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ORIGINAL PAPER

Sinorhodobacter hungdaonensis sp. nov. isolated from activated sludge collected from a municipal wastewater treatment plant

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Abstract A novel bacterium, strain L3^T, was isolated from an activated sludge sample retrieved from a municipal wastewater treatment plant in Huangdao, China. On the basis of 16S rRNA gene sequence similarity studies, strain L3^T was affiliated to the genus *Sinorhodobacter*, being most closely related to *Sinorhodobacter ferrireducens* (98.0 %). The 16S rRNA gene sequence similarity of strain L3^T to other related species, *Thioclava atlantica* DLFJ1-1^T (96.5 %), *Rhodobacter capsulatus* ATCC 11166^T (96.3 %), *Paenirhodobacter enshiensis* DW2-9^T (96.3 %) and *Rhodobacter viridis* JA737^T (96.0 %) is less than 96.5 %. Chemotaxonomic characterization further supported classification of the strain to the genus *Sinorhodobacter*. The major polar lipid profile consists of diphosphatidyglycerol, phosphatidylglycerol and phosphatidylethanolamine. The major fatty acids are $C_{18:1}$ ω7c (66.3 %), $C_{16:0}$ (12.9 %) and $C_{18:0}$ (8.0 %). The major quinone is Q-10. The G+C content of the genomic DNA of strain L3^T is 68.0 mol %. DNA–DNA relatedness value between L3^T and the closely related type strain *S. ferrireducens* SgZ-3^T was 35.2 %. Based on these results, a new species *Sinorhodobacter huangdaonensis* is proposed. The type strain is L3^T (= CGMCC 1.12963^T = KCTC 42823^T).

Keywords Activated Sludge · Polyphasic · Rhodobacteraceae · *Sinorhodobacter huangdaonensis* · New species

Introduction

The proposal of new genus *Sinorhodobacter* was based on the differential phenotypic including the lack of phototrophic growth and the absence of bacteriochlorophyll α and carotenoids, phylogenetic, and chemotaxonomic properties of the in type strain SgZ-3^T (Yang et al. 2013). However, the proposed genus and species names are yet to be validated.

During an investigation of the bacterial composition of activated sludge from a municipal wastewater treatment plant, we isolated strain L3^T, which showed morphological characteristics typical to the genus *Sinorhodobacter*. In this paper, we report the taxonomic characterization of strain L3^T as a novel species of the genus *Sinorhodobacter*. An emended description of the genus *Sinorhodobacter* is also proposed.

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Materials and methods

Bacterial strain isolation and growth conditions

Strain L3^T was isolated from an activated sludge sample, which was collected from a municipal wastewater treatment plant in Qingdao, China (36°35′ 16.04″N 120°10′ 40.89″E) in October 2014. The sediment sample was well-mixed at room temperature, serially diluted, and plated on yeast extract-malt extract agar [ISP 2, medium 2 of the International Streptomyces Project (Küster 1959)]. After 5 days of incubation at 30 °C, the strain L3^T was isolated from a single colony and purified on ISP 2 medium.

Type strain *S. ferrireducens* SgZ-3^T was used for comparative purposes in this study and was kindly provided by Prof. Yuan of Guangdong Institute of Eco-Environmental and Soil Sciences, Guangzhou, China.

Phenotypic and chemotaxonomic analysis

Cell growth under anaerobic and photo-organoheterotrophic conditions was tested according to the method described by Yang et al. (2013). The in vivo absorption spectrum of intact bacteria cells in 60 % sucrose (w/v) and the absorption spectrum of pigments extracted with acetone were recorded using UV-2450 Visible spectrophotometer (Shimadu, Japan). Cultural and morphological characteristics of strain L3^T were observed on tryptic soy agar (TSA, Oxoid) or in tryptic soy broth (TSB, Oxoid) following incubation for 2–7 days at 30 °C. Cell morphology was investigated using a transmission electron microscope (JEM-1400, JEOL). The Gram-reaction was determined by the conventional Gram staining method (Smibert and Krieg 1994). Media and procedures used to study physiological and biochemical features (growth conditions, catalase, β-galactosidase, urease, oxidase, esterase, arginine dihydrolase, tryptophan dehydrogenase, gelatin hydrolysis, H_2S production, nitrate reduction, acid formation, and carbon assimilation) were as described elsewhere (Dong and Cai 2001). The reduction of Fe^{3+} was monitored by measuring the change in the total Fe^{2+} concentration (Wu et al. 2010). Biomass for fatty acid analysis was collected by scraping colonies from TSA medium after strain L3^T was grown at 30 °C for 3 days. Biomass for other chemotaxonomic studies was obtained after strain L3^T was grown TSB at 30 °C for 3–7 days in a rotary shaker (model, brand). Standard analytical procedures were used to extract and analyze the isomeric forms of quinones (Collins et al. 1985, 1977; Tamaoka 1986) and polar lipids (Collins and Jones 1980; Minnikin et al. 1979). Fatty acids were analyzed using the standard MIDI (Microbial Identification, Sherlock version 6.0) procedure (Sasser, 1990) and an Agilent GC 6890 gas chromatograph. The resulting profiles were identified using the database TSBA6, version 6.0. The G+C content of genomic DNA was determined by using the HPLC method (Jin and Komagata 1984).

Phylogenetic analyses

Extraction of genomic DNA was performed as described by Chun and Goodfellow (1995). PCR amplification of the 16S rRNA gene was performed using a bacterial universal primer set (27F and 1492R) (Lane 1991). The PCR product was purified using the TIANgel Midi Purification Kit DP209 (Beijing, China), ligated into the PMD19-T vector and transformed into *Escherichia coli* DH5α using a PMD19-T cloning kit (Takara Bio). The 16S rRNA gene sequence was determined by Sangon Biotech (Shanghai, China). The isolate was identified using the EzTaxon server on the basis of 16S rRNA sequence data (Kim et al. 2012). Phylogenetic trees were constructed using MEGA version 6.0 (Tamura et al. 2013) based on approximately 1350 nucleotides from each related type strain (retrieved from the GenBank/EMBL/DDBJ database) mentioned in this study. *Rhodospirillum rubrum* ATCC 11170^T was used as an out-group. The neighbour-joining (NJ) method (Saitou and Nei 1987), maximum-parsimony (MP) (Fitch 1971) and maximum-likelihood (ML) (Felsenstein 1981) were used for phylogenetic analysis. The topologies of the phylogenetic trees were determined using bootstrap analyses (Felsenstein 1985) based on 1000 replications.

DNA–DNA relatedness

The DNA G+C content was determined using the thermal denaturation method (Marmur and Doty 1962) using *Escherichia coli* K12 as calibration standard. DNA–DNA hybridizations between isolate L3^T and *S. ferrireducens* SgZ-3^T were performed using the liquid

renaturation method (De Ley et al. 1970) and according to modification described by Huss et al. (1983). DNA–DNA hybridizations were carried out in 2× SSC at 81 °C. Both experiments were performed at 260 nm with a model Lambda 35 UV/VIS spectrometer equipped with a Peltier System (PTP 1+1) (Perkin–Elmer). The hybridization value was calculated from triplicate experiments.

Results and discussion

Analysis of the almost-full length 16S rRNA gene sequence (1435 bp) confirmed that strain L3^T belongs to the genus *Sinorhodobacter*. It formed a coherent cluster (bootstrap value of 100 %) with type strain *Sinorhodobacter ferrireducens* (98.0 %), and grouped together with *Thioclava atlantica* DLFJ1-1^T (96.5 %), *Rhodobacter capsulatus* ATCC 11166^T (96.3 %), *Paenirhodobacter enshiensis* DW2-9^T (96.3 %) and *Rhodobacter viridis* JA737^T (96.0 %) as shown on the phylogenetic trees (NJ tree (Fig. 1), MP tree (Supplementary Fig. S1) and ML tree (Supplementary Fig. S2)). The 16S rRNA gene sequence similarity between this strain and other phylogenetically related species is <96.0 %.

The morphological and physiological properties of the new bacterium are given in species description and summarized in Table 1. The G+C content of the genomic DNA of strain L3^T is found to be 68.0 mol %. The DNA–DNA hybridization experiments revealed that strain L3^T shared 35.2 ± 5.1 % DNA relatedness with *S. ferrireducens* SgZ-3^T. This value is notably lower than the 70 % cut-off value recommended for species differentiation (Wayne et al. 1987); this result confirmed that L3^T can be considered as a distinct species of the genus *Sinorhodobacter*.

The chemotaxonomic characteristics include the presence of the principal quinone Q-10, which is typical quinone reported for the genera *Sinorhodobacter* (Yang et al. 2013), *Rhodobacter* (Imhoff et al. 1984) and *Thioclava* (Sorokin et al. 2005); the major fatty acids such as $C_{18:1}$ ω7c (66.3 %, 66.7 %), $C_{16:0}$ (12.9 %, 11.7 %), $C_{18:0}$ (8.0 %, 6.8 %), $C_{10:0}$ 3-OH (4.3 %, 5.7 %), $C_{18:0}$ 3-OH (4.3 %, 3.7 %) and $C_{12:0}$ 3-OH (1.2 %, 1.5 %), which are detected in both strain L3^T and *S. ferrireducens* SgZ-3^T.

The polar lipid profile of strain L3^T was found to contain diphosphatidyglycerol, phosphatidylglycerol, phosphatidylethanolamine, unidentified aminophospholipid and aminolipid, and lacking diphosphatidyglycerol. This phospholipids composition is characteristic for bacteria of the genus *Sinorhodobacter* (Fig. S3). The lack of diphosphatidylglycerol is a distinct feature of strain L3^T and *S. ferrireducens* SgZ-3^T and could be used to distinguish bacteria of this genus from other phylogenetically related bacteria (Dan et al. 2013; Lai et al. 2014; Raj et al. 2012; Sorokin et al. 2005). Consequently, strain L3^T and SgZ-3^T demonstrate genus level polar lipid production characteristics; thus, these results support the assignment of strain L3^T to the genus *Sinorhodobacter*.

Based on the results of the phenotypic, phylogenetic, chemotaxonomic analysis and DNA–DNA relatedness, it can be concluded that strain L3^T represents a novel species within the genus *Sinorhodobacter*, for which we propose the name *Sinorhodobacter huangdaonensis* sp. nov.

Emended description of the genus *Sinorhodobacter*

Iron may or may not be reduced from Fe^{3+} to Fe^{2+} by members of this genus. The major polar lipid profile contains the compounds diphosphatidyglycerol, phosphatidylglycerol and phosphatidylethanolamine.

Description of *Sinorhodobacter huangdaonensis* sp. nov.

Sinorhodobacter huangdaonensis (huang.dao.neńsis. N.L. masc. adj. Huangdaonensis of Huangdao, a district of Qingdao city in Shandong province, PR China, where the type strain was first isolated).

Cells are motile, Gram-negative, and ovoid to short rod-shaped, about 0.3–0.6 μm wide and 0.8–1.5 μm long (Fig. 2). Aerobic. Chemoorganotroph. On TSA medium forms smooth grey–white colonies with regular edges and are 2–3 mm in diameter after 72 h of incubation at 30 °C. Growth does not occur via anoxygenic photosynthesis. The growth occurs in media with 0–5 % (w/v) NaCl (optimum 0–1 %), at 15–35 °C (optimum 30 °C) and at pH 5.0–7.0 (optimum pH 6.0). Both bacteriochlorophyll α and carotenoids are absent (Supplementary Figs. S4, S5). Positive for catalase, β-galactosidase, urease, arginine dihydrolase, tryptophan dehydrogenase, hydrolysis of gelatin, H_2S production and nitrate reduction and they

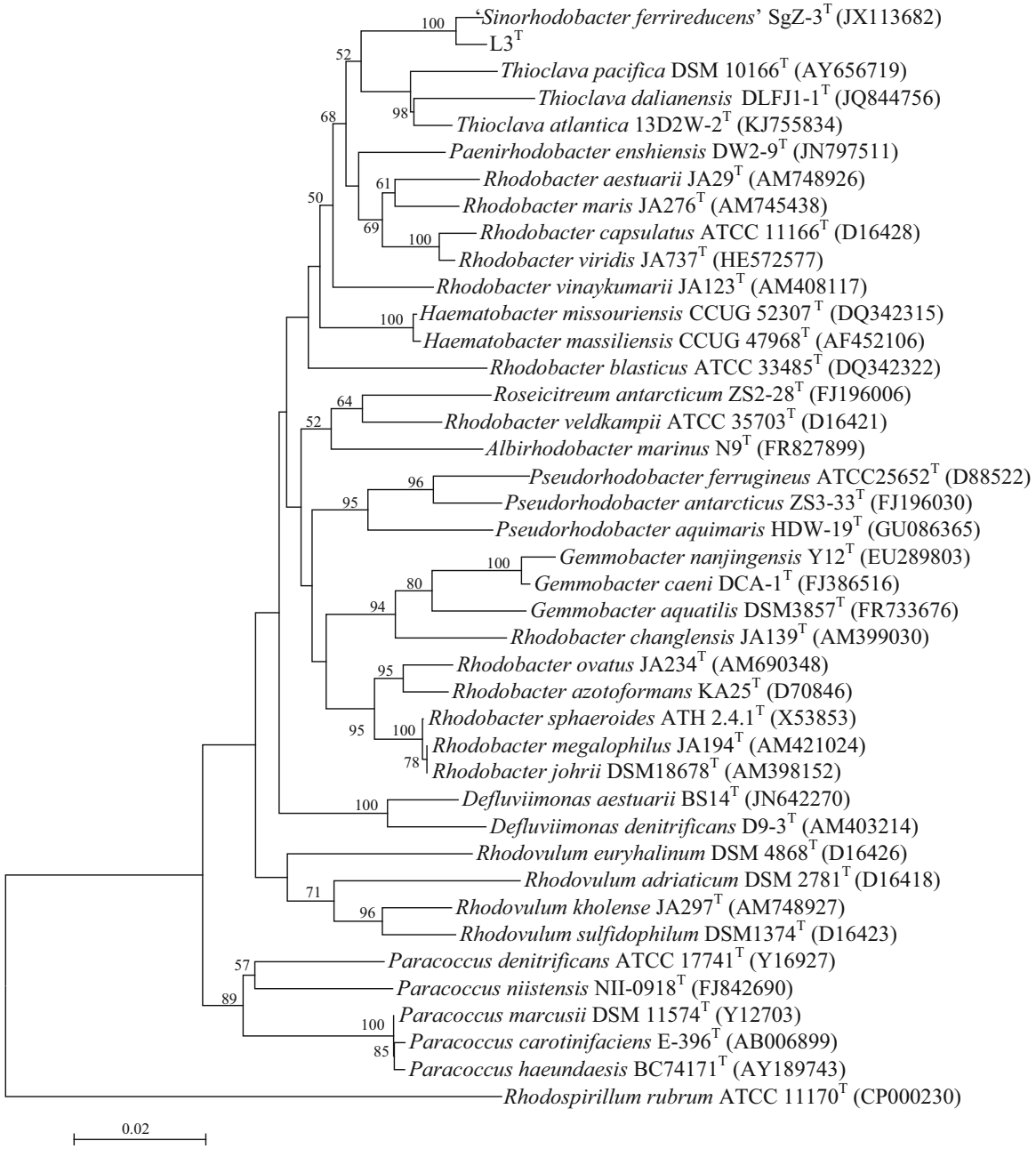

Fig. 1 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain L3^T and representatives of other related taxa. Bootstrap values (Interior Branch Test of Phylogeny, bootstrap = 1000) 50 % are shown at the *branch points*. *Bar*, 0.02 substitutions per nucleotide position

are able to ferment D-glucose. Negative for Fe^{3+} reduction, indole production, acetoin production, oxidase and esterase. Assimilate L-serine, lysine, ornithine, D-maltose, D-saccharose and citric acid. Principal fatty acids are C18:1 ω7c, C16:0 and C18:0 and the major quinone is Q-10. The major polar lipid profile contains diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The G+C content of genomic DNA of the type strain is 68.0 mol %.

Table 1 Comparative phenotypic characteristics of strain $L3^T$ and *S. ferrireducens* $SgZ\text{-}3^T$

	$L3^T$	$SgZ\text{-}3^T$
Cell size (μm)	0.3–0.6 × 0.8–1.5	0.6–1.0 × 1.0–2.0
Fe(III)reducing	−	+
Growth Tm (°C)	15–35	20–40
pH range	5.0–7.0	6.0–8.0
Optimum pH	6.0	7.0
Hydrolysis of gelatin	+	−
Assimilation of		
Inositol	−	+
L-Alanine	−	W
L-Serine	+	W
Lysine	+	−
Ornithine	+	−
L-Arabinose	−	+
Propionic acid	−	+
Valeric acid	−	+
Trisodium citrate	−	+
DNA G+C content (mol %)	68.0	68.6

All data are from this study and were obtained in the same experiment under identical growth conditions

+ positive; − negative; *W* weakly positive

The identical phenotypic characteristics of strain $L3^T$ and *S. ferrireducens* $SgZ\text{-}3^T$ are Anoxygenic photosynthesis (−), Cell shape (Ovoid to rod-shaped), Flagella (Peritrichous), Motility (+), Nitrate reduction (+), Gram staining (−), Growth in 3 % NaCl (+), Oxidase (−), β-galactosidase (+), Urease (+), Tryptophan dehydrogenase (+), Catalase (+), Esterase (Tween 80) (−), Arginine dihydrolase (+), Indole production (−), VP test (−), H_2S production (+); Acid formation from: D-glucose (+), D-Melibiose (−) and L-Arabinose (−); Assimilation of: D-Maltose (+), D-Mannitol (−), D-Saccharose (+), Rhamnose (−), Ctric acid (+), 3-Hydroxybenzoic acid (−), Amygdalin (−)

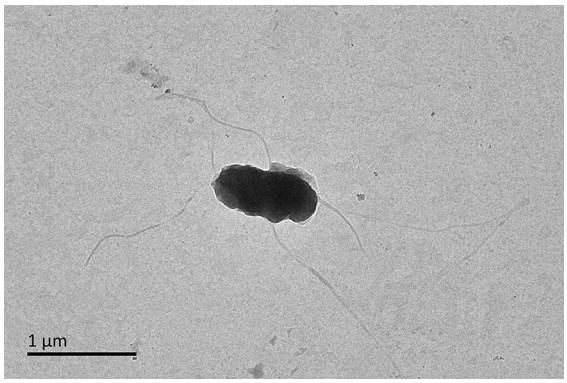

Fig. 2 Transmission electron micrographs of strain $L3^T$ cells showing cell morphology, shape and the flagella

The type strain $L3^T$ (=CGMCC 1.12963^T = KCTC 42823^T), isolated from an activated sludge sample collected from a municipal wastewater treatment plant. The GenBank accession number for the 16S rRNA gene sequence of *S. huangdaonensis* $L3^T$ is KU042973.

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