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Spirochaeta smaragdinae sp. nov., a new mesophilic strictly anaerobic spirochete from an oil field

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Abstract

An obligately anaerobic spirochete designated strain SEBR 4228^T (T = type strain) was isolated from an oil field of Congo, Central Africa. The strain grew optimally with a sodium chloride concentration of 5% (sodium chloride concentration growth range 1.0–10%) at 37°C (growth temperature range 20–40°C) and pH of 7.0–7.2 (pH growth range pH 5.5–8.0). Strain SEBR 4228^T grew on carbohydrates (glucose, fructose, ribose, D-xylose, galactose, mannitol and mannose), glycerol, fumarate, peptides and yeast extract. Yeast extract was required for growth and could not be replaced by vitamins. It reduced thiosulfate and sulfur, to H_2S. Glucose was oxidised to lactate, acetate, CO_2 and H_2S in the presence of thiosulfate but in its absence lactate, ethanol, CO_2 and H_2 were produced. Fumarate was fermented to acetate and succinate. The G+C content of strain SEBR 4228^T was 50%. Strain SEBR 4228^T was spiral shaped measuring 5–30 by 0.3–0.5 μm and was motile with a corkscrew-like motion. Electron microscopy revealed the presence of periplasmic flagella in a 1-2-1 arrangement. Strain SEBR 4228^T possessed features typical of the members of the genus *Spirochaeta*. 16S rRNA sequence analysis revealed that it was closely related to *Spirochaeta bajacaliforniensis* (similarity 98.6%). The lack of DNA homology with *S. bajacaliforniensis* (38%), together with other phenotypic differences, indicated that strain SEBR 4228^T is a new species, which we have designated *Spirochaeta smaragdinae*. The type strain is SEBR 4228^T (= DSM 11293).

Keywords: Spirochaetaceae; *Spirochaete*; Taxonomy; Oil field

1. Introduction

Members of the genus *Spirochaeta* are facultatively or strictly anaerobic free-living spirochetes and colonise a variety of mesophilic and thermal aquatic environments with neutral or alkaline pH [1–3,16–18]. With the exception of *Spirochaeta thermophila* [2], all have been isolated from environments associated with extensive plant or algal matter [3]. It has been assumed that in such environments *Spirochaeta* species grow by fermenting soluble sugars

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which are derived from the enzymatic activities of plant polysaccharide-degrading microbes. However, *S. thermophila*, which has an optimum growth temperature of 65°C, the highest known for any member of the genus *Spirochaeta*, degrades cellulose and was isolated from a hot spring devoid of high organic input. This paper reports on a new haloanaerobic carbohydrate fermenting strain, designated SEBR 4228^T (T = type strain), isolated from a production water sample collected from a Congo offshore oil field. Strain SEBR 4228^T reduced thiosulfate to H_2S and this constitutes the first report on such a trait for a member of the family Spirochaetaceae. In addition, the phenotypic, genotypic, and phylogenetic properties indicated that strain SEBR 4228^T was related to members of the genus *Spirochaeta* but was also distinct enough from all other species to be designated a new species, *Spirochaeta smaragdinae* sp. nov.

2. Materials and methods

2.1. Source of strains

Samples were collected from the production waters of the Emeraude oil fields in Congo, namely E153 and BB325. The in situ temperature of the oil fields was 35°C. The pH was measured at normal atmospheric pressure and determined to be pH 6.7. *Spirochaete bajacaliforniensis* was purchased from ATCC (ATCC 35968) and used for DNA relatedness studies.

2.2. Media and growth conditions

Medium SEM was used for enrichment and contained (g l^{-1}) biotrypcase 15, yeast extract 10, glucose 2.5 and also the following minerals: $MgCl_2 \cdot 6H_2O$ 7.4, Na_2SO_4 2.0, NH_4Cl 0.04, $CaCl_2 \cdot 2H_2O$ 3.8, KCl 0.9, NaCl 40.2, $NaHCO_3$ 0.7, $SrCl_2$ 0.007 and $FeCl_2$ 0.001, which reflected the composition of the production waters of the oil fields. The medium was boiled to remove oxygen, 1 g l^{-1} cysteine added as a reducing agent, the pH adjusted to 7.0 and the medium dispensed under an oxygen-free gas mixture of N_2:CO_2:H_2 (85:5:10) into Hungate tubes or serum vials.

