Tistlia consotensis gen. nov., sp. nov., an aerobic, chemoheterotrophic, free-living, nitrogen-fixing alphaproteobacterium, isolated from a Colombian saline spring

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A Gram-negative, aerobic, mesophilic, non-spore-forming, chemotrophic, chlorophyll-lacking, nitrogen-fixing bacterium, designated strain USBA 355^T, was isolated from the saline spring 'Salado de Consotá' situated in the Colombian Andes. The non-flagellated cells of strain USBA 355^T were straight to slightly curved rods (0.6–0.7 × 3.0–3.5 µm). Growth occurred optimally at 30 °C (growth temperature range between 20 and 40 °C), at pH 6.5–6.7 (pH growth range between 5.0 and 8.0) and at 0.5 % NaCl (w/v) (range between 0 and 4 %). The major quinone present was Q-10 and the predominant fatty acids identified were C_{19:0} cyclo ω8c, C_{18:1} ω7c and C_{18:0}. The G + C content of the chromosomal DNA was 71 ± 1 mol%. 16S rRNA gene sequence analysis indicated that strain USBA 355^T formed a distant phylogenetic line of descent with members of the genus *Thalassobaculum*, family *Rhodospirillaceae*, class *Alphaproteobacteria* (90 % gene sequence similarity). Comparison of the phylogenetic, chemotaxonomic and physiological features of strain USBA 355^T with all other members of the family *Rhodospirillaceae* suggested that it represents a novel genus and species for which the name *Tistlia consotensis* gen. nov., sp. nov. is proposed. The type strain of the type species is USBA 355^T (=JCM 15529^T=KCTC 22406^T).

The family *Rhodospirillaceae* currently comprises 21 genera of varying metabolic and nutritional properties and includes photoheterotrophs, photoautotrophs and chemoheterotrophs. The chemoheterotrophs include the facultative anaerobic genera *Skermanella*, *Telmatospirillum*, *Caenispirillum*, *Thalassobaculum* and *Nisaea* (Sly & Stackebrandt, 1999; Sizova *et al.*, 2007; Yoon *et al.*, 2007; Zhang *et al.*, 2008; Urios *et al.*, 2008) and the aerobic and microoxic genera *Azospirillum*, *Conglomeromonas*, *Magnetospirillum*, *Thalassospira*, *Tistrella*, *Inquilinus* and *Defluviicoccus* (Tarrand *et al.*, 1978; Skerman *et al.*, 1983; Schleifer *et al.*, 1991; Coenye *et al.*, 2002; López-López *et al.*, 2002; Shi *et al.*, 2002; Maszenan *et al.*, 2005). Some members of the family are known to change from one nutritional mode to another depending on the availability of light and the concentration of oxygen and carbon sources. This strategy is exemplified by members of the genera *Rhodocista*, *Rhodospira*, *Roseospira*, *Rhodovibrio*, *Rhodospirillum* and *Phaeospirillum* (Kawasaki *et al.*, 1992; Pfennig *et al.*, 1997; Imhoff *et al.*, 1998). Other genera of

the family, such as members of the genus *Azospirillum*, even have the ability to fix nitrogen (Xie & Yokota, 2005a). In this paper, the isolation and characterization of a novel aerobic, chemoheterotrophic, nitrogen-fixing bacterium, strain USBA 355^T, is reported. This new strain was isolated from samples collected from the saline spring, Salado de Consotá, located in the Colombian Andes (64° 40' 43.1" N, 75° 31' 34.3" W). Salado de Consotá is a neutral pH spring with a salt content of 4.5 % (w/v). The dominant ions in the spring water are Na⁺, Ca²⁺ and Cl⁻ (Tistl, 2004).

