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ORIGINAL PAPER

Description of *Niveispirillum fermenti* gen. nov., sp. nov., isolated from a fermentor in Taiwan, transfer of *Azospirillum irakense* (1989) as *Niveispirillum irakense* comb. nov., and reclassification of *Azospirillum amazonense* (1983) as *Nitrospirillum amazonense* gen. nov

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Abstract A taxonomic study was carried out on a novel aerobic bacterial strain (designated CC-LY736^T) isolated from a fermentor in Taiwan. Cells of strain CC-LY736^T were Gram-stain negative, spiral-shaped and motile by means of a monopolar flagellum. Strain CC-LY736^T shared the greatest degree of 16S rRNA gene sequence similarity to *Azospirillum irakense* DSM 11586^T (97.2 %), *Rhodocista centenaria* JCM 21060^T (96.3 %) and *Rhodocista pekingensis* JCM 11669^T (96.1 %). The major fatty acids were $C_{16:0}$, $C_{16:1}$ ω5c, $C_{19:0}$ cyclo ω8c, $C_{18:1}$ ω7c/$C_{18:1}$ ω6c, $C_{16:0}$ 3-OH and $C_{18:1}$ 2-OH. The predominant polar lipids included phosphatidylcholine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidyldimethylethanolamine and two unidentified glycolipids. The common major respiratory quinone was ubiquinone Q-10 and predominant polyamines were *sym*-homospermidine and putrescine. The DNA G+C content of strain CC-LY736^T was 67.6 ± 0.1 mol %. During phylogenetic analysis, strain CC-LY736^T formed a unique phyletic lineage associated with *Rhodocista* species. However, the combination of genetic, chemotaxonomic and physiological data clearly indicated that strain CC-LY736^T was a novel representative of the family *Rhodospirillaceae*. Based on the polyphasic comparison, the name *Niveispirillum fermenti* gen. nov., sp. nov. is proposed; the type strain of the type species is CC-LY736^T (= BCRC 80504^T = LMG 27263^T). In addition, the reclassifications of *Azospirillum irakense* as *Niveispirillum irakense* comb. nov. (type strain KBC1^T = ATCC 51182^T = BCRC 15764^T = CIP 103311^T), and *Azospirillum amazonense* as *Nitrospirillum amazonense* gen. nov., sp. nov. (type

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence of strain CC-LY736^T is JX843283. In this study, the GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence of strains *Azospirillum amazonense* BCRC 14279^T, *A. halopraeferens* DSM 3675^T, *A. irakense* DSM 11586^T, *A. lipoferum* DSM 1691^T, *A. canadense* LMG 23617^T were GU256437, GU256439, GU256440, GU256441 and HM636056, respectively. A transmission electron micrograph of cells of strain CC-LY736^T, polar lipid profile and polyamine compositions of all strains used in this study are available as supplementary material with the online version of this paper.

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strain Am14^T = ATCC 35119^T = BCRC 14279^T = DSM 3787^T) are proposed based on the polyphasic taxonomic data obtained in this study.

Keywords *Azospirillum irakense* · *Azospirillum amazonense* · *Niveispirillum fermenti* · *Niveispirillum irakense* · *Nitrospirillum amazonense*

Introduction

The family *Rhodospirillaceae*, affiliated to the subclass *Alphaproteobacteria* currently accommodates 34 bacterial genera that were isolated and described from various geographical locations. The members of nitrogen-fixing genus *Azospirillum* of the family *Rhodospirillaceae* are distributed in natural environments such as soils of tropical, subtropical and temperate regions, where they are frequently associated with grasses, cereals and crops (Bally et al. 1983; Döbereiner and Day 1976; Kirchhof et al. 1997; Ladha et al. 1987; Patriquin et al. 1983), discarded road tar, oil-contaminated soil and fermentative tanks (Young et al. 2008; Lin et al. 2009, 2012, 2013) and a sulfide spring (Lavrinenko et al. 2010). The other spiral-shaped phototrophic purple non-sulfur bacteria were included in the genus *Rhodospirillum* (Trüper and Imhoff 1989; Favinger et al. 1989; Kawasaki et al. 1993; Gest and Favinger 2001) before the proposal of the species *Rhodocista centenaria* (Kawasaki et al. 1992; Euzéby and Kudo 2001) and *Rhodospira trueperi* (Pfennig et al. 1997). The genus *Rhodocista* was established based on the results of 16S rRNA gene sequences, unique cyst-producing morphology and ubiquinone Q-9 as predominant respiratory quinone (Kawasaki et al. 1992). As new phototrophic bacterial strains were isolated and more information on the phenotypes and genotypes (particularly 16S rRNA gene sequences) of these and other previously described strains became available, it was evident that the original description of some of the genera within the family *Rhodospirillaceae* needed revision.

Materials and methods

Bacterial strains and growth conditions

While investigating bacterial diversity in a fermentative tank in central Taiwan (24°12′ N, 120°67′ E) located near a greenhouse set up at the National Chung Hsing University, Taichung. Bacteria were isolated from the fermentation medium; approximately 100 µl fermentation broth were spread on nutrient agar (NA, Hi-Media) by using the standard ten-fold dilution plating technique. After three days of aerobic incubation on nutrient agar (NA, Himedia) at 30 °C, strain CC-LY736^T formed a cream-colored colony, which was isolated, purified and preserved at −80 °C as glycerol suspension.

The following type strains *Azospirillum amazonense* BCRC 14279^T (= DSM 2787^T) (Magalhães et al. 1983; Falk et al. 1985, 1986); *Azospirillum brasilense* BCRC 12270^T (Tarrand et al. 1978); *Azospirillum doebereinerae* DSM 13131^T (Eckert et al. 2001); *Azospirillum fermentarium* BCRC 80505^T (Lin et al. 2013); *Azospirillum formosense* BCRC 80273^T (Lin et al. 2012); *Azospirillum irakense* BCRC 15764^T (= DSM 11586^T) (Khammas et al. 1989); *Azospirillum lipoferum* BCRC 12213^T (type species; Tarrand et al. 1978); *Azospirillum oryzae* JCM 21588^T (Xie and Yokota 2005); *Azospirillum picis* DSM 19922^T (Lin et al. 2009); *Azospirillum rugosum* DSM 19657^T (Young et al. 2008); *Azospirillum zeae* LMG 23989^T (Mehnaz et al. 2007b); *Inquilinus limosus* LMG 20952^T (type species; Coenye et al. 2002); *Rhodocista centenaria* JCM 21060^T (type species; Kawasaki et al. 1992); *Rhodocista pekingensis* JCM 11669^T (Zhang et al. 2003) and *Skermanella aerolata* DSM 18479^T (type species; Weon et al. 2007) were purchased for the purpose of direct comparative taxonomy. All these strains were grown on NA at 30 °C for 2 days, unless specified otherwise.

