Nitrosophilus kaiyonis sp. nov., a hydrogen-, sulfur- and thiosulfate-oxidizing chemolithoautotroph within "*Campylobacteria*" isolated from a deep-sea hydrothermal vent in the Mid-Okinawa Trough

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Abstract

A novel bacterium, strain MOT50^T, was isolated from the chimney structure at the Iheya North field in the Mid-Okinawa Trough. The cells were motile short rods with single polar flagellum. Growth was observed between 40 and 65ºC (optimum, 52ºC), at pH values between 5.0 and 7.1 (optimum, pH 6.1) and in the presence of 2.0–4.0% NaCl (optimum, 2.5%). The isolates utilized molecular hydrogen, thiosulfate, or elemental sulfur as the sole electron donor. Thiosulfate, elemental sulfur, nitrate, and molecular oxygen are utilized as the sole electron acceptor. Ammonium is required as a nitrogen source. Thiosulfate, elemental sulfur, sulfate, or sulfite serves as a sulfur source for growth. The G+C content of the genomic DNA was 28.9%. Phylogenetic analysis based on the 16S rRNA gene sequences indicated that strain MOT50^T belonged to the genus *Nitrosophilus* of the class "*Campylobacteria*", and its closest relative was *Nitrosophilus labii* HRV44^T (97.20%). On the basis of the phylogenetic, phylogenetical and molecular characteristics, it is proposed that the organism represents a novel species within the genus *Nitrosophilus*, *Nitrosophilus kaiyonis* sp. nov. The type strain is MOT50^T (=JCM 39187^T =KCTC 25251^T).

Introduction

The class "*Campylobacteria*" (homotypic synonym *Epsilonproteobacteria* Garrity *et al*., 2006) is known as a dominant bacterial group in hydrothermal environments that can account for more than 80% of microbial communities in the chimney structures (Nakagawa *et al*., 2006; Muto *et al*., 2017). Members of this group play an important ecological role as primary producers in the deep-sea hydrothermal ecosystems (Nakagawa *et al*., 2006, Muto *et al*., 2017). After the first cultivation of thermophilic "*Campylobacteria*" from deep-sea hydrothermal vents (Campbell *et al.*, 2001), taxonomically diverse isolates have been successfully obtained with the development of cultivation techniques, and their physiological features have been described (Zhang *et al*., 2018). In addition, recent *in situ* incubation experiment has revealed genus specific primary production traits among hydrothermal vent *Campylobacteria* (McNichol *et al.*, 2022). Further efforts to isolate and characterize novel campylobacterial strains from deep-sea hydrothermal environments could help to better elucidate the ecophysiological traits of taxa among *Campylobacteria*.

The class "*Campylobacteria*" consists of 225 validly described species, including subspecies, 18 of which are thermophilic species according to the List of Procaryotic Names with Standing in Nomenclature (LPSN) at the time of writing (21 June 2022). Although thermophilic species have been detected in global deep-sea hydrothermal environments (Nakagawa *et al*., 2005b; Takai *et al*., 2004; Voordeckers *et al*., 2005), 4 out of 6 thermophilic genera (e.g., *Nitratiruptor*, *Nitrosophilus,* *Hydrogenimonas,* and *Lebetimonas*) have been described with two or single species. To better

understand the evolution and physiological diversity of the thermophilic "*Campylobacteria*", it is necessary to obtain thermophilic "*Campylobacteria*" and investigate their physiological and genomic characteristics. In this study, we report the newly thermophilic campylobacterium, strain MOT50^T, belonging to the genus *Nitrosophilus* based on its physiological and genomic characterization.

Materials and Methods

Sample collection, enrichment, and purification

Strain MOT50^T was isolated from the C0016B chimney structure at the Iheya North field in the Mid-Okinawa Trough (27º 47' 26.9" N, 126º 53' 47.4" E), at a depth of 993 m, by *ROV Hyper Dolphin* during the KY14-01 scientific cruise aboard *R/V Kaiyo* in January 2014. The interior part of the chimney sample was mixed anaerobically with 25 ml sterilized seawater containing 0.05% (w/v) neutralized sodium sulfide in 100 ml glass bottles (Schott Glaswerke, Mainz, Germany) soon after the vehicle was recovered. The bottle was then tightly sealed with a butyl-rubber stopper under a 100% N_2 gas phase (0.2 MPa) and stored at 4ºC until use. For enrichment, 100 µl of the resultant slurry was inoculated into 15 ml test tubes containing 3 ml MMJHS (Takai *et al*., 2003). MMJHS medium contained 1 g $NaNO_3$, 1 g $NaHCO_3$, 1 g $Na_2S_2O_3 \cdot 5H_2O$, 10 g S^0 per liter MJ synthetic seawater. The gas phase (0.2 MPa) of MMJHS medium was H_2/CO_2 (80:20, v/v). Growth of thermophiles was observed after one day at 50ºC. Strain MOT50^T was isolated using the dilution-to-extinction technique (Takai & Horikoshi, 2000) with MMJHS medium at 50ºC. The purity was confirmed with a routine microscopic examination and by repeated partial sequencing of 16S rRNA gene using several PCR primers (Lane, 1991).

Cell morphology and growth characteristics

Cells were observed with the ZEISS Axiophot microscope (Carl Zeiss Co., Oberkochen, Germany). For transmission electron microscope, cells grown in MMJHS medium at 52ºC in the late exponential phase were stained with EM Stainer (Nisshin EM Co., Ltd, Tokyo, Japan). Electron micrographs were obtained using JEM-1011 transmission electron microscope (JEOL, Tokyo, Japan).

Growth of the novel isolates was measured by direct cell counts after staining with 4', 6-diamidino-2-phenylindole (DAPI) (Porter & Feig, 1980). To determine optimum temperature, pH and NaCl concentrations, cultures were grown in 3 ml MMJHS medium in various conditions.

The isolate was tested for the ability to grow on combinations of a single electron donor and acceptor. MJ synthetic seawater containing 0.1% (w/v) $NaHCO_3$ was used as the basal medium. To examine the growth on hydrogen as an electron donor, H_2/CO_2 (80:20) was used as the gas phase. In an attempt to examine the growth on thiosulfate (0.1%, w/v) or elemental sulfur (S^0) (1%, w/v) as an electron donor, N_2/CO_2 (80/20) was used as the gas phase. Nitrate (0.1%, w/v), thiosulfate (0.1%,

w/v), nitrous oxide (33%, v/v), sulfate (0.1%, w/v), elemental sulfur (1%, w/v) and molecular oxygen (0.1, 1 and 2%, v/v) were tested for potential electron acceptors. The growth was confirmed by measuring the OD_{620} (Microplate Reader, Infinite F200, Tecan, Manndorf, Switzerland) or cell increase via microscopy.