Water samples were collected aseptically from the Salado de Consotá spring in 2006 by filling sterile glass containers to the brim. The samples were capped tightly, placed on ice and transported to our laboratory in Bogotá. Enrichments were initiated by inoculating a 2 ml water sample in 10 ml filter-sterilized saline spring water which had been amended with 0.1 % (w/v) starch (Sigma) and 0.02 % (w/v) yeast extract (Sigma). Turbidity was observed after 10 days incubation at 37 °C. Subsequent phase-contrast microscopy (Eclipse 50i; Nikon) revealed the presence of curved and rod-shaped cells. Several colonies developed

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain USBA 355^T is EU728658.

from serial dilutions of the enrichment culture streaked onto the same medium fortified with 2 % (w/v) noble agar (Sigma) after 3 days incubation at 37 °C. A beige and slightly raised, circular, mucoid colony (1 mm diameter) was selected and the culture derived from this, designated strain USBA 355^T, was then used for further studies. Strain USBA 355^T was routinely cultured in a basal medium (BM) supplemented with 20 mM D-glucose and 0.1 % (v/v) yeast extract. BM contained (l⁻¹ deionized water): 1 g NH₄Cl, 0.3 g K₂HPO₄, 0.3 g KH₂PO₄, 3 g MgCl₂·6H₂O, 0.1 g CaCl₂·2H₂O, 0.1 g KCl, 23 g NaCl and 1 ml Zeikus' trace-element solution (Zeikus *et al.*, 1979); the pH of the medium was adjusted to 7.1 with 1 M NaOH. Cells were preserved at -20 °C in BM supplemented with 20 % (v/v) glycerol.

Samples were prepared for electron microscopy as described previously (Patel *et al.*, 1985). Cells of strain USBA 355^T occurred singly or in pairs and were slightly curved to straight rods (0.6–0.7 × 3.0–3.5 µm). Although tumbling motility was observed, neither flagella nor axial filaments were evident by electron microscopy. Cells of strain USBA 355^T stained Gram-negative but electron micrographs of ultrathin sections revealed a typical Gram-positive cell-wall ultrastructure (Fig. 1). Spores were not observed and cells were not resistant to heating at 90 °C for 5 min.

Characterization studies were performed in BM. Cell growth was monitored at 580 nm and all tests were

Fig. 1. Transmission electron micrograph of a thin section of a cell of strain USBA 355^T revealing a typical Gram-positive cell-wall ultrastructure. CM, cytoplasmic membrane; P, cell wall. Bar, 200 nm.

performed in duplicate with the strain subcultured at least once under the same experimental conditions before use. The pH range for growth was tested in BM in which the pH was adjusted to between pH 4.0 and 9.0 with stock solutions of NaHCO₃ (10 %, w/v), Na₂CO₃ (10 %, w/v) and citrate buffer (1 M). Strain USBA 355^T grew optimally at pH 6.5–6.7 (range 5.0–8.0). The temperature optimum was 30 °C (range 20–40 °C) and the optimum NaCl concentration for growth was 0.5 % (w/v) (range 0–4 % NaCl, w/v). Unless indicated otherwise, all subsequent growth experiments were conducted using these optimal conditions.

Strain USBA 355^T was able to use glucose or peptone as a sole carbon source but the addition of 0.1 % (w/v) yeast extract increased the cell yield and was thus added to BM in order to determine the nutritional spectrum of the new isolate. Monosaccharides, disaccharides, polysaccharides, peptides and sugar alcohols were tested at a final concentration of 0.2 % (w/v), organic acids, alcohols and amino acids were tested at a final concentration of 20 mM and aromatic compounds were tested at a final concentration of 5 mM. An increase in optical density of more than 0.1 OD unit relative to controls (which lacked carbon sources, but included 0.1 % yeast extract) was regarded as a positive reaction for growth on the substrate. Strain USBA 355^T grew on a range of substrates and these are given in more detail in the species description.

The strain USBA 355^T did not grow anaerobically using the method of Baena *et al.* (1998) and was catalase-negative using the method of Smibert & Krieg (1994). Strain USBA 355^T was sensitive to ampicillin and streptomycin at 10 µg ml⁻¹ and to tetracycline, chloramphenicol and penicillin at concentrations of 50 µg ml⁻¹.

