Sphaerochaeta globosa gen. nov., sp. nov. and Sphaerochaeta pleomorpha sp. nov., free-living, spherical spirochaetes

Kirsti M. Ritalahti,1,2 Shandra D. Justicia-Leon,3,4 Kathleen D. Cusick,1 Natalia Ramos-Hernandez,3,4 Michael Rubin,3,4 Jessica Dornbush3,4 and Frank E. Löfler1,2,5

Correspondence
Frank E. Löfler
frank.loeffler@utk.edu

1Department of Microbiology, University of Tennessee, Knoxville, TN 37996, USA
2Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA
3School of Biology, Georgia Institute of Technology, Atlanta, GA 30332, USA
4School of Civil and Environmental Engineering, Georgia Institute of Technology, Atlanta, GA 30332, USA
5Department of Civil and Environmental Engineering, University of Tennessee, Knoxville, TN 37996, USA

Free-living bacteria with spherical cells 0.5–2.5 μm in diameter were isolated from freshwater sediment. 16S rRNA gene sequence analysis placed the new isolates within the phylum Spirochaetes (‘spirochaetes’). The isolates never displayed a helical morphology or motility. Growth occurred in the presence of 100 mg ampicillin l⁻¹ in complex and defined mineral salts medium amended with vitamins, yeast extract and monosaccharides, disaccharides or soluble starch as fermentable substrates. Two distinct isolates, designated Buddyᵀ and Grapesᵀ, exhibited doubling times of 21 ± 2 and 15 ± 1 h in glucose-amended medium and grew at 15–37 and 15–30 °C. Optimum growth was observed between 25 and 30 °C and pH 6.5–7.5, with no growth below pH 5 or above pH 10. Hexose and pentose fermentation yielded ethanol, acetate and formate as major end products. Growth was strictly fermentative and anaerobic, but the isolates tolerated brief oxygen exposure. Nitrate, sulfate, thiosulfate and carbon dioxide were not used as electron acceptors, but soluble Fe(III) was reduced to Fe(II) in glucose-amended medium. The DNA G+C base contents of isolates Buddyᵀ and Grapesᵀ were 45.5–46.4 and 47.0–49.2 mol%, respectively. Phospholipid fatty acid (PLFA) profiles contained large proportions of C₁₄ : ₀ and C₁₆ : ₀ straight-chain saturated fatty acids; C₁₆ : ₁ω7c and C₁₆ : ₁₁₀9c dominated the mono-unsaturated PLFAs in isolate Grapesᵀ, whereas isolate Buddyᵀ also possessed C₁₈ : ₁₁₀5c, C₁₈ : ₁₁₀7c and C₁₈ : ₁₁₀9c fatty acids. Branched monoenoic acids accounted for up to 12.4 and 30 % of the total PLFA in isolates Grapesᵀ and Buddyᵀ, respectively. Based on their unique morphological features and the phylogenetic distance from their closest relatives, we propose the new genus, Sphaerochaeta gen. nov., to accommodate the new isolates within the novel species Sphaerochaeta globosa sp. nov. (type strain Buddyᵀ = DSM 22777ᵀ = ATCC BAA-1886ᵀ) and Sphaerochaeta pleomorpha sp. nov. (type strain Grapesᵀ = DSM 22778ᵀ = ATCC BAA-1885ᵀ). Sphaerochaeta globosa is the type species of the genus.

Novel, free-living bacteria with pleomorphic, spherical morphologies form a phylogenetically coherent, monophyletic clade arising from the family Spirochaetaceae. Two isolates were obtained from reductively dechlorinating consortia initiated from sediments of the Red Cedar River (Okemos, MI, USA), and were preliminarily described as ‘non-spiral spirochaetes’ (Ritalahti & Löfler, 2002) and subsequently as free-living, pleomorphic spirochaetes (Ritalahti & Löfler, 2004). The isolates are non-motile.

Abbreviations: PLFA, phospholipid fatty acid; TCE, trichloroethene; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains Buddyᵀ and Grapesᵀ are respectively AF357916 and AF357917.