For subsequent studies, a different medium, designated medium SGM, was used. It contained (g l^{-1}) NH_4Cl 1, K_2HPO_4 0.3, KH_2PO_4 0.3, $MgCl_2$ 0.2, $CaCl_2$ 0.1, NaCl 50, KCl 0.2, cysteine 0.5, yeast extract (Difco Laboratories, Detroit, MI) 1, biotrypcase (Biomérieux, Crapone, France) 1, resazurin 0.001, and 10 ml of trace mineral element solution [4]. The medium was adjusted to pH 7.3 with KOH rendered anaerobic and dispensed under oxygen-free nitrogen using Hungate's technique [5].

2.3. Enrichment and isolation

To initiate enrichment cultures, 2 ml samples from E153 and BB325 were inoculated into medium SEM followed by incubation at 30°C. Several pure cultures were obtained by repeatedly streaking on SEM agar plates (2.5% Noble agar, Difco) in an anaerobic chamber (La Calhène, Vélizy, France). One of the isolates, designated strain SEBR 4228^T, was studied further.

2.4. pH, temperature and sodium chloride ranges for growth

For pH studies, Hungate tubes containing pre-reduced medium SGM were adjusted to the desired pH by injecting $NaHCO_3$ or Na_2CO_3 from sterile 10% anaerobic stock solutions. Growth of strain SEBR 4228^T was tested at temperatures ranging from 20 to 45°C. To determine salt requirement for growth, sodium chloride was weighed directly into the tubes. The medium was subsequently dispensed into the tubes as described above.

2.5. Substrate utilisation

Substrates were added from sterile stock solutions to SGM medium at a final concentration of 20 mM. The utilisation of thiosulfate (20 mM) and sulfur (2%) as electron acceptors was tested in biotrypcase lacking SGM medium that contained 5 g l^{-1} yeast and 20 mM glucose. The vitamin requirements of strain SEBR 4228^T were tested using Widdel's vitamins [6]

2.6. Analytical techniques

Unless indicated, all experiments were performed in duplicate. Bacterial growth was measured at 660 nm by inserting anaerobic Hungate tubes directly into a Shimadzu model UV 160A spectrophotometer. Volatile fatty acids and alcohols were detected on a SP-1000 column (1% H_3PO_4 on Chromosorb WAW) mounted in a Delsi series 30 chromatograph equipped with a flame ionisation detector and a Delsi Integrator. The column was operated at 150°C and nitrogen was used as carrier gas. Hydrogen was detected using a Carbosphere SS (60/80 mesh) column mounted on a Girdel series 30 gas chromatograph equipped with a thermal conductivity detector. Glucose, lactate, and formate were detected by high-performance liquid chromatograph and sulfide was determined photometrically as collodial CuS [7,8]. Microscopic examination of the cultures was undertaken with a Nikon microscope. Electron microscopy was performed as previously described [9].

2.7. DNA base composition

DNA was isolated and purified by hydroxyapatite chromatography. The guanine-plus-cytosine (G+C) content of the DNA was determined at the DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) by the method of Meshbah et al. [10]. Nonmethylated λ DNA (Sigma) was used as the standard.

2.8. DNA relatedness

DNA relatedness was determined at the DSM. DNA was isolated by chromatography on hydroxylapatite by the procedure of Cashion et al., 1977 and DNA-DNA hybridisation carried out according to DeLey [11] using a Gilford 2600 spectrophotometer equipped with a Gilford 2527-R thermoprogrammer and plotter.

2.9. 16S rRNA 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 [12]. The 16S rRNA gene sequence which we determined was manually aligned with reference sequences of various members of the domain *Bacteria* using the editor 'ae2' [13]. Reference sequences were obtained from the Ribosomal Database Project [13], and that of *S. alkalica* was extracted from EMBL database (accession number X93927). Positions of sequence and alignment uncertainty were omitted from the analysis. The pairwise evolutionary distances based on 1168 unambiguous nucleotides were computed using the method of Jukes and Cantor and dendrograms constructed from these distances using the neighbour-joining method, both of which form part of the PHYLIP suite of programs [14].

2.10. Nucleotide sequence accession number

The nucleotide sequence for the 16S rRNA gene of isolate SEBR 4228^T has been deposited in GenBank under accession number U80597.

3. Results

3.1. Isolation and cellular properties

Translucent colonies with regular edges and 0.5 mm in diameter developed on agar plates after a 2 week incubation at 37°C. Well isolated single colonies were picked to obtain several pure cultures and a strain designated SEBR 4228^T was retained for further characterisation. The cells of strain SEBR 4228^T were spiral shaped, measured 0.3–0.5 by 5–30 μm (Fig. 1a) and were motile with a corkscrew-like motion. Electron microscopy of thin sections of SEBR 4228^T revealed that the strain possessed a multilayered crenulating Gram-negative cell envelope consisting of an outer membrane and an inner membrane adjoining the cytoplasmic membrane (Fig. 1b). Sillons, which are the points of contact between the protoplasmic cylinder, the inner membrane and the outer membrane, were also observed (Fig. 1b). The arrangement of the flagella was 1-2-1 (Fig. 1c). Enlarged spherical bodies typical of spirochetes were also observed (Fig. 1d).