Morphological tests and biochemical characterization

Cell morphology of strain CC-LY736^T, including presence of flagella was determined by placing the cells (1–2 days old) on a carbon-coated copper grid followed by staining with aqueous solution of 0.2 % (w/v) uranyl acetate for 5–10 s, brief air-drying and observation under a transmission electron microscope (JEOL JEM-1400). Gram-staining was performed as described by Murray et al. (1994). Growth was tested using nutrient broth (NB, Hi-Media) at temperature 20–50 °C (at 5 °C intervals) and at pH 5–10 (at 1 unit intervals). Salt tolerance was determined by cultivating the organism in nutrient broth (Hi-Media) supplemented with 0–5 % (w/v) NaCl (at 1 % intervals).

Catalase activity was determined by assessing bubble production by cells in 3 % (v/v) H_2O_2 and oxidase activity was determined by using 1 % (w/v) *N*,*N*,*N*,*N*,-tetramethyl-1,4-phenylenediamine reagent (bioMérieux, France). Intracellular poly-β-hydroxybutyrate (PHB) granules were detected with different methods, including Sudan Black staining (Schlegel et al. 1970) and Nile blue A staining (Ostle and Holt, 1982), which result in dark blue or fluorescent granules. DNase test was conducted by using DNase test agar (Hi-Media). Carbon source utilization pattern were determined by using Biolog GN2 MicroPlate (bioMérieux, France). Nitrate reduction, indole production, activity of β-galactosidase and urease, hydrolysis of esculin and gelatin, and assimilation of 12 substrates were tested with API 20 NE strips (bioMérieux, France). The activities of various enzymes were determined by using API ZYM system (bioMérieux, France).

Nitrogen-fixing capability

Acetylene-reduction assay described by Hardy et al. (1973) was used to assay the nitrogen-fixing capability. Vials (30 ml) containing 10 ml semisolid nitrogen-free broth (NFB) medium (Reinhold et al. 1987) were inoculated with strain CC-LY736^T, *A. irakense* BCRC 15764^T and *A. brasilense* BCRC 12270^T, sealed with rubber septa and incubated at 30 °C in the dark incubator. After 72 h, 10 % (v/v) of the air phase was replaced with acetylene (Koch and Evans, 1966) and the vials were re-incubated. The amount of ethylene was measured for a total of 24 h by using a gas chromatograph (FID Gas Chromatograph, HITACHI, Model 163) equipped with a flame-ionization detector and a packed column (1.0 m × 2.0 mm i.d., steel column packed with Porapak-T 80–100). Conditions of analysis were: carrier gas, nitrogen; flow rate, 35 ml h^{-1}; temperature of the flame ionization detector, 110 °C; column temperature, 65 °C.

Genomic DNA preparation and PCR amplification

Bacterial genomic DNA was isolated by using UltraClean^{TM} Microbial Genomic DNA Isolation Kit (MO BIO, USA) by following manufacturer's instructions. An almost full-length of 16S rRNA gene was amplified by using bacterial universal primers 1F (5′-GAG TTT GAT CAT GGC TCA GA-3′) and 9R (5′-AAG GAG GTG ATC CAA CCG CA-3′); the primers 3F (5′-CCT ACG GGA GGC AGC AG-3′), 5F (5′-AAA CTC AAA TGA ATT GAC GGG G-3′) and 4R (5′-TTA CCG CGG CTG CTG GCA C-3′) were used for sequencing reaction (Edwards et al. 1989). Gene sequencing was performed by using the Bigdye terminator kit (Heiner et al. 1998), and nucleotide sequence of PCR product was determined by an automatic DNA sequencer (ABI PRISM 310, Applied Biosystems, CA, USA) (Watts and MacBeath 2001). Obtained DNA sequences were then assembled using the Fragment Assembly System program from the Wisconsin Package (GCG 1995).

Azospirillum-specific PCR and *nif*H gene detection

The specific primer pair for the genus *Azospirillum* was designed and discussed by Lin et al. (2011). The genomic DNA of novel strain CC-LY736^T as well as reference strains (including *A. oryzae* JCM 21588^T; *A. brasilense* BCRC 12270^T; *A. canadense* LMG 23617^T; *A. doebereinerae* DSM 13131^T; *A. fermentarium* BCRC 80505^T; *A. formosense* BCRC 80273^T; *A. halopraeferens* DSM 3675^T; 6, *A. largimobile* DSM 9441^T; *A. lipoferum* BCRC 12213^T; *A. melinis* LMG 23364^T; *A. picis* DSM 19922^T; *A. rugosum* DSM 19657^T; *A. thiophilum* DSM 21654^T; *A. zeae* LMG 23989^T) were screened by using a primer pair Azo494-F (5′-GGC CYG WTY AGT CAG RAG TG-3′) and Azo756-R (5′-AAG TGC ATG CAC CCC RRC GTC TAG C-3′) that can selectively amplify an *Azospirillum*-specific DNA fragment of size 263 bp. On the other hand, Gene *nif*H (nitrogenase reductase) was amplified to confirm the existence of sym gene.

The *nifH* gene was amplified by PCR using the primer set PolF (5′-TGC GAY CCS AAR GCB GAC TC-3′)/PolR (5′-ATS GCC ATC ATY NTC RCC GGA-3′) and the conditions were described previously by Poly et al. (2001). The expected size of the product was about 360 bp. Another primer set Zehrf (5′-TGY GAY CCN AAR GCN GA-3′)/Zehrr (5′-AND GCC ATC ATY TCN CC-3′) was used as described by Zehr and McReynolds (1989). The amplified patterns were screened by electrophoresis in 1.0 % (w/v) agarose gel, stained with ethidium bromide, visualized under UV radiation, and photographed.