A supplementary figure and table are available with the online version of this paper.
and replicate in round forms, thus breaking with the prevailing dogma of spirochaete biology, wherein ‘all spirochaetes are helical or spiral shaped microorganisms with internal organelles of motility called periplasmic flagella’ (Paster & Canale-Parola, 1980; Paster & Dewhirst, 2000). Such bacteria belong to the class Spirochaetes, order Spirochaetales, which includes the families Brachyspiraceae (genera Brachyspira and Serpulina), Leptospiraceae (genera Leptonema and Leptospira) and Spirochaetaceae (genera Borrelia, Brevinema, Cristispira, Spiroptera, Treponema and Spirochaeta) (Paster & Dewhirst, 2000). Based on taxonomic comparisons with other spirochaete lineages, the new isolates warrant a new genus designation. We assert that strains GrapesT and BuddyT are members of a seventh genus, for which we propose the name Sphaerochaeta gen. nov., within the Spirochaetaceae.

Sediment samples from the Red Cedar River were collected for the purpose of establishing reductively dechlorinating consortia. Microcosms were amended with 0.05–0.1 mM trichloroethene (TCE) as electron acceptor (He et al., 2005). Repeated transfers to defined, completely synthetic, reduced (0.2 mM l-cysteine and 0.2 mM Na2S·9H2O) and bicarbonate-buffered dechlorination medium (Sung et al., 2003) amended with TCE, hydrogen and acetate yielded non-methanogenic, ethene-producing cultures. The enrichment process included six consecutive transfers in dechlorination medium amended with 50 or 100 µg ampicillin ml⁻¹, a peptidoglycan biosynthesis inhibitor. Isolates were obtained from serially diluted culture suspensions in semi-solid (0.6 %, w/v, low-melting-point agarose), quarter- or half-strength tryptic soy broth (TSB) medium using the dilution-to-extinction principle (Löffler et al., 2005). This process was repeated before isolated colonies were transferred to Balch tubes with 15 ml half-strength modified TSB (mTSB) medium consisting of 7.4 g TSB l⁻¹ (quarter-strength), 85.6 mM NaCl, 1 g yeast extract l⁻¹, 1.8–5 g D-glucose l⁻¹ (10–30 mM), the Wolin vitamin mixture (Wolin et al., 1963) and 1 mM DTT as reductant. Isolates were maintained (2 %, v/v, transfers) in mTSB or the mineral salts dechlorination medium reduced with 1 mM DTT and amended with yeast extract, glucose and vitamins. In semisolid mTSB, strain GrapesT formed compact, non-pigmented colonies, 1–1.5 mm in diameter, with irregular, convoluted surfaces after 3 weeks of incubation, which reached 2–4 mm in diameter after 2 months. Strain BuddyT produced round, loose, pale colonies, 0.2–0.5 mm in diameter after 2 weeks of incubation, which grew into lentil-shaped colonies 0.5–2 mm in diameter and about 0.2 mm thick in the centre of the colonies after extended incubation periods of several months. During exponential growth in liquid medium, individual cells of strain GrapesT elicited primarily spherical morphologies, 0.5–2.5 µm in diameter, with many cells displaying elongated or crescent shapes and characteristic protrusions (Fig. 1a). Cells also occurred as clusters or aligned along threads like strings of pearls (Fig. 1b). In static mTSB incubations, cells of strain GrapesT settled and adhered to the glass walls of the culture vessels, unlike strain BuddyT. In growing cultures of strain BuddyT, one to ten round to oval cells, 0.5–1.2 µm in size, assembled on or within the perimeter of spherical, bubble-like structures, 3–30 µm in diameter (Fig. 2a). Acridine orange staining suggested that only the cells, and not the encapsulating structure, contained DNA (Fig. 2b). Scanning electron microscopy performed with strain BuddyT corroborated the round cell morphology, and many cells demonstrated the presence of a hook-like feature (Fig. 2c, arrowhead). During transition to stationary phase, cells of the new isolates became smaller and separated into individual cells, and clusters (GrapesT) or larger encapsulated structures (BuddyT) were rare. Smaller, individual cells were also common during growth in low-nutrient medium; however, the characteristic aggregates occurred, albeit smaller. The presence of ampicillin (50, 100 and 250 µg ml⁻¹) or rifamycin SV (30 and 60 µg ml⁻¹) had no effect on growth or on colony morphology in liquid or semi-solid media. Additionally, the isolates were resistant to carbenicillin (50 and 250 µg ml⁻¹) and vancomycin (20 and 100 µg ml⁻¹). Sensitivity was observed to kanamycin, erythromycin, chloramphenicol and tetracycline. Growth occurred in full-strength and diluted mTSB medium and was stimulated by monosodium glutamate (2 g l⁻¹) and Casamino acids (10 g l⁻¹). For the two isolates, lag times in D-glucose-amended mineral salts medium ranged from 2–3 (GrapesT) to 7–14 (BuddyT) days, and two to three shorter lag times were observed in mTSB. In half-strength mTSB
Fig. 2. Cells of strain Buddy\textsuperscript{T} visualized by phase-contrast microscopy (a), confocal microscopy following acridine orange staining (b), critical-point-dried scanning electron microscopy preparation (c) and TEM cross-section (d). In (a), (b) and (d), a membranous structure that encapsulates one to ten cells of strain Buddy\textsuperscript{T} is highlighted. In (c), a hook-like protrusion is indicated by an arrowhead.