3.2. Growth and metabolic properties

Strain SEBR 4228^T was a strictly anaerobic chem-

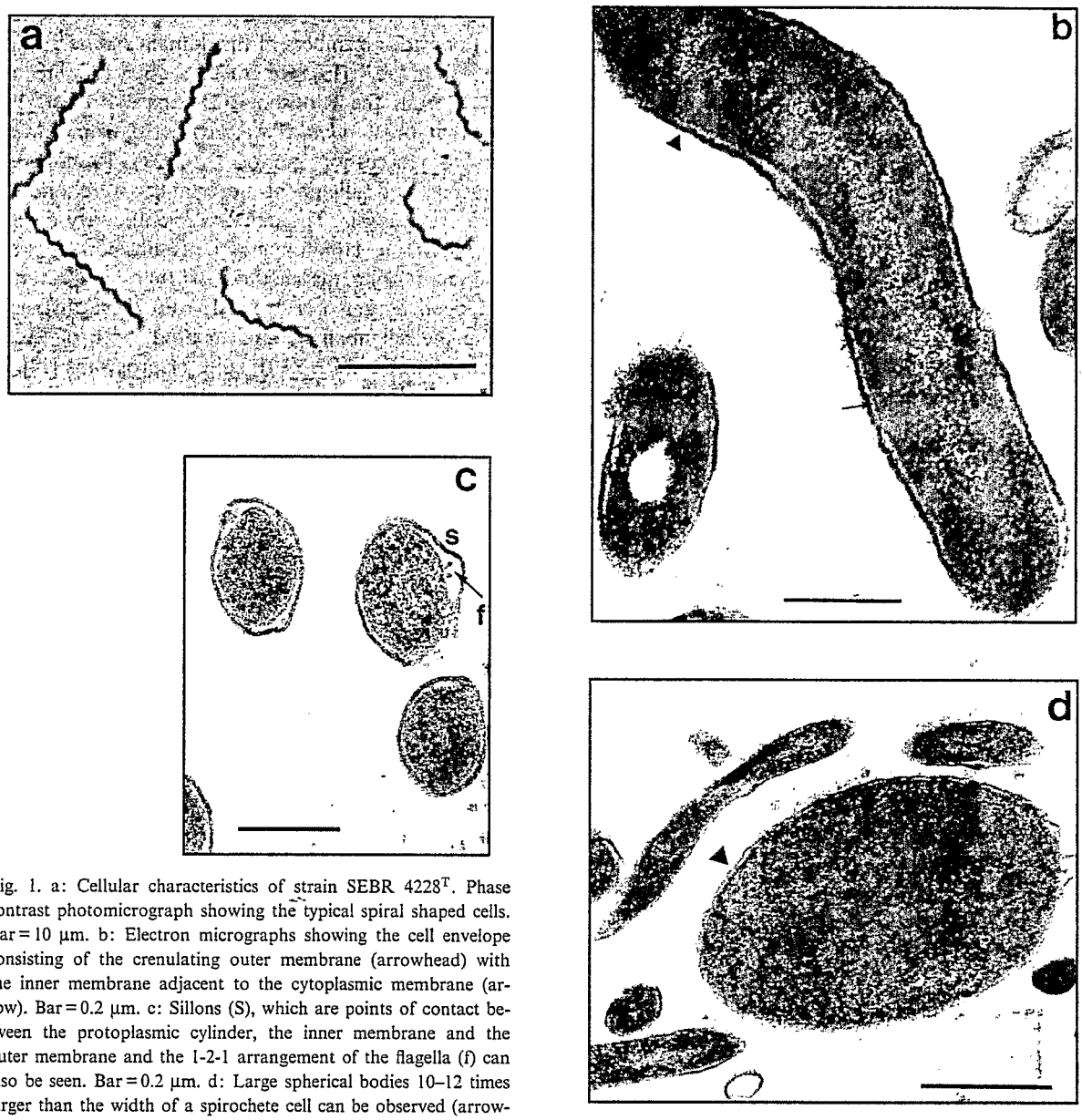

Fig. 1. a: Cellular characteristics of strain SEBR 4228^T. Phase contrast photomicrograph showing the typical spiral shaped cells. Bar = 10 μm. b: Electron micrographs showing the cell envelope consisting of the crenulating outer membrane (arrowhead) with the inner membrane adjacent to the cytoplasmic membrane (arrow). Bar = 0.2 μm. c: Sillons (S), which are points of contact between the protoplasmic cylinder, the inner membrane and the outer membrane and the 1-2-1 arrangement of the flagella (f) can also be seen. Bar = 0.2 μm. d: Large spherical bodies 10–12 times larger than the width of a spirochete cell can be observed (arrowhead). Bar = 0.5 μm.