The novel strain did not grow chemolithotrophically on 10 mM thiosulfate, on 1 % (w/v) elemental sulfur or on 5 mM sulfide with 0.1 % (v/v) NaHCO₃ under aerobic dark conditions.

In order to test the use of nitrogen sources, the novel strain was cultured aerobically in BM but without chemical or organic nitrogen sources. Strain USBA 355^T was able to utilize 0.1 % (w/v) urea, 10 mM nitrate and 2.5 mM glutamate, but not 10 mM nitrite.

The ability to fix dinitrogen was tested on nitrogen-free semisolid malate (NFb) enrichment medium (Baldani & Döbereiner, 1980) and acetylene reduction activity was measured as described previously by Witty & Minchin (1988), using *Azospirillum forbidum* ATCC 29707^T as the reference strain. Further, the presence of the *nifH* gene fragment was determined by using a nested PCR to increase sensitivity. The amplification was conducted with the primers Set 1 FGPH19/PolR and Set 2 AQER/PolF (Simonet *et al.*, 1991; Poly *et al.*, 2001) as described by Diallo *et al.* (2004). The novel strain showed very high acetylene reduction activity and was found to possess the *nifH* gene.

Respiratory lipoquinone analyses were carried out by the Identification Service and Dr B. J. Tindall, DSMZ, Braunschweig, Germany, and showed that ubiquinone Q-10 was the most dominant (95 %) quinone in strain USBA 355^T. Lower concentrations of Q-9 (5 %) were also present. These data support the affiliation of strain USBA 355^T as a member of the class *Alphaproteobacteria* since a characteristic of this group is the possession of ubiquinone Q-10 as the major quinone (Xie & Yokota, 2005b). Fatty acid analysis performed by the identification services of the DSMZ showed that the major cellular fatty acids of strain USBA 355^T were C_{19:0} cyclo ω 8c (38 %), C_{18:1} ω 7c (26 %) and C_{18:0} (18 %). Fatty acids 11 methyl C_{18:1} ω 7c (0.7 %), C_{20:2} ω 6.9c (1.2 %) and C_{18:0} iso (1 %) were also present but in lower concentrations. The hydroxy fatty acids identified were C_{18:1} 2-OH (3.7 %), C_{18:0} 3-OH (3.6 %), C_{16:1} ω 7c/C₁₅ iso 2-OH (1.5 %) and C_{14:0} 3-OH/C_{16:1} iso I (0.7 %). These data suggest that strain USBA 355^T possesses a unique fatty acid profile amongst members of the family *Rhodospirillaceae* (Table 1).

Aerobic liquid cultures of strain USBA 355^T were colourless but the microbial pellet presented a brownish-pink tinge. The absorption spectra (Cintra20, GBC) of a cell pellet suspended in a sucrose solution (Pfennig & Trüper, 1992) or after ethanol extraction (Yurkov & van Gemerden, 1993) showed a single absorption peak at 398 nm, which indicated the absence of bacteriochlorophyll *a*.

DNA of strain USBA 355^T was prepared by using the whole genome amplification method (Ogg & Patel, 2009) and the G + C content of the DNA was determined by the thermal denaturation (*T*_m) method (Marmur & Doty, 1962) and was calculated to be 71 ± 1 mol%.

The methods used for the purification of the DNA, PCR amplification, sequencing of the 16S rRNA gene and phylogeny were as described previously (Redburn & Patel, 1993; Woo *et al.*, 1997; Spanevello *et al.*, 2002). The partial sequences that were generated were assembled using BioEdit v5.0.1 (Hall, 1999) and the consensus sequence of 1430 nucleotides was corrected manually for errors. The most closely related sequences in GenBank (version 152) and the Ribosomal Database Project II (RDP; release 10) identified using BLAST (Altschul *et al.*, 1997) were extracted, aligned and manually adjusted according to the 16S rRNA secondary structure obtained from RDP using BioEdit. Nucleotide ambiguities were omitted and evolutionary distances were calculated by using the Jukes and Cantor option (Jukes & Cantor, 1969) in TREECON (Van de Peer *et al.*, 1997). Phylogenetic trees were constructed from evolutionary distances using the neighbour-joining method (Saitou & Nei, 1987). The tree topology was re-examined by the bootstrap method (1000 replications) of resampling (Felsenstein, 1985).