(without stimulants), doubling times of 15.3±0.6 and 20.9±2.3 h were observed for strains Grapes\textsuperscript{T} and Buddy\textsuperscript{T}, respectively. The maximum cell density attained was dependent on the amount of glucose added, rather than the strength of the mTSB medium. In quarter-strength mTSB with 10 mM glucose, strain Buddy\textsuperscript{T} reached an OD\textsubscript{600} of ~0.1, whereas cultures of strain Grapes\textsuperscript{T} reached an OD\textsubscript{600} of 0.2–0.3. Other hexoses that supported growth included D-galactose and D-fructose, while D-mannose was used by strain Grapes\textsuperscript{T} but not strain Buddy\textsuperscript{T}. Other saccharides that supported growth of both isolates included the pentoses D-xylose and L-arabinose, the disaccharide sucrose and the trisaccharides raffinose and melezitose as well as soluble starch. Strain Buddy\textsuperscript{T} also grew with lactose, sucrose and the trisaccharides raffinose and melezitose as well as soluble starch. Strain Buddy\textsuperscript{T} also grew with lactose and maltose, while strain Grapes\textsuperscript{T} did not. Glucose and xylose fermentation yielded acetate, formate and ethanol, which are characteristic spirochaete carbohydrate fermentation products. H\textsubscript{2}–CO\textsubscript{2} homocatogenic growth was not observed in mineral salts medium with a H\textsubscript{2}/CO\textsubscript{2} (80:20, v/v) headspace. Citrate (10 mM), ethanol (1.7 and 170 mM), methanol (2.5 and 250 mM) and glycerol (1.4 and 140 mM) were not used by either isolate, nor was growth observed with the insoluble substrates glycogen, xylan, cellulose, cellulobiose or chitin in liquid medium or supplied at 0.1 g l\textsuperscript{−1} suspended in semi-solid mineral salts medium. Electron acceptors including nitrate (1–5 mM), nitrite (1–5 mM), sulfate (10 mM), fumarate (5 mM) and thiosulfate (10 mM) were not reduced in mineral salts medium amended with glucose, propionate, acetate or hydrogen as electron donors. Soluble Fe(III) (as ferric citrate; 5 mM) was reduced to Fe(II) in defined medium amended with 10 mM glucose; however, growth with Fe(III) as electron acceptor could not be demonstrated. Neither isolate dechlorinated TCE and the dechlorinating activity in the enrichments was attributed to Dehalococcoides sp. strain FL2 (He et al., 2005). The results of substrate utilization experiments were verified in at least two independent experiments. Growth was measured by an increase in OD\textsubscript{600}, microscopic cell counts and analysis of substrate utilization and/or product formation.