16S rRNA gene sequence analysis and phylogenetic analysis

The 16S rRNA gene sequence data were submitted to EzBioCloud server (EzTaxon-e Database, Kim et al. 2012) and NCBI GenBank for BLAST search. Subsequently, closely related 16S rRNA gene sequences were retrieved and aligned by using the CLUSTAL_X (1.83) program (Thompson et al. 1997). Unfortunately, sequence data of several type strains of *Azospirillum* that are available in GenBank were ambiguous due to many unidentified bases that leads to erroneous phylogenetic distinctions of some *Rhodospirillaceae* representatives Therefore, the 16S rRNA genes of several *Azospirillum* species were re-sequenced and submitted to NCBI earlier (Lin et al. 2011, 2012). The phylogenetic analysis was performed using MEGA 5 software (Molecular Evolutionary Genetics Analysis, version 5) (Tamura et al. 2011) and the topology of the resultant trees were evaluated by Neighbor-Joining (Saitou and Nei 1987), Maximum Likelihood (Felsenstein 1981) and Maximum Parsimony (Fitch 1971) methods. The bootstrap values (Felsenstein 1985) were computed after 1,000 replications.

Sequence deposition

Ambiguous 16S rRNA gene sequences of *A. amazonense* BCRC 14279^T (X79735), *A. canadense* LMG 23617^T (DQ393891), *A. halopraeferens* DSM 3675^T (Z29618), *A. irakense* DSM 11586^T (Z29583), *A. lipoferum* DSM 1691^T (M59061) were replaced with newly obtained 16S rRNA gene sequences during current phylogenetic analysis.

DNA–DNA hybridization analysis

DNA–DNA hybridization assay was conducted between strain CC-LY736^T and *A. irakense* BCRC 15764^T using DIG DNA labeling and detection kit (Roche Diagnostics; Cat. No.11 093 657 910) according to the manufacturer's protocol. Chromosomal DNA of strain CC-LY736^T was used to construct hybridization probe by labelling with digoxigenin–11-dUTP (DIG). The experiment was carried out in triplicate for each sample.

Fatty acid methyl ester analysis

Fatty acid methyl esters (FAME) were prepared, separated and identified according to the standard protocol (Paisley 1996) of the Microbial Identification System (MIDI) (Sasser 1990) by gas chromatograph (Agilent 7890A) fitted with a flame ionization detector. The cultures were grown under the same condition on NA plates at 30 °C. After 48 h cells were harvested from the plate and subjected to saponification, methylation and extraction (Miller 1982). Identification and comparison were made by using the Aerobe (RTSBA6) database of the MIDI System (Sherlock version 6.0).

Cellular polyamines and polar lipids analyses

Polyamines were extracted as described by Scherer and Kneifel (1983), analyzed by high performance liquid chromatography (HPLC). The dansyl derivatives were separated by using a Hitachi L-2130 equipped with Hitachi L-2200 autosampler, Hitachi L-2485 fluorescence detector (excitation at 360 nm and emission at 520 nm), and a reverse-phase C18 column (Phenomenex® Synergi Fusion-RP80, 250 × 4.60 mm, 4 µm particle size). Polar lipids were extracted and analyzed by two-dimensional TLC, and isoprenoid quinones were purified by the methods according to Minnikin et al. (1984) and analyzed by HPLC as described by Collins (1985).

Determination of the predominant ubiquinone and DNA base composition

Ubiquinone is an essential component of electron transfer system in the plasma membrane of prokaryotes. The isoprenoid quinones were purified by the methods according to Minnikin et al. (1984). Extraction and assay of quinone was routinely performed according to the HPLC method described by Collins (1985). For the analysis of DNA G+C content, DNA samples were prepared and degraded enzymatically into nucleosides as described by Mesbah et al. (1989). The nucleoside mixtures obtained were then separated and analyzed via HPLC (Hitachi L-2130 chromatograph equipped with Hitachi L-2200 autosampler, Hitachi L-2455 diode array detector, and a reverse-phase C18 column (Phenomenex® Synergi 4 µ Fusion-RP80 250 × 4.60 mm)).

Results and discussion

Phenotypic and biochemical characterization

Strain CC-LY736^T is Gram-stain negative, spiral shaped, 2.0–2.3 μm in length and 0.6–0.8 μm in diameter. PHB granules were observed under light microscopy and visualized by UV illumination after directly staining growing bacteria on plates containing Nile blue A. The cell morphology of strain CC-LY736^T is shown in Fig. S1 and the phenotypic characteristics are given in the genus and species description. Strain CC-LY736^T did not growth in nutrient broth supplemented with 3 % NaCl (w/v), which is different with strain *Niveispirillum irakense* BCRC 15764^T. Nitrate reduction was observed in strain CC-LY736^T, but *Niveispirillum irakense* BCRC 15764^T and *Nitrospirillum amazonense* BCRC 14279^T did not reduce this compound. Urea hydrolysis activity and indole production were negative. The new strain was able to utilize utilize α-D-lactose, D-sorbitol, acetic acid, cis-aconitic acid, γ-hydroxybutyric acid, succinic acid, succinamic acid, D-alanine, glycyl-L-aspartic acid, L-histidine, L-leucine and D-serine as carbon sources; assimilation of D-glucose, N-acetyl-glucosamine and D-maltose, which distinguished from other related type species. Distinctive phenotypic characteristics for the strains of the novel group and the type strains of the phylogenetically closest species are shown in Table 1.