oorganotrophic halophilic bacterium which required 5% NaCl for optimal growth. Growth was completely inhibited in the presence of 11% NaCl. The optimal growth temperature was 37°C and the temperature range for growth was 20–40°C. The optimum pH was 7.0 with growth occurring between pH 5.5 and 8.0.

Fructose, galactose, D-xylose, D-glucose, maltose, ribose, D-mannose, mannitol, glycerol, yeast extract, biotrypcase and fumarate were used as substrates, but not D-arabinose, rhamnose, sorbose, L-xylose, sucrose, maltose, acetate, butyrate, propionate, pyruvate, lactate and casamino acids. Yeast extract is required for growth on substrates and cannot be replaced by a vitamin mixture. Fumarate was fermented to acetate and succinate. H_2 and ethanol

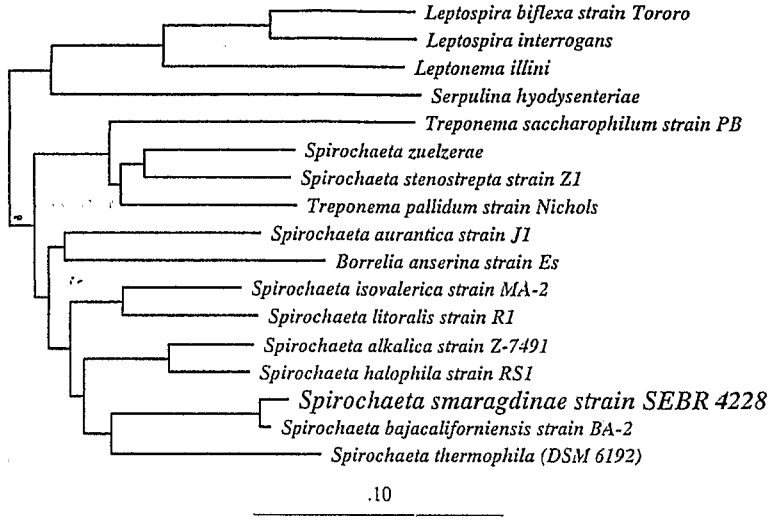

Fig. 2. Dendrogram showing the position of strain SEBR 4228^T within the the radiation of the members of the order Spirochaetales and related genera. Bar indicates evolutionary distance.

were produced as minor end-products and lactate as a major end-product from glucose fermentation. The doubling time for strain SEBR 4228^T in the presence of glucose, yeast extract and biotrypcase under optimal conditions was 25 h. Thiosulfate and elemental sulfur but not sulfate were reduced to sulfide. The end-products formed from glucose oxidation in the presence of thiosulfate were lactate, acetate, CO_2 and H_2S.

3.3. DNA base composition

The G+C content of DNA from strain SEBR 4228^T as determined by HPLC was 50 mol%.

3.4. 16S rRNA sequence studies and DNA relatedness

Using 10 primers, a nearly complete sequence of the 16S rRNA gene of strain SEBR 4224^T was determined. The sequence (positions 8–1541, *Escherichia coli* numbering according to Winker and Woese [15]) was aligned and phylogenetic analysis performed with representatives of the various phyla of the domain *Bacteria*. The analysis indicated that strain SEBR 4228^T was a member of the order Spirochaetales. Further sequence alignments and phylogenetic analysis with members of this phylum indicated that the closest relative of strain SEBR 4228^T was *S. bajacaliforniensis* (similarity 98.6%). A dendrogram generated by the neighbour-joining method from the evolutionary distance matrix of Jukes and Cantor showing this relationship is shown in Fig. 2. DNA-DNA hybridisations indicated that strain SEBR 4228^T was only distantly related to *S. bajacaliforniensis* (38%).

