Description of Oleispirillum naphthae gen. nov., sp. nov., a potential nitrogen-fixing bacterium of the order Rhodospirillales, and proposal of Oleispirillaceae fam. nov.

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Abstract

A microaerophilic, gram-negative, motile, and spiral-shaped bacterium, designated Y-M2^T, was isolated from oil sludge of Shengli oil field. The optimal growth condition of strain Y-M2^T was at 25°C, pH 7.1, and in the absence of NaCl. The major polar lipid was phosphatidylethanolamine. The main cellular fatty acid was iso $C_{17:0}$ 3-OH. It contained Q-9 and Q-10 as the predominant quinones. The DNA G + C content was 68.1 mol%. Strain Y-M2^T shared the highest 16S rRNA gene sequence similarity with *Telmatospirillum siberiense* 26-4b^T (90.13%). Phylogenetic analyses based on 16S rRNA gene and genomes showed that strain Y-M2^T formed a distinct cluster in the order *Rhodospirillales*. Genomic analysis showed that Y-M2^T possesses a complete nitrogen fixation cluster which was phylogenetically close to that of methanogene. The nitrogenases genes *nif* cluster were present in all N_2-fixing strains in the order *Rhodospirillales*. Phylogeny, phenotype, chemotaxonomy, and genomic results demonstrated that strain Y-M2^T represents a novel species of a novel genus in a novel family *Oleispirillaceae* fam. nov. in the order *Rhodospirillales*, for which the name *Oleispirillum naphthae* gen. nov., sp. nov. was proposed. The type strain is Y-M2^T (= CCAM 827^T = JCM 34765^T).

Introduction

Members of the order *Rhodospirillales*, affiliated with the class *Alphaproteobacteria* within the phylum *Pseudomonadota*, are ubiquitous in soil (Cordeiro et al., 2017, Divyasree et al., 2015), air (Yoo et al., 2008), water (Baik et al., 2013, Yang et al., 2019) , anode biofilms (Zhou et al., 2013), crude oil-contaminated soil (Wu et al., 2021, Young et al., 2008), respiratory secretions (Coenye et al., 2002), and other niches (Dziuba et al., 2016, Humrighouse et al., 2016, Tikhonova et al., 2019, Yoon et al., 2007). The order *Rhodospirillales* comprises 12 validly published families which were reclassified by Hördt et al. in 2020 (Hordt et al., 2020). However, six more novel families were separated from the existing families within this order according to the phylogenomic analyses (ref). The family *Rhodospirillaceae* was splitted into family "*Dongiaceae*" (*Dongia*, *Aliidongia*, and *Hypericibacter*), "*Oceanibaculaceae*" (*Oceanibaculum*), and current *Rhodospirillaceae* (*Pararhodospirillum*, *Phaeovibrio*, *Rhodospira*, *Rhodospirillum* and *Roseospirillum*) (Koziaeva et al., 2023). Most members of this order are chemoorganoheterotrophic and/or photoorganoheterotrophic (Guyoneaud et al., 2002, Kalyan Chakravarthy et al., 2007, Urdiain et al., 2008). It also contains free-living nitrogen-fixing bacteria, such as the genus *Azospirillum* in the family *Azospirillaceae* (Eckert et al., 2001, Tikhonova, et al., 2019, Xie and Yokota, 2005). Most of them were obtained from plant roots-related environments, including the facultative anaerobes *A. oryzae* COC8^T (Xie and Yokota, 2005) and *A. lipoferum* Sp 7^T (Tarrand et al., 1978, Zhou, et al., 2013). There were several nitrogen-fixing bacteria within *Azospirillum* (*A. palustre* B2^T, *A. oleiclasticum* RWY-5-1-1^T and *A. rugosum* IMMIB AFH-6^T) and aerobic *Oleiliquidispirillum nitrogeniifigens* 64-1^T in the family *Rhodospirillaceae* isolated from oil-related environments, including oil-contaminated soil and oil production mixtures (Tikhonova, et al., 2019, Wu, et al., 2021, Young, et al., 2008), indicating members of *Rhodospirillales* probably involved nitrogen cycling in oilfield.

Biological nitrogen fixation, in which microorganisms convert atmospheric nitrogen gas (N_2) to ammonia (NH_3), is an important mechanism to support bio-available nitrogen for organisms. Additionally, it plays a critical role in the global nitrogen cycle. It has been shown that this process is mainly mediated by three different nitrogenases, molybdenum-iron nitrogenase (Nif, Mo-Fe), vanadium-iron nitrogenase (Vnf, V-Fe), and iron-only nitrogenase (Anf, Fe-Fe) (Jasniewski et al., 2020, Parsons et al., 2021). Nif is constructed by two protein components: an electron delivery component (NifH, encoded by *nifH*) and a catalytic component (NifD and NifK, encoded by *nifD* and *nifK*, respectively) (Jasniewski, et al., 2020). Nif is the most important nitrogen fixation protein and is widely distributed in archaea (phylum *Euryarchaeota*) and thirteen phyla in the bacterial domain, primarily including *Proteobacteria*, *Firmicutes*, *Cyanobacteria*, and *Bacteroidetes* (Jasniewski, et al., 2020), which have been found in a variety of environments ranging from marine (Affourtit et al., 2001, Dong et al., 2022), rhizosphere (Mahmud et al., 2020, Zhang et al., 2021), freshwater sediments (Affourtit, et al., 2001), and oilfields (Sizova et al., 2007, Wu, et al., 2021). Genomic studies have revealed that nitrogen fixation-related genes constitute a nitrogen fixation cluster, such as the 17 *nif* genes (*nifQ*, *nifA*, *nifL*, *nifH*, *nifD*, *nifK*, *nifT*, *nifY*, *nifE*, *nifN*, *nifX*, *nifS*, *nifU*, *nifW*, *nifZ*, *nifM*, and *nifF*) distributed in a 49-kb region in *Pseudomonas stutzeri* A1501^T (Yan et al., 2008). Previous studies have demonstrated that all nitrogen fixation bacteria in the order *Rhodospirillales* carry *nif* gene clusters, and no *vnf* or *anf* cluster has been identified in these species (FL. et al., 2020, Sizova, et al., 2007, Tikhonova, et al., 2019, Wu, et al., 2021, Xie and Yokota, 2005, Young, et al., 2008, Zhou, et al., 2013).