Nitrogen-fixing capability and *nif*H gene detection

Strains CC-LY736^T, *A. irakense* BCRC 15764^T and *A. brasilense* BCRC 12270^T were able to reduce acetylene to ethylene with a mean value of 0.4, 0.8 and 21.4 nmol ethylene h^{-1} (10^8 cells) at 30 °C, respectively. The free-living nitrogen fixing activity was also compared with our previously described novel bacteria namely *A. formosense* CC-Nfb-7^T (Lin et al. 2012), *A. picis* IMMIB TAR-3^T (Lin et al. 2009) and *A. rugosum* IMMIB AFH-6^T (Young et al. 2008), and the free-living nitrogen fixing activities were 25, 93 and 18 nmol ethylene h^{-1}, respectively. It demonstrates that the nitrogen-fixing ability of strains CC-LY736^T and *A. irakense* BCRC 15764^T was related lower than the other free-living nitrogen-fixing *Azospirillum* species. Furthermore, the PCR amplicon of *nif*H gene in strain CC-LY736^T was not observed by using the PolF/PolR or Zehrf/Zehrr primer sets in this study.

16S rRNA gene sequence analysis

The 16S rRNA gene sequence of strain CC-LY736^T showed highest pair-wise similarity to *A. irakense* DSM 11586^T (97.2 %), *R. centenaria* JCM 21060^T (96.3 %), *R. pekingensis* JCM 11669^T (96.1 %), *A. rugosum* DSM 19657^T (91.8 %), *A. doebereinerae* DSM 13131^T (91.6 %), *A. formosense* BCRC 80273^T (91.6 %) and *A. picis* DSM 19922^T (91.6 %). The bacterial genera such as *Skermanella* and *Ochrobactrum* shared relatively lower (<91.1 %) sequence similarity to strain CC-LY736^T. The Neighbor-Joining phylogenetic tree based on 16S rRNA gene sequences showed that the type strains of *Azospirillum* largely constituted one subcluster within the family *Rhodospirillaceae*, except for *A. irakense* with *A. amazonense*, same result was also emphasized with Maximum Likelihood and Maximum Parsimony methods (Fig. 1). The pairwise 16S rRNA gene sequence similarity data of strain CC-LY736^T and other representatives of the family *Rhodopsirillaceae* are shown in Table 2. Low similarity data (<92 %) obtained for *Azospirillum* representatives and strain CC-LY736^T, *A. amazonense* BCRC 14279^T and *A. irakense* BCRC 15764^T suggested that none of those strains belonged to the genus *Azospirillum*. Therefore, further detailed studies were carried out to investigate most likely taxonomic positions of strain CC-LY736^T, *A. amazonense* DSM 2787^T and *A. irakense* DSM 11586^T.

Azospirillum-specific PCR detection

The genus-specific primer pair (Azo494-F/Azo756-R) selectively amplified 263 bp *Azospirillum*-specific fragments from representative strains of large *Azospirillum* subcluster (including *A. oryzae* JCM 21588^T; *A. brasilense* BCRC 12270^T; *A. canadense* LMG 23617^T; *A. doebereinerae* DSM 13131^T; *A. fermentarium* BCRC 80505^T; *A. formosense* BCRC 80273^T; *A. halopraeferens* DSM 3675^T; 6, *A. largimobile* DSM 9441^T; *A. lipoferum* BCRC 12213^T; *A. melinis* LMG 23364^T; *A. picis* DSM 19922^T; *A. rugosum* DSM 19657^T; *A. thiophilum* DSM 21654^T; *A. zeae* LMG 23989^T). The 263 bp DNA fragment was not detected in strains CC-LY736^T, *A. amazonense* BCRC 14279^T and *A. irakense* BCRC 15764^T. These data also clearly suggested a possible earlier misclassification of *A. amazonense* BCRC 14279^T and *A. irakense* BCRC 15764^T.

Table 1 Physiological differences between strain CC-LY736^T and other representatives of the family *Rhodospirillaceae*. Taxa: 1, CC-LY736^T; 2, *Niveispirillum irakense* BCRC 15764^T; 3, *Nitrospirillum amazonense* BCRC 14279^T; 4, *Azospirillum lipoferum* BCRC 12213^T; 5, *Azospirillum fermentarium* BCRC 80505^T; 6, *Azospirillum brasilense* BCRC 12270^T; 7, *Azospirillum formosense* BCRC 80273^T; 8, *Azospirillum picis* DSM 19922^T; 9, *Rhodocista pekingensis* JCM 11669^T; 10, *Rhodocista centenaria* JCM 21060^T; 11, *Skermanella aerolata* DSM 18479^T; 12, *Inquilinus limosus* LMG 20952^T

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Growth in 3 % NaCl	−	+	−	−	+	−	−	−	−	NA	+	+
Nitrate reduction	+	−	−	+	+	−	+	+	+	+	−	−
Urease	−	+	+	+	+	+	−	+	+	+	+	−
Indole production	−	−	−	−	−	−	−	−	+	−	−	−
Carbon source												
Tween 40	−	−	−	+	+	+	+	−	+	+	+	+
D-cellobiose	−	+	+	−	−	−	−	−	−	−	−	+
D-fructose	−	+	−	+	+	+	+	+	−	−	−	+
α-D-lactose	+	−	−	−	−	−	−	−	−	−	−	+
Maltose	−	+	+	−	−	−	−	−	−	−	−	−
D-sorbitol	+	−	−	−	+	−	−	+	−	−	−	+
Acetic acid	+	+	−	−	+	−	−	−	−	−	−	+
Cis-aconitic acid	+	+	−	−	−	+	+	+	−	−	−	+
Citric acid	−	+	−	+	+	−	−	−	−	−	−	−
γ-hydroxybutyric acid	+	+	−	−	−	−	−	−	−	−	−	−
Succinic acid	+	+	−	+	+	+	+	+	−	−	−	+
Succinamic acid	+	+	−	−	+	−	−	−	−	−	−	+
D-alanine	+	+	−	−	−	−	+	−	−	−	−	+
Glycyl-L-aspartic acid	+	+	−	−	−	−	−	−	−	−	−	+
L-histidine	+	+	−	+	−	−	−	+	−	−	−	−
L-leucine	+	+	−	+	−	−	+	+	−	−	−	−
D-serine	+	+	−	−	−	−	−	−	−	−	−	−
Glycerol	−	−	−	+	−	+	+	+	−	−	−	+
Enzymatic activities												
Alkaline phosphatase	+	+	−	+	+	+	+	+	+	+	+	+
Lipase (C14)	−	−	−	−	−	−	−	−	+	+	−	−
Leucine arylamidase	+	+	−	+	+	+	+	+	+	+	+	+
Valine arylamidase	+	+	−	+	+	+	−	+	+	+	+	+
Cystine arylamidase	+	+	−	+	+	+	−	+	+	+	−	+
Trypsin	+	+	−	−	+	−	−	−	+	+	−	−
α-chymotrypsin	+	−	+	−	−	−	−	−	+	+	−	−
α-galactosidase	−	−	+	−	−	−	−	+	−	−	−	−
β-galactosidase	−	−	+	−	−	−	−	−	−	−	−	+
α-glucosidase	−	−	+	−	−	−	−	−	+	+	−	−
β-glucosidase	+	−	+	+	+	+	+	+	−	−	+	−
N-acetyl-β-glucosaminidase	−	−	−	−	−	−	−	−	−	−	−	+
α-mannosidase	−	−	+	−	−	−	−	−	−	−	−	−