4. Discussion

Strain SEBR 4228^T is a free-living spirochete, has a Gram-negative envelope with a crenulated outer membrane outer layer and possesses a 1-2-1 flagella arrangement and large spherical bodies. These features are characteristics of the members of the genus *Spirochaeta* with the exception of *S. plicatilis* which contains 18–25 flagella. Strain SEBR 4228^T is a strictly anaerobic, moderately halophilic, carbohydrate-fermenting spirochete and therefore resembles *S. litoralis* and *S. bajacaliforniensis* [16,17] but not *S. alkalica*, *S. africana*, and *S. asiatica* which are alkalophiles [18] or *S. halophila* which is a facultative anaerobe [19]. Its affiliation to *S. litoralis* and *S. bajacaliforniensis* is confirmed by the similar G+C content of their DNA (50%) but phylogeny based on 16S rRNA sequence analysis indicates that strain SEBR 4228^T is more closely related to *S. bajacali-*

Table 1
Phenotypic differences between *Spirochaeta smaragdinae* and *Spirochaete bajacaliforniensis*

Phenotypic characteristic	*S. smaragdinae*[a]	*S. bajacaliforniensis*[b]
Environment	marine sediments	oil field
Size (μm)	0.3–0.5×5–30	0.2–0.3×15–45
Optimum pH for growth	7.5	7.0
Optimum NaCl (M) for growth	0.85	0.48
Substrates used:		
Arabinose	–	+
Maltose	–	+
Rhamnose	–	+
Sorbose	–	+
Mannose	+	–
Xylose	+	–
Glycerol	+	–
Pyruvate	–	+
End-products from glucose fermentation	lactate, ethanol, CO_2, H_2	acetate, ethanol, CO_2, H_2

[a]Results from this study.
[b]Results from Fracek and Stolz [15].

forniensis (98.6% similarity) than to *S. litoralis* (83% similarity) or any other members of the spirochetes including the treponemes. Despite its close phylogenetic relationship, strain SEBR 4228^T exhibits marked phenotypic differences (Table 1). In addition, DNA-DNA hybridisation studies indicate a very poor homology (38%) between the genomes of the two strains. Based on the characteristics, strain SEBR 4228^T is a novel species on the genus *Spirochaeta* which we have designated *S. smaragdinae* sp. nov.

Previously, members of the order Spirochaetales have frequently been isolated from thermal, mesophilic, marine hypersaline and alkaline environments [1–3,17–19]. In this paper we extend their known habitat to include the deep subsurface waters of oil fields. In addition, we have also shown that *S. smaragdinae* is capable of reducing thiosulfate to sulfide and this constitutes the only report of this occurrence amongst the members of the order Spirochaetales. Further studies need to be undertaken in order to understand the distribution of this trait in other members of the genus *Spirochaeta* and its effect on organic matter oxidation in their natural terrestrial marine and subsurface ecosystems.

4.1. Description of *Spirochaeta smaragdinae* sp. nov.

Spirochaeta smaragdinae (sma.rag.di.nae. M. L. masc. smaragdus, emerald; gen. smaragdinae, from Emerald, the name of the oil field in Congo, Central Africa). Spirochetes with corkscrew-like motility which produce translucent colonies with regular edges and a diameter of 0.5 mm after 2 weeks incubation at 37°C. Spiral cells that are 0.3–0.5 by 5–30 μm. Cells stain Gram-negative and are chemoorganotrophic obligately anaerobic members of the domain *Bacteria*. The isolate contains a 1-2-1 flagella arrangement, a typical characteristic of most members of the genus *Spirochaeta*. The optimum temperature for growth is 37°C with growth occurring between 20°C and over 40°C. The optimum pH is 7.0 with growth occurring between pH 5.5 and 8.0. Obligately halophilic. The optimum NaCl concentration for growth is 5% and the NaCl concentration range for growth is 1.0–10%. Doubling time under optimal conditions is about 25 h in the presence of glucose and thiosulfate. Growth occurs in the presence of fructose, galactose, D-xylose, D-glucose, ribose, D-mannose, mannitol, glycerol, yeast extract, biotrypcase and fumarate, but not with D-arabinose, rhamnose, sorbose, L-xylose, sucrose, maltose, acetate, butyrate, propionate, pyruvate, lactate and casamino acids. Yeast extract is required for growth and cannot be replaced by a vitamin mixture. Fumarate was fermented to acetate and succinate. H_2 and ethanol were produced as minor end-products and lactate as a major end-product from glucose fermentation. Thiosulfate and elemental sulfur were reduced to sulfide. The end-products formed from glucose oxidation in

the presence of thiosulfate were lactate, acetate, CO_2 and H_2S. The G+C content of the DNA is 50% (as determined by HPLC). Isolated from an oil-injection water. The type strain is SEBR 4228^T (=DSM 11293).

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