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TAXONOMIC DESCRIPTION

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Segnochrobactrum spirostomi gen. nov., sp. nov., isolated from the ciliate *Spirostomum yagiui* and description of a novel family, *Segnochrobactraceae* fam. nov. within the order *Rhizobiales* of the class *Alphaproteobacteria*

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Abstract

A bacterial strain, designated Sp-1^T, was isolated from the heterotrich ciliate *Spirostomum yagiui* collected from a reservoir located in Ulsan, Republic of Korea. Cells of Sp-1^T were Gram stain-negative, rod-shaped, non-spore-forming, non-motile and contained poly-β-hydroxybutyrate granules. Phylogenetic analyses based on 16S rRNA gene sequences indicated that Sp-1^T constituted a distinct phylogenetic lineage within different families in the order *Rhizobiales* with a pairwise sequence similarity of 95% to the species of the genus *Ochrobactrum: Ochrobactrum anthropi* ATCC 49188^T and *Ochrobactrum cytisi* ESC1^T (family *Brucellaceae*). The major cellular fatty acids were $C_{19:0}$ cyclo $\omega 8c$ (44.4%) and $C_{16:0}$ (32.1%). The identified sole isoprenoid quinone was ubiquinone-10 (Q-10). The major polar lipids produced were phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, an unidentified aminolipid, two unidentified phospholipids and three unidentified lipids. The genome size was about 5.4 Mbp and the DNA G+C content was 68.2 mol%. Sp-1^T exhibited the highest average nucleotide identity value of 76.6% and *in silico* DNA–DNA hybridization value of 22.1% with *Pseudoxanthobacter soli* DSM 19599^T (family *Xanthobacteraeae*). This strain is distinguishable from closely related members of the order *Rhizobiales* by its differential phenotypic, chemotaxonomic, genomic and phylogenetic characteristics. On the basis of evidence from polyphasic taxonomic analysis, we concluded that Sp-1^T represents a novel species in a novel genus within the order *Rhizobiales*, for which the name *Segnochrobactrum spirostomi* gen. nov., sp. nov. is proposed. The type strain is Sp-1^T (=KCTC 62036^T=JCM 32162^T). We also describe a novel family, *Segnochrobactraceae* fam. nov., to encompass the proposed novel genus and species.

Symbiotic relationship exists between protists and diverse microorganisms, having great influence on their ecology and evolution [1]. Ciliated protists are considered as the great consumers of different microbes, including bacteria, fungi, algae and other protists, in the aquatic environment, and can also evolve various associations under certain circumstances [2–4]. Their coexistence synergistically affects each other's physiology and metabolism [5]. The symbiotic relationship between protists and bacteria is also beneficial, allowing both partners to take advantage by development of new characteristics, adaptation to new environments as well as an important condition favoring their spread in the environment [6]. To date, at least 200 ciliate species containing bacterial symbionts have been reported, which is likely to be only a minuscule piece of a whole [1]. Most of the bacterial symbionts belong to the four classes of the phylum *Proteobacteria*, namely *Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria,* and *Deltaproteobacteria* [7]. The members of the order *Rhizobiales* within the class *Alphaproteobacteria* are highly diverse in nature, ranging from free living to symbiotic and found in diverse habitats including seawater, marine sediments, activated sludge, soil and in association with plants, animals and humans. Several members of this order are pathogenic for humans, animals and plants [8]. Different kinds of bacterial

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Keywords: *Segnochrobactrum*; novel family; novel genus; novel species; ciliate-host; *Spirostomum yagiui*.

Abbreviations: ANI, average nucleotide identity; BI, Bayesian inference; DDH, DNA–DNA hybridization; EMB, eosin methylene blue; ME, minimum evolution; ML, maximum likelihood; MP, maximum parsimony; NJ, neighbor-joining; WGS, whole-genome sequencing.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *atpD* gene sequences of (*Segnochrobactrum spirostomi*) strain Sp-1^T are MF370560 and MK478370, respectively. The genome sequence of Sp-1^T has been deposited at DDBJ/ENA/GenBank under the accession number VWNA00000000. The GenBank/EMBL/DDBJ accession number for the 18S rRNA gene sequence of *Spirostomum yagiui* is MH460446.

Two supplementary figures and three supplementary tables are available with the online version of this article.

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symbionts with *Holospora*-like bacteria have been found in species of the genus *Spirostomum* [4, 9]. In an attempt to study the culturable microbial diversity associated with the ciliate, a novel bacterial strain, Sp-1^T, was isolated from the heterotrich ciliate *Spirostomum yagiui*.