Phylogenetic analysis revealed that strain USBA 355^T was most closely related to members of the genus *Thalassobaculum*, family *Rhodospirillaceae*, class *Alphaproteobacteria*, phylum

Proteobacteria, with a 16S rRNA gene sequence similarity value of 90 % (Fig. 2).

In addition to the large phylogenetic distance separating strain USBA 355^T from members of the genus *Thalassobaculum*, strain USBA 355^T could be differentiated from this genus on the basis that it could not grow under anaerobic conditions, was catalase-negative and, despite being motile, did not appear to possess flagella. The salt tolerance range for growth of the novel strain was lower (0–4 %) compared with 1–10 % (w/v) for members of the genus *Thalassobaculum*. The DNA G + C content of the novel strain was also higher (71 mol% versus 68 mol%; Zhang *et al.*, 2008). Strain USBA 355^T could also be differentiated from other chemoheterotrophic, aerobic, nitrogen-fixing genera, such as *Azospirillum* (Tarrand *et al.*, 1978), in the family *Rhodospirillaceae* by its fatty acid composition. Members of the genus *Azospirillum* contain a large amount of C_{18:1} ω 7c, C_{16:1} ω 7c, C_{16:0} and the major hydroxyl fatty acids C_{14:0} 3-OH and C_{16:0} 3-OH (Xie & Yokota, 2005a). The salt tolerance range distinguished strain USBA 355^T (<4 %, w/v) from the genera *Thalassospira* (<10 %) (López-López *et al.*, 2002), *Inquilinus* (<6 %) (Coenye *et al.*, 2002) and *Tistrella* (<1 %) (Shi *et al.*, 2002). The novel strain was distinguishable from some phylogenetically related phototrophic bacteria such as members of the genera *Roseospira* and *Rhodovibrio* by the absence of bacteriochlorophyll *a* and intracellular photosynthetic membranes (Imhoff *et al.*, 1998; Guyoneaud *et al.*, 2002). The low level of 16S rRNA gene sequence similarity between strain USBA 355^T and all other members of the family *Rhodospirillaceae*, together with the differential phenotypic properties (Table 1), suggest that the new isolate represents a novel species of a new genus for which the name *Tistlia consotensis* gen. nov., sp. nov. is proposed.

Description of *Tistlia* gen. nov.

Tistlia (Tist'li.a. N.L. fem. n. *Tistlia* named after Tistl, honouring Michael Tistl, a geologist for his rediscovery of the Salado de Consotá saline spring).

Cells are strictly aerobic, slightly curved to straight rods which do not possess pili or form spores. Gram-reaction is negative. Q-10 is the predominant ubiquinone and C_{19:0} cyclo ω 8c, C_{18:1} ω 7c and C_{18:0} are the dominant fatty acids. 16S rRNA gene sequence analysis places the genus *Tistlia* in the family *Rhodospirillaceae*, class *Alphaproteobacteria*, phylum *Proteobacteria*. The type species is *Tistlia consotensis*.

Description of *Tistlia consotensis* sp. nov.

Tistlia consotensis (con.so.ten'sis. N.L. fem. adj. *consotensis* pertaining to Salado de Consotá saline spring, Risaralda-Colombia, the geographical location from which the organism was first isolated).