Materials and methods

Isolation and cultivation

Strain Y-M2^T was isolated from oil sludge collected from Shengli oil field in Shandong province, China (37°54'N, 118°33'E) using a traditional dilution method with 96-well microplate as described previously (Zhang et al., 2018). Mineral medium containing 9.0 g NaCl, 0.5 g KCl, 0.3 g NH_4Cl, 0.2 g KH_2PO_4, 3.0 g $MgCl_2{\cdot}6H_2O$, 0.15 g $CaCl_2{\cdot}2H_2O$, 0.5 g l-cysteine hydrochloride, 1 ml 2 × TE284 buffer, and 0.001 g resazurin in 1000 ml distilled water was used for isolation. The medium was prepared under anaerobic conditions with 99.999% nitrogen as a protective atmosphere and was sterilized for 30 min at 121 °C. Samples were pretreated in 96-well plates using a series of dilutions with mineral media containing short-chain fatty acids, glucose, yeast extract, and tryptone, subsequently were cultured at 25 °C in rectangular jar using AnaeroGen (Thermo Scientific, USA) for oxygen removal. After 7–30 days of incubation, pure cultures of strain Y-M2^T were identified by sequencing the 16S rRNA gene that were amplified with the primer 27F/1492R (Weisburg et al., 1991). The purified strain Y-M2^T was cultured at 25 °C under microaerobic conditions (10% oxygen) in Reasoner's 2 (R2A) broth containing 0.25 g tryptone, 0.5 g casein hydrolysate, 0.5 g yeast extract, 0.5 g soluble starch, 0.3 g K_2HPO_4, 0.1 g $MgSO_4$, 0.3 g sodium pyruvate, 0.25 g peptone, 0.5 g glucose, and 1000 ml distilled water. The medium was prepared and dispensed under 99.999% N_2 with the addition of 10% oxygen, and sterilized for 15 min at 121 °C.

Genomic sequencing and phylogenetic analyses

A commercial bacterial genomic DNA extraction kit (Magen, Guangzhou, China) was used to extract the genomic DNA of strain Y-M2^T. The draft genome of strain Y-M2^T was sequenced and assembled using the Illumina NovaSeq sequencing platform (Beijing novogene Technology Co., Ltd., Beijing, China). The SPAdes strategy was used to assemble the genome, and the resulting N20, N50, and N90 values were 461,460, 216,590, and 128,336, respectively. The universal bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Weisburg, et al., 1991) were used to amplify the 16S rRNA gene fragments for preliminary analysis using Ezbiocloud (https://www.ezbiocloud.net/identify). The entire 16S rRNA sequence was used for further phylogenetic analyses. The 16S rRNA gene sequences of strains with a close taxonomic relationship were downloaded from the NCBI (https://www.ncbi.nlm.nih.gov/) and LPSN databases (http://www.bacterio.net/). All sequences were aligned using MUSCLE (Edgar, 2004), and the phylogenetic trees were constructed using the software FastTree with default parameters (Price et al., 2010), and IQ-TREE with '-m TEST -bb 1000 -alrt 1000' (Nguyen et al., 2015).

A total of 447 genomes belonging to the order *Rhodospirillales* and 23 genomes of type strains belong to the order *Rickettsiales* in class *Alphaproteobacteria* were downloaded from the NCBI database. These genomes were predicted by Prodigal (V2.6.3) (Hyatt et al., 2010) and annotated by using KofamKOALA with default parameters (Aramaki et al., 2020). Paired genomic average nucleotide identity (ANI) and average amino acid identity (AAI) were calculated using OrthoANIu (Yoon et al., 2017) and Compare M (https://github.com/dparks1134/CompareM), respectively. Percentages of conserved proteins (POCP) between two microbial genomes were calculated according to the method described previously(Yang et al., 2021). Genomes quality and single-copy marker genes were retrieved using CheckM (https://github.com/Ecogenomics/CheckM/wiki).

The phylogenetic of concatenated alignments of multiple single-copy marker genes, not only makes additional remarks for 16S rRNA gene phylogenies, but also provide better phylogenetic resolution at the family level, so a genome tree based on concatenated alignments of single-copy genes of *Rhodospirillales* was inferred using IQ-TREE with '-m TEST -bb 1000 -alrt 1000' (Nguyen, et al., 2015). Phylogenomic trees based on 92 bacterial core genes (UBCG) was constructed by UBCG pipeline (Na et al., 2018).The genome was also used to construct a genome tree based on the GTDB database (release r95) with the software GTDB-Tk (Parks et al., 2018) with optimization by ETE3 (Huerta-Cepas et al., 2016). The entire evolutionary trees were visualized using the Evolview website (https://www.evolgenius.info/evolview/#/treeview).

A global distribution of Y-M2^T and relative strains was analysed based on 16S rRNA gene and genomes, a total 12 available 16S rRNA gene sequences and 6 available genomes were download from NCBI and Sliva database, and visualized using the R (version 3.6.3) according to the location information.

The 16S rRNA gene sequence of strain Y-M2^T were submitted to GenBank with accession numbers MZ270535.1 and the genome shotgun project of strain Y-M2^T has been deposited at DDBJ/ENA/GenBank under the accession JAQAZG000000000.

Morphology, physiology, and chemotaxonomy

For morphology tests, the strain Y-M2^T was incubated at 25 °C in R2A medium for 7 days. Cell size, shape, and flagella were assessed using a scanning electron microscope (JEOL JEM-1400 Plus, Tokyo, Japan) and a transmission electron microscope (JEOL JEM-1230, Tokyo, Japan). A Gram Stain Kit and a Spore Stain Kit (Solarbio, Beijing, China) were used to assess gram staining and spore formation, respectively.