Heterotrophic growth was tested in MMJHS medium without $NaHCO_3$ under a gas phase of 100% H_2 (0.3% MPa). Each of the following potential carbon sources was tested: yeast extract, peptone, tryptone, casamino acids, D(+)-glucose, galactose, sucrose, fructose, lactose, maltose, starch (all 0.1%, w/v), formate, acetate, glycerol, tartrate, malate, succinate, propionate, lactate, pyruvate (all 10 mM), methanol (0.05%, w/v), ethanol (0.1%, v/v) and 2-propanol (0.2%, v/v). In addition, to assess the utilization of these organic compounds as an energy source, substrates were added to medium excluded thiosulfate (0.1%, w/v) and elemental sulfur (1%, w/v) from MMJHS medium under a N_2/CO_2 (80/20) gas phase (0.3 MPa).

Potential nitrogen and sulfur sources required for growth were tested. To determine the nitrogen sources utilization, NH_4Cl (0.025%, w/v), $NaNO_3$ (0.1%, w/v) and $NaNO_2$ (0.1% w/v) were examined in MMJHS medium lacking all nitrogen sources, under a H_2/CO_2 (80:20) gas phase (0.3 MPa). To determine the sulfur sources utilization, sulfate (0.34%, w/v), thiosulfate (0.1%, w/v), sulfite (0.1 and 1%, w/v) and elemental sulfur (1%, w/v) were examined in MMJHS medium in which sulfur compounds were removed and replaced with the chloride salts under an H_2/CO_2 (80/20) gas phase (0.3 MPa).

Molecular phylogenetic analysis based on 16S rRNA gene sequences

The 16S rRNA gene was amplified by PCR using primers Eubac 27F and 1492R (Lane, 1991). The nearly complete 16S rRNA gene sequence (1,420 bp) of strain MOT50^T was obtained by direct sequencing of both strands. The 16S rRNA gene sequence was analyzed using the BLAST search algorithm with all nucleotides (Altshul *et al.*, 1997). In order to determine the phylogenetic position of the strain, the other thermophilic campylobacterial sequences were retrieved from the Silva database (Quast *et al*., 2013), and the NCBI RefSeq database (https://www.ncbi.nlm.nih.gov/) and then aligned using Silva Incremental Aligner v1.2.11 (Pruesse *et al*., 2012). A phylogenetic tree was constructed using the neighbor-joining method (Saitou *et al*., 1987) with the MEGAX software (Kumar *et al*., 2018) using 1,134 homologous sequence positions for each organism. Bootstrap analysis was conducted using 1,000 replications to provide a confidence result for the phylogenetic tree topology.

Genome sequencing and assembly

Genomic DNA of strain MOT50^T was extracted from the cells grown in MMJHS medium with Wizard genomic DNA purification kit (Promega, Madison, Wisconsin, USA) according to the

manufacturer's protocol. The genome sequencing was performed using both Oxford Nanopore Technology (ONT) MinION and Illumina MiSeq platforms. For the ONT sequencing, the library was prepared using the Rapid Barcoding Sequence kit (Oxford Nanopore Technologies, Oxford, UK) according to the standard protocol provided by the manufacturer. The library was made by mixing DNA samples with Fragment Mix RB01-12. The constructed library was loaded into the Flow Cell (R9.4.1) on a MinION device and performed a 48-hour sequencing run with MinKNOW3.6.0 software. Basecalling for ONT reads was performed with Guppy v3.4.4 (Oxford Nanopore Technologies). Illumina reads were combined with ONT reads for hybrid assembly with Unicycler v0.4.7 (Wick *et al*., 2017), with default parameters. The whole genome sequence was annotated with DFAST (Tanizawa *et al*., 2018). Metabolic pathways were confirmed using BlastKOALA (Kanehisa *et al*., 2016) and sulfur oxidizing pathway was visualized by KEGG mapper (Kanehisa & Sato, 2020). In order to survey genes responsible for sulfur reducing metabolisms (*psrABC* and *sud*), homology search equipped with in silico MolecularCloning (In Silico Biology, Inc., Yokohama, Japan) was performed with default setting using amino acid sequences (WS0116-0118 and WS1629) from *Wolinella succinogenes* DSM 1740^{T} (BX571656.1) as reference sequences (Yamamoto *et al*., 2010). To compare the related strains of strain MOT50^{T}, complete genome sequences of *Nitratiruptor tergarcus* MI55-1^{T} (Nakagawa *et al*., 2005b) and *Hydrogenimonas thermophila* EP1-55-1%^{T} (Takai *et al*., 2004) were also obtained in the same way.

Calculation of genome sequence similarities

To determine the taxonomic position of strain MOT50^{T}, genome-based taxonomic indices were calculated with related strains. *In silico* DNA-DNA hybridization (DDH) values of strain MOT50^{T} against *Nitrosophilus alvini* EPR55-1^{T} (Shiotani *et al*., 2020), *Nitrosophilus labii* HRV44^{T} (Fukushi *et al*, 2020), *Nitratiruptor tergarcus* MI55-1^{T} (Nakagawa *et al*., 2005b), *Nitratiruptor* sp. SB155-2 (Nakagawa *et al*., 2007) and *Hydrogenimonas thermophila* EP1-55-1%^{T} (Takai *et al*., 2004) were calculated using the Genome-to-Genome Distance Calculator version (Meier-Kolthoff *et al*., 2013) with the BLAST+ alignment tool. Results were based on recommended formula2, which is independent of genome length and is robust against using draft genomes. Average nucleotide identity (ANI) and average amino acid identity (AAI) values were calculated by using the EZ BioCloud (Yoon *et al*., 2017) and the aai.rb script (https://github.com/lmrodriguezr/enveomics) (Rodriguez-R & Konstantinidis, 2016), respectively. The genome sequences of *Nitrosophilus alvini* EPR55-1^{T}, *Nitrosophilus labii* HRV44^{T} and *Nitratiruptor* sp. SB155-2, were retrieved from NCBI RefSeq database (https://www.ncbi.nlm.nih.gov/).

In order to determine the phylogenetic position of the strain, the other campylobacterial sequences were retrieved from the NCBI RefSeq database (https://www.ncbi.nlm.nih.gov/) and

constructed a phylogenetic tree based on Campbell's 139 single-copy genes (Campbell *et al*., 2013) using the Anvi'o v5.5.0 (Eren *et al*., 2015). The workflow of this analysis was based on the workflow (http:// merenlab.org/2017/06/07/phylogenomics/).

Pan-genomic analysis

Pan-genomic analysis was conducted with 22 deep-sea campylobacterial genomes using the Anvi'o v5.5.0 (Eren *et al*., 2015). The workflow of this analysis was based on the workflow (http:// merenlab.org/2017/06/07/phylogenomics/). COG-annotated speices-specific gene clusters were exported from pangenome (anvi-summarize) for the gene functional analysis. Genomes used in this study is shown in supplementary Table S1.

Nucleotide sequence accession number

The nucleotide sequence of the 16S rRNA gene and the complete genome sequence of strain MOT50^T have been deposited in the GenBank/ EMBL/DDBJ under accession numbers LC716068 and AP025696, respectively. Complete genome sequences of *Nitratiruptor tergarcus* MI55-1^T and *Hydrogenimonas thermophila* EP1-55-1%^{T} have been deposited in the GenBank/ EMBL/DDBJ under accession numbers AP026671-AP026672 and AP026673-AP026676, respectively.

Results

Cell morphology and growth characteristics

Cells of MOT50^T were Gram-negative rods (2.0 μm long and 1.0 μm in wide) (Fig. 1). The cells were motile by means of the polar flagellum. Spore formation was not observed under any laboratory conditions.