Samples of *Spirostomum yagiui* were collected from a freshwater reservoir in Jeong gol, Daehak-ro, Nam-gu, Ulsan, Republic of Korea (35°32′35.2″N 129°14′51.2″E). Initially, the ciliate cells were identified as *Spirostomum yagiui* by morphological analyses [10]. A monoclonal xenic culture of the ciliate *Spirostomum* was established and maintained using the method described previously [11–14]. Ciliate cells from monaxenic culture were transferred into sterilized source water without food for starving for 8–9 h. To remove bacterial contamination or non-symbiotic bacteria, the starved ciliate cells were washed with sterile source water containing the antibiotics penicillin G at 5000 U ml^{-1}, neomycin sulfate at 50 µg ml^{-1} [15] and streptomycin at 4000 µg ml^{-1} [16]. Finally starved sterile ciliate cells were washed several times with sterilized source water and used for further analyses.

The starved, sterile ciliate cells from the *Spirostomum* culture were used for the identification of ciliate-associated culturable bacteria. The ciliate cells were mechanically lysed using short pulses of vortexing, and spread on nutrient agar (NA), tryptic soy agar (TSA), and MuellerHinton agar (MHA) plates (all from Sigma-Aldrich), and incubated at 20 °C, 25 °C and 37 °C for 15 days. Bacterial growth appeared after 5 days of incubation at 25 °C. A colony on TSA at 25 °C was selected for further characterization. Strain Sp-1^T was maintained on TSA plates in a refrigerator at 4 °C and preserved in LuriaBertani broth supplemented with 30% (v/v) glycerol at −80 °C.

Morphological, biochemical and physiological tests were performed with Sp-1^T grown on TSA medium for 7 days incubation at 28 °C. Colony morphology on NA, TSA, MHA, MacConkey agar, and eosin methylene blue (EMB) agar medium (all from Sigma–Aldrich) was checked after growth aerobically for 7 days at 28 °C. The two most closely related species of the genus *Ochrobactrum* (family *Brucellaceae*), the type strains of *O. anthropi* ATCC 49188^T=KACC 11936^T and *O. cytisi* ESC1^T for phenotypic characterization and fatty acid analyses and the type strains of two closely related species, *Pseudoxanthobacter soli* CC4^T=DSM 19599^T (family *Xanthobacteraeae*) and *Kaistia adipata* Chj404^T=KCTC 12095^T (family *Rhizobiaceae*) for comparative analyses of phenotypic properties were selected as reference strains. Growth of SP-1^T and reference strains was observed on NA, TSA, MHA, MacConkey agar, and EMB agar medium (all from Sigma–Aldrich) after incubation for 15 days at 28 °C. Cell morphology of Sp-1^T and KACC 11936^T were observed using an optical microscope (Axio Imager A1; Carl Zeiss) with a differential interference contrast device using 24 h culture in tryptic soya broth (TSB). Gram type of Sp-1^T and reference strains were examined using a Gram staining kit (bioMérieux) according to the manufacturer's instructions. The test for motility was carried out according to MacFaddin's [17] method. Cells grown in TSB and acetic acid medium (g l^{-1}:10.9 $CH_3COONa.3H_2O$, 1.6 NH_4Cl, 0.8 K_2HPO_4, 0.3 KH_2PO_4, 0.4 $MgSO_4$.7H2O) were tested for the poly-β-hydroxybutyrate granule accumulation using an optical microscope (Axio Imager A1; Carl Zeiss) with a differential interference contrast device after staining of the cells with Sudan black. Poly-β-hydroxybutyrate granule accumulation was also observed under a fluorescence microscope (Axioskop 2 plus; Carl Zeiss) after the staining of the cells with acridine orange [18]. For checking the growth temperature range and the optimal growth temperature cultures were incubated at 4–46 °C on NA and TSA medium for up to 15 days. The tolerable range of pH and optimum pH were determined on NA at pH 4.0–12.0 (in increments of 0.5 pH units) adjusted by the addition of 10 M NaOH or HCl with citric acid/sodium citrate buffer (for pH 4.0–5.0), KH_2PO_4/K_2HPO_4 buffer (pH 5.5–8.0), $NaHCO_3$/Na_2CO_3 buffer (pH 8.5–10), Na_2CO_3/NaOH buffer (pH 10.5–11), and KCl/NaOH buffer (pH 11.5–12) prior to sterilization. The tolerance to NaCl was tested on NA supplemented with different salt concentrations (0–6%, w/v at intervals of 0.5%) at 28 °C for up to 15 days incubation. The catalase activity was assessed by bubble production on slide (drop) and direct colony methods after the addition of 3% (v/v) hydrogen peroxide. The oxidase activity was confirmed using an oxidase reagent (REF55635; bioMérieux) according to the manufacturer's instructions and using Kovacs' solution [19] as described previously [20, 21]. The oxygen requirements of Sp-1^T were tested using thioglycolate broth (Sigma-Aldrich) in accordance with the manufacturer's recommendations. The following tests were performed with the procedures described previously [20–23]: presence of spores; temperature tolerance; nitrate reduction; decarboxylase-dehydrolase activity; urease activity; gelatinase; indole production; methyl red and Voges–Proskauer reactions; H_2S production; reactions on Kligler iron agar (KIA); hydrolysis of starch, aesculin, casein, Tween 20 and 80. Acid production from carbohydrates was tested in OF basal medium [24] supplemented with 1% (w/v) of the desired carbohydrate solutions. Sole carbon source utilization medium (pH 7.2) contained the following, supplemented with filter-sterilized 1% carbon source (%): $(NH_4)_2SO_4$, 0.2; K_2HPO_4, 0.024; $MgSO_4.7H_2O$, 0.024; KCl, 0.01; yeast extract, 0.01; agarose, 1.5. Negative control media did not contain any added carbon source. A positive control culture was grown on TSA medium. Acid production and carbon source utilization were recorded after 7 and 15 days incubation at 28 °C. API 20E test strips (bioMérieux) was used to assess the β-galactosidase activity (ONPG), tryptophan deaminase activity and citrate utilization after incubation of up to 7 days at 28 °C following the manufacturer's instructions using a saline solution (0.85%) for the preparation of the inocula. The susceptibility of the novel strain Sp-1^T to various antibiotics was determined using inhibition zone diameters around the discs according to the Kirby–Bauer disc diffusion susceptibility test protocol [25] on MHA plates (Sigma-Aldrich) at 28 °C for 2 and 5 days using the following antibiotic discs: ampicillin (10 µg), gentamicin (10 µg), kanamycin (30 µg), penicillin G (10 U), streptomycin (10 µg), doxycycline (30 µg), and tetracycline (3 µg). Susceptibility to the vibriostatic compound O/129 (150

