et al.*, 1986) and belongs to cluster XI, which includes *Clostridium lituseburense* and its related taxa (Collins *et al.*, 1994). This cluster is taxonomically heterogeneous, containing non-spore forming species

Table 1. Fermentation of glucose in the presence and absence of thiosulfate by strain SEBR 4211^T

Culture conditions	Amount of substrate used (mM)	End product formed (mM)					Ratio of acetate produced/sugar consumed
		Acetate	Butyrate	CO ₂ *	H ₂ *	H ₂ S	
Glucose	5.0	1.6	4.0	10.2	15.2	0	0.32
Glucose + thiosulfate†	9.9	12.2	3.0	20.4	7.1	11.5	1.23

* Millimolar equivalents.

† Sodium thiosulfate was added at a final concentration of 20 mM.

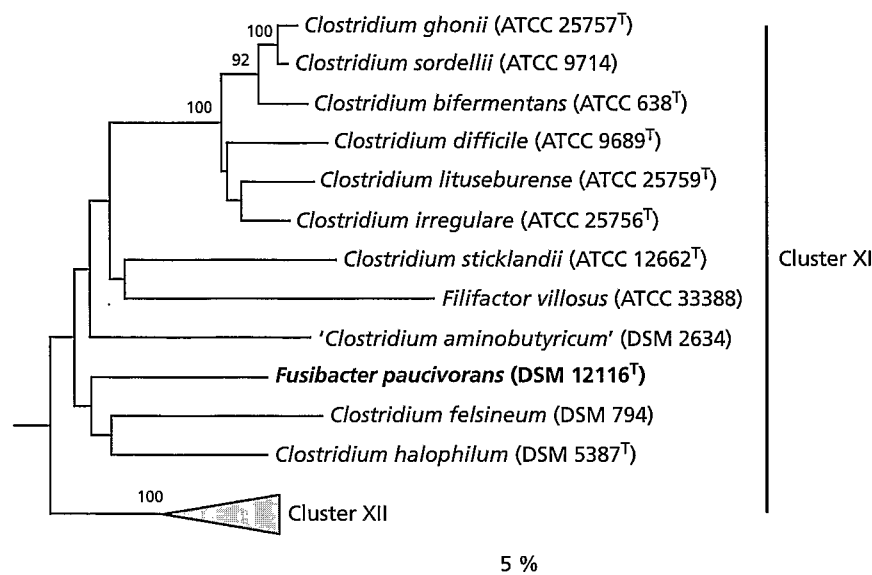


Fig. 3. Phylogenetic dendrogram indicating the position of *Fusibacter paucivorans* strain SEBR 4211^T (DSM 12116^T) as a member of cluster XI within the radiation of representatives of the low G+C content Gram-positive bacteria. The tree was constructed as described in Methods and the two clusters, cluster XI and cluster XII (shown as a triangle), are defined as described by Collins *et al.* (1994). Sequences were extracted from the Ribosomal Database Project, version 5.0 (Maidak *et al.*, 1996). Bootstrap values are expressed as a percentage of 100 replications and are shown at branching points. Only values above 90% were considered significant and reported. The scale bar indicates 5 nucleotide substitution per 100 nucleotides.

such as *Peptostreptococcus anaerobius* and *Eubacterium tenue*. The closest phylogenetic relatives of strain SEBR 4211^T are *C. halophilum* (90% similarity) (Fendrich *et al.*, 1990) followed by '*C. aminobutyricum*' and *C. felsineum* (Cato *et al.*, 1986) (mean similarity of 89%). However, '*C. aminobutyricum*' has not yet been taxonomically validated and its status remains unknown (Collins *et al.*, 1994). In addition to the significant phylogenetic difference (10%), the following genotypic and phenotypic traits distinguish strain SEBR 4211^T from its two nearest taxonomically validated phylogenetic neighbours: (i) strain SEBR 4211^T failed to produce spores; (ii) the G+C content of DNA from strain SEBR 4211^T is 43 mol% while that of *C. halophilum* and *C. felsineum* DNA are 27 and 26 mol%, respectively; (iii) the substrate range of strain SEBR 4211^T is very narrow; (iv) the end products of carbohydrate fermentation are different; in contrast to strain SEBR 4211^T, ethanol and lactate are produced by *C. halophilum* whereas butanol is produced by *C. felsineum*.

Strain SEBR 4211^T, when cultured on glucose in the presence of thiosulfate, alters the flow of electrons from butyrate to acetate with concomitant increases in H₂S production. The use of thiosulfate as an electron acceptor causes a shift in the flow of electrons, favouring H₂S production as already reported for the *Thermoanaerobacter* species (Fardeau *et al.*, 1996). In the case of strain SEBR 4211^T, this resulted in channeling of the electrons away from butyrate to acetate, thereby increasing its concentration.

Based on the phylogenetic, phenotypic and genotypic characteristics presented above, we propose placing strain SEBR 4211^T as a new species of a new genus within the *Clostridiales* as *Fusibacter paucivorans*, gen. nov., sp. nov.

Description of *Fusibacter* gen. nov.

Fusibacter (Fu.si.bac'ter. M.L. n. *fusum* -us a spindle, Gr. dim. n. *bakterion* a small rod, M.L. neut. n. *Fusibacter* a small spindle-shaped rod).

Cells are spindle-shaped rods (1 × 2–5 μm). The cells occur singly or in pairs and possess one to four peritrichous flagella. Cells stain Gram-positive. Chemo-organotrophic and obligately anaerobic member of the domain *Bacteria*. Uses carbohydrates but not peptides, Casamino acids or gelatin. Acetate and butyrate are produced from glucose fermentation. Uses thiosulfate and sulfur as electron acceptors during glucose fermentation. H₂S is produced from thiosulfate and sulfur reduction. The G+C content of the DNA is 43 mol% (as determined by HPLC). The type species is *Fusibacter paucivorans*.

Description of *Fusibacter paucivorans* sp. nov.

Fusibacter paucivorans [pau.ci.vo'rans. L. adj. *paucus* few; L. part adj. *vorans* devouring, eating (L. v. *vorare* to devour, to eat); M.L. part. adj. *paucivorans* intended to mean a bacterium that utilizes few substrates].

Round colonies (diameter, 1 mm) are present after 8 d incubation at 30 °C. Morphology as for genus *Fusibacter*. The optimum temperature for growth is 37 °C, temperature range between 20 and 45 °C. The optimum pH is 7.3, growth occurs between pH 5.7 and pH 8.0. Halotolerant; the optimum sodium chloride concentration for growth is between 0 and 3% (w/v), growth occurs at NaCl concentrations ranging between 0 and 10%. Utilizes cellobiose, fructose, glucose, D-mannitol and D-ribose but not arabinose, galactose, lactose, maltose, mannose, sorbose, sucrose, rhamnose, trehalose, xylose, acetate, butyrate, lactate or propionate. Requires yeast extract for growth. The end products of glucose fermentation in the presence

of yeast extract and bio-Trypticase are butyrate, acetate, CO₂ and H₂. Isolated from an oil-producing well. The type strain is SEBR 4211^T (= DSM 12116^T).

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