Growth of strain Y-M2^T at different temperatures (15, 20, 25, 30 and 37 °C), pH values (4.1, 5.1, 6.0, 7.1, 8.3, 9.3, 10.3), and NaCl concentrations (0–70 g NaCl l^{-1} at intervals of 10 g NaCl l^{-1}) was determined in R2A broth by measuring the OD_{600} value with a spectrometer (DU730, Beckman Coulter, Inc., California, USA). The pH values were adjusted to pH 4.1-10.3 with the sterilized anoxic HCl or NaOH solutions and were buffered to a final concentration of 20 mM: Na_2HPO_4-citric acid (pH 4.1 and pH 5.1), MES (pH 6.0), PIPES (pH 7.1), Tris (pH 8.3), and CHES (pH 9.3 and pH 10.3). The final pH was determined using a pH meter (HORIBA, Tokyo, Japan).

To investigate the physiological features of strain Y-M2^T, API 20NE and API 20E kits (BioMérieux, Lyon, France) were used according to the manufacturer's instructions. API 20NE and API 20E were incubated for 3 days at 28 °C and 37 °C. The protocols of Microbial Identification Inc. (MIDI) and gas chromatography (Aglient 6990, Aglient, California, USA) were used to identify fatty acids using Sherlock software (version 6.3) (M, 1990). Respiratory quinones were detected in strain Y-M2^T using a previously described protocol (BJ, 1990, Komagata K, 1988). Polar lipids were extracted using a chloroform/methanol system and were analysed using one- and two-dimensional thin-layer chromatography (TLC) following the method described by Kates et al. (Kates, 1986).

Results and discussion

General phenotypic and chemotaxonomic characteristics

Cells of strain Y-M2^T were gram-negative, spiral-shaped with motility with monotrichous flagella, 0.8–3.0 μm in length, and 0.2–0.4 μm in width (Fig S1). Growth was observed at 20–30 °C, pH 7.1, without NaCl, and at 2%–10% O_2. Optimal growth occurred at 25 °C, pH 7.1, without NaCl, and in presence of 10% O_2.

In API 20E tests, strain YM2^T showed positive reactions for gelatinase and pyruvate (Table 1). In API 20NE tests, positive reactions for arginine and urease, and negative reactions for nitrate reduction (Table 1). The strain Y-M2^T containedQ-9 and Q-10 as the predominant quinones (14.2% and 85.2%, respectively). The major fatty acids were iso $C_{17:0}$ 3-OH (26.1%), Summed Feature 8 (15.3%), Summed Feature 3 (13.3%), and iso $C_{15:0}$ 3-OH (12.6%), which were different with the closest related strain containing $C_{18:1}$ *ω7c*, $C_{16:0}$, and $C_{17:0}$ as the major fatty acids (Table 1). The polar lipids profiling was comprised of phosphatidylethanolamine, aminolipid and aminophospholipidase as the main polar lipids (Fig S2).

Genome sequencing

The draft genome sequence of strain Y-M2^T was 3,214,613 bp, contained 2903 open reading frames, 27 contigs, three rRNAs (one each of 5S rRNA, 16S rRNA, and 23S rRNA), 48 tRNA, and 69 ncRNA. The DNA G+C content was 68.07 mol%. (Table S1).

Phylogenetic and phylogenomic analyses

The 16S rRNA gene sequence similarity revealed that strain Y-M2^T was most closely related to *T. siberiense* 26-4b1^T (91.1%) and *Magnetospirillum gryphiswaldense* MSR-1^T (90.6%) in the family "*Magnetospirillaceae*" (formerly belonged to *Rhodospirillaceae*) of the order *Rhodospirillales*. Based on the threshold sequence identity of 16S rRNA genes for separating new genera (94.5%), strain Y-M2^T is proposed to represent a novel genus in order *Rhodospirillales*. The maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences showed that strain Y-M2^T located in the clade containing species of the family "*Novispirillaceae*" and *Rhodospirillaceae*, but formed an independent branch which was well separated from all published families of the order *Rhodospirillales* (Fig 1). Phylogenomic trees reconstructed using GTDB-tk (Fig 2) and using concatenated alignment of 92 core genes (Fig S3) further confirmed that strain Y-M2^T is independent of "*Magnetospirillaceae*", "*Novispirillaceae*", *Rhodospirillaceae*, as well as other *Rhodospirillales* families, indicating it may represent a new family in order *Rhodospirillales*.

The average 16S rRNA gene sequence similarities between strain Y-M2^T and species within "*Magnetospirillaceae*", "*Novispirillaceae*", *Rhodospirillaceae* were ≤90.6% (Table S2), which was close to the minimum sequence identity for distinguishing families (87.65%). The AAI values between strain Y-M2^T and the genera of these three families were ≤58.65% (Table S2), below the family delineating threshold (60%) of order *Rhodospirillales* (Koziaeva, et al., 2023), supporting the proposal of a novel family. ANI and POCP values were less than 71.83% and 48.03%, respectively (Table S2).

Differential characteristics between strain YM2^T and the closely related families

Comparison of the phenotypic characteristics between strain YM2^T and the closely related type strain *T. siberiense* 26-4b^T were presented in Table 1. The representative species of the order *Rhodospirillales* are mesophilic, and containQ-9 and/or Q-10 as the predominant quinones, with the exception of *Rhodovibrio salinarum* DSM 915^T, which contains MK-10. However, there were many differences distinct strain YM2^T from its closely related species *T. siberiense* 26-4b^T, as well as the other families within *Rhodospirillales* in phenotypic characteristics, such as the differences of gelatinase activity, pyruvate utilization (Table 1). The cellular fatty acid composition between Y-M2^T and *T. siberiense* 26-4b^T was different (Table 1). Furthermore, the physiological comparison revealed that strain Y-M2^T has differences in sulfur and nitrate metabolism with the representative species of family "*Magnetospirillaceae*", "*Novispirillaceae*", and *Rhodospirillaceae* (Table 2). Meanwhile, strain Y-M2^T could be distinguished by the urease from "*Magnetospirillaceae*", and by cell shape from most of representative species in *Rhodospirillaceae*. Therefore, on the basis of phylogenetic analyses lower genomic indices and physiological traits, we proposed that strain Y-M2^T represents a novel family in the order *Rhodospirillales*.