In half-strength mTSB with 10 mM glucose, isolates Grapes\textsuperscript{T} and Buddy\textsuperscript{T} grew at 15–30 and 20–37 °C, respectively, with optimum growth at 20–25 °C (Grapes\textsuperscript{T}) and 30 °C (Buddy\textsuperscript{T}). No growth was observed below 10 °C, but cultures remained viable at 4 °C for at least 3 years, and transfer cultures incubated at 25 °C grew after a 4–6 day lag period. The pH optimum ranged from 6.5 to 7.5, but cultures incubated at pH 5 and pH 10 lost viability (i.e. no growth occurred following transfers to growth medium at pH 7). Both isolates tolerated oxygen (i.e. air) exposure (i.e. shaking of open culture vessels at 200 r.p.m.) for up to 8 h, and resumed growth following transfers; however, the lag times before the onset of growth in mTSB increased to 5–7 days (Grapes\textsuperscript{T}) or several weeks (Buddy\textsuperscript{T}), depending on inoculum size and duration of exposure to air. Cultures became non-viable after 24 h of air exposure. Both isolates tested oxidase- and catalase-negative, and growth in mTSB required a reductant, suggesting that both isolates are strict anaerobes.

During exponential growth, cells stained Gram-negative. Transmission electron microscopy (TEM) of a cross-section of a single cell of strain Grapes\textsuperscript{T} demonstrated features of a Gram-negative cell wall (Fig. 1c, d). Fig. 1(d) shows the inner cell wall and a double membrane with a periplasmic space between the cell wall and outer membrane. TEM of cross-sections of strain Buddy\textsuperscript{T} suggested that several cells share a common outer membrane during exponential growth (Fig. 2d). Cell-wall analyses, performed by the DSMZ using chromatographic separation techniques as described previously (MacKenzie, 1987; Rhuland et al., 1955), did not detect daminopimelic acid in cell hydrolysates; however, only 0.2 mg polymeric substance was obtained from 3.5 g (wet weight) biomass from strain Grapes\textsuperscript{T}, precluding detailed investigations. GC-MS analysis identified leucine, isoleucine, lysine and other amino acids, but daminopimelic acid and ornithine were absent. Ornithine is the predominant peptidoglycan cross-link in spirochaetes (Joseph et al., 1973), suggesting that the new isolates lack these structural elements, possibly promoting pleomorphy.

Isolates Buddy\textsuperscript{T} and Grapes\textsuperscript{T} grown in half-strength mTSB amended with 10 mM glucose were analysed for phospholipid fatty acid (PLFA) profiles by Microbial Insights (http://www.microbe.com; Rockford, TN, USA) using established protocols (White et al., 2005). Similar to other spirochaetes (Franzmann & Rohde, 1992; Kondo & Ueta,
Sphaerochaeta gen. nov.: free-living, pleomorphic spirochaetes

1972; Livermore & Johnson, 1974), both isolates contained large proportions of even-carbon-number, straight-chain saturates, which accounted for 44 % (GrapesT) and 31 % (BuddyT) of the total PLFA (Supplementary Table S1, available in IJSEM Online). The major saturated PLFAs were C14:0 (26.4 and 13.4 mol% in isolates GrapesT and BuddyT, respectively) and C16:0 (12.9–16.9 mol%). Mono-unsaturated fatty acids made up 39–42 % of the cellular fatty acids. Of the mono-unsaturated PLFAs, C16:1 was the most abundant, and accounted for 90 % of the monoenoic acids in strain GrapesT, while strain BuddyT had a slightly smaller total amount of monoenoic acids, and C16:1 and C18:1 were equally abundant. Branched monoenoic acids (br-C15:1, br-C17:1 and br-C19:1) accounted for nearly 30 % and 12.4 % of the total PLFA in BuddyT and GrapesT, respectively. These amounts correlated inversely with the abundance of straight-chain mono-unsaturated C16:1 fatty acids, which were higher in GrapesT and lower in BuddyT (Supplementary Table S1). Odd-carbon-number iso-branched and anteiso-branched PLFAs were not detected.

Motility was not observed during any stage of growth or under non-growth conditions in liquid or semi-solid (0.1–0.6 % low-melting-point agarose) medium under various substrate, pH and temperature regimes. Silver nitrate staining never revealed flagella (i.e. periplasmic flagella) in either isolate. Primers targeting flaA (Li et al., 2008) failed to produce amplicons with template DNA from either isolate, whereas DNA from a Sphaerochaeta stenostreptta strain yielded amplicons of the expected size.

