Spirochaeta sinaica sp. nov., a halophilic spirochaete isolated from a cyanobacterial mat

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A strain of free-living obligately anaerobic, halophilic spirochaete, SLT, was isolated from a sample of a cyanobacterial mat of the hypersaline Solar Lake, Sinai shore. The strain had motile helical cells, 0.35–0.40 × 6–10 μm. Strain SLT exhibited high resistance to NaCl among known halophilic spirochaetes growing at NaCl concentrations from 2 to 12 % (optimum growth at 7 %). The strain grew at temperatures from 10 to 32 °C (optimum at 28 °C) and pH from 6 to 8.5 (optimum at pH 7.0–7.5). Carbohydrates, but not alcohols, organic acids or nitrogenous compounds (peptone, yeast extract and amino acids), were used as energy substrates for growth. Ethanol, acetate, lactate, H2 and CO2 were the products of glucose fermentation. Sulfide was produced in the presence of S0 or thiosulfate in the medium. The DNA G+C content was 44.7 mol%. Based on 16S rRNA gene sequence analysis, strain SLT clustered within the genus Spirochaeta, exhibiting 94.2 and 93.7 % similarity with its closest relatives, Spirochaeta bajacaliforniensis DSM 160554T and Spirochaeta smaragdinae DSM 11293T, respectively; similarity with other species did not exceed 86 %. The phenotypic and chemotaxonomic characteristics of the strain, as well as the results of phylogenetic analysis support the classification of strain SLT as representing a novel species of the genus Spirochaeta, for which the name Spirochaeta sinaica sp. nov. is proposed. The type strain is SLT (=DSM 14994=UNIQEM U 783).

The spirochaetes are presently classified in the class Spirochaetes in the order Spirochaetales and are divided into three major phylogenetic groups or families. The first family, Spirochaetaceae, contains species of seven genera. The genus Spirochaeta of the family Spirochaetaceae comprises free-living, non-pathogenic obligately or facultatively anaerobic, motile bacteria with helical cells and periplasmic flagella. Coccolid-shaped bacteria, which are phylogenetically most closely related to Spirochaeta and were previously considered as members of this genus (Spirochaeta cocoides) (Dröge et al., 2006), have recently been reclassified, along with two new isolates, as species of an independent genus Sphaerochaeta, namely Sphaerochaeta cocoides, Sphaerochaeta pleomorpha and Sphaerochaeta globosa (Abt et al., 2012; Ritalahti et al., 2012).

Comparison of 16S rRNA gene sequences shows that some species of the genus Spirochaeta form a cluster interspersed with symbiotic species of the genus Treponema (Leschine et al., 2006).

Most Spirochaeta species exhibit low levels of 16S rRNA gene sequence similarity with each other (80–88 %). In other bacterial phyla, such low 16S rRNA gene sequence similarities are usually reported for organisms belonging to different families. Therefore, there remains taxonomic uncertainty regarding the genera Spirochaeta and Treponema. Many other spirochaetes, including the type species of the genus

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Abbreviation: DMA, dimethyl acetal.

The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequence of strain SLT is KC261846.
Spirochaeta, Spirochaeta plicatilis, have never been isolated in pure culture or even characterized by 16S rRNA gene sequence analysis. Further research is therefore necessary to broaden the circle of representatives of the genus and where they are found in nature. Spirochaetes are widespread in sulfide-rich freshwater and marine sediments, in sulfide springs, and in marine coastal bacterial mats (Dubinina et al., 1993a, b; Berlanga et al., 2008; Harris et al., 2013). The presence of various spirochaete species in other ecological niches such as the bottom sediments of alkaline lakes or the marshes of hypersaline and brackish bodies of water and mineral springs has been reported in numerous studies. These data have been summarized by Leschine et al. (2006). In the present work we report on the isolation, characterization and taxonomic description of a new halophilic spirochaete from the microbial communities of a hypersaline habitat.