Strain MOT50^T grew at temperatures between 40 and 65ºC, showing optimum growth at 52ºC (doubling time, 2 h). No growth was observed below 35ºC or above 70ºC. Growth occurred between pH 5.0 and 7.1, with optimum growth at pH 6.1. No growth was observed below pH 4.6 or above 7.8. The isolate grew at concentrations in the range 2.0–4.0% (w/v) NaCl, with optimum growth at 2.5% NaCl. No growth was observed in concentrations below 1.5% NaCl or above 5.0% NaCl (Supplementary Fig. S1). Growth characteristics of strain MOT50^T were similar to those of *Nitrosophilus labii* HRV44^T (Table 1).

Strain MOT50^T was able to utilize thiosulfate (0.1%, w/v), elemental sulfur (1%, w/v), or H_2 as the sole electron donor. Thiosulfate (0.1%, w/v), elemental sulfur (1%, w/v), nitrate (0.1%, w/v), nitrous oxide (33%, v/v) or molecular oxygen (0.1%, 1% and 2%, v/v) could serve as the sole electron acceptor. N_2O consumption was observed only when H_2 was provided as the electron donor. The combination of H_2 and nitrate resulted in the highest cell growth. The isolate was unable to use any organic compounds examined in this study as energy or carbon sources. MOT50^T was able to utilize

ammonium (0.025%, w/v) and nitrate (0.1%, w/v) as a nitrogen source. The isolate was able to utilize thiosulfate (0.1%, w/v) and elemental sulfur (1%, w/v) as a sulfur source.

Phylogenetic analysis based on 16S rRNA gene sequences

The 16S rRNA gene sequence of MOT50^T shared the highest similarity with that of *Nitrosophilus labii* HRV44^T (97.20%), followed by that of *Nitrosophilus alvini* EPR55-1^T (95.07%). These values were below the threshold for species definition (98.7%) based on 16S rRNA gene sequences (Yarza *et al*., 2014). The phylogenetic analysis also showed that the isolate was a member of the genus *Nitrosophilus* (Fig. 2).

Genome properties

A single complete circular contig of MOT50^T with a length of 1,769,550 bp was obtained by Unicycler, and 1,798 contig sequences (CDSs) were predicted (Supplementary Fig. S2). This genome size was the smallest among previously sequenced genomes of *Nitrosophilus* spp., i.e., *Nitrosophilus alvini* EPR55-1^T (1,807,889 bp and 1,797 CDSs) (Shiotani *et al*., 2020), and *Nitrosophilus labii* HRV44^T (chromosome; 1,990,315 bp and 2,050 CDSs, plasmid; 102,672 bp and 128 CDSs) (Fukushi *et al*., 2020). The G+C content of MOT50^T genome was 28.9%, which is similar to that of *Nitrosophilus labii* HRV44^T (33.4%) (Table 1). MOT50^T had a complete set of Sox genes (*soxABXYZ*) responsible for sulfur oxidation. Strain MOT50^T had homologues of *psrABC* encoding polysulfide reductase and of *sud* encoding sulfide dehydrogenase (Klimmek *et al*., 1998).

Taxonomic placement of MOT50^T based on the genomic analyses

In silico DDH values of strain MOT50^T against *Nitrosophilus alvini* EPR55-1^T, *Nitrosophilus labii* HRV44^T, *Nitratiruptor tergarcus* MI55-1^T, *Nitratiruptor* sp. SB155-2 and *H. thermophila* EP1-55-1%^{T} were 14.5%, 20.0%, 19.8%, 19.7%, and 17.6%, respectively, which were below the threshold of bacterial species boundary (Meier-Kolthoff *et al*., 2013). In addition, ANI values of strain MOT50^T against *Nitrosophilus alvini* EPR55-1^T, *Nitrosophilus labii* HRV44^T, *Nitratiruptor tergarcus* MI55-1^T, *Nitratiruptor* sp. SB155-2 and *H. thermophila* EP1-55-1%^{T} were 71.6%, 75.9%, 72.8%, 72.3% and 70.3%, respectively. These values are also below a threshold of 95.0% ANI similarity for the definition of bacterial species (Richter & Rosselló-Mora, 2009). These results also support the proposal that strain MOT50^T is a new species within the class "*Campylobacteria*". AAI values of the novel isolate against *Nitrosophilus alvini* EPR55-1^T, *Nitrosophilus labii* HRV44^T, *Nitratiruptor tergarcus* MI55-1^T, *Nitratiruptor* sp. SB155-2 and *H. thermophila* EP1-55-1%^{T} were 69.9%, 75.7%, 68.7%, 67.7% and 58.8%, respectively. The values against *Nitrosophilus* and *Nitratiruptor* species were above the threshold of the genus-level boundary of "*Campylobacteria*" (60–62%) (Shiotani *et al*., 2020). The phylogenetic tree based on nucleotide

sequences of Campbell's 139 SCGs showed that the isolate was a member of the genus *Nitrosophilus* (Fig. 3).

Pan-genomic analysis

The pangenome of members of "*Campylobacteria*" isolated from deep-sea hydrothermal environments revealed a total of 13,859 gene clusters comprising 46,679 genes in which 424 gene clusters with 9,328 genes were represented SCGs, and 38 gene clusters with 943 genes were core genome (Fig. 4). The species-unique genes were also found among the genus *Nitrosophilus*; *N. labii* HRV44^T had approximately 1.6 times higher number of unique gene clusters (337 gene clusters with 367 genes) than other two species, *N. kaiyonis* MOT50^T (199 gene clusters with 201 genes) and *N. alvini* EPR55-1^T (197 gene clusters with 201 genes). COG functional annotation of species-unique gene clusters among the genus *Nitrosophilus* showed that a relative abundance of genes involved in cell wall/membrane/envelope biogenesis (M) in strain MOT50^T was higher (18.8% in without uncharacterized categories) than those in *N. labii* HRV44^T (6.4%) and *N. alvini* EPR55-1^T (10.1%) (Supplementary Fig. S3). Genes related to defense mechanisms (V) (9.0%), mobilome: prophages, transposons (X) (19.4%), and replication, recombination and repair (L) (28.6%) in strain HRV44^T were higher than those in strain MOT50^T (2.4%, 9.8%, and 13.8%, respectively) and strain EPR55-1^T (3.4%, 0%, and 17.9%, respectively).

Discussion

Although more than 20 years have passed since the first report on thermophilic members of "*Campylobacteria*", novel thermophilic campylobacterial species, i.e., *Lebetimonas natsushimae* (Nagata *et al*., 2017), *Nitrosophilus labii* (Fukushi et al., 2020), *Nitrosophilus pacificus* (Shiotani et al., 2020), *Hydrogenimons urashimensis* (Mino *et al*., 2021), still have been isolated from deep-sea hydrothermal environments, and their characterizations update physiological traits of these genera. For example, *L. natsushimae* is able to utilize formate as its electron donor, that trait has not not been observed in other *Lebetimonas* species. *Nitrosophilus labii* is the first described species in "*Campylobacteria*" that can reduce exogenous N_2O to N_2. Contiued efforts to isolate and cultivate novel species are great worth not for elsidating microbial ecological roles in the deep-sea hydrothermal vent ecosystem but also for finding microbial resources that could contribute to application studies. In this study, we successfully isolated a novel thermophilic strain, MOT50^T, from a chimney structure collected at the Iheya North field in the Mid-Okinawa Trough, and characterized physiological and genomic features of the strain, and propose it as a novel species belonging to the genus *Nitrosophilus*.