The two isolates had distinct rep-PCR profiles (Rademaker et al., 2000), which remained consistent over time, suggesting genome stability. The DNA G+C base contents of strains BuddyT and GrapesT were respectively 45.5–46.4 and 47.0–49.2 mol% (determined as described by Mesbah et al., 1989). Phylogenetic analysis based on 1500 positions of the 16S rRNA gene revealed that the sequences of strains BuddyT and GrapesT were 95.5 % similar by pairwise alignment performed with Geneious Pro 5.0.2 software and the EzTaxon tool (http://www.eztaxon.org; Chun et al., 2007). Additional isolation efforts yielded isolate JEL1 (GenBank accession no. EU169845 (Chung & Rittmann, 2007), AF349763 (Gu et al., 2004), AJ249227 and AF349763), a methanogenic, glucose-fed bioreactor with highly abundant ‘sphaerochaet’ 16S rRNA gene sequences, but with a predominance of round cells (Fernandez et al., 2000), and a cellulose- and methanol-utilizing digester (EF586024, EF559111) (Supplementary Fig. S1). Phylogenetic affiliation with the sphaerochaetes was determined by BLAST analysis against the GenBank nr database and the RDP (Cole et al., 2005). Unrooted phylogenetic trees of 16S rRNA gene sequences of sphaerochaete type strains (Fig. 3, Supplementary Fig. S1) were generated using the Geneious software and the PhyML tree plugin (Drummond et al., 2009; Guindon & Gascuel, 2003). Alignment algorithms utilized MUSCLE with the neighbour-joining or UPGMA clustering method and CLUSTAL W weighting scheme (1352 aligned bases). All approaches yielded similar results, with isolates BuddyT, GrapesT, JEL1 and TQ1 forming a distinct cluster within the family Spirochaetaceae (Fig. 3). Supplementary Fig. S1 highlights 16S rRNA gene clone sequences related to those of the new isolates and aligned with representative Sphaerochaeta and Treponema sequences.

Organisms with <98.5 % 16S rRNA gene sequence similarity have been found to share less than 95 % average nucleotide identity and therefore would not belong to the same species (Cole et al., 2010; Stackebrandt et al., 2002). The closest relative with confirmed sphaerochaete-characteristic motility and spiral morphology is Sphaerochaeta smaragdinae DSM 11293T (GenBank accession no. U80597), a free-living, anaerobic, mesophilic, carbohydrate-fermenter (Magot et al., 1997), which exhibits 85.1 and 85.2 % 16S rRNA gene sequence similarity to BuddyT and GrapesT, respectively. Phylogenetic analysis based on the comparison of sequences with >1200 16S rRNA gene nucleotide positions available in the RDP (Cole et al., 2005) demonstrated that the new isolates, along with a number of environmental clone sequences, formed a monophyletic cluster arising from the genus Sphaerochaeta (Fig. 3). The genus Sphaerochaeta comprises facultatively or obligately anaerobic, free-living sphaerochaetes that are often isolated from aquatic or marine environments, including deep-sea sediments (Canale-Parola, 1984). Only two isolates, Sphaerochaeta cocoides SPN1T (GenBank accession no. AJ698092; about 11 % 16S rRNA gene sequence divergence from the novel isolates) obtained from the termite hindgut (Dröge et al., 2006) and Sphaerochaeta sp. ACE-P (M87055; 13.3 % sequence divergence), an unusual, obligately psychrophilic, wall-less sphaerochaete from Ace Lake, Antarctica (Franzmann & Dobson, 1992), have been described with round rather than spiral morphology. As shown in Fig. 3 and Supplementary Fig. S1, the new isolates share a common line of descent with Sphaerochaeta but exhibit >10 % 16S rRNA gene sequence divergence. Based on the unique taxonomic features, we propose that the new isolates belong to a new genus, Sphaerochaeta gen. nov., as the type strains of two novel species, Sphaerochaeta pleomorpha sp. nov. and Sphaerochaeta globosa sp. nov. As non-motile sphaerochaetes with round cells have been described as members of the...
genera *Spirochaeta* and *Sphaerochaeta*, future comparative analyses that include more isolates may justify reclassification of *Spirochaeta coccoides* SPN1T and *Spirochaeta* sp. ACE-P as members of the genus *Sphaerochaeta*.