Exhibits the following properties in addition to those given in the genus description. Cells reveal a Gram-positive

Table 1. Phenotypic characteristics of strain USBA 355^T and some genera of the family *Rhodospirillaceae*

Taxa: 1, USBA 355^T; 2, *Rhodospira* (Pfennig *et al.*, 1997); 3, *Rhodovibrio* (Imhoff *et al.*, 1998); 4, *Roseospira* (Imhoff *et al.*, 1998); 5, *Rhodocista* (Kawasaki *et al.*, 1992); 6, *Rhodospirillum* (Imhoff *et al.*, 1998); 7, *Phaeospirillum* (Imhoff *et al.*, 1998); 8, *Thalassospira* (López-López *et al.*, 2002); 9, *Deftuivococcus* (Maszenan *et al.*, 2005); 10, *Tistrella* (Shi *et al.*, 2002); 11, *Inquilinus* (Coenye *et al.*, 2002); 12, *Azospirillum* (Tarrand *et al.*, 1978); 13, *Skermanella* (Sly & Stackebrandt, 1999); 14, *Telmatospirillum* (Sizova *et al.*, 2007); 15, *Caenispirillum* (Yoon *et al.*, 2007); 16, *Thalassobaculum* (Zhang *et al.*, 2008); 17, *Magnetospirillum* (Schleifer *et al.*, 1991; Sakane & Yokota, 1994); 18, *Nisaea* (Urios *et al.*, 2008). +, Positive; –, negative; v, variable; ND, no data available; CH, chemoheterotrophic; PH, photoheterotrophic; CL, chemolithotrophic; AE, aerobic; AN, anaerobic; MO, microoxic; FA, facultatively anaerobic; O, oxic.

Characteristic	1	2	3	4	5	6	7	8	9
Habitat	Saline spring	Salt marsh, sediment mats	Seawater salterns	Sulfur spring	Freshwater, wastewater	Freshwater	Stagnant and anoxic freshwater	Seawater	Sludge
Flagella	–	+	+	+	+	+	+	+	–
DNA G + C content (mol%)	71	65.7	66–67	66.6	69.9	63–66	60.5–65.3	54.7	66
Preferred metabolism	CH AE	PH AN	PH AN	PH AN	PH AN	PH AN	PH AN	CH AE	CH AE
Alternative metabolism		CH MO	CH MO	CH MO	CH MO		CH MO		
Growth factors	–	+	+	+	+	+	+	–	–
Na ⁺ requirement	–	+	+	+	–	–	–	+	–
Salt tolerance (% w/v)	<4	5	3–24	Up to 15	ND	ND	ND	Up to 10	ND
Salt optimum (% w/v)	0.5	2	3.5	4–7	ND	ND	ND	ND	ND
pH optimum	6.5–6.7	7.3–7.5	7–8	7.0	6.8	6.5–7.5	7.3	ND	7.5–8.0
Temperature optimum (°C)	30	25–30	35–42	30–35	31–45	25–35	25–30	13	25–30
Catalase activity	–	ND	ND	ND	ND	ND	ND	+	+
NO ₃ [–] to NO ₂ [–]	+	ND	ND	ND	ND	ND	ND	–	+
NO ₃ [–] as N source	+	ND	ND	ND	–	ND	ND	+	ND
Growth on carbohydrates	+	–	–	Only fructose	–	v	+	+	+
Major quinones	Q-10, Q-9	Q-7, MK-7	Q-10, MK-10	ND	Q-9	Q-10, RQ-10	Q-9, MK-9	ND	ND
Major fatty acids	C _{19:0} cyclo ω8c, C _{18:1} ω7c, C _{18:0}	C _{18:1} , C _{16:0} , C _{14:0} , C _{18:0} , C _{16:1}	C _{18:1} , C _{18:0} , C _{16:0} , C _{14:0}	ND	ND	C _{18:1} , C _{16:1} , C _{16:0} , C _{14:0}	C _{18:1} , C _{16:1} , C _{16:0} , C _{18:0}	C _{18:1} cis ω7, C _{16:0} , C _{16:0} cis ω7	ND