Table 1 continued

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
API 20NE (assimilation)												
D-glucose	+	+	+	+	–	+	–	+	–	–	+	+
L-arabinose	–	+	+	+	–	+	+	+	–	–	+	+
D-mannose	+	+	+	+	–	–	–	–	+	–	+	+
D-mannitol	–	+	–	+	–	–	–	+	–	–	+	+
N-acetyl-glucosamine	+	+	–	+	+	–	–	+	–	–	–	+
D-maltose	+	+	–	–	–	–	–	–	–	+	–	–
Potassium gluconate	–	+	–	+	–	+	+	+	+	+	+	+
Capric acid	–	+	–	+	+	–	–	–	–	–	–	–
Malic acid	+	+	–	+	+	+	+	+	+	+	+	+
Trisodium citrate	–	–	–	+	–	–	–	–	–	–	–	–
Phenylacetic acid	–	–	–	+	–	–	–	+	–	–	–	–
DNA G+C content	67.6 ± 0.1	64–67	67–68	69–70	69.6 ± 0.1	69–71*	64–66	68.7	68.8*	69–70	65*	69.7*

* Data of DNA G+C content were obtained from Coenye et al. (2002); Zhang et al. (2003); Mehnaz et al. (2007a); Weon et al. (2007)

DNA–DNA hybridization analysis

The DNA–DNA relatedness values of strain CC-LY736^T with *A. irakense* BCRC 15764^T was 51.2 ± 2.3 %. Strain CC-LY736^T showed less DNA–DNA homology with *A. irakense* BCRC 15764^T. Using the established molecular criteria for species-level relatedness described by Wayne et al. (1987), strain CC-LY736^T shows less DNA–DNA homology with other closest related species, which supports its genomic distinction as a separate species.

Cellular fatty acid analysis

The FAME profiles in strain CC-LY736^T and *A. irakense* BCRC 15764^T were similar, both strains contain $C_{16:1}\omega5c$, $C_{19:0}$ cyclo ω8c, $C_{16:0}$ 3-OH, $C_{18:1}$ 2-OH and $C_{18:1}$ $\omega7c/C_{18:1}$ $\omega6c$ as major (>5 % of the total) fatty acids. Strain CC-LY736^T and *A. irakense* BCRC 15764^T were distinguished from genera *Azospirillum*, *Rhodocista* and *Skermanella* based on the FAME features. *A. amazonense* BCRC 14279^T also accommodated similar major fatty acids of strain CC-LY736^T and *A. irakense* BCRC 15764^T except for $C_{16:1}\omega5c$, which was found in minor amounts in the former. On the other hand, FAME profile of *A. lipoferum* BCRC 12213^T was distinct when compared to strain CC-LY736^T, *A. amazonense* BCRC 14279^T and *A. irakense* BCRC 15764^T as it accommodated $C_{17:1}\omega6c$, $C_{18:1}$ 2-OH, summed features 2, 3 and 8 in major amounts. Similarly, members of the genus *Rhodocista* possessed $C_{14:0}$, $C_{16:1}\omega5c$ and summed feature 8 in major amounts. There were clear qualitative and quantitative differences in terms of several fatty acids within *Rhodospirillaceae* members, irrespective of the presence of summed feature 8 in significant (>25 %) amounts. The details of fatty acid profiles of strain CC-LY736^T and other *Rhodospirillaceae* representatives are given in Table 3.

Cellular polar lipid analysis

Strain CC-LY736^T contained phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylmethylethanolamine (PME), and phosphatidyldimethylethanolamine (PDE), which were in line with that of *A. irakense* BCRC 15764^T. In addition, strain CC-LY736^T contained two unidentified glycolipids (GL1–2) that were absent in *A. irakense* BCRC 15764^T. Interestingly, *A. amazonense* BCRC 14279^T possessed DPG and three unidentified aminolipids (AL1–3), three unidentified phospholipids (PL1–3), an unidentified glycolipid (GL) and an unidentified lipid (L) besides lacking other polar lipids that were found commonly in strain CC-LY736^T and *A. irakense* BCRC 15764^T. In contrast, other *Azospirillum* species ($n = 4$) possessed PC, PE, PG, PDE and AL in common, but consistently lacked PME. Similarly, members of the genera *Rhodocista* and