**Description of Sphaerochaeta gen. nov.**

*Sphaerochaeta* [Sphae.ro.chae.ta Gr. n. sphaira (Latin transliteration sphaera), a sphere; Gr. fem. n. chaiteˆ (Latin transliteration chaeta), long flowing hair; N.L. fem. n. Sphaerochaeta round cells along threads of hair, indicative of a round morphology with a derivation from spirochaetal ancestry].

Spherical cells, 0.4–2.5 μm in diameter. Pleomorphic; cells of different sizes and shapes are observed during growth. Spiral morphology not observed. Cells are non-motile, without flagella, periplasmic or otherwise. Cells stain Gram-negative and do not have ornithine or lysine peptidoglycan cross-links. Mesophilic. Neutrophilic. Chemoheterotrophic anaerobes; fermentative growth is observed with carbohydrates including pentose and hexose monosaccharides, disaccharides and soluble starch. End products of glucose fermentation include acetate, formate and ethanol. Growth is stimulated by yeast extract, Casamino acids and vitamins. DNA G + C content is 45.4–48 mol%. Oxidase- and catalase-negative. Phylogenetic position is in the family *Spirochaetaceae*, order *Spirochaetales*, class *Spirochaetes* of the phylum *Spirochaetes*. *Sphaerochaeta globosa* is the type species.

**Description of Sphaerochaeta globosa sp. nov.**

*Sphaerochaeta globosa* (glo.bo.sa. L. fem. adj. globosa sphere-shaped, reflecting the characteristic morphology, wherein round cells form on spherical 'bubbles' during exponential growth). Has the following properties in addition to those described for the genus. Cells in exponential growth phase occur as spherical aggregates, 3–25 μm in diameter, comprising one to ten cells. A membranous structure surrounds cell aggregates. Cells carry a single, hook-like protrusion. Prefers low-nutrient conditions. Fermentable substrates include D-galactose, D-glucose, D-fructose, D-xylose, sucrose, maltose, lactose, raffinose, melezitose and soluble starch. L-Arabinose is used poorly; D-mannose is not used for growth, nor are glycogen, cellulose, chitin or xylan. Major cellular fatty acids are C14 : 0, C16 : 0, C16 : 1 ω7c, C18 : 1 ω7c, C18 : 1 ω9c and br-C17 : 1 ω7c (Supplementary Table S1). Grows at 20–35 °C, with irreversible inactivation above 40 °C. The DNA G + C content of the type strain is 46.5 ± 0.4 mol%.

The type strain, BuddyT (=ATCC BAA-1886T =DSM 22777T), was isolated from black, anoxic sediments of the Red Cedar River in Okemos, MI, USA (42° 41’ 49.72” N 84° 22’ 43.67” W).

**Description of Sphaerochaeta pleomorpha sp. nov.**

*Sphaerochaeta pleomorpha* (ple’o.mor’pha. Gr. adv. pleon more; Gr. n. morphe shape, form; N.L. adj. pleomorpha having many shapes and sizes, pleomorphic, reflecting the characteristic morphological progression during phases of growth).

Has the following properties in addition to those described for the genus. Chains of cells, 0.8–2 μm in size, predominate during exponential growth. Cells carry a single, hook-like protrusion. Fermentable substrates..
The type strain, Grapes (ATCC BAA-1885T = DSM 22778), was isolated from black, anoxic sediments of the Red Cedar River in Okemos, MI, USA (42° 41’ 49.72” N 84° 22’ 43.67” W).

ACKNOWLEDGEMENTS

We are indebted to John Breznak for encouragement and many helpful discussions, and Notha M. Mesbah for determining the G+C content of isolates Buddy and Grapes. Appreciation to all the microbiologists who participated in imaging, particularly Shirley Owens, Jeanette Taylor, and the late Rob Apkarian. Thanks also to Jarrod Pollock for help with the iron analysis. This material is based upon work supported by the National Science Foundation under grant no. 0919251.

REFERENCES