Physiological characterization and advanced polyphasic taxonomy including genome comparison revealed strain MOT50^T is a novel bacterial species of the genus *Nitrosophilus*. Temperature, pH, and NaCl concentration ranges for growth of strain MOT50^T are similar to those of *Nitrosophilus labii* HRV44^T, whereas energy metabolic traits of strain MOT50^T are similar to those of *Nitrosophilus alvini* EPR55-1^T. Strain MOT50^T is able to utilize elemental sulfur and thiosulfate as both electron donors and acceptors. This energy metabolic trait has never been reported in any other thermophilic "*Campylobacteria*". Key genes responsible for sulfur oxidation and reduction were found in the genome of MOT50^T, supporting the ability to utilize reduced sulfur compound as both electron donors and acceptors. In comparison with other species within the genus *Nitrosophilus*, strain MOT50^T is able to utilize a relatively wide variety of inorganic compounds (i.e., molecular hydrogen, thiosulfate, or elemental sulfur) as the sole energy source. Although we did not evaluate which combination of substances strain MOT50^T prefers when those are present at the same time, this characteristic might help strain MOT50^T to adapt to fluctuating environmental conditions surrounding deep-sea hydrothermal vents.

Species-specific differences in gene content and its function commonly related to a specific metabolism, virulence, antibiotic resistance mechanisms, or other environmental adaptation (Costa *et al.*, 2020). COG functional annotation of gene clusters unique to each *Nitrosophilus* species showed that genes involved in the category M (cell wall/membrane/envelope biogenesis) accounted for higher proportion in strain MOT50^T than in strain HRV44^T or strain EPR55-1^T, suggesting an advantage role against physical and chemical stresses (Jiang *et al*., 2019) for strain MOT50^T. Also, genes involved in the categories V (defense mechanisms), X (mobilome: prophages, transposons), and L (replication, recombination and repair) in strain HRV44^T were more abundant than those in strain MOT50^T and strain EPR55-1^T, suggesting that gene exchange and recombination are important in strain HRV44^T and these functions are responsible for strategies in response to the environment factors (Jiang *et al.*, 2019).

Description of *Nitrosophilus kaiyonis* sp. nov.

Nitrosophilus kaiyonis (ka.i.yo'nis. N.L. gen. n. *kaiyonis*, of *Kaiyo*, the name of the Japanese retired research ship used for the exploration of deep ocean).

Cells are Gram-negative, motile and short-rod shape and a mean length of 2.0 μm and width of 1.0 μm. The temperature range for the growth is 40–65ºC (optimum, 52ºC; doubling time, 2h). The pH range for the growth is 5.0–7.1 (optimum, pH 6.1). NaCl in the concentration range for the growth is 2.0–4.0% (optimum, 2.5%). Strictly chemolithoautotrophic growth occurs with molecular hydrogen, thiosulfate, or elemental sulfur as the sole electron donor. Thiosulfate, elemental sulfur,

nitrate, and molecular oxygen are utilized as the sole electron acceptor. Ammonium is required as a nitrogen source. Thiosulfate, elemental sulfur, sulfate, or sulfite serves as a sulfur source for growth. The size of the complete genome is 1,769,550 bp. The GC content of DNA is 28.9%. The type strain, MOT50^T (=JCM 39187^T =KCTC 25251^T) was isolated from a deep-sea hydrothermal vent in the Mid-Okinawa Trough.

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Figure Legends

Fig. 1 Electron micrograph of a negatively stained cell of strain MOT50^T. Scale bar represents 1.0 μm.

Fig. 2 Phylogenetic tree based on 16S rRNA gene sequences. Phylogenetic tree of the members of "*Campylobacteria*" isolated from deep-sea hydrothermal environments by the neighbor-joining method using 1,134 homologous sequence positions with TN93+G+I model. Numbers at branches are bootstrap values (%) based on 1,000 replications.

Fig. 3 Phylogenetic tree of the members of "*Campylobacteria*" isolated from deep-sea hydrothermal environments based on nucleotide sequences of Campbell's 139 SCGs.

Fig. 4 The pan-genomic analysis of members of "*Campylobacteria*" isolated from deep-sea hydrothermal environments. Bars represent the occurrence of gene clusters in a given genome. The heatmap matrix in the upper right corner indicates the average nucleotide identity (ANI) over 70%. The tree above the heatmap was constructed based on the presence/absence of gene clusters for each strain. Genomes are ordered as layers using a tree based on ANI values matrix. The species names of thermophiles and mesophiles are distinguished by red and black colors, respectively.

503 **Table 1.** Comparison of physiological characteristics of MOT50^T with species of "*Campylobacteria*" isolated from deep-sea hydrothermal environments

Characteristics	1	2	3	4	5	6	7	8	9	10	11
Origin	Mid-Okinawa Trough	East Pacific Rise	Mid-Okinawa Trough	Mid-Okinawa Trough	Mid-Okinawa Trough	Central Indian Ridge	East Pacific Rise	Mid-Atlantic Ridge	Mid-Okinawa Trough	Mid-Okinawa Trough	Mid-Okinawa Trough
Temperature range (ºC)	40-65	50-60	45-60	40-55	37-65	35-65	30-55	45-70	28-40	10-40	10-40
Temperature optimum (ºC)	52	60	53	55	55	55	40	55	37	28-30	25
pH range	5.0-7.1	5.4-8.6	5.4-6.4	5.4-6.9	ND	4.9-7.2	6.0-9.0	4.5-7.5	5.6-7.6	5.0-9.0	4.5-9.0
pH optimum	6.1	6.6	6.0	6.4	ND	5.9	7.0	5.5	7.0	6.5-7.0	6.5
NaCl range (%, w/v)	2.0-4.0	2.4-3.2	2.0-4.0	1.5-4.0	ND	1.6-5.6	2.0-5.0	1.0-4.0	1.5-3.5	1.0-6.0	1.6-6.0
NaCl optimum (%, w/v)	2.5	2.4	2.5	2.5	ND	3.2	3.0	3.0	3.0	4.0	4.0
Electron donor	H_2, $S_2O_3^{2-}$, S^0	H_2	H_2	H_2	H_2, S^0, S^{2-}, $S_2O_3^{2-}$	H_2	H_2, formate	H_2	H_2	$S_2O_3^{2-}$, S^0	$S_2O_3^{2-}$, S^0, S^{2-}
Electron acceptor	NO_3^-, S^0, $S_2O_3^{2-}$, O_2 (up to 1.0%), N_2O	NO_3, $S_2O_3^{2-}$, O_2, S^0, N_2O	NO_3^-, N_2O, S^0, O_2 (up to 1.0%)	NO_3^-, S^0, O_2 (up to 0.7%)	NO_3^-, O_2	NO_3^-, O_2, S^0	S^0	NO_3^-, S^0	NO_3^-, O_2	NO_3^-, O_2	O_2
Carbon sources other than CO_2	-	-	-	-	ND	-	formate	-	-	-	-
Nitrogen sources	NH_4^+	NH_4^+	NH_4^+, NO_3^-	NH_4^+ , NO_3^-	ND	NH_4^+, NO_3^-	NO_3^-, NH_4^+, peptone, yeast extract	ND	NH_4^+, NO_3^-	NH^{4+}	ND
Sulfur sources	$S_2O_3^{2-}$, S^0, SO_4^2	$S_2O_3^{2-}$, S^0, SO_3^{2-}	S^0, SO_4^{2-}	S^0	ND	ND	ND	ND	S^0	ND	ND
DNA G+C content (mol%)	28.9	37.7	33.4	36.9	39.7	33.6	33.5	25.6	35.5	48.0	35.2