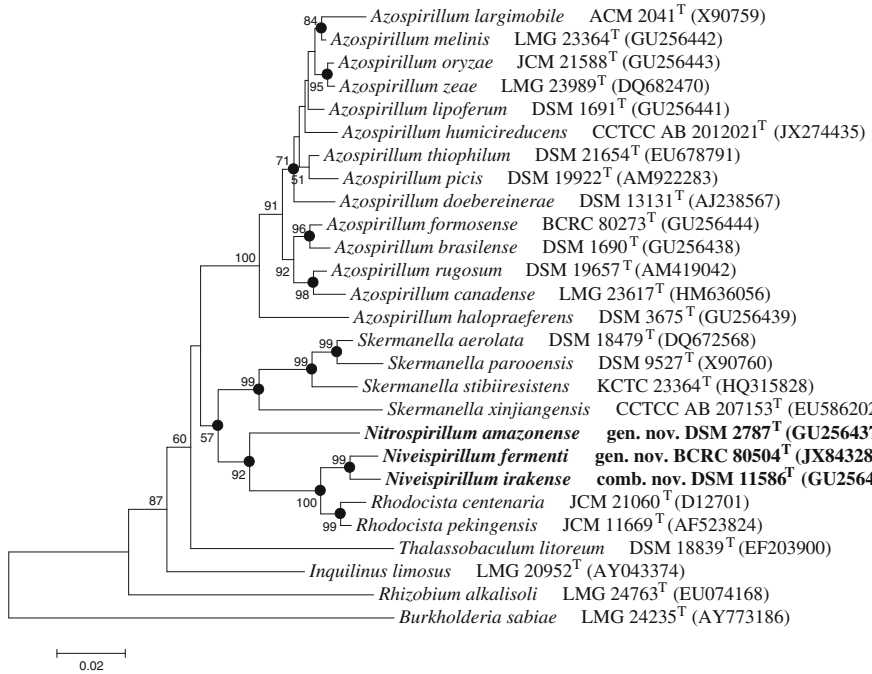

Fig. 1 Phylogenetic analysis of *Niveispirillum, Nitrospirillum* and other representatives of the family *Rhodospirillaceae* based on 16S rRNA gene sequences. Distances and clustering were performed by using Neighbor-Joining method with the software package MEGA 5. Filled circles indicate that the corresponding nodes were also recovered in the tree constructed based on Maximum Likelihood and Maximum Parsimony algorithm. Bootstrap values (>50 %) based on 1,000 replications are shown at the branching points. *Rhizobium alkalisoli* LMG 24763^T and *Burkholderia sabiae* LMG 24235^T were used as outgroups. Bar, 0.02 substitutions per nucleotide position

Skermanella also lacked PME, but accommodated other polar lipids in significant amounts. Taken together, polar lipid profile clearly distinguished strain CC-LY736^T, *A. amazonense* BCRC 14279^T and *A. irakense* BCRC 15764^T from each others as well as from other *Rhodospirillaceae* representatives. The details of polar lipid profiles of strain CC-LY736^T and other *Rhodospirillaceae* representatives are given in Table 4 and Fig. S2.

Cellular polyamine analysis

The distribution of polyamines within various members of the family *Rhodospirillaceae* is given in Fig. S3. Irrespective of their quantitative variations, putrescine and *sym*-homospermidine were two important polyamines detected consistently in *Rhodospirillaceae* representatives. Strain CC-LY736^T possessed major amounts of *sym*-homospermidine and moderate amounts of putrescine. However, *A. irakense* BCRC 15764^T contained both *sym*-homospermidine and putrescine in predominant amounts. In contrast, *A. amazonense* BCRC 14279^T accommodated major amounts of putrescine and moderate amounts of *sym*-homospermidine. The polyamine data presented here revealed that *sym*-homospermidine is most likely to be a predominant polyamine in *Azospririllum* species, whereas putrescine could be a major polyamine in the members of the genera *Rhodocista* and *Skermanella*.

Cellular respiratory quinone and DNA base composition

Strain CC-LY736^T and the reference strains (except for *Rhodocista*, which possessed ubiquinone Q-9 as a major respiratory quinone) contained ubiquinone Q-10 as the predominant respiratory quinone. The DNA G+C content of strain CC-LY736^T was 67.6 ± 0.1 mol %, a value that was well within the range (64.0–70.0 mol %) reported for the representatives of the family *Rhodospirillaceae*.

Table 2 Pairwise 16S rRNA gene sequence similarity between strain CC-LY736^T, *Niveispirillum irakense* DSM 11586^T, *Nitrospirillum amazonense* DSM 2787^T and other representatives of the family *Rhodospirillaceae*

	Azospirillum ($n = 14$)	*Inquilinus* ($n = 1$)	*Rhodocista* ($n = 2$)	*Skermanella* ($n = 4$)
Nitrospirillum amazonense DSM 2787^T	85–91	88	91, 93	89–93
Niveispirillum irakense DSM 11586^T	85–91	89	95, 97	89–91
Niveispirillum fermenti CC-LY736^T	90–92	88	96	90–91

The number of species (n) of a corresponding genus is given in parenthesis

In conclusion, based on the phylogenetic, physiological, chemotaxonomic and phenotypic characteristics, strain CC-LY736^T should be classified as a novel genus of the family *Rhodospirillaceae*, for which we propose the name *Niveispirillum fermenti* gen. nov. sp. nov.; the type strain of the type species is CC-LY736^T (= BCRC 80504^T = LMG 27263^T). We also reclassify *Azospirillum irakense* as *Niveispirillum irakense* comb. nov., and *Azospirillum amazonense* as *Nitrospirillum amazonense* gen. nov., sp. nov.

Description of *Niveispirillum* gen. nov

Niveispirillum (Ni.ve.i.spi.ril'lum. L. adj. niveus snow-white; L. fem. n. spirillum spiral; N.L. neut. n. Niveispirillum snow-white spiral)

Cells are Gram-stain negative and show positive reaction for catalase and oxidase. The polyamine profile consists of *sym*-homospermidine and putrescine. The predominant (>30 %) fatty acids are $C_{18:1}$ ω7c/$C_{18:1}$ ω6c. The major polar lipids are DPG, PG, PC, PE, PME, PDE and an unidentified aminolipid (AL1). The predominant quinone is ubiquinone Q-10. The type species is *Niveispirillum fermenti*.