Biogeography

A global analysis of biotope based on 16S rRNA gene sequences and genomes showed that only 12 available 16S rRNA gene sequences (sharing similarity with Y-M2^T > 92%) and 6 available genomes (genome identities with Y-M2^T was > 74, 74, and 64% for ANI, AAI, and POCP) were found in public database, and only 3 16s rRNA sequences and 3 genomes have location information. The preliminary results revealed that members in YM2^T clade were just detected in oil reservoirs, soil, marine hydrothermal sulfide sediment and water (Fig 3), the rarity in natural environments probably due to slow growth and sensitivity to NaCl concentration and pH variation.

Nitrogen fixation function based on comparative genomicanalysis

According to analysis of the Y-M2^T genome, we found that Y-M2^T contains the *nif* family genes *nifH, nifD,* and *nifK*, which are key genes related to nitrogen fixation. *nifH* encodes Fe protein, which is a homodimer bridged by an inter-subunit (4Fe-4S) cluster that serves as the obligate electron donor for the MoFe protein, *nifDK* encodes Mo-nitrogenase, which is composed of dinitrogenase (MoFe protein) and dinitrogenase reductase (Fe protein) (Garcia et al., 2020). *nifH, nifD*, and *nifK* act as marker genes for predicting the nitrogen fixation capabilities of microbes (Hartmann LS, 2010, Normand and Bousquet, 1989, Normand P, 1992). Therefore, we speculate that strain Y-M2^T has ability to fix atmospheric nitrogen to ammonium.

To elucidate the adaptive mechanisms of the *nif* gene of Y-M2^T, we conducted a comparative genomic analysis of the chromosomal regions flanking the *nif* gene clusters contained within a contig. We found that the *nif* cluster genes *nifH*, *nifD*, and *nifK* were present in all N_2-fixing strains in the order *Rhodospirillales*, whereas partial genes of the *anf* and *vnf* clusters were only found in *M. fulvum* DSM 113^T, *M. molischianum* DSM120^T, *Rhodospirillum rubrum* ATCC 11170^T, and *T. siberiense* 26-4b^T. Further analysis revealed that the *anfG* gene mediates crosstalk between the nitrogenases (Pence et al., 2021) *nifH* and *nifK*, for which the assignments were similar to the archaea *Methanosarcina activorans* C2A^T (Fig 4).

The maximum likelihood tree was constructed by concatenation sequence of *nifHDK* genes, and the type of *nifHKD* was distincted to four distinct lineages (Fig 5). Lineage I, II and III was most related to the genus *Azospirillum, Magnetospirillum* and *Roseospirillum*. Interestingly, strain Y-M2^T, as well as several species of the family *Thalassospiraceae* and *Rhodospirillaceae* were placed to the lineage IV which also contained the archaea belonging to the genera *Methanosarcina*, *Methanocella*, *Methanocaldococcus*, *Methanococcus* and *Methanothermobacter*, implying the homology of their nitrogenases (Fig 5). In addition, most of bacteria and archaea in lineage IV were isolated from anaerobic or methanogenic environments (Fig 5). Previous study has revealed that the distribution of nitrogenase genes was crucially impacted by horizontal gene transfer (HGT) events, the acquisition of *nif* by *Firmicutes* possibly through HGT events with methanogen ancestral (Boyd et al., 2011). Therefore, the phylogenetic relationship among strain Y-M2^T, *Thalassospiraceae, Rhodospirillaceae*

species and archaea in the *nifHDK* phylogenetic tree suggested the HGT events possibly occurred between *Rhodospirillales* and methanogens in anoxic environments (Fig 5).

To further analyse *nifHDK* lineage IV, we constructed the *nif* gens organization of based on encoding genes sequences, the *nif* clusters in the lineage IV could be classified to three distinct types (Fig 4). The type I *nif* gene cluster was the most common nitrogenase in bacteria (Fig 4). Compared to the type I, the type II had an additional *anfG* gene inserted between *nifH* and *nifK*, whereas type III had genes *nifN* and *nifE* which replaced *nifT* in type I (Fig 4). Surprisingly, the organization of *nif* genes showed that *T. siberiense* 26-4b1^T contained all types of *nif* cluster described above, which referred that it may play an important role in the evolution of the *nif* genes in the order *Rhodospirillales*.

Conclusion

In conclusion, strain Y-M2^T belongs to the order *Rhodospirillales* and represents a novel genus in a novel family based on the results of phylogenetic analyses, genome relatedness, as well as the observed differences in physiological traits, for which the name *Oleispirillum naphthae* gen. nov. sp. nov. within *Oleispirillaceae* fam. nov. is proposed. The type strain is Y-M2^T.

Declarations

Description of *Oleispirillaceae* fam. nov.

Oleispirillaceae (O.le.i.spi.ril.la.ce'ae. N.L. neut. n. *Oleispirillum* a bacterial genus; -aceae suffix to denote a family; N.L. fem. pl. n. *Oleispirillaceae* the *Oleispirillum* family).

The description of the family is based on the type genus *Oleispirillum*. This family is affiliated with the order *Rhodospirillales* in the class *Alphaproteobacteria*. The type and only genus is *Oleispirillum*.

Descriptions of *Oleispirillum* gen. nov., and *Oleispirillum naphthae* gen. nov., sp. nov.

Cells are microaerophilic, gram-negative, and mesophilic. Motile, spiral-shaped cells with flagella. Cell size is 0.8–3.0×0.2–0.4 μm. Growth is observed at 20–30℃ (optimal temperature 25℃), pH 7.1 (optimal pH 7.1) without NaCl. Positive for gelatinase and pyruvate in API 20E test, and positive for arginine, urease in API 20NE test. The predominant cellular fatty acids are Summed Feature 3, iso C15:0 3-OH, Summed Feature 8 and iso C17:0 3-OH. Polar lipids include phosphatidylethanolamine (PE), aminolipid (AL) and aminophospholipidas (APL). The predominant quinone are Q-9 and Q-10.

The type strain Y-M2^T (=CCAM 827^T=JCM 34765^T) was isolated from Oil sludge. The G+C content of the genomic DNA of strainY-M2^T is 68.1%.

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Supplementary data

Supplementary materials related to this article can be found in the online version.

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Tables

Table 1 Differential phenotypic characteristics of Y-M2^T compared with representative species of each family within the order *Rhodospirillales*.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Habitat	Oil sludge	Mesotrophic fen	Mud	Vinegar plant	Wheat	Biofilter	Marine macroalga	Freshwater	Salterns and salt lakes	Soil, freshwaters	Ma sh
Gram reaction	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND
Morphology	Spiral	Vibrio, spirilla	Helical spirilla	Rod	Vibroid	Coccoid	Spiral	Rod	Vibrioid, spiral	Six-pronged stars	Sp
Motility	-	+	+	ND	ND	ND	+	-	+	-	+
Spore-forming	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cells (μm)	0.8-3.0×0.4-0.6	Diameter 0.2-0.6	0.2-0.4×3-4	0.6-0.9×1.0-4.0	1.0-1.7	1.5-2.1	0.5-0.6×2.5-5.0	0.68-0.92×1.16-1.91	0.8-0.9×1.0-3.5	Diameter 0.7-3.0	0.3 0.5 4.0
Tempreture (optimum)(oC)	20-30(25)	4-30(25-28)	18-38	30	37	15-45(30–35)	4-40(25)	18-37(30-35)	20-45(42)	(28-30)	6-4
pH (optimum)	7.1	4–7(5.7–6.0)	1.5-1.5	(4-6)	5.7-6.8	5.5–11.0(8)	3.5-9.5(3)	ND	(7.5-8.0)	neutral	6.0
NaCl (optimum,%,w/v)	-	ND	ND	ND	-	ND	0.3-10	ND	3-24(8-12)	up to 1	0.5
DNA G+C content (mol%) *	68.07	61.5-64.0	67.2	56.2-57.2	69-70	60.2-60.4	51.1	ND	69.1	69.3-72.9	48
API 20NE											
Arginine	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Reduction of nitrate	-	-	ND	-	+ (to nitrite)	+	+(to N2O)	+	ND	ND	+ (nit
Urease	+	ND	ND	ND	ND	+	-	W	ND	ND	ND
Gelatin hydrolysis	ND	ND	-	ND	ND	ND	ND	+	ND	ND	
Oxidase	ND	-	+	-	+	ND	ND	+	ND	+	+
Catalase	ND	-	+	+	ND	+	ND	-	ND	+	W
Fatty acid	Summed feature 8, Summed feature 3, iso $C_{15:0}$ 3-OH, iso $C_{17:0}$ 3-OH	$C_{18:1}$ *ω7c*, $C_{16:0}$, $C_{17:0}$	Summed feature 8, Summed feature 3, $C_{16:0}$	ND	ND	ND	$C_{18:1}$ *ω7c*, $C_{16:1}$ *ω7c*, $C_{16:0}$, $C_{18:0}$, $C_{19:0}$ cyclo *ω8c*	ND	$C_{18:1}$, $C_{18:0}$	ND	C_1 C_1 C_1
Quinone	Q9, Q10	ND	Q10	Q-9	ND	ND	ND	ND	Q-10, MK-10	ND	Q-

1, strain Y-M2^T (this study); 2, *T. siberiense* 26-4b^T (Sizova, et al., 2007); 3, *M. gryphiswaldense* MSR-1^T (FL., et al., 2020, Garrity et al., 2005); 4, *A. aceti* NBRC 14818^T (Garrity, et al., 2005); 5, *A. lipoferum* DSM 1691^T (Eckert, et al., 2001); 6, *G. roseus* D2-3^T (Foesel et al., 2007); 7, *K. laminariae* LD81^T (Wiese et al., 2009); 8, *R. massiliensis* 521^T (Garrity, et al., 2005); 9, *R. salinarum* DSM 9154^T (Garrity, et al., 2005); 10, *S. humosa* DSM 5900^T (Garrity, et al., 2005); 11, *T. pusilla* DSM 6293^T (Yoon and Kang, 2018); 12, *T. litoreum* CL-GR58^T (Zhang et al., 2008); 13, *T. lucentensis* QMT2^T (López-López et al., 2002); 14, *Z. compransoris* LMG 5821^T (Garrity, et al., 2005); +, positive; –, negative; W, weak; ND, not determined. Summed feature 3, $C_{16:1}$ *ω7c* and/or $C_{16:1}$ *ω6c*; Summed feature 8, $C_{18:1}$ *ω7c* and/or $C_{18:1}$ *ω6c*.

Table 2 Differential phenotypic characteristics of Y-M2^T compared with phylogenetically closed families within the order *Rhodospirillales.*

Characteristics	*Oleispirillaceae*	*Rhodospirillaceae*	*"Novispirillaceae"*	*"Magnetospirillaceae"*
genus	1	14	5	5
Oxygen requirement	Microaerophilic	Strictly aerobic; aerobic; facultatively anaerobic;	Anaerobic	Aerobic; microaerobic
shape	Spiral	Rod; curved rod; vibrioid or spiral	Helical	Spirillum; vibrioid to spiral
Motility	+	+/-	+	+/-
Gram-stain	-	-	-	-
Flagellation	single polar	single polar; bipolar tufts of flagella (two to five fibrils);	single polar; bipolar	polar or subpolar flagella
spore-forming	-	-	-	-
oxidase	-	+/-	+/-	+/-
Catalase	-	+/-	+	+/-
Growth temperature (℃):				
Range	20-30	10–45	10–47	4–55
Optimum	25	25-40	25–37	25-45
NaCl concertration for growth (g/L):				
Range	0	0–15%	0-12 %	0-3%
Optimum	0	0-8%	0.5-8%	-
pH				
range for growth	7.1	5.0-11.0	6.5 and 10.0	4.0-10.0
Optimum	7.1	7.0-9.0	7.0–8.0	5.7-7.5
Gelatin hydrolysis	+	+/-	-	-
urease	+	+/-	+/-	-
H2S production	ND	-	+/-	+
Electron acceptors + :	ND	Sulfate; nitrate	Nitrate	Nitrate
utilization of sugars	+	+	+	+
utilization of amino acids	+	+	+	-
utilization of organic acids	+	+	+	+
Respiratory quinones	Q9; Q10	Q-7; MK-7; Q8; RQ8; Q9; Q10	Q-9; Q-10	Q-9; MK-9; Q-10
Genome size (Mb)	3.2	2.1-5.4	2.4-4.6	3.8-6.2
DNA G+C content (mol%)	68.1	59.1-69.3	62.4-70.0	61.6-66.4

1, *Oleispirillaceae* fam. nov. (this study); 2, "*Magnetospirillaceae*" (FL., et al., 2020, Imhoff et al., 1998, Koziaeva, et al., 2023, Sizova, et al., 2007, Thrash et al., 2010); 3, "*Novispirillaceae*" (Humrighouse et al., 2016, Lai et al., 2009, Yoon et al., 2007, Yoon, et al., 2007); 4, *Rhodospirillaceae* (Anil Kumar et al., 2008, Chen et al., 2018, Dar Jean et al., 2016, Imhoff, et al., 1998, Kim et al., 2019, Lakshmi et al., 2014, Lakshmi et al., 2011, Lin et al., 2021, Pfennig et al., 1997, Tang et al., 2019, Wang et al., 2020, Wang et al., 2019, Wang et al., 2019). +, positive; −, negative; W, weak; ND, not determined.

Figures

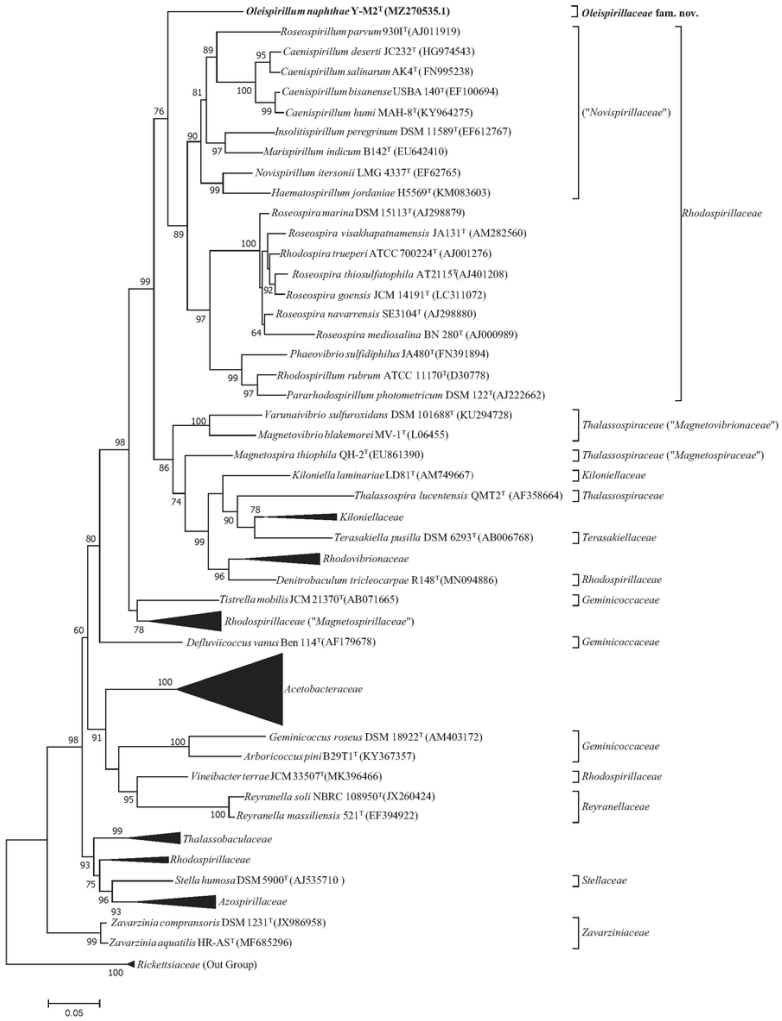

Figure 1

Phylogenetic trees of strain Y-M2^T and *Rhodospirillales* members based on 16S rRNA genes. Species of the genus *Rickettsia* within order *Rickettsiales* were used as an out-group. Bootstrap percentages are based on 1000 replications.

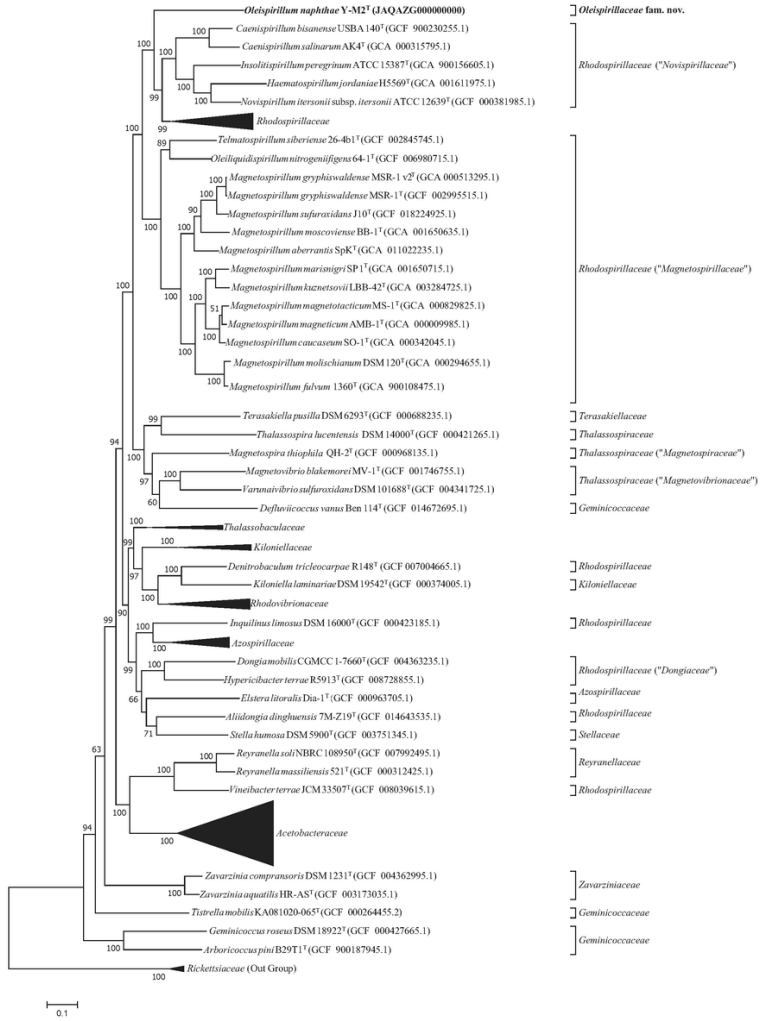

Figure 2

Phylogenomic analyses of *Rhodospirillales* members based on 120 marker genes reconstructed by GTDB-tk.

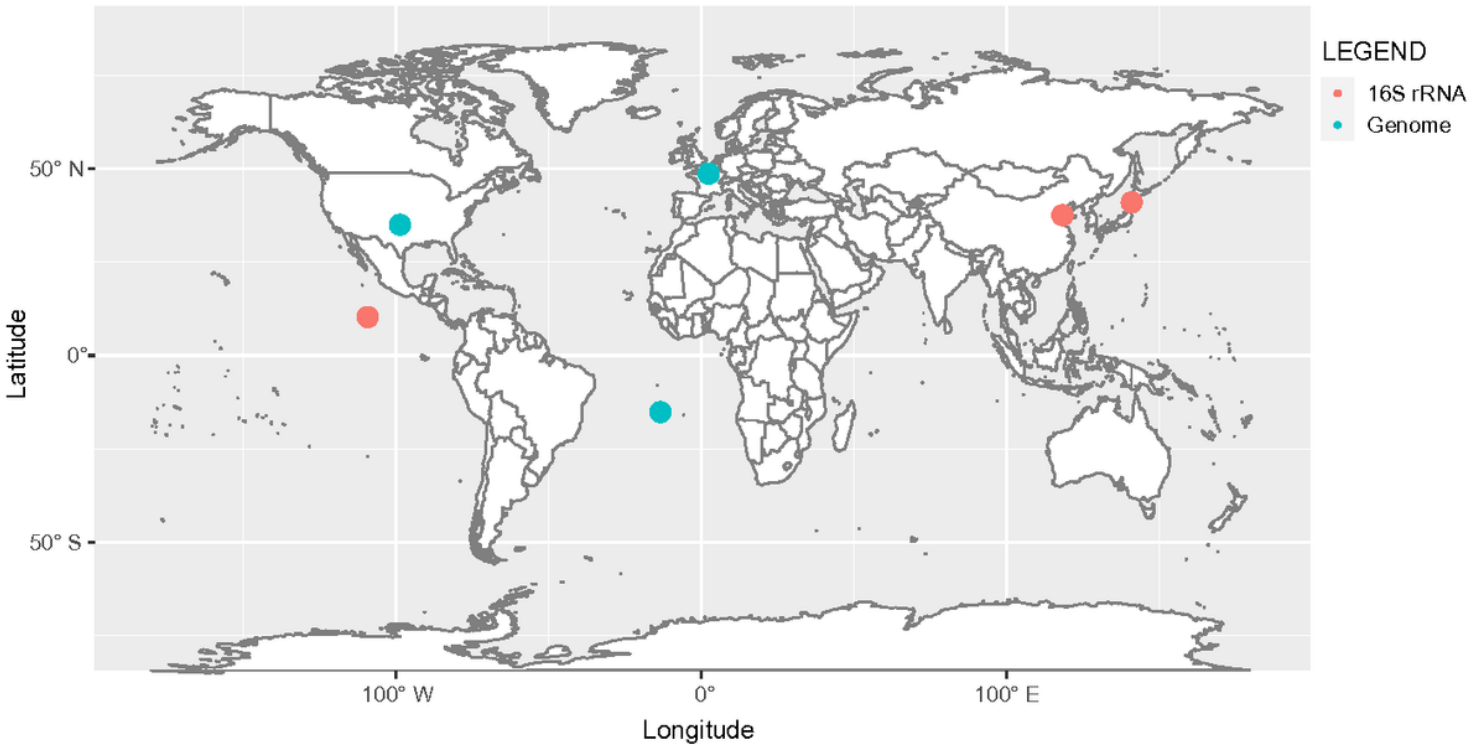

Figure 3

Global distribution of members in family "*Oleispirillaceae*". Node shape and colour indicate the type and source of data. Circle and Square indicate the 16S sequence and genome, respectively; red, black, blue, green represent the data source: oil reservoirs, soil, water and marine hydrothermal sulfide sediment.