μg; Oxoid) was measured on TSA (Sigma-Aldrich) plates at 28 °C for 2 and 5 days.

The ciliate host was identified as *Spirostomum yagiui* based on morphological properties [10]. The morphological observations were congruent with the results obtained with the phylogenetic analyses of the 18S rRNA gene sequences (data not shown).

The cells of both strains were rod-shaped, Gram-staining-negative and non-spore forming. Cells of Sp-1^T were 1.5–3.1 μm in length and 0.8–1.2 μm in width whereas those of KACC 11936^T were 1.5–2.6 μm length and 0.7–0.9 μm width. Detailed phenotypic characteristic of the culturable bacterial strain Sp-1^T associated with *Spirostomum yagiui* are included in Table S1, (available in the online version of this article) and in the genus and species descriptions. Several phenotypic characteristics differentiated Sp-1^T from other phylogenetically closely related genera are presented in Table S1. Moreover, comparison of different phenotypic characteristics of Sp-1^T and reference strains (ATCC 49188^T, ESC1^T, CC4^T and Chj404^T) is provided in Table 1. Sp-1^T was sensitive to the vibriostatic agent O/129, ampicillin (10 μg), gentamicin (10 μg), kanamycin (30 μg), penicillin G (10 U), streptomycin (10 μg), doxycycline (30 μg), and tetracycline (3 μg).

Extraction of total genomic DNA from Sp-1^T was conducted using the REDExtract-N-Amp Tissue PCR Kit (Sigma) following the manufacturer's recommendations. The amplification of the 16S rRNA gene with the PCR was carried out using the universal bacterial primer set 27F and 1492R [26]. The PCR using a high-fidelity TaKaRa ExTaq DNA polymerase Kit (TaKaRa Bio-medicals) according to the manufacturer's instructions was accomplished as described by Gong *et al.* [7]. The PCR product was purified and directly sequenced in both directions using an ABI 3730 automatic sequencer (Macrogen), using the same pair of PCR primers for sequencing to obtain the almost complete sequence of the 16S rRNA gene. The *atpD* gene was amplified using the set of primers atpD-273F and atpD-771R [27]. For phylogenetic analyses, the obtained sequences of the 16S rRNA and *atpD* genes of Sp-1^T were compared with those in GenBank by using BLASTn [28] to get the idea its phylogenetic neighbours. The 16S rRNA gene sequence was also employed by comparing it with those of the type strains of the species with validly published names in the EzBioCloud database server [29] to identify its phylogenetic neighbours and calculate the pairwise 16S rRNA gene sequence similarity. The 16S rRNA and *atpD* gene sequences of related taxa were retrieved from the GenBank database. For the sequences of 16S rRNA and *atpD* genes of Sp-1^T and related taxa, the alignment was performed using the ClustalW feature, version 1.6, in MEGA software, version 6.06 [30]. The position of Sp-1^T in the phylogenetic consensus trees based on the aligned 16S rRNA and *atpD* gene sequences was determined by reconstructing it using the neighbor-joining (NJ) [31], minimum evolution (ME) [32], and maximum parsimony (MP) [33] methods using MEGA, version 6.06 [30], the maximum likelihood (ML) method using PhyML, version 3.0 [34], and the Bayesian inference (BI) method using MrBayes, version 3.2.1 [35]. The evolutionary distances were calculated according to DNA substitution model Kimura's two-parameter model with pair-wise deletion [36] using MEGA, version 6.06 [30]. The confidence levels of the nodes in the NJ, ME, MP, and ML trees were assessed using the bootstrap method with 1000 replications [37].