504 ND, not determined; -, negative.

505 1, Strain MOT50^{T}; 2, *Nitrosophilus alvini* EPR55-1^{T} (Shiotani *et al*, 2020); 3, *Nitrosophilus labii* HRV44^{T} (Fukushi *et al*, 2020); 4, *Nitratiruptor tergarcus*
506 MI55-1^{T} (Nakagawa *et al*., 2005b); 5, *Nitratiruptor* sp. SB155-2 (Nakagawa *et al*., 2007); 6, *Hydrogenimonas thermophila* EP1-55-1%^{T} (Takai *et al*.,
507 2004); 7, *Nautilia profundicola* AmH^{T} (Smith *et al*., 2008); 8, *Caminibacter mediatlanticus* TB-2^{T} (Voordeckers *et al*., 2005); 9, *Nitratifractor salsuginis*
508 E9I37-1^{T} (Nakagawa *et al*., 2005b); 10, *Sulfurovum lithotrophicum* 42BKT^{T} (Inagaki *et al*., 2004); 11, *Sulfurimonas autotrophica* OK10^{T} (Inagaki *et al*.,
509 2003).

Supplementary Information

Nitrosophilus kaiyonis sp. nov., a hydrogen-, sulfur- and thiosulfate-oxidizing chemolithoautotroph within "*Campylobacteria*" isolated from a deep-sea hydrothermal vent in the Mid-Okinawa Trough

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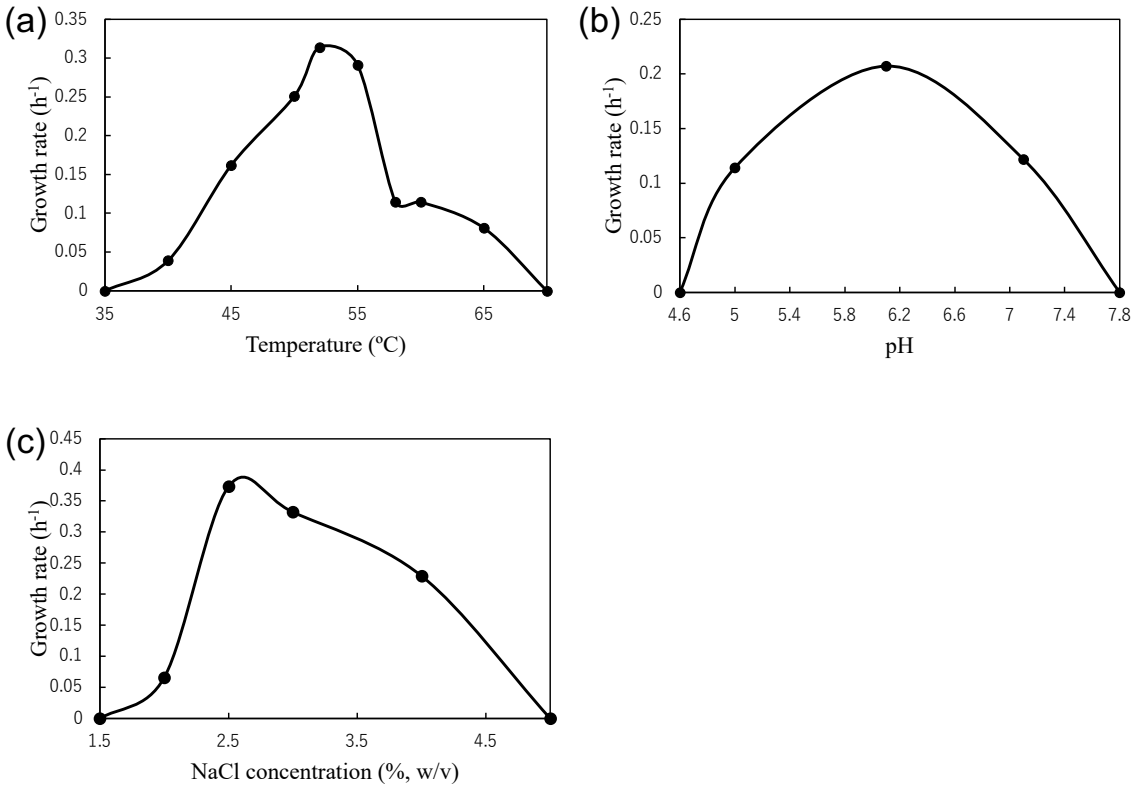
Figure legends

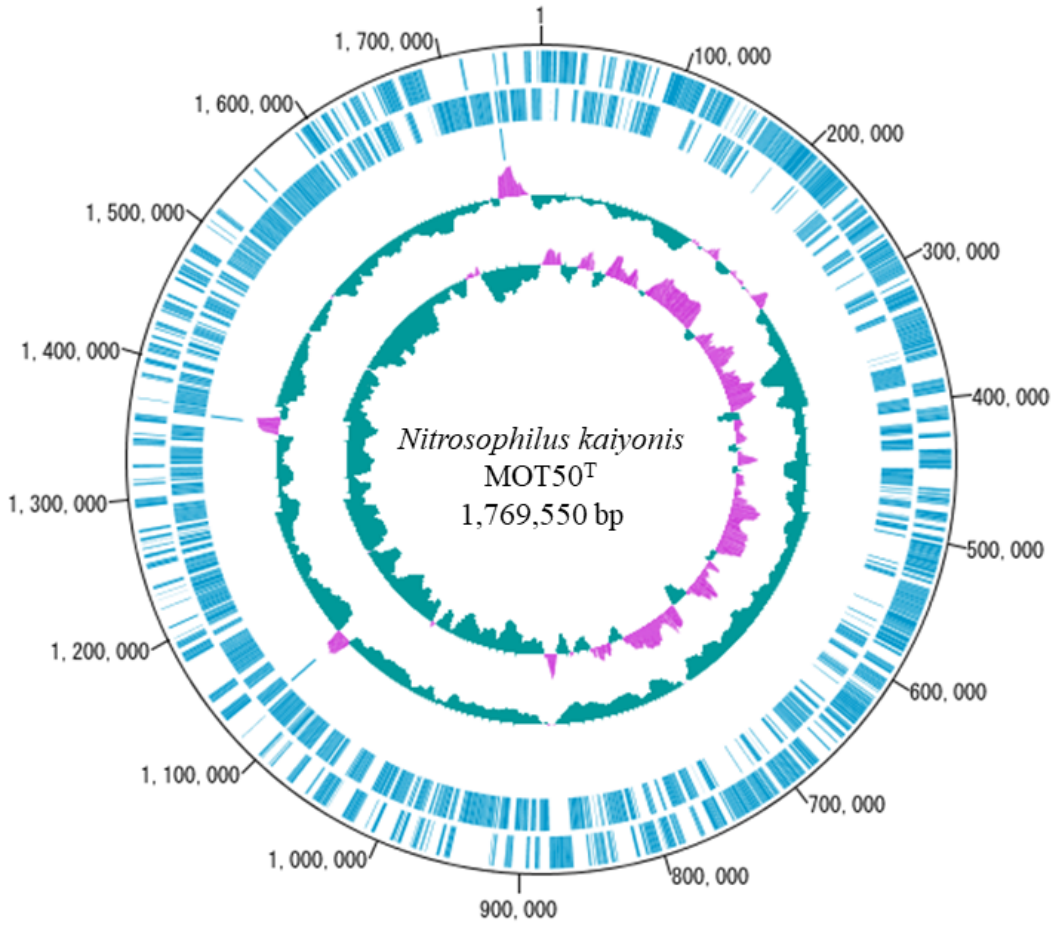
Supplementary Fig. S1. The effect of temperature (a), pH (b), and NaCl concentration (c) on the growth of strain MOT50^T.