Description of *Niveispirillum fermenti* sp. nov

Niveispirillum fermenti (fer.men'ti. L. neut. gen. n. fermenti, of a fermentation process)

Cells are spiral-shaped, which are 0.6–0.8 μm in width and 2.0–2.3 μm in length and contain a monopolar flagellum. Colonies appear cream-colored, circular and smooth when grown on R2A agar. Growth occurs at temperature 20–40 °C, pH 5.0–9.0 and <2 % (w/v) NaCl. The accumulation of intracellular granules (PHB) is observed. In the GN2 Biolog MicroPlate, cells can utilize α-D-lactose, D-melibiose, β-methyl-D-glucoside, D-sorbitol, acetic acid, cis-aconitic acid, D-galactonic acid lactone, γ-hydroxybutyric acid, itaconic acid, succinic acid, succinamic acid, D-alanine, glycyl-L-aspartic acid, L-histidine, L-leucine, L-pyroglutamic acid, D-serine, L-serine, urocanic acid, putrescine, 2-aminoethanol, 2,3-butanediol, D,L-α-glycerol phosphate and α-D-glucose-1-phosphate as sole carbon source; other carbon sources are not utilized. Nitrate and nitrite are reduced. In the API ZYM strip, positive for alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase and β-glucosidase; negative for other reactions. The major fatty acids are $C_{16:0}$, $C_{16:1}$ ω5c, $C_{19:0}$ cyclo ω8c, $C_{18:1}$ ω7c/$C_{18:1}$ ω6c, $C_{16:0}$ 3-OH and $C_{18:1}$ 2-OH. The polar lipids are DPG, PG, PC, PE, PME, PDE, AL1, PL and GL1-2. The predominant quinone is ubiquinone Q-10 and DNA G+C content of the type strain CC-LY736^T is 67.6 ± 0.1 mol %.

Type strain is CC-LY736^T (= BCRC 80504^T = LMG 27263^T) isolated from a fermentor in Taiwan.

Description of *Niveispirillum irakense* comb. nov

Niveispirillum irakense (i.ra.ken'se. N.L. neut. adj. irakense, pertaining to Iraq, where the organism was first isolated)

Gram-stain negative, aerobic, curved rods or S-shaped, motile with winding or snake-like movements. Catalase- and oxidase-positive. The growth temperature ranges from 20 to 35 °C, pH 5.5–8.5 (optimum pH is 6.5) and tolerates up to 3 % (w/v) NaCl. A monopolar flagellum is observed when the cells are grown in a liquid medium. Intracellular granules (poly-β-hydroxybutyrate) are observed. The

Table 3 Comparison of the cellular fatty acid contents (%) of strain CC-LY736^T and. other representatives of the family *Rhodospirillaceae*

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12
Saturated												
$C_{10:0}$	–	1.2	3.5	–	–	–	–	–	–	–	–	–
$C_{12:0}$	tr	4.2	–	–	3.6	–	–	tr	1.6	–	–	–
$C_{14:0}$	1.9	1.2	2.4	tr	1.4	–	–	1.6	–	–	–	–
$C_{15:0}$	–	–	–	1.4	–	–	–	–	–	–	–	–
$C_{16:0}$	7.3	2.1	10.0	4.3	13.6	4.1	4.4	7.7	4.2	5.9	11.8	1.4
$C_{17:0}$	tr	–	–	tr	–	–	–	–	–	–	–	1.0
$C_{18:0}$	tr	–	1.5	tr	–	–	–	tr	–	–	–	1.6
Unsaturated												
$C_{15:1}$ iso G	–	–	–	–	–	–	–	–	–	–	–	1.1
$C_{16:1}$ ω5c	8.6	5.2	2.6	–	–	–	–	–	6.1	1.9	–	–
$C_{16:1}$ ω9c	tr	–	–	–	–	–	–	–	–	–	1.5	–
$C_{16:1}$ ω11c	–	–	–	–	–	–	–	–	–	–	1.6	–
$C_{17:0}$ cyclo	1.5	tr	tr	–	–	–	–	–	–	–	–	–
$C_{17:1}$ ω6c	–	–	–	7.1	–	–	–	tr	5.3	1.5	–	–
$C_{17:1}$ ω8c	–	–	–	3.4	–	–	–	–	1.9	–	–	–
$C_{18:1}$ ω5c	tr	1.3	–	–	–	–	–	–	–	–	–	–
Branched												
$C_{18:0}$ iso	–	–	–	–	–	–	–	–	–	–	2.5	–
$C_{19:0}$ cyclo ω8c	14.8	6.2	14.1	1.6	5.8	–	–	6.8	–	–	–	9.1
Hydroxy												
$C_{10:0}$ 3-OH	–	–	–	–	–	–	–	–	–	–	–	2.1
$C_{14:0}$ 2-OH	4.5	5.9	4.8	–	–	–	–	–	–	–	–	–
$C_{16:0}$ 2-OH	tr	–	1.5	–	–	–	–	–	–	1.3	–	–
$C_{16:0}$ 3-OH	7.3	11.6	15.5	4.3	4.2	3.7	4.2	9.6	3.2	5.3	1.8	3.1
$C_{17:0}$ 2-OH	–	–	–	–	–	–	–	–	–	–	–	1.6
$C_{17:0}$ 3-OH	–	–	–	tr	–	–	–	–	–	–	–	1.5
$C_{18:0}$ 2-OH	–	–	–	–	–	–	–	–	–	–	–	1.6
$C_{18:0}$ 3-OH	tr	–	–	tr	–	–	–	–	–	–	–	4.3
$C_{18:1}$ 2-OH	6.5	11.6	8.5	5.5	1.4	5.9	6.9	1.0	3.9	7.2	1.2	18.9
Summed feature 2	1.0	10.4	2.1	5.9	5.5	4.8	5.8	10.9	3.9	2.9	5.4	–
Summed feature 3	4.8	2.6	1.3	6.5	12.5	13.1	15.1	6.6	11.7	3.5	7.5	–
Summed feature 5	–	1.1	–	–	–	–	–	–	–	–	–	–
Summed feature 8	36.4	31.3	27.6	53.4	46.7	60.2	56.4	51.83	51.4	53.1	64.9	45.5

Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system

Summed feature 2: $C_{14:0}$ 3-OH/$C_{16:1}$ iso I

Summed feature 3: $C_{16:1}$ ω7c/$C_{16:1}$ ω6c

Summed feature 5: $C_{18:0}$ ante/$C_{18:2}$ ω6,9c

Summed feature 8: $C_{18:1}$ ω7c/$C_{18:1}$ ω6c

Taxa: 1, CC-LY736^T; 2, *Niveispirillum irakense* BCRC 15764^T; 3, *Nitrospirillum amazonense* BCRC 14279^T; 4, *Azospirillum lipoferum* BCRC 12213^T; 5, *Azospirillum fermentarium* BCRC 80505^T; 6, *Azospirillum brasilense* BCRC 12270^T; 7, *Azospirillum formosense* BCRC 80273^T; 8, *Azospirillum picis* DSM 19922^T; 9, *Rhodocista pekingensis* JCM 11669^T; 10, *Rhodocista centenaria* JCM 21060^T; 11, *Skermanella aerolata* DSM 18479^T; 12, *Inquilinus limosus* LMG 20952^T. tr, <1 %; –, not detected