The 16S rRNA gene sequence comparison against the EzBioCloud database revealed that Sp-1^T was most closely related to *O. anthropi* ATCC 49188^T and *O. cytisi* ESC1^T (from the family *Brucellaceae*) with 95% 16S rRNA gene sequence similarity. The novel strain was also related to the members of the order *Rhizobiales: Brucella* with maximum of 94.8% 16S rRNA gene sequence similarity and *Mycoplana* with 94.7% (from the family *Brucellaceae*), *Pseudoxanthobacter* with 94.8% (*Xanthobacteraeae*), *Aquamicrobium* with 94.8%, *Mesorhizobium* and *Tianweitania* with 94.7% and *Phyllobacterium* with 94.3% (*Phyllobacteriaceae*) and *Ensifer* with 94.4% (*Rhizobiaceae*). The 16S rRNA gene sequence similarities to the type strains of the other species in the order *Rhizobiales* were below 94.3%. A sequence similarity value of 95% or lower for two 16S rRNA genes is widely used as a cut-off value for assigning a strain to a particular genus [38]. Consequently, similarities between Sp-1^T and closely related species indicated that Sp-1^T should be treated as a novel taxon beyond the genus level. Phylogenetic consensus trees (Figs 1 and S1) based on the 16S rRNA and *atpD* gene sequences confirmed a fair affiliation between Sp-1^T and the members of the order *Rhizobiales* with validly published names. Although Sp-1^T shared the highest 16S rRNA gene sequence similarity with the members of the family *Brucellaceae*, in the phylogenetic consensus tree it constituted an independent evolutionary lineage within a cluster containing the members of the family *Xanthobacteraeae: Xanthobacter*, *Pseudoxanthobacter*, *Labrys*, *Pseudolabrys*, *Azorhizobium*, *Ancylobacter* and *Starkeya*, and the family *Rhizobiaceae: Kaistia* within the order *Rhizobiales* (Fig. 1). The 16S rRNA gene sequence similarity values to related taxa (95% or less) and the distinct phylogenetic position indicated that Sp-1^T could be treated as a unique taxonomic representative of a novel genus of a novel family within the order *Rhizobiales*. Previously, the housekeeping gene *atpD* has been evaluated as a phylogenetic tool in the taxonomic characterization of rhizobia [27]. Phylogenetic analysis of the *atpD* gene sequence indicated that Sp-1^T formed a robust clade with *P. soli* DSM 19599^T (family *Xanthobacteraeae*), having 90.9% sequence similarity (Fig. S1). The sequence similarities between Sp-1^T and members of different genera within the order *Rhizobiales* were low enough to prevent the assignment of strain Sp-1^T to any of the recognized species or genera.

The whole-genome sequencing and analyses were performed at ChunLab (Seoul, Republic of Korea). The genomic DNA (gDNA) was isolated from the cultured type strain (Sp-1^T) (sample volume ≥0.75 ml) using the FastDNASpin Kit for Soil (MP biomedicals). The integrity of gDNA was checked by running an agarose gel electrophoresis and gDNA was quantified using Quant-IT PicoGreen (Invitrogen). The

Table 1. Characteristics that differentiate strain Sp-1ᵀ from the type strains of *Ochrobactrum anthropi*, *O. cytisi*, *Pseudoxanthobacter soli* and *Kaistia adipata*

Taxa: 1, Strain Sp-1ᵀ; 2, *Ochrobactrum anthropi* ATCC 49188ᵀ=KACC 11936ᵀ; 3, *O. cytisi* ESC1ᵀ; 4, *Pseudoxanthobacter soli* CC4ᵀ=DSM 19599ᵀ; 5, *Kaistia adipata* Chj404ᵀ=KCTC 12095ᵀ; All data were from this study unless indicated. +, Positive; w+, weakly positive; –, negative, ND, not detected.