Characteristic	10	11	12	13	14	15	16	17	18
Habitat	Wastewater	Cystic fibrosis patients	Soil, root, freshwater	Lake water	Mesotrophic Siberian fen	Sludge	Seawater	Freshwater ponds, swamps	Seawater
Flagella	+	ND	+	+	+	+	+	+	+
DNA G + C content (mol%)	67.5	70.9	67–71	67	61.5–64	70	68	64–71	60
Preferred metabolism	CH AE	CH AE	CH AE	CH FA	CH AN or MO	CH AE	CH FA	CH MO-O	CH AE
Alternative metabolism					CL O	CH AN with NO ₃			
Growth factors	+	ND	v	–	ND	–	–	–	–
Na ⁺ requirement	–	ND	–	–	ND	–	+	–	–
Salt tolerance (% w/v)	<1	<6	<5	<2	ND	<5	<10	ND	<6
Salt optimum (% w/v)	ND	ND	ND	ND	ND	0.5	2–4	ND	2
pH optimum	7.4	ND	5.8–6.8*	ND	5.7–6.5	7.0–8.0	8.0	7.0	6
Temperature optimum (°C)	30	ND (25–42)	34–37	28	25–28	37	30–35	30	30
Catalase activity	+	+	v	+	–	+	+	v	+
NO ₃ [–] to NO ₂ [–]	+	ND	+	+	ND	+	+	–	+
NO ₃ [–] as N source	ND	ND	+	ND	ND	ND	ND	ND	ND

Table 1. cont.

Characteristic	10	11	12	13	14	15	16	17	18
Growth on carbohydrates	+	–	+	+	+	Only ribose	Ribose, sucrose	–	+
Major quinones	Q-10	ND	Q-10	Q-10	ND	Q-10	Q-10	Q-10	Q-10
Major fatty acids	C _{18:1} ω7c, C _{18:1} 2-OH, C _{14:0} 3-OH, C _{16:0} 3-OH, C _{18:1} ω7c 11 methyl	ND	C _{18:1} ω7c, C _{16:1} ω7c, C _{16:0} , C _{14:0} 3-OH, C _{16:0} 3-OH	ND	C _{18:1} ω7c, C _{17:0} cyclo, C _{18:0} , C _{14:0} , C _{16:1} ω7c	C _{18:1} ω7c	C _{18:1} ω7c, C _{16:0} , C _{17:0} , C _{19:0} cyclo ω8c	C _{18:1} , C _{16:1} , C _{16:0} , C _{14:0} 3-OH, C _{16:0} 3-OH	C _{18:1} ω7c, C _{16:1} ω7c, C _{16:0}

*Except for *Azospirillum halopraeferans*, which has an optimal temperature of 41 °C and an optimal pH of 8.0.

cell-wall ultrastructure. Cells are 0.6–0.7 × 3.0–3.5 μm, multiply by binary fission and show tumbling motility. Mesophilic and slightly halophilic with optimum growth occurring at 30 °C (growth temperature range 20–40 °C), pH 6.5–6.7 (pH growth range 5.0–8.0) and salinity of 0.5 % (w/v) (NaCl growth range 0–4 %). Chemoheterotrophic. Cells grow on glucose or peptone as a sole carbon source. Yeast extract is not required for growth but increases biomass yields. Grows on pyruvate, butyrate, succinate, glucose, mannose, xylose, galactose, arabinose, trehalose,

cellobiose, lactose, sucrose, rhamnose, fructose, maltose, peptone, Casamino acids, tryptone, peptidase, gelatin, arginine, alanine, leucine, isoleucine, valine, glutamate, glycerol, inositol and starch, but not on formate, acetate, methanol, lactate, citrate, α-ketoglutarate, ribose, raffinose, methionine, threonine, lysine, glycine, histidine, Tween 80, ethyl oleate, olive oil, benzoate or cinnamate. Urea, nitrate and glutamate can serve as sole nitrogen sources. Sensitive to ampicillin, streptomycin, chloramphenicol, tetracycline and penicillin.

Fig. 2. Dendrogram showing the phylogenetic position of strain USBA 355^T and the closest relatives. GenBank accession numbers are given parentheses. Bootstrap values >90 % from 1000 replicates are shown. Bar, 0.1 nucleotide changes per 100 nucleotides.

The type strain, USBA 355^T (=JCM 15529^T=KCTC 22406^T), was isolated from the Salado de Consotá saline spring, Colombia. The DNA G+C content of the type strain is 71 ± 1 mol%.

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