Supplementary Fig. S2. Graphical circular map of strain MOT50^T genome. Tracks from inside to outside indicate G+C skew, G+C content, rRNA, reverse strand CDS, and forward strand CDS, respectively.

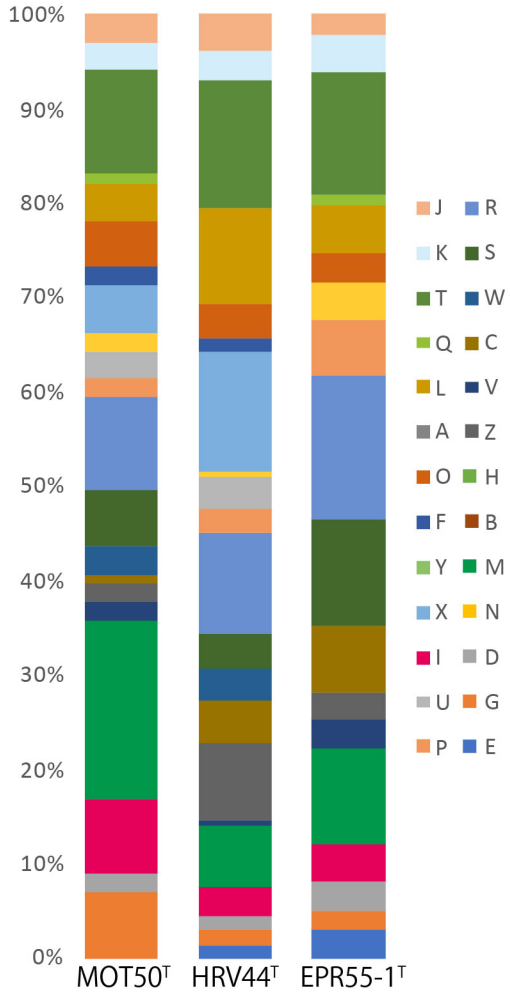
Supplementary Fig. S3. Functional categories of species-specific gene clusters in *Nitrosophilus* species. Category abbreviations are as follows: C, energy production and conversion; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport and catabolism; X, mobilome: prophages, transposons; A, RNA processing and modification; B, chromatin structure and dynamics; J, translation, ribosomal structure and biogenesis; K, transcription; L, replication, recombination and repair; D, cell cycle control, cell division, chromosomal partitioning; M, cell wall/membrane/envelope biogenesis; N, cell motility; O, posttranslational modification, protein turnover, chaperones; T, signal transduction mechanisms; U, intracellular trafficking, secretion, and vesicular transport; V, defense mechanisms; W, extracellular structures; Z, cytoskeleton; R, general function predicted only; S, function unknown.



Supplementary Fig. S1.



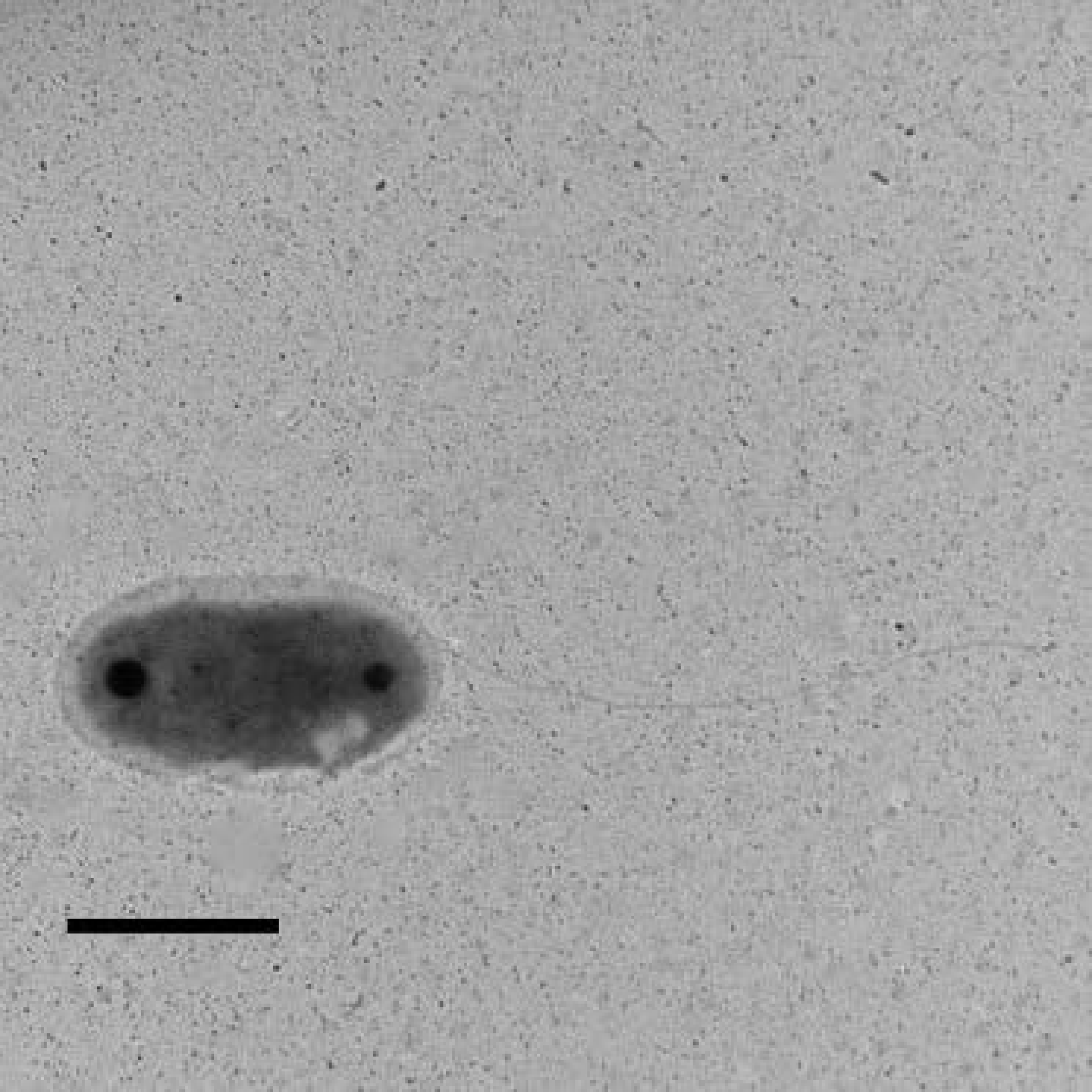
Supplmentary Fig. S2.