Strain SL<T> was isolated from a sample of cyanobacterial mat material collected from ~2 m depth at the littoral of the meromictic Solar Lake, Sinai shore of the Gulf of Elat. This lake has high salinity, varying from 10 to 20 % NaCl depending on the season and considerably exceeding that of inflowing Red Sea water (4 %) (Krumbein & Cohen, 1977). Spirochaetes were isolated in liquid medium under anaerobic conditions in Hungate tubes. The medium contained the following (g l<sup>-1</sup>): NaCl (50), NH₄Cl (0.3), CaCl₂·2H₂O (0.3), MgCl₂·6H₂O (3), DL-glucose (1), Bacto yeast extract (1), Bacto peptone (1), KH₂PO₄ (1) and K₂HPO₄ (1). Prior to inoculation, the following components were added per litre of the medium as sterile solutions: 1 ml of trace elements solution (Pfennig & Lippert, 1966), 5 mg vitamin B₁₂, 50 mg rifampicin and 10 % Na₂S.₉ H₂O to 0.5 mM. The procedure used for preparation of the medium is described in detail in the supplementary material to Dubinina et al. (2011). For determination of the spectrum of utilized carbon sources, glucose was replaced by other compounds. Medium with 0.3–0.5 % Difco agar was used to obtain bacterial colonies, which were then transferred to liquid medium. All experiments for determination of the range and optimal concentration of substrates, and NaCl, pH and temperature were conducted in the same medium. Resistance to rifampicin was defined after addition of the antibiotic solution following its ultrafiltration (pore diameter 0.2 μm) at concentrations of 10, 50, 100 and 200 μg ml<sup>-1</sup> to the medium.

Cell morphology was studied using a phase-contrast microscope (NU-2; Zeiss) and a JEM-100C transmission electron microscope at an accelerating voltage of 80 kV.

The effect of NaCl was determined in media with 0–15 % NaCl (in 0.5 % increments). The temperature range and optimum for growth were determined by cultivation at 5–45 °C (increments of 5 °C) and at 25–35 °C (increments of 2 °C), respectively. The pH range for growth was determined between pH 6.0 and 8.5 (increment of 0.5 pH units). Growth was determined by an optical density spectrophotometric reading at 600 nm of 3-day cultures. Capacity for aerobic growth was determined under optimal temperature, pH and salinity in 100 ml vials half-filled with the medium and containing 1 or 5 % O₂ in the gas phase.

The products of glucose fermentation were measured by GLC (Chrom-5). Gaseous metabolic products were measured by GC (LKHM80). Details of these analytical procedures are given by Dubinina et al. (2004), as is the protocol for measuring the enzyme activity of cell-free preparations. All experiments were repeated three times.

For extraction and analysis of cellular fatty acids, strains were incubated in their respective media and under individual growth conditions. Cells of strain SL<T> were harvested from a broth culture grown in the medium described above and incubated at 30 °C for 16 days. Analysis was conducted using the Microbial Identification System (MIDI) Sherlock software version 6.1 (method and peak naming table: ANAER6) as described by Sasser (1990).

Genomic DNA was extracted according to Marmur (1961). The genomic DNA G+C content of strain SL<T> was determined by thermal denaturation (T<sub>sm</sub>) (Owen & Lapage, 1976). The 16S rRNA gene sequence was determined as described by Rainey et al. (1996) and an alignment was generated using CLUSTAL X software (Thompson et al., 1997). An evolutionary-distance matrix was calculated using the Jukes and Cantor algorithm (Jukes & Cantor, 1969). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Neg, 1987) and maximum-parsimony (Fitch, 1971) methods. Bootstrap analyses were based on 1000 resamplings. The analyses were conducted using MEGA version 4 (Tamura et al., 2007). The 16S rRNA gene sequence of strain SL<T> (1444 nt) was obtained and a phylogenetic tree was reconstructed based on compared sequences of related species of the genera Spirochaeta and Sphaerochaeta (Fig. 1).

Strain SL<T> formed a stable cluster together with Spirochaeta bajacaliforniensis DSM 160554<sup>T</sup> and Spirochaeta smaragdinae DSM 11293<sup>T</sup>, which was supported by a 100 % bootstrap value. Highest levels of sequence similarity were found with Spirochaeta bajacaliforniensis DSM 160554<sup>T</sup> and Spirochaeta smaragdinae DSM 11293<sup>T</sup>, 94.2 and 93.7 %, respectively. Similarity to other species in the genus was less than 86 %.

Cells of strain SL<T> were Gram-stain-negative, helical, motile, and showed rotation, undulation and gliding motility typical of spirochaetes. Helical cells were 0.35–0.40 × 6–10 μm in size. In the stationary growth phase or under unfavourable micro-oxic conditions, rounded cells (spheroplasts) were formed (Fig. 2). Electron microscopy confirmed the presence of structural features typical of those seen in other spirochaetes. The protoplasmic cylinder was surrounded by the outer membrane and axial flagella arrangement within the periplasmic space (Fig. 3).