Characteristics	1	2	3	4	5
Cell shape and size (µm) (length × diameter)	1.5–3.1×0.8–1.2	1.5–2.6×0.7–0.9	ND	2.0–2.2×0.2–0.3*	0.7–0.9†
Motility	–	+	+	+	–
Optimum growth temperature range (°C)	25–30	20–37	25–30	37	37
Growth on MacConkey agar	–	+	+	+	+
Colonies' texture on TSA	Smooth	Smooth	Mucoid	Smooth	Smooth
Accumulation of poly-β-hydroxybutyrate acid	+	–	–	+	–
Reduction of nitrates	–	+	+	+	+
Indole production	–	–	+	–	–
Urease	+	+	–	+	+
Voges–Proskauer	–	–	+	+	–
Citrate utilization	+	+	+	–	–
ONPG	–	–	–	–	+
Hydrolysis of:					
Gelatin	–	–	–	–	+
Starch	–	–	–	–	+
Aesculin	+	–	+	–	+
Tween 20	+	–	–	–	–
Lysine decarboxylase	+	–	–	–	–
Arginine dihydrolase	+	–	–	+	–
Ornithine decarboxylase	+	–	–	w+	–
Acid production from:					
D-glucose	+	+	+	+	–
Sucrose	–	+	+	–	–
D-mannitol	–	–	+	+	–
L-arabinose	+	+	+	–	–
Maltose	–	+	+	–	+
D-mannose	+	+	+	–	–
D-galactose	+	w+	+	+	–
myo-Inositol	w+	–	w+	–	–
D-sorbitol	+	–	–	–	–
Cellobiose	–	+	+	–	–
L-rhamnose	+	w+	+	–	–
Glycerol	–	+	+	–	–
Utilization of:					

Continued

Table 1. Continued

Characteristics	1	2	3	4	5
Sucrose	–	+	+	–	+
D-mannitol	w+	+	+	+	+
Maltose	–	+	+	–	+
Lactose	–	–	–	–	+
Cellobiose	w+	+	+	–	+
Melibiose	–	–	w+	–	+
D-amygdalin	–	+	–	–	–
DNA G+C content (%; from whole-genome sequencing)	68.2	57.2‡	56.4§	68.4*	67.4†

*Data from Arun *et al.* [56]
†Data from Im *et al.* [57]
‡Data from Holmes *et al.* [58]
§Data from Zurdo-Piñeiro *et al.* [59]

sequencing libraries were then prepared according to the manufacturer's instructions with 20 kb template preparation using BluePippinSize-Selection System using PacBio DNA Template Prep Kit 1.0. The libraries were quantified using Quant-IT PicoGreen (Invitrogen) and quality was measured using a high-sensitivity DNA chip (Agilent Technologies). Subsequently the libraries were sequenced using a PacBio RS-II platform. The whole genome of Sp-1^T was reconstructed and assembled *de novo* with PacBio SMRT Analysis 2.3.0 using the HGAP2 protocol (Pacific Biosciences). The average nucleotide identity (ANI) and *in silico* DNA–DNA hybridization (DDH) were calculated by using Average Nucleotide Identity calculator [39] and Genome-to-Genome Distance Calculator [40], respectively. For the determination of the G+C content of the genomic DNA, Sp-1^T was grown on TSA at 28 °C for 5 days. Genomic DNA was prepared from the strain using the REDExtract-N-Amp Tissue PCR Kit (Sigma). The DNA G+C content of Sp-1^T was determined by the reversed-phase HPLC [41]. DNA from *Escherichia coli* (Sigma) was used as a standard and for calibration. HPLC analysis was performed with two replications for the type strain. The mean of the two values was quoted as DNA G+C content (mol%).

The genome size was about 5.4 Mbp with five contigs (all >1000 bp, N50 was 4137067 bp). The genomic DNA G+C content of Sp-1^T calculated from the genome sequence was found to be 68.2 mol%, which was in very good agreement with the value (68.1 mol%,) obtained by reversed-phase HPLC. The DNA G+C content is higher than that of the members of the closely related families *Brucellaceae*, *Rhizobiaceae* and *Phyllobacteriaceae* [42–45]. Though the DNA G+C content of Sp-1^T was within the range reported for members of the family *Xanthobacteraeae* [46], this value is lower than that of members of the phylogenetically closely related genus *Pseudoxanthobacter* and higher than that of members of the genus *Pseudolabrys* (Table S1). The sequence similarity of the 16S rRNA gene revealed that the members of the family *Brucellaceae* were the nearest neighbours identified, while the 16S rRNA gene tree indicated that Sp-1^T was phylogenetically close to the members of *Rhizobiaceae* and *Xanthobacteraeae*. However, genomic data (Table S2) and phylogenetic analyses based on *atpD* genes (Fig. S1) indicated the nearest neighbour to be *P. soli* DSM 19599^T of the family *Xanthobacteraeae* with the highest ANI value of 76.6% and DDH value of 22.1%. The ANI value and *in silico* DDH value were significantly lower than the threshold value (ANI, 94–96% and *in silico* DDH 70%) for prokaryotic species delineation [47, 48].