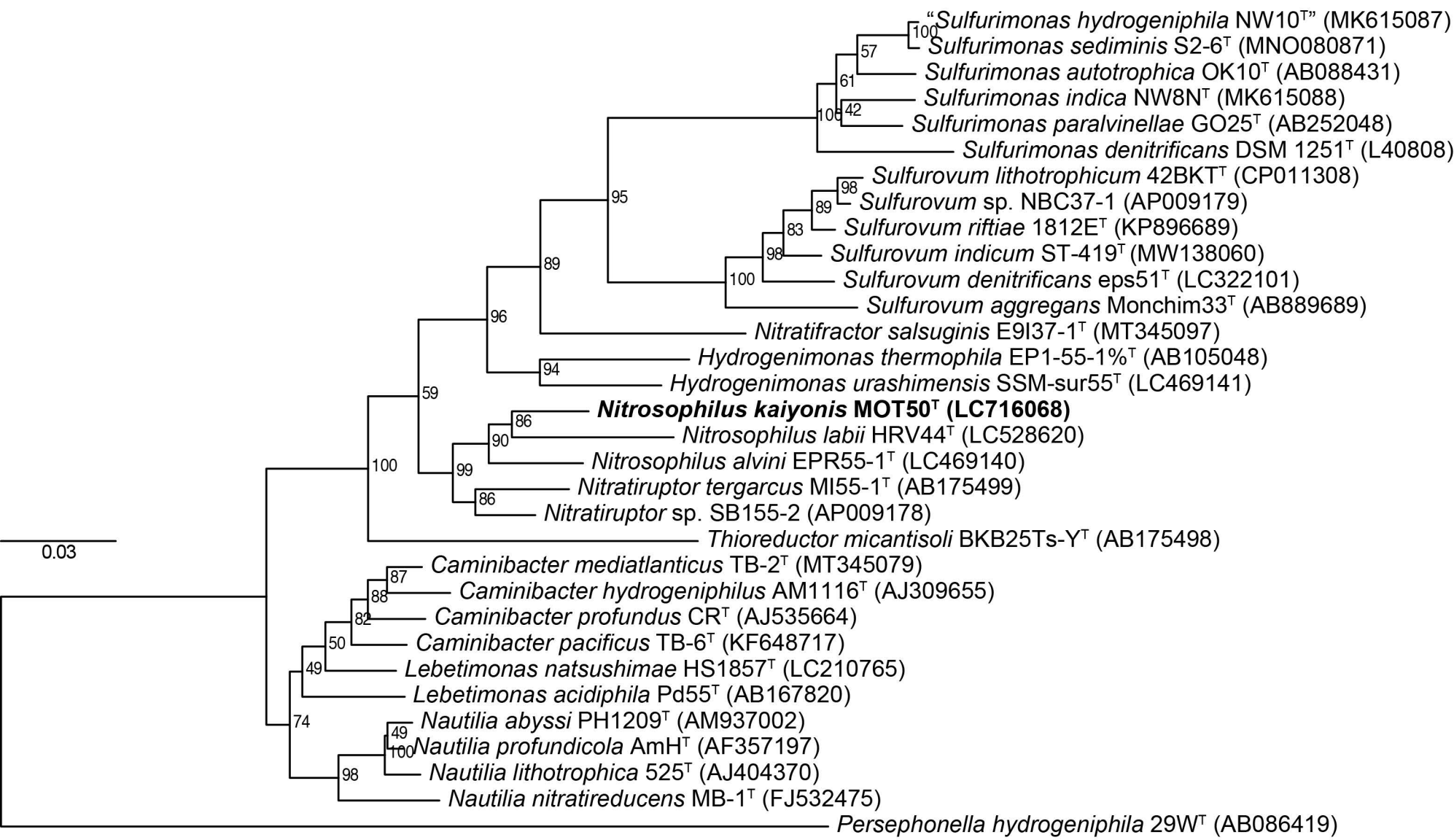


Supplmentary Fig. S3.

Supplementary Table S1. List of genomes used in this study

Genus	Species	Strain	Assembly level	# of contigs	# of chromosomes and plasmids	Genome size (bp)	G+C content (%)	Accession number
Nitrosophilus	*kaiyonis*	MOT50	Complete genome	1	1	1,769,550	28.9	AP025696
Nitrosophilus	*labii*	HRV44	Complete genome	2	2	2,092,987	33.4	GCF_014466985.1
Nitrosophilus	*alvini*	EPR55-1	Complete genome	1	1	1,807,889	37.7	GCF_015100395.1
Nitratiruptor	*tergarcus*	DSM 16512	Complete genome	2	2	1,940,884	36.9	AP026671-AP026672
Nitratiruptor	sp.	SB155-2	Complete genome	1	1	1,877,931	39.7	GCF_000010325.1
Hydrogenimonas	*thermophila*	EP1-55-1%	Complete genome	4	4*	2,551,102	33.6	AP026673-AP026676
Hydrogenimonas	*urashimensis*	SSM-sur55	Complete genome	2	2	2,286,687	52.8	GCF_016593255.1
Lebetimonas	*natsushimae*	HS1857	Contig	8	-	1,639,064	30.4	GCF_002335445.1
Caminibacter	*pacificus*	TB6	Complete genome	2	2	1,871,566	34.0	GCF_005083985.2
Caminibacter	*mediatlanticus*	TB-2	Complete genome	1	1	1,685,887	25.6	GCF_005843985.1
Nautilia	*profundicola*	AmH	Complete genome	1	1	1,676,444	33.5	GCF_000021725.1
Nitratifractor	*salsuginis*	DSM 16511	Complete genome	1	1	2,101,285	53.9	GCF_000186245.1
Sulfurimonas	*denitrificans*	DSM 1251	Complete genome	1	1	2,201,561	34.5	GCF_000012965.1
Sulfurimonas	*autotrophica*	DSM 16294	Complete genome	1	1	2,153,198	35.2	GCF_000147355.1
Sulfurimonas	*paralvinellae*	GO25	Complete genome	2	2	2,078,871	38.5	GCF_014905135.1
Sulfurimonas	*sediminis*	S2-6	Complete genome	1	1	2,320,257	37.3	GCF_014905115.1
Sulfurimonas	*hydrogeniphila*	NW10	Complete genome	1	1	2,342,011	37.3	GCF_009068765.1
Sulfurimonas	*indica*	NW8N	Contig	52	-	2,093,483	36.8	GCF_009192995.1
Sulfurovum	sp.	NBC37-1	Complete genome	1	1	2,562,277	37.1	GCF_000010345.1
Sulfurovum	*lithotrophicum*	ATCC BAA-797	Complete genome	1	1	2,217,891	44.3	GCF_000987835.1
Sulfurovum	*indicum*	ST-419	Complete genome	1	1	2,209,694	42.5	GCF_014931715.1
Sulfurovum	*riftiae*	1812E	Contig	72	-	2,374,692	45.7	GCF_001595645.1
Persephonella	*hydrogeniphila*	DSM15103	Contig	19	-	1,998,302	35.1	GCF_900215515.1