Cells were catalase- and oxidase-negative. The organism was an obligate anaerobe, not growing in liquid medium in the presence of 1 % O₂ in the gas phase. The strain was obligately halophilic, and exhibited the highest resistance to NaCl among known halophilic spirochaetes, growing at NaCl...
concentrations from 2 to 12 % (optimum at 7 %). Growth occurred at 10–32 °C (optimum at 27 °C) and at pH 6.5–8.5 (optimum at pH 7.0–7.5). A comparison of the morphological and physiological properties of strain SL T and the type strains of species of the genus Spirochaeta is given in Table 1.

**Fig. 1.** 16S rRNA-based neighbour-joining phylogenetic tree of a Spirochaeta cluster indicating the position of strain SL T. 16S rRNA gene sequences of type strains were used for the dendrogram. Bootstrap values greater than 70 % (1000 resamplings) are shown. Bar, 2 substitutions per 100 nt. The root was determined by the inclusion of the type strain of Brevinema andersonii as an out-group organism. All clusters were confirmed in the maximum-parsimony tree (data not shown).

**Fig. 2.** Phase-contrast micrograph of cells of strain SL T. Coccoid bodies are indicated by the arrow. Bars, 5 μm.
The strain was resistant to rifampicin (up to 100 µg ml\(^{-1}\) of medium).

The compounds used as growth substrates were carbohydrates: adonitol, cellobiose, dextran, D-fructose, galactose, galacturonic acid, D-glucose, glycogen, lactose, laminarin, levulose, maltose, melibiose, raffinose, rhamnose, D-ribose, sucrose, trehalose, starch and D-xylose. No growth occurred with alginic acid, L-arabinose, cellulose, dulcitol, inositol, D-mannitol or sorbitol, as well as with organic acids and alcohols, peptone, yeast extract or amino acids. Glucose was fermented with formation of ethanol, acetate, lactate, H\(_2\) and CO\(_2\). Ammonium salts, peptone, yeast extract and casein hydrolysate were used as nitrogen sources, while urea and nitrates were not.

Sulfide formation was detected in the presence of S\(_0\) or thiosulfate in the medium with glucose. A similar process has been previously reported for *Spirochaeta smaragdinae* (Magot *et al.*, 1997) and *Spirochaeta perfilievii* (Dubinina *et al.*, 2004, 2011). The biochemical mechanism of this process studied in *Spirochaeta perfilievii* (Dubinina *et al.*, 2004) was similar to that reported for some anaerobic bacteria (Janssen and Morgan, 1992), where sulfur reduction acted as an electron sink preventing the inhibitory effect of the H\(_2\) produced.

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**Table 1.** Distinguishing characteristics between strain SL\(^T\) and phylogenetically related species of the genus *Spirochaeta*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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</thead>
<tbody>
<tr>
<td>Cell diameter (µm)/cell width (µm)</td>
<td>0.35–0.40</td>
<td>0.2–0.3</td>
<td>0.3–0.5</td>
<td>0.4</td>
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<tr>
<td>Cell length (µm)</td>
<td>6–10</td>
<td>15–45</td>
<td>5–30</td>
<td>15–30</td>
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<tr>
<td>Temperature range (optimum) (°C)</td>
<td>10–32 (28)</td>
<td>(36)</td>
<td>20–40 (37)</td>
<td>(35–40)</td>
</tr>
<tr>
<td>pH range (optimum)</td>
<td>6.0–8.5 (7.0)</td>
<td>6.0–9.0 (7.5)</td>
<td>5.5–8.0 (7.5)</td>
<td>7.0–8.4 (7.5)</td>
</tr>
<tr>
<td>NaCl range (optimum) (%)</td>
<td>2–12 (7)</td>
<td>2–4 (3)</td>
<td>1–10 (5)</td>
<td>3–7 (4–5)</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>44.7</td>
<td>50.1</td>
<td>50.0</td>
<td>62.0</td>
</tr>
<tr>
<td>Reduction of S(_0) or S(_2)O(_3)(^{2–}) to H(_2)S, glucose fermentation</td>
<td>+</td>
<td>OA</td>
<td>OA</td>
<td>OA</td>
</tr>
<tr>
<td>Relationship to O(_2)*</td>
<td>OA</td>
<td>OA</td>
<td>OA</td>
<td>FA</td>
</tr>
<tr>
<td>Source of isolation</td>
<td>Cyanobacterial mat</td>
<td>Marine (laminated sediments)</td>
<td>Oilfield, marine sediments</td>
<td>Lake bottom sediment</td>
</tr>
<tr>
<td>Carbon and energy source:</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>L-Arabinose</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>D-Xylose</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>D-Maltose</td>
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<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>D-Mannitol</td>
<td>–</td>
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<td>Rhamnose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<td>D-Fructose</td>
<td>+</td>
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<tr>
<td>Glycerol</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<td>Pyruvate</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>End products from glucose fermentation</td>
<td></td>
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<tr>
<td>Acetate</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Ethanol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Lactate</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+(\dagger)</td>
</tr>
<tr>
<td>H(_2)</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>CO(_2)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

\(\dagger\) Small amount of lactate.