For fatty acid profile analysis, cells of SP-1^T, *O. anthropi* ATCC 49188^T and *O. cytisi* ESC1^T were grown on tryptic soy broth agar at 28 °C for 5 days . Cellular fatty acids were prepared according to the standard protocol described by the Microbial Identification System (MIDI) and analyzed by gas chromatography using the Sherlock Microbial Identification System (version 6.3) in combination with the TSBA6 6.21 library [49]. For analysis of isoprenoid quinone and polar lipids, cells of Sp-1^T were grown at 28 °C on TSA for 5 days. Extracted isoprenoid quinones were analyzed by HPLC as described by Tamaoka *et al.* [50]. The polar lipids were extracted then examined by two-dimensional chromatography [51].

The major cellular fatty acids detected in Sp-1^T were $C_{19:0}$ cyclo ω8*c* (44.4%) and $C_{16:0}$ (32.1%); other cellular fatty acids are listed in Table S3. Major cellular fatty acids had a characteristic pattern in Sp-1^T, which was distinct from that of the vast majority of the genera within the families *Brucellaceae*, *Xanthobacteraeae*, *Rhizobiaceae* and *Phyllobacteriaceae* in the order *Rhizobiales*, as the major cellular fatty acids of these genera were $C_{18:1}$ω7*c* (Table S1) [42–44, 46]. Sp-1^T was also differentiated from the other genera within the families *Brucellaceae* and *Xanthobacteraeae* by the absence of major fatty acids $C_{18:1}$ and $C_{16:1}$ [42, 46] and from the genera *Pseudoxanthobacter* and *Pseudolabrys* by the absence of the minor fatty acids $C_{19:0}$, iso-$C_{15:0}$, iso-$C_{15:0}$ 3-OH, iso-$C_{17:1}$ω7*c* and