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Sulfurimonas paralvinellae GO25T (AB252048)
Sulfurimonas denitrificans DSM 1251T (L40808)
Sulfurovum lithotrophicum 42BKTT (CP011308)
Sulfurovum sp. NBC37-1 (AP009179)
Sulfurovum riftiae 1812ET (KP896689)
Sulfurovum indicum ST-419T (MW138060)
Sulfurovum denitrificans eps51T (LC322101)
Sulfurovum aggregans Monchim33T (AB889689)
Nitratifractor salsuginis E9I37-1T (MT345097)
Hydrogenimonas thermophila EP1-55-1%T (AB105048)
Hydrogenimonas urashimensis SSM-sur55T (LC469141)
Nitrosophilus kaiyonis MOT50T (LC716068)
Nitrosophilus labii HRV44T (LC528620)
Nitrosophilus alvini EPR55-1T (LC469140)
Nitratiruptor tergarcus MI55-1T (AB175499)
Nitratiruptor sp. SB155-2 (AP009178)
Thioreductor micantisoli BKB25Ts-YT (AB175498)
Caminibacter mediatlanticus TB-2T (MT345079)
Caminibacter hydrogeniphilus AM1116T (AJ309655)
Caminibacter profundus CRT (AJ535664)
Caminibacter pacificus TB-6T (KF648717)
Lebetimonas natsushimae HS1857T (LC210765)
Lebetimonas acidiphila Pd55T (AB167820)
Nautilia abyssi PH1209T (AM937002)
Nautilia profundicola AmHT (AF357197)
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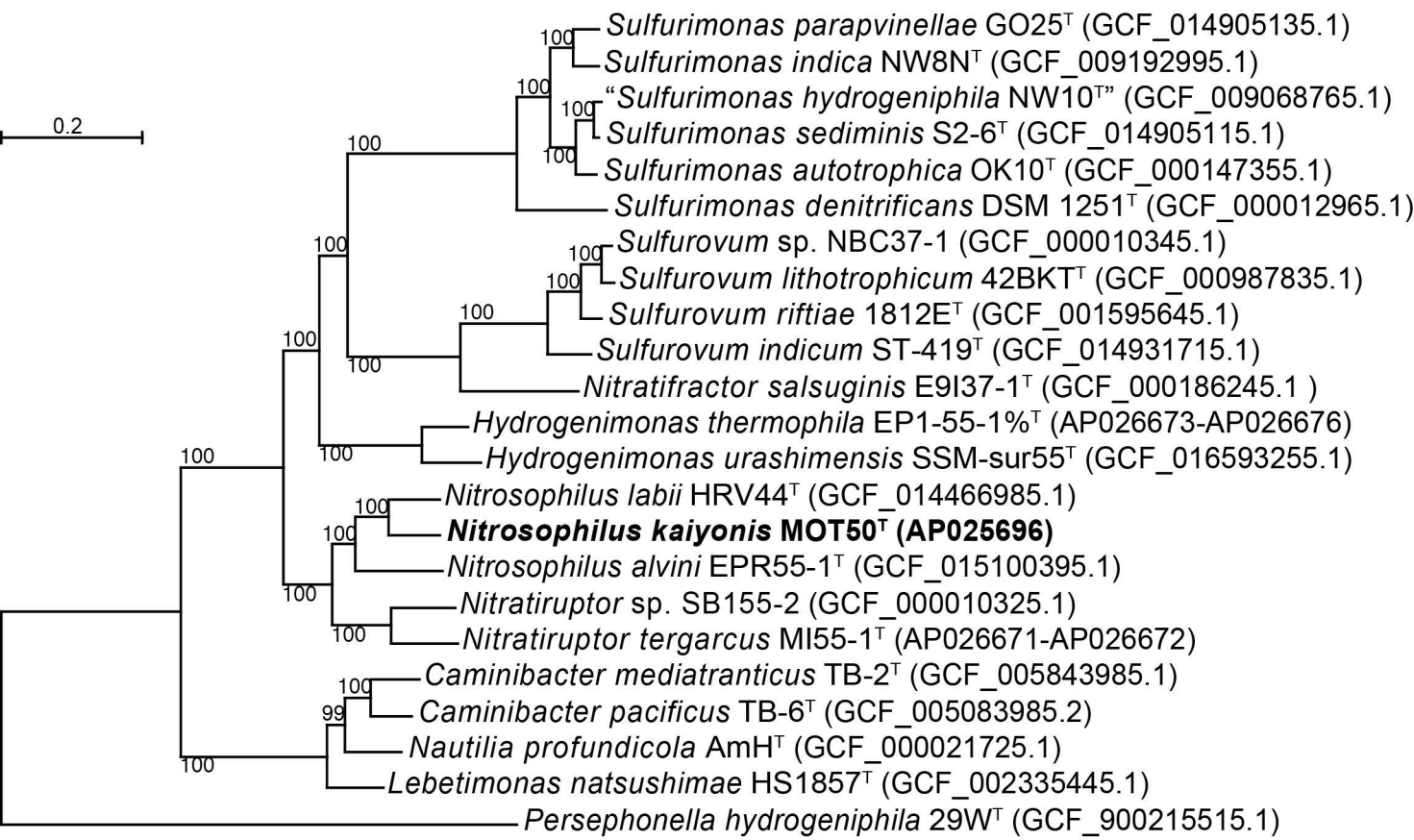
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Sulfurimonas sediminis S2-6T (GCF_014905115.1)
Sulfurimonas autotrophica OK10T (GCF_000147355.1)
Sulfurimonas denitrificans DSM 1251T (GCF_000012965.1)
Sulfurovum sp. NBC37-1 (GCF_000010345.1)
Sulfurovum lithotrophicum 42BKTT (GCF_000987835.1)
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Nitrosophilus labii HRV44T (GCF_014466985.1)
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Nitrosophilus alvini EPR55-1T (GCF_015100395.1)
Nitratiruptor sp. SB155-2 (GCF_000010325.1)
Nitratiruptor tergarcus MI55-1T (AP026671-AP026672)
Caminibacter mediatranticus TB-2T (GCF_005843985.1)
Caminibacter pacificus TB-6T (GCF_005083985.2)
Nautilia profundicola AmHT (GCF_000021725.1)
Lebetimonas natsushimae HS1857T (GCF_002335445.1)
Persephonella hydrogeniphila 29WT (GCF_900215515.1)