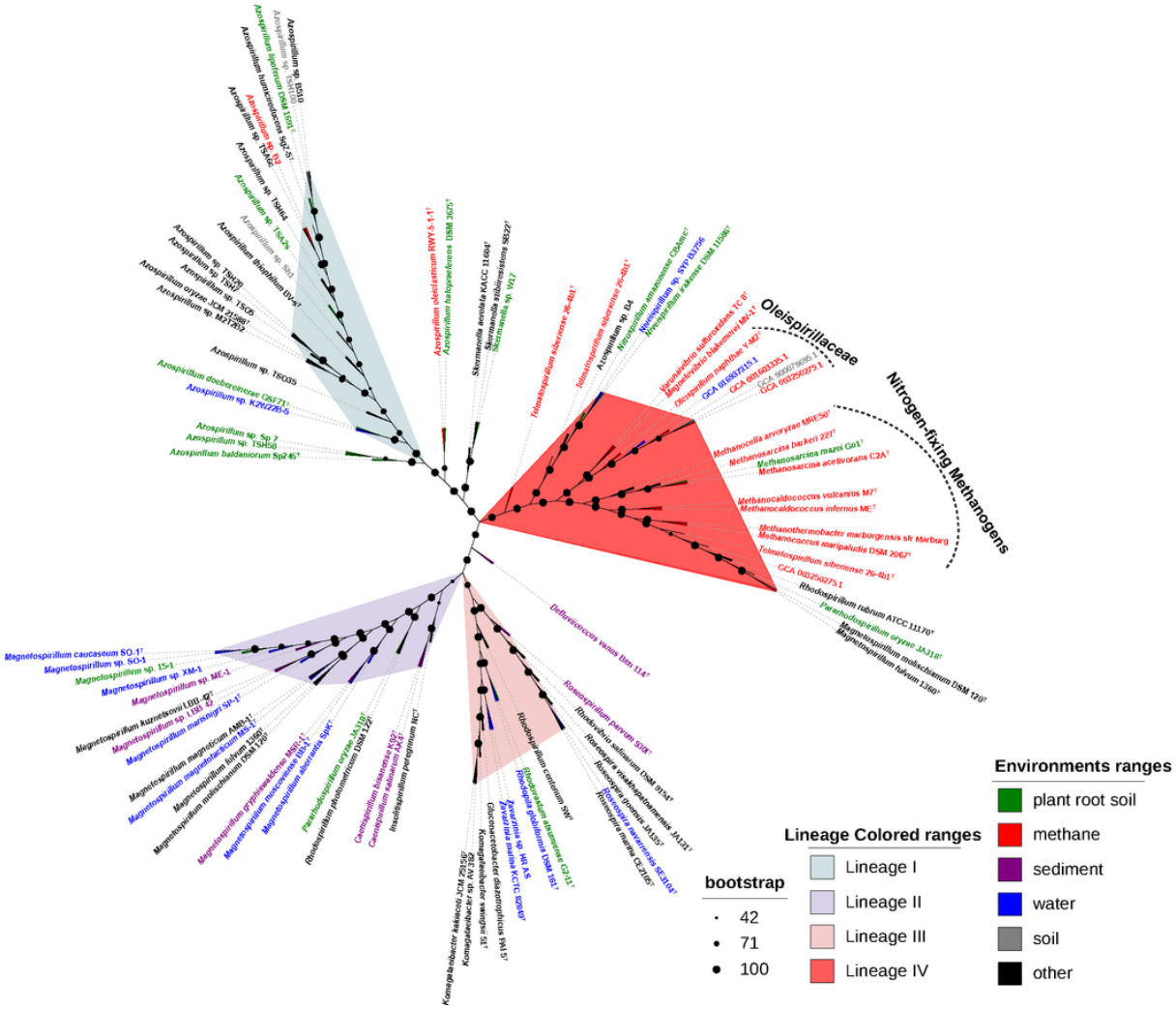

Figure 4

The maximum likelihood tree was constructed by concatenation of *nifHDK* genes. This tree includes the genomes of 45 nitrogen fixing *Rhodospirillales* species and 8 archaea nitrogen-fixing species from a variety of environments. Nodes with bootstrap values are marked with different size black dots. The colors of the labels and clades indicate the environment where the strain was isolated and different lineages of *nifHDK* concatenation, respectively.

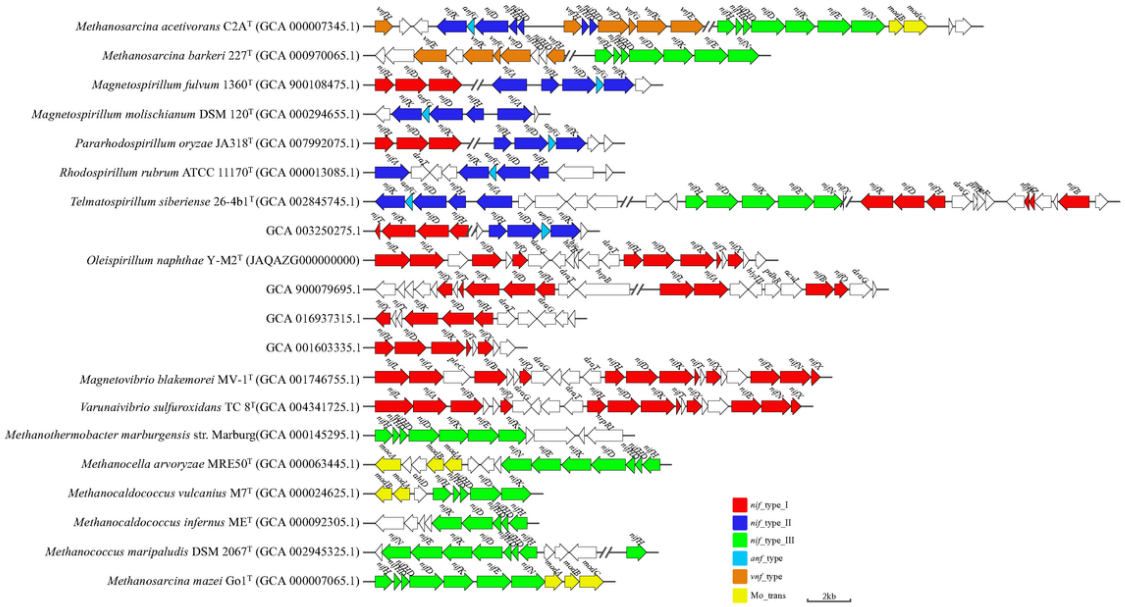

Figure 5

Organization of *nif*, *vnf*, *anf,* and *nif*-like genes in N_2-fixing strains of *Rhodospirillales* species and 8 archaea nitrogen-fixing species contain in lineage IV in fig 3. The three *nif* cluster types, *nif*_type_I, *nif*_type_II, and *nif*_type_III, are marked with red, blue and green, respectively. The *anf*_type and *vnf*_type genes were marked with light blue and brown, respectively. The gene relative to the Mo transform is marked with yellow.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigS1.jpg
- SupplementaryFigS2.jpg
- SupplementaryFigS3.jpg
- Supplementarymaterials.docx