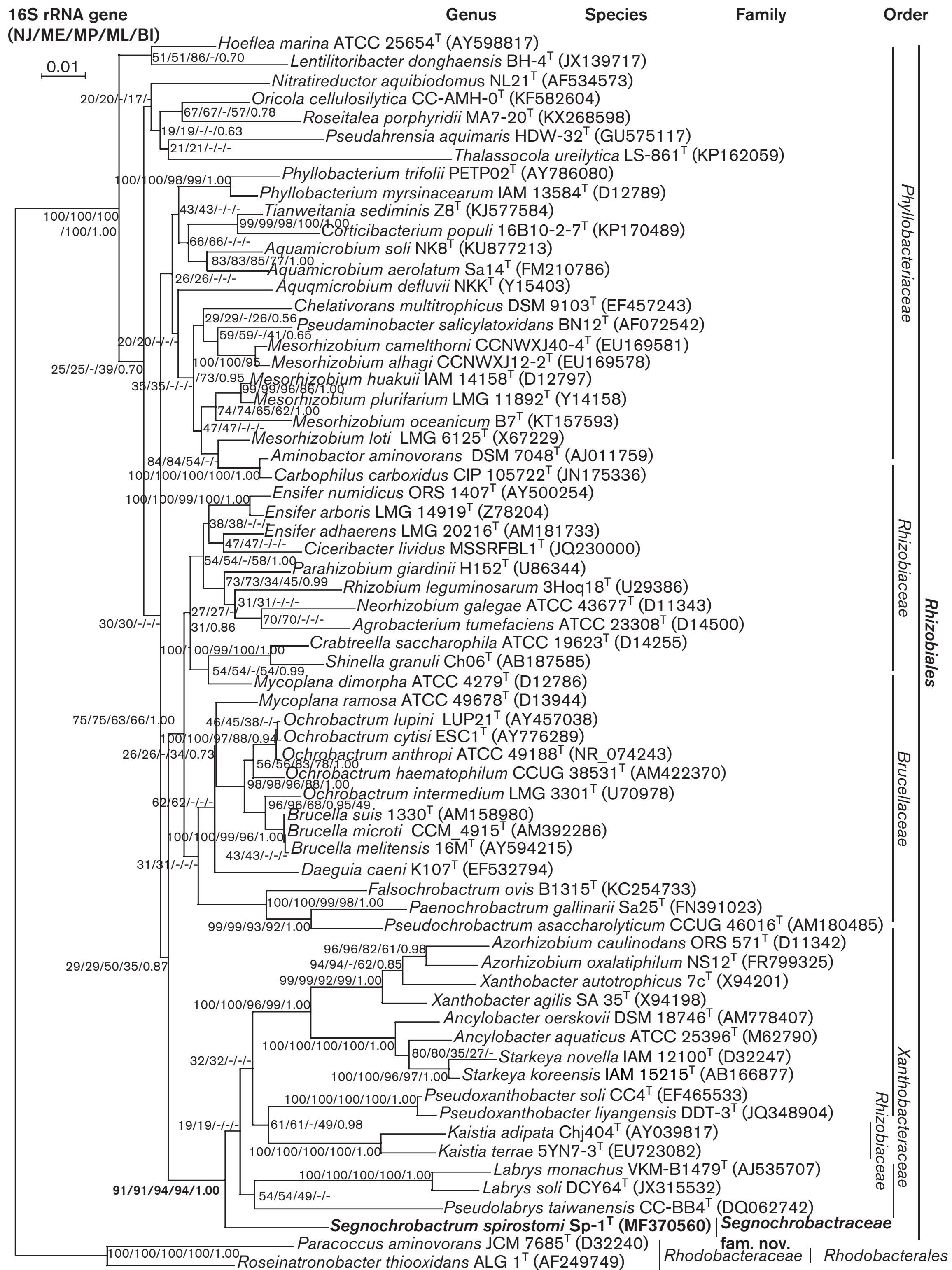

Fig. 1. Phylogenetic consensus tree based on 16S rRNA gene sequences showing the phylogenetic relationship of strain Sp-1^T among the most closely related species of the order *Rhizobiales*. The tree was reconstructed based on the neighbor-joining (NJ), minimum evolution (ME), maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) methods, and the numbers at the nodes represent bootstrap values for the NJ, ME, MP and ML analyses (based on 1000 resamplings) and posterior probability for the BI analyses. *Paracoccus aminovorans* JCM 7685^T (D32240) and *Roseinatronobacter thiooxidans* ALG 1^T (AF249749) of the family *Rhodobacteraceae* of the order *Rhodobacterales* served as the multiple outgroups. The sequence of Sp-1^T is indicated in bold type. Bar, 0.01 substitutions per nucleotide position.

iso-$C_{17:0}$ [52, 53]. Furthermore, the cellular fatty acid profile readily distinguished Sp-1^T from the phylogenetically closest relatives *O. anthropi* ATCC 49188^T and *O. cytisi* ESC1^T in the amount of $C_{19:0}$ cyclo *ω*8*c* and $C_{16:0}$, which were higher in the novel isolate, the amount of summed feature 8, which was lower, the presence of several minor fatty acids, such as $C_{16:0}$ 3-OH, $C_{16:1}$ω11*c* and summed feature 5, and the absence of several, such as $C_{17:0}$ and $C_{18:1}$ 2-OH (Table S3). Sp-1^T had ubiquinone-10 (Q-10) as the solitary isoprenoid quinone and phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, an unidentified aminolipid, two unidentified phospholipids, and five unidentified lipids as polar lipids (Fig. S2). The ubiquinone system of Sp-1^T is consistent with those of the examined genera of the families *Brucellaceae*, *Xanthobacteraeae*, *Rhizobiaceae* and *Phyllobacteriaceae* in the order *Rhizobiales*, in which Q-10 was the sole or major ubiquinone [42–44, 46]. Although, phosphatidylcholine, phosphatidylglycerol and phosphatidylethanolamine found in Sp-1^T were also frequently detected in the majority of genera within the families *Brucellaceae*, *Xanthobacteraeae*, *Rhizobiaceae* and *Phyllobacteriaceae* as the major polar lipids [42–44, 46], Sp-1^T can be distinguished from the members of the most closely related genera *Ochrobactrum* and *Phyllobacterium* by the absence of diphosphatidylglycerol and phosphatidyl methylethanolamine (Table S1). The unidentified aminolipid is considered to be specific for the members of the genus *Ochrobactrum* [54] but it was not detected in SP-1^T. The absence of diphosphatidylglycerol serves to distinguish it from the members of the genera *Brucella*, *Pseudoxanthobacter*, *Kaistia*, *Aquamicrobium* and *Mesorhizobium* and the absence of phosphatidyl methylethanolamine and unidentified glycolipids distinguish it from the members of the genera *Pseudoxanthobacter* and *Tianweitania* (Table S1) [44]. Comparing the major polar lipids reported previously for the members of the genus *Kaistia* [55], the results for the novel isolate were different in several cases, including the presence of phosphatidylcholine and the absence of hydroxyphosphatidylethanolamine, phosphatidylserine and unidentified aminophospholipids.

The low level of 16S rRNA and *atpD* gene sequences similarity, independent phylogenetic position, relatively low ANI and *in silico* DDH values and differences in several phenotypic properties, cellular fatty acid compositions, polar lipid profile and DNA G+C content between Sp-1^T and its closest phylogenetic neighbours strongly indicate that Sp-1^T is not closely affiliated with any recognized taxa. Therefore, we suggest that Sp-1^T is representative of a novel species in a novel genus within a novel family of the order *Rhizobiales*, for which the name *Segnochrobactrum spirostomi* gen. nov., sp. nov., of the new family *Segnochrobactraceae* fam. nov. is proposed.