Table 4 Comparison of the polar lipid profiles of strain CC-LY736^T (= BCRC 80504^T) and other representatives of the family *Rhodospirillaceae*

Organism	Polar lipid										
	PC	PE	PG	DPG	PME	PDE	APL	AL	PL	GL	L
Niveispirillum fermenti BCRC 80504^T	+	+	+	+	+	+	+	+	+	+	–
Niveispirillum irakense BCRC 15764^T	+	+	+	+	+	+	+	+	+	–	–
Nitrospirillum amazonense BCRC 14279^T	–	–	–	+	–	–	–	+	+	–	–
Azospirillum brasilense BCRC 12270^T	+	+	+	+	–	+	–	+	+	–	–
Azospirillum picis DSM 19922^T	+	+	+	+	–	+	+	+	+	–	–
Azospirillum rugosum DSM 19657^T	+	+	+	+	–	+	–	+	–	–	–
Azospirillum formosense BCRC 80273^T	+	+	+	–	–	+	–	+	+	–	–
Azospirillum oryzae JCM 21588^T	+	+	+	+	–	+	–	+	+	–	–
Rhodocista pekingensis JCM 11669^T	+	+	+	+	+	+	+	+	–	+	–
Rhodocista centenaria JCM 21060^T	+	+	+	+	+	+	+	+	–	+	–
Skermanella aerolata DSM 18479^T	+	+	+	+	–	–	+	+	–	–	+

PC phosphatidylcholine, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerol, *DPG* diphosphatidylglycerol, *PME* phosphatidylmethylethanolamine, *PDE* phosphatidyldimethylethanolamine, *AL* aminolipid, *GL* glycolipid, *APL* unidentified aminophospholipid, *PL* unidentified phospholipid, *L* unidentified lipid. Symbol: **+**, positive; **−**, negative

predominant quinone is Q-10. In the GN2 Biolog MicroPlate, cells can utilize dextrin, adonitol, L-arabinose, D-arabitol, D-cellobiose, D-fructose, L-fucose, D-galactose, α-D-glucose, maltose, D-mannitol, D-mannose, L-rhamnose, sucrose, D-trehalose, acetic acid, citric acid, L-histidine, L-leucine, L-ornithine, L-proline, and D-serine as sole carbon source; other substrates are not utilized. Nitrate is reduced to nitrite. In the API 20NE system, cells assimilate D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid and malic acid. In the API ZYM system, positive for alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase and trypsin. The DNA G+C content of the type strain KBC1^T is 64–67 mol %.

Type strain is KBC1^T (= ATCC 51182^T = BCRC 15764^T = CIP 103311^T) isolated from rice rhizosphere in Iraq.

Description of *Nitrospirillum* gen. nov

Nitrospirillum (Ni.tro.spi.ril'lum. Gr. n. nitron soda; L. neut. n. spirillum spiral; N.L. neut. n. Nitrospirillum soda spiral)

Cells are Gram-stain negative, diazotrophic and show positive reaction for catalase and oxidase. The polyamine patterns include major amounts of putrescine and moderate amounts of *sym*-homospermidine. The predominant (>27 %) fatty acids are $C_{18:1}$ ω7c/$C_{18:1}$ ω6c. The polar lipid profile constitutes major amounts of DPG, three unidentified phospholipids (PL1–3) and two unidentified aminolipids (AL2–3). The predominant quinone is ubiquinone Q-10. The type species is *Nitrospirillum amazonense*.

Description of *Nitrospirillum amazonense* sp. nov

Nitrospirillum amazonense (a.ma.zon.en'se N.L. neut. adj. amazonense, pertaining to the Amazon region of Brazil)

Cells are spiral-shaped, which are 0.9–1.0 μm in width and 3.5 μm in length. Colonies appear cream-colored, circular and smooth when grown on R2A agar. Optimal growth occurs at temperature 35 °C, pH 5.7–7.8 and <3 % (w/v) NaCl. In the GN2 Biolog MicroPlate, cells are able to utilize α-cyclodextrin, dextrin, D-cellobiose, α-D-glucose, maltose and D-melibiose as sole carbon source; other carbon sources are not utilized. Nitrate and nitrite are not reduced. Assimilate D-glucose, L-arabinose and D-mannose. In the API ZYM strip, positive for esterase (C4), esterase lipase (C8), α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase and α-

mannosidase; other reactions are negative. The major fatty acids are $C_{16:0}$, $C_{19:0}$ cyclo ω8c, $C_{16:0}$ 3-OH, $C_{18:1}$ 2-OH and $C_{18:1}$ ω7c/$C_{18:1}$ ω6c. The polar lipid profile constitutes DPG, three unidentified phospholipids (PL1–3) and three unidentified aminolipids (AL1–3), an unidentified lipid (L) and glucolipid (GL) each. The predominant quinone is ubiquinone Q-10 and DNA G+C content of the type strain $Am14^T$ is 67–68 mol %.

Type strain is $Am14^T$ (= ATCC 35119^T = BCRC 14279^T = DSM 3787^T) was isolated from grass rhizosphere in Brazil.

Emended description of the genus *Rhodocista* Kawasaki et al. 1992

The genus description of *Rhodocista* is given by Kawasaki et al. (1992) with following amendments: both nitrate and nitrite are reduced. The predominant (>51 %) fatty acids are $C_{18:1}$ ω7c/$C_{18:1}$ ω6c. The major polar lipids are PC, PG, DPG, PE, PDE, PL and two unidentified aminolipids (AL2 and AL4). Polyamine profile contains major amounts of putrescine and moderate amounts of *sym*-homospermidine. The range of DNA G+C content is 68.8–70 mol %.

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