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Thiomicrorhabdus heinhorstiae sp. nov. and *Thiomicrorhabdus cannonii* sp. nov.: novel sulfur-oxidizing chemolithoautotrophs isolated from the chemocline of Hospital Hole, an anchialine sinkhole in Spring Hill, Florida, USA

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Repositories: The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and genome sequences of strain HH1^T are MZ029054 and GCA_013391765.1. The genome is also available from the Integrated Microbial Genomes & Microbiomes (IMG; https://img.jgi.doe.gov/), genome ID # 2901320023. Strain HH1^T has been deposited at the DSMZ-German Collection of Microorganisms and Cell Cultures (=DSM 111584^T) and ATCC (=ATCC TSD-240^T). The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and genome sequences of strain HH3^T are MZ029089 and GCA_013391695.1. The genome is also available from IMG, genome ID # 2873448755. Strain HH3^T has been deposited at the DSMZ (=DSM 111593^T) and ATCC (=ATCC TSD-241^T).

Abstract

Two sulfur-oxidizing, chemolithoautotrophic aerobes were isolated from the chemocline of an anchialine sinkhole located within the Weeki Wachee River of Florida. Gram-stain-negative cells of both strains were motile, chemotactic rods. Phylogenetic analysis of the 16S rRNA gene and predicted amino acid sequences of ribosomal proteins, average nucleotide identities, and alignment fractions suggest the strains HH1^T and HH3^T are novel species belonging to genus *Thiomicrorhabdus*. The genome G+C fraction of HH1^T is 47.8 mol% with a genome length of 2.61 Mb, whereas HH3^T has a G+C fraction of 52.4 mol% and 2.49 Mb genome length. Major fatty acids of the two strains included $C_{16:1}$, $C_{18:1}$, $C_{16:0}$, with the addition of $C_{10:0\ 3\text{-}OH}$ in HH1^T and $C_{12:0}$ in HH3^T. Chemolithoautotrophic growth of both strains was supported by elemental sulfur, sulfide, tetrathionate, and thiosulfate, and HH1^T was also able to use molecular hydrogen. Neither strain was capable of heterotrophic growth or use of nitrate as a terminal electron acceptor. Strain HH1^T grew from pH 6.5 - 8.5, with an optimum of 7.4, whereas strain HH3^T grew from pH of 6 - 8 with an optimum of 7.5. Growth was observed between 15 - 35˚C with optima of 32.8˚C for HH1^T and 32˚C for HH3^T. HH1^T grew in media with [NaCl] 80 – 689 mM, with an optimum of 400 mM, while HH3^T grew at 80 – 517 mM, with an optimum of 80 mM. The name *Thiomicrorhabdus heinhorstiae* sp. nov. is proposed, and the type strain is HH1^T (=DSM 111584, ATCC TSD-240). The name *Thiomicrorhabdus cannonii* sp. nov is proposed, and the type strain is HH3^T (=DSM 111593, ATCC TSD-241).

The genera *Thiomicrospira (T.), "Thiosulfativibrio"* (*"Tsv."*)*, "Thiosulfatimonas"* (*"Tss."*), *Thiomicrorhabdus (Tmr.), Hydrogenovibrio (H.),* and *Galenea (G.)* cluster together within the *Thiotrichales* of the *Gammaproteobacteria* [1-3]. They are commonly detected either by sequencing or cultivation from a variety of sulfidic environments, including hydrothermal vents, brackish lakes, marine sediments, hot springs, and soda lakes (reviewed in [1-9].

These organisms typically use reduced sulfur species as electron donors, with a few species capable of using molecular hydrogen [4, 10-12] or ferrous iron [10, 11, 13, 14]; reviewed in [1]. Molecular oxygen is the only electron acceptor supporting their growth, except in *Tmr. sediminis* (reviewed in [1, 3-7]). Members of these genera grow chemolithoautotrophically using the transaldolase-variant of the Calvin-Benson-Bassham cycle [2, 15, 16]. Most are unable to grow heterotrophically (e.g., [3, 17, 18], although for some, growth yields can be increased with the addition of organic compounds, suggesting mixotrophy is possible [19], and *H. thermophilus* is capable of *bona fide* heterotrophic growth [20]. The majority of members of these genera are mesophilic (28 to 32˚C optima) neutralophiles (pH 7.0 to 8.5 optima; reviewed in [1]).