DESCRIPTION OF *SEGNOCHROBACTRUM* GEN. NOV.

Segnochrobactrum (Segn.o.chro.bac′trum. L. adj. *segnis*, slow, lazy, inactive; N.L. neut. n. *Ochrobactrum* a bacterial genus name; N.L. neut. n. *Segnochrobactrum* the slow, lazy, inactive *Ochrobactrum*).

Cells are Gram-stain negative, aerobic, rod-shaped, non-motile and non-spore-forming. Positive for catalase and oxidase but negative for nitrate reduction. Poly-*β*-hydroxybutyrate accumulation in the cell is observed. The major cellular fatty acids are $C_{19:0}$ cyclo ω8*c* and $C_{16:0}$. The only isoprenoid quinone is identified as ubiquinone-10 (Q-10). The polar lipids are comprised of phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, an unidentified aminolipid, two unidentified phospholipids and five unidentified lipids. The DNA G+C content of the type strain of the species is 68.2 mol% [by whole-genome sequencing (WGS)]. Based on 16S rRNA gene sequence analysis, the genus is affiliated within the family *Segnochrobactraceae* under the order *Rhizobiales*. The type species is *Segnochrobactrum spirostomi*.

DESCRIPTION OF *SEGNOCHROBACTRUM SPIROSTOMI* SP. NOV.

Segnochrobactrum spirostomi (spi.ro.sto′mi. N.L. gen. n. *spirostomi* of *Spirostomum,* the generic name of the animal-like protist, the source of the organism).

The species exhibits the following characteristics along with those described for the genus description. Cells are Gram-stain negative, non-spore-forming, aerobic and non-motile. Cells are rods approximately 0.76–1.20 µm in width and 1.52–3.07 µm in length after 24 h of incubation in TSB at 28 °C. They occur singly, in pairs and irregularly in short chains. Cells are found to contain poly-*β*-hydroxybutyrate accumulating granules. Growth occurs on NA, TSA and EMB agar plates but not on MacConkey agar. Growth on NA, TSA and EMB plates appears after 2 days of incubation at 28 °C. Colonies on NA and TSA (about 1 mm in diameter) are observed to be circular, low convex with entire edges, smooth surfaces, opaque and cream colored after 7 days at 28 °C. Colonies produced on EMB agar are convex, with smooth surfaces and pale pink. The cells are unable to grow at over 40 °C but able to survive being heated at 60 °C for 30 min. Acid is produced from glucose without producing gas. The cells exhibit positive reaction for catalase and oxidase but negative ones for ONPG and nitrate reduction. Aesculin and Tween 20 are hydrolyzed, but gelatin and casein are not. Positive for citrate utilization, urease activity, arginine dihydrolase, lysine-, ornithine-, methionine- and leucine decarboxylases but negative for Voges–Proskauer, methyl red, tryptophan deaminase activity and H_2S production. The other biochemical and physiological characteristics are shown in (Tables 1 and S1). The major cellular fatty acids are $C_{19:0}$ cyclo ω8*c* and $C_{16:0}$. The diagnostic ubiquinone-10 (Q-10) is recognized as the only isoprenoid quinone and phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, an unidentified aminolipid, two unidentified phospholipids, and three unidentified lipids as dominant polar lipids.

The type strain Sp-1^T (=KCTC 62036^T=JCM 32162^T) was isolated from a monoclonal culture of the heterotrich ciliate *Spirostomum yagiui* collected from a freshwater reservoir in Ulsan, Republic of Korea. The DNA G+C content of the type strain is 68.2 mol% (by WGS). The GenBank/EMBL/DDBJ/PIR accession numbers for the 16S rRNA gene, *atpD* gene and genome sequences of (*Segnochrobactrum spirostomi*) Sp-1^T are MF370560, MK478370 and VWNA00000000, respectively. The GenBank/EMBL/DDBJ accession number for the 18S rRNA gene sequence of *Spirostomum yagiui* is MH460446.

DESCRIPTION OF *SEGNOCHROBACTRACEAE* FAM. NOV.

Segnochrobactraceae (Segn.o.chro.bac.tra.ce′ae. N.L. neut. n. *Segnochrobactrum* type genus of the family; suffix. -*aceae* ending to denote a family; N.L. fem. pl. n. *Segnochrobactraceae* the family of the genus *Segnochrobactrum*).

The family *Segnochrobactraceae* comprising solely the genus *Segnochrobactrum* at present is a novel member of the order *Rhizobiales*. The description of this family is the same as that given for the genus *Segnochrobactrum*. The type genus of the family is *Segnochrobactrum*.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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