\(\dagger\) OA, obligate anaerobe; FA, facultative anaerobe.
The predominant fatty acids of strain SL\textsuperscript{T} were saturated branched-chain acids such as iso-C\textsubscript{15} : 0 (42.6\%), iso-C\textsubscript{15} : 0 dimethyl acetal (DMA) (26.6\%), anteiso-C\textsubscript{15} : 0 DMA (6.2\%) and anteiso-C\textsubscript{15} : 0 (3.9\%), as well as straight-chain acids such as C\textsubscript{14} : 0 (4.6\%) and C\textsubscript{16} : 0 (4.0\%), and additionally an unidentified fatty acid which is most likely iso-C\textsubscript{15} : 0 aldehyde (summed feature 3; 6.3\%) (Table 2; see footnote).

The G+C content of the genomic DNA of strain SL\textsuperscript{T} was 44.7 mol\% (\(T_m\)). This is significantly lower than in its phylogenetically closest relatives: Spirochaeta smaragdinae DSM 11293\textsuperscript{T}, 50.0 mol\%; Spirochaeta bajacaliforniensis DSM 16054\textsuperscript{T}, 50.1 mol\%; Spirochaeta halophila DSM 10522\textsuperscript{T}, 62.0 mol\% (Table 1).

Comparative characterization of the phenotypic and chemotaxonomic properties of strain SL\textsuperscript{T} and phylogenetically most closely related species of the genus Spirochaeta is presented in Table 1. According to the results of 16S rRNA gene sequencing, as well as significant differences in the DNA G+C content, fatty acid composition and phenotypic characteristics, strain SL\textsuperscript{T} differs markedly from closest related Spirochaeta species and may be described as representing a novel species, for which the name Spirochaeta sinaica sp. nov. is proposed.

**Description of Spirochaeta sinaica sp. nov.**

* Spirochaeta sinaica (s.i.n’a.i.ca. N.L. fem. adj. sinaica pertaining to the Sinai shore, from where the type strain was isolated).

Cells are Gram-stain-negative, motile, helical and show rotation, undulation and gliding motility typical of spirochaetes. Helical cells are 0.35–0.40 \(\times\) 6–10 \(\mu\)m in size. Rounded cells (spheroplasts) are formed during the stationary growth phase or under unfavourable cultivation conditions. Saccharolytic, halophilic obligate anaerobe. Growth occurs with 2–12\% NaCl in the medium (optimum 7\% NaCl). The temperature range for growth is 10–32\°C (optimum at 28\°C) and the pH range is 6.0–8.5 (optimum at pH 7.0–7.5). Resistant to rifampicin (up to 100 \(\mu\)g ml\textsuperscript{−1}). Catalase- and oxidase-negative. Carbohydrates (adonitol, cellobiose, dextrin, D-fructose, galactose, galacturonic acid, DL-glucose, glycojen, lactose, laminarin, levulose, maltose, melibiouse, raffinose, rhamnose, D-ribose, sucrose, trehalose, starch and D-xylose) may be used as sole sources of carbon and energy. Alginic acid, L-arabinose, cellobiose, dulcitol, inositol, D-mannitol and sorbitol, as well organic acids and alcohols, peptone, yeast extract or amino acids are not utilized. Glucose is fermented with formation of ethanol, acetate, lactate, \(\text{H}_2\) and \(\text{CO}_2\). Fermentation of glucose in the presence of \(\text{S}_2\) or thiosulfate results in formation of sulfide. Cellular fatty acids consist mainly of branched-chain saturated acids: iso-C\textsubscript{15} : 0 and iso-C\textsubscript{15} : 0 DMA.

The DNA G+C content of the type strain is 44.7 mol\%. The type strain, SL\textsuperscript{T} (= DSM 14994\textsuperscript{T} = UNIQUEM U 783\textsuperscript{T}), was isolated from a cyanobacterial mat sample of Solar Lake (Sinai shore).

**Acknowledgements**

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