Though members of *Thiomicrorhabdus*, *Hydrogenovibrio,* and *Thiomicrospira* have been isolated from a diverse array of sulfidic habitats (described above), they had never been isolated from sinkholes. Since a single sinkhole can provide a variety of electron donors and acceptors, including reduced sulfur species, along with a variety of physical conditions (temperature, pH, salinity) [21], we reasoned that it might harbor novel sulfur oxidizing chemolithoautotrophs. Here we describe two new species cultivated from a stratified, sulfidic sinkhole, and propose the names *Thiomicrorhabdus heinhorstiae* sp. nov. and *Thiomicrorhabdus cannonii* sp. nov. for these organisms.

HABITAT AND ISOLATION

Strains HH1^T and HH3^T were isolated from the chemocline of Hospital Hole, a vertically stratified sinkhole in Florida, USA, with inputs from the Weeki Wachee River and saltwater intrusion from below, located at 28.53˚N, 82.62˚W [21]. Four strata are apparent: a surface layer of water from the Weeki Wachi River (1-3 m deep) above the halocline, a brackish hypoxic layer (3-21 m deep), a cloudy chemocline (3 cm to 6 m mixing zone centered around 25 m depth), and a higher-salinity anoxic layer below the chemocline, ending at a debris mound at *c.* 40 m depth. The chemocline is centered just below the ingress of saltwater from active conduits from the Upper Floridan Aquifer [21]. Typically, the waters below the chemocline contain *c.* 100 µM total sulfide, and those within the chemocline *c.* 5 µM [21].

In December 2018, scientific divers collected samples from the chemocline with sterile 50-ml polypropylene centrifuge tubes. Chemocline water samples were analyzed as in [21]. Salinities for these samples suggest mixing of fresh and saltwater (Table 1). Though nitrate concentrations were not measured for these particular samples, prior samples from this site had nitrate concentrations of ~13 µM [21]. Two samples were set aside for cultivating microorganisms and stored overnight at 4˚C. The following morning, they were diluted 1:100 by volume with thiosulfate-supplemented artificial seawater [22], with NaCl lowered to 9.5 g l^{-1}, pH 7.5 (½TASW), and incubated unshaken at 20˚C. Once turbid, cultures were spread as two dilution series on solid ½ TASW medium. Many small colonies were visible after one week, and 10 colonies ultimately from each sample were streaked to isolation on ½ TASW solidified with 1.5% w/v Fisher Bioreagents agar. Five colonies from each sample were selected for 16S rRNA

gene sequencing. Within each sample, all five 16S rRNA gene sequences had 100% identity but were distinct from those from the other sample.

Unless otherwise stated, cultures were propagated in solid or liquid ½TASW under a headspace of air at 20˚C. Liquid cultures were agitated at 100 rpm with a New Brunswick Scientific Excella E24 Incubator Shaker. Frozen stocks were prepared by adding sterile glycerol (15% v/v) to exponential-phase liquid cultures, flash-freezing with liquid nitrogen, and storing at -80˚C.

PHENOTYPIC AND CHEMOTAXONOMIC CHARACTERIZATION

Colonies of strains HH1^T and HH3^T on ½TASW plates are small (< 1 mm diameter) and white, likely from elemental sulfur deposition, though the products of thiosulfate oxidation were not characterized in this study (Fig. 1A, 1B). When cultivated on swim plates (0.3% w/v agar; [23]), rings form and expand, indicating that these organisms are chemotactic and motile. Gram-stain-negative cells are rod-shaped, with maximum dimensions in transmission electron micrographs of 2.9 × 0.7 µm (HH1^T) and 2.8 × 0.8 µm (HH3^T). Dark inclusions of approximately 0.12 µm in diameter, likely carboxysomes, are apparent in cells cultivated in chemostats under dissolved inorganic carbon limitation (Fig. 1C, 1D).

To identify the range of conditions permitting growth, cells were cultivated in ½TASW at 5-55°C, 0 – 2.6 M NaCl, and pH 5.0 – 8.5. 10 ml liquid cultures were incubated for 72 h in an incubator shaker, and growth was determined turbidometrically (λ = 440 nm). Cultures often turned milky, likely due to elemental sulfur production during growth on thiosulfate, making it difficult to distinguish growth extent under these conditions, so additional experiments (described below) were needed to determine optimal conditions.

For temperature and NaCl optima, 50 µl cultures in ½ TASW supplemented with pH indicator phenol red (0.0005% w/v) were incubated in sterile 200 µl PCR tubes in a thermocycler that maintained steady temperature over the course of the experiment. For temperature optima experiments, the gradient feature of the thermocycler was used to create a range of temperatures. For NaCl optima experiments, cultures were maintained at 25˚C. The apparent rates of proton extrusion were calculated from the time, in hours, necessary for the cultures to turn from magenta (pH 8) to yellow (pH 6.8).

Optimal pH values and oxygen tensions were determined by monitoring growth as [^{14}C]-bicarbonate incorporation into biomass (0.2 µCi ml^{-1}; 0.02 µCi µmol^{-1}). For both pH and oxygen experiments, cells were cultivated in 5-ml liquid ½TASW. Cultures to determine pH optima were grown in sterile 50 ml polypropylene centrifuge tubes, while cultures at different oxygen partial pressures were incubated in sealed 100 ml glass serum bottles, with a range of oxygen tensions in the headspace generated with mixtures of argon, air, and oxygen (1 atm total pressure). After incubation in an incubator shaker at 25°C for 24 h, 1 ml portions were acidified with 0.5 ml glacial acetic acid, and [^{14}C]-bicarbonate incorporation was measured via scintillation counting [24]. To provide further evidence for optimal oxygen tensions, cells were stab-inoculated into ½ TASW slush agar tubes (0.5% w/v bacteriological agar) to observe their position relative to the surface of the culture.

Optima were calculated from 3rd order polynomial curves fitted to the data. Maximum specific growth rate coefficients (μ_{MAX}) were determined from washout kinetics of cells cultivated in chemostats under optimal conditions [25-27]

[^{14}C]-bicarbonate incorporation by strains HH1^T and HH3^T was highest at oxygen concentrations of 5 – 21% in the headspace (Fig. 2A, 2B). Low [^{14}C]-bicarbonate incorporation by strain HH3^T was not improved by extending the length of the incubation beyond 24 h (values were low after two and seven days). Both strains HH1^T and HH3^T grew as plates below the surface of slush agar tubes (Fig. 1E, 1F), with HH1^T positioning itself approximately 1 mm below the surface, and HH3^T approximately 1.5 – 2 mm, suggesting that both are microaerophiles. This observation is consistent with genome sequences from these organisms (described below), which include genes encoding *cbb*$_3$-type cytochrome *c*-oxidases (E.C. 7.1.1.9) in both organisms, which typically have high affinities for O_2 [28].

Both strains are mesophiles, with optimal temperatures for growth of 32.8 and 32.0˚C, respectively (Fig. 2C). Temperature coefficients (Q_{10}) calculated from Arrhenius plots [25] are 1.05 (HH1^T) and 1.99 (HH3^T). Both strains are neutralophiles, with optimal growth at 7.4 (HH1^T) and 7.5 (HH3^T; Fig. 2D). Strain HH1^T was moderately halophilic (optimum at 0.41 M), while HH3^T grew best at 0.08 M, the lowest [NaCl] tested, and the lowest NaCl optimum for any member of *Thiomicrorhabdus* (Fig. 2E; Table 2). Maximum specific growth rate constants were determined at 25˚C, pH 7.5, 20 mM thiosulfate, with 0.41 M (HH1^T) or 0.08 M (HH3^T) NaCl, and were found to be 0.29 ± 0.04 h^{-1} (HH1^T) and 0.21 ± 0.01 h^{-1} (HH3^T).

Both strains could use elemental sulfur (flowers-of-sulfur, >99% α-cyclooctasulfur; 0.5% w/v), thiosulfate (20 mM), or tetrathionate (5 mM) as electron donors for chemolithoautotrophic growth. Growth on sulfide was also possible but was only observed as turbid layers in gradient tubes [29]. Sulfite, thiocyanate (7 mM), ammonium (10 mM), or nitrite (10 mM) did not support chemolithoautotrophic growth. Strain HH1^T grew on molecular hydrogen (1% headspace) when ½ASW was supplemented with Fe(II) and Ni(II) [30], but HH3^T did not. Growth on molecular hydrogen as an electron donor is uncommon among members of *Thiomicrorhabdus* (Table 2)*;* thus far, *Tmr. hydrogeniphila* is the only other member to do so [11].

For tests to determine carbon and nitrogen sources, all ionic species were provided as their sodium or chloride salts. Cells were grown in ½ASW medium (no thiosulfate) to determine whether organic compounds could serve as carbon sources and electron donors. For testing nitrogen sources, thiosulfate was provided as the electron donor (½TASW). Neither strain was able to use any of the organic carbon compounds tested as carbon source and electron donor for heterotrophic growth in liquid culture; growth in liquid ½ASW medium (without thiosulfate) was not supported by yeast extract and tryptone (as a 1:10 dilution of lysogeny broth), glyceraldehyde (20 mM), D-arabinose (6 mM), D-glucose (10 mM), D-fructose(10 mM), D-rhamnose (10 mM), D-sucrose (5 mM), acetate (10 mM), pyruvate(10 mM), citrate (10 mM), 2-oxoglutarate (5 mM), succinate (10 mM), malate (10 mM), oxaloacetate (10 mM), ethanol (25 mM), propan-2-ol (10 mM), glycerol (10 mM), or D-mannitol (5 mM). No methylotrophic growth was apparent on any of the one-carbon (C_1) species provided: monomethylammonium, dimethylsulfoxide (20 mM), formate (10 mM), formaldehyde (2 mM) or methanol (50 mM). As nitrogen sources, both strains used ammonium (7 mM) and L-glutamine (3.5 mM). HH1^T could also use nitrite, nitrate, monomethylammonium, and L-cysteine (7 mM for each). Neither strain

could use EDTA (3.5 mM), L-serine (7 mM), L-glycine (7 mM), L-aspartate (7 mM), or molecular nitrogen. Anaerobic growth at the expense of nitrate was not observed in either strain.

To identify the dominant cellular fatty acids and respiratory quinones, cells were grown in flasks of ½TASW liquid medium. Cells were harvested by centrifugation (Sorvall GSA rotor, 4000 × g, 4˚C, 20 min), and stored at -80˚C. Fatty acids and quinones were extracted and analyzed by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, as described in [31, 32]. For both strains, the dominant fatty acids are in keeping with those of closely affiliated species (Table 3). Palmitic ($C_{16:0}$), palmitoleic ($C_{16:1}$), and vaccenic ($C_{18:1}$) acids are dominant. Odd-chain fatty acids ($C_{17:0}$; $C_{17:1}$) are also present, while hydroxylated fatty acids ($C_{10:0\ 3\text{-}OH}$) are particularly abundant HH1^T. For both strains, ubiquinone-8 (UQ-8) is the dominant respiratory quinone, as is typical for the *Thiotrichales.*

GENOMIC CHARACTERIZATION

DNA was extracted from cells using CTAB [33]. Genome sequencing was provided by MicrobesNG (http://www.microbesng.uk), and protocols used for library preparation, sequencing via Illumina HiSeq, and trimming are described online (https://microbesng.com/documents/5/MicrobesNG_Methods_Document_-_PDF.pdf). 592,666 and 862,434 reads were produced from strains HH1^T and HH3^T, respectively, and were assembled into scaffolds (strain HH1^T: 102-fold average coverage, 97 scaffolds, 26924 nt avg scaffold length, 2.61 Mb total length, 47.8% G+C fraction, 2550 genes; strain HH3^T: 162-fold average coverage, 62 scaffolds, 40,233 nt avg scaffold length, 2.49 Mb total length, 52.4% G+C fraction, 2422 genes). These sequences were annotated via the IMG/ER pipeline [34], and are publicly available (HH1^T: IMG genome ID #2901320023, Genbank GCA_013391765.1; HH3^T: IMG genome ID #2873448755, Genbank GCA_013391695.1).

Genome sequence data for these two strains have many parallels with members of genera *Thiomicrospira*, *Thiomicrorhabdus*, and *Hydrogenovibrio*. Genes for enzymes and complexes necessary for using reduced sulfur species are present in the genome, including bacterial sulfide: quinone oxidoreductase (EC 1.8.5.4, *sqr*), sulfide-cytochrome-*c* reductase (flavocytochrome *c*, EC 1.8.2.3, *fccAB*), and the enzymes of the Lu-Kelly cycle of thiosulfate oxidation ("Sox complex", *soxXYZABCD*: L-cysteine *S*-thiosulfotransferase, EC 2.8.5.2, *soxAX*; *S*-sulfosulfanyl-L-cysteine sulfohydrolase, EC 3.1.6.20, *soxB*; *S*-disulfanyl- L-cysteine oxidoreductase, EC 1.8.2.6, *soxCD*; and the thiosulfate-binding protein *soxYZ*). Strain HH1^T carries genes encoding both a group 1d and sensory class 2b [NiFe] hydrogenase (EC 1.12.99.6, *hyaABC* and *hupUV,* as classified using HydDB; [35]). Strain HH1^T also carries genes encoding enzymes necessary for assimilatory sulfate reduction, which make it possible for this organism to grow by using H_2 as its electron donor in the absence of reduced sulfur species (sulfate adenylyltransferase, EC 2.7.7.4, *cysDN*; adenylsulfate kinase, EC 2.7.1.25, *cysC*; phosphoadenosine phosphosulfate reductase (thioredoxin), EC 1.8.4.8, *cysH*; assimilatory sulfite reductase (NADPH, EC 1.8.1.2, *cysIJ*)). Both strains carry genes for the high-affinity *cbb*$_3$-type cytochrome *c* oxidase (EC 7.1.1.9, *ccoNOQP*).

Both strains carry genes encoding the transaldolase-variant of the Calvin-Benson-Bassham cycle [36-38], with three types of ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO; EC 4.1.1.39): both carboxysomal (IAc) and cytosolic (IAq) types of the form IA isozyme (*cbbLS)*, and one form II isozyme (*cbbM)*. Encoded downstream from the carboxysome loci are

multisubunit DIC-accumulating complexes [39, 40]; the presence of genes encoding both carboxysomes and these complexes suggests these organisms express CO_2-concentrating mechanisms when grown in the presence of low concentrations of CO_2 [41]. Indeed, inclusions resembling carboxysomes are abundant when cells are grown under dissolved inorganic carbon limitation (Fig. 1). The inability of these organisms to use multicarbon compounds for heterotrophic growth is consistent with the presence of an incomplete form of Krebs' cycle, lacking genes encoding enzymes to convert 2-oxoglutarate to succinyl-CoA ('Smith's horseshoe'; [42, 43]). As for members of *Thiomicrospira, Thiomicrorhabdus,* and *Hydrogenovibrio,* genes encoding malate dehydrogenase (NAD^+; EC 1.1.1.37) are absent, though genes encoding malate dehydrogenase (quinone) are present (EC 1.1.5.4, *mqoB*; [2, 18]).

The presence of genes encoding enzymes responsible for nitrogen metabolism is also consistent with the results from cultivating these organisms. Nitrogenase genes are absent, while genes encoding ferredoxin-nitrate reductase (EC 1.7.7.2, *narB*) and nitrite reductase (NADH; EC 1.7.1.15, *nasB*) are present in strain $HH1^T$. Strain $HH3^T$ has genes encoding cyanase (EC 4.2.1.104, *cynS*), suggesting cyanate could serve as a nitrogen source.

As previously observed for other taxonomically affiliated organisms [2], these strains are poised to sense and respond to changes in their environment. Chemotaxis and motility are facilitated by a large number of genes encoding methyl-accepting chemotaxis proteins (10 in $HH1^T$, 19 in $HH3^T$), and GGDEF/EAL-domain proteins and histidine kinase/response regulators are well-represented in these genomes.

Strain $HH3^T$ has a prophage encoded in its genome in a ~32 kb region spanning IMG gene id's 2873448806 to 2873448853. This region includes genes encoding a lambda repressor-like predicted transcriptional regulator as well as structural components of phage particles, including phage-related tail fiber proteins, head proteins, baseplates, and sheaths. Analyses in PHASTER [44] placed top matches to the genes in this region to prophages found primarily in other members of *Gammaproteobacteria*—those in *Vibrio* spp. being the most common matches (top matches for 15 of the 49 prophage genes).

PHYLOGENETIC AND GENOMIC ANALYSES

16S rRNA (*rrs)* gene sequences of strains $HH1^T$ and $HH3^T$ affiliate them with the genera *Thiomicrorhabdus*, *Hydrogenovibrio,* and *Thiomicrospira* (Fig. 3). Closest pairwise matches for $HH1^T$ are $HH3^T$ (95.25% identity) and *Thiomicrorhabdus xiamensis* (94.87% identity). The closest pairwise match for $HH3^T$ is *Thiomicrorhabdus aquaedulcis* (95.56% identity; Table 4). On the basis of the Stackebrandt threshold for species (98.7% 16S rRNA gene identity; [45]), and the Yarza cut-off for the rank of genus (94.50% 16S rRNA gene identity [46]), which we have used previously [1, 47], $HH1^T$ and $HH3^T$ represent members of genus *Thiomicrorhabdus.* Based on the Yarza median for rank of family (<92.25%; [1, 12, 46]), the genera *Galenea*, *Thiomicrorhabdus,* and *Hydrogenovibrio* are members of the same family, while *Thiomicrospira* is in a different family.

For genome-level comparisons, genome sequences are available for the type strains of the type species of the genera *Thiomicrospira* (*Thiomicrospira pelophila* DSM 1534^T) and *Hydrogenovibrio* (*Hydrogenovibrio marinus* MH-110^T). As the equivalent strain for

Thiomicrorhabdus (*Thiomicrorhabdus frisia* JB-A2^T) has yet to be genome sequenced, data from *Tmr. frisia* Kp2 was used. The 16S rRNA gene sequence of this strain has 99.3% identy to that of *Tmr. frisia* JB-A2^T. Digital DNA-DNA hybridization (dDDH) values for comparisons of strains HH1^T and HH3^T against other species are all <70% (Table 5), consistent with both strains being distinct from these species [48]. The highest dDDH values were within genera *Thiomicrorhabdus* and *Hydrogenovibrio,* but with no affiliation close enough to indicate that they are members of extant species of either genus. Phylogenetic analysis based on an alignment of 53-ribosomal-protein-amino-acyl-sequence concatamers generated using the rMLST database [49] includes strains HH1^T and HH3^T in a strongly-supported clade with *Thiomicrorhabdus,* (Fig. 4). Genome-level comparisons with the type species of genera *Thiomicrorhabdus, Hydrogenovibrio,* and *Thiomicrospira* via average nucleotide identities of orthologous genes (ANI) and alignment fractions of orthologous genes (AF), as described in [50], also suggest closest affiliation with *Thiomicrorhabdus* (Fig. 5; Table 2, Table 5). AF values place both strains among members of *Thiomicrorhabdus,* while their ANI values (Table 2) are a bit lower than those for other members of this genus. Indeed, their ANI values are slightly higher when compared to *H. marinus* than *Tmr. frisia* (Fig. 5; Table 2, Table 5). However, their ANI and AF values both have best matches with members of *Thiomicrorhabdus* (Table 5). Whether compared to *H. marinus* or *Tmr. frisia*, their ANI values are slightly lower than the boundary previously suggested for these genera (71.98 and 70.85%, respectively; [50]). Recently described members of two newly proposed genera (albiet without validly published names at this time), *"Thiosulfativibrio zosterae"* (*"Tsv. zosterae")* and "*Thiosulfatimonas sediminis" ("Tss. sediminis"* [3] also fall among HH1^T, HH3^T, and other members of *Thiomicrorhabdus* (Fig. 5), suggesting that membership within *Thiomicrorhabdus* may need to be revised as more strains are isolated and characterized. For now, based on their phenotypes (Fig. 1, Table 2), positions on the rMLST tree (Fig. 4), AF values, and top matches based on dDDH, ANI and AF values (Table 2, Table 5), strains HH1^T and HH3^T are most closely affiliated to *Thiomicrorhabdus.* As such, we propose that each of these strains represents a novel species of *Thiomicrorhabdus;* we propose *Thiomicrorhabdus heinhorstii* sp. nov. for which the type strain is HH1^T, and *Thiomicrorhabdus cannonii* sp. nov. for which the type strain is HH3^T.

DESCRIPTION OF *THIOMICRORHABDUS HEINHORSTIAE* SP. NOV.

Thiomicrorhabdus heinhorstiae (hein.hor′sti.ae. N.L. gen. n. *heinhorstiae,* of or pertaining to Heinhorst, named to honor Professor Sabine Heinhorst (*b*. 1952), microbiologist at University of Southern Mississippi who made significant contributions to study of the structure and function of carboxysomes in autotrophic *Bacteria*).

Cells are motile, chemotactic rods of 1.9-2.9 μm long and 0.5-0.7 μm diameter and contain 120 nm-diameter polyhedral bodies resembling carboxysomes, the genes for which are also present in the genome. On ½ TASW plates grown under air, colonies are white with powdery deposits likely to be elementary sulfur, circular, entire and < 1 mm in diameter. On plates supplemented with phenol red, colonies are yellowish owing to acid production during thiosulfate oxidation. Moderately halophilic, neutralophilic mesophile. Growth occurred at 15 - 35 ℃, pH 6.5 - 7.5, and at 80 – 689 mM NaCl with optimal growth at 32.8 ℃, pH 7.4, and at 410 mM NaCl. Vitamins are not required for growth. Obligate aerobes growing optimally under 5 - 21% *v/v* molecular oxygen. Obligate chemolithoautotrophs using thiosulfate, elemental sulfur, sulfide,

tetrathionate, and molecular hydrogen as electron donors but not sulfite, thiocyanate, ammonium or nitrite. Heterotrophic growth was not observed in liquid ½ ASW broth supplemented with the following potential carbon sources: diluted lysogeny broth, glyceraldehyde, D-arabinose, D-glucose, D-fructose, D-rhamnose, sucrose, acetate, pyruvate, citrate, 2-oxoglutarate, succinate, malate, oxaloacetate, ethanol, *iso*-propanol, glycerol, D-mannitol, monomethylammonium, dimethylsulfoxide, formate, formaldehyde, or methanol. Nitrogen sources used during growth on thiosulfate were ammonium, nitrate, nitrite, L-glutamine, monomethylammonium and L-cysteine, but EDTA, L-serine, glycine, L-aspartate and molecular nitrogen could not be used. Dominant fatty acids in biomass grown on thiosulfate are palmitoleic acid ($C_{16:1}$), vaccenic acid ($C_{18:1}$), palmitic acid ($C_{16:0}$) and 3-hydroxycapric acid ($C_{10:0\ 3\text{-}OH}$). Dominant respiratory quinone is ubiquinone-8 (UQ-8). Genes encoding the high-affinity *cbb*$_3$-type cytochrome *c* oxidase (EC 7.1.1.9) are present in the genome, which is consistent with isolation site. G+C fraction of genomic DNA is 47.8 mol% (from genome sequence), with a genome size of 2.61 Mbp containing 2,550 genes of which 2,485 are predicted to be protein-coding.

The type strain, HH1^T (=DSM 111584^T; =ATCC TSD-240^T), was isolated from the chemocline of Hospital Hole, an anchialine sinkhole in the Weeki Wachee River (Spring Hill, Florida, USA).

The GenBank accession number for the 16S rRNA gene and whole genome sequences of strain HH1^T are MZ029054 and GCA_013391765.1, respectively. The IMG genome ID for the whole genome sequence of strain HH1^T is 2901320023.

DESCRIPTION OF *THIOMICRORHABDUS CANNONII* SP. NOV.

Thiomicrorhabdus cannonii (can.no′ni.i. N.L. gen. n. *cannonii*, of or pertaining to Cannon, named to honor Professor Gordon C. Cannon (*b*. 1953), microbiologist at University of Southern Mississippi who made significant contributions to study of the structure and function of carboxysomes in autotrophic *Bacteria*).

Cells are motile, chemotactic rods of 1.5-2.8 μm long and 0.6-0.8 μm diameter and contain 120 nm-diameter polyhedral bodies resembling carboxysomes, the genes for which are also present in the genome. On ½ TASW plates grown under air, colonies are white with powdery deposits likely to be elementary sulfur, circular, entire and < 1 mm in diameter. On plates supplemented with phenol red, colonies are yellowish owing to acid production during thiosulfate oxidation. Moderately halotolerant neutralophilic mesophile. Growth occurred at 15 - 35 °C, pH 6.0 – 8.0, and at 80 – 517 mM NaCl with optimal growth at 32.0 °C, pH 7.5, and at 80 mM NaCl. Vitamins are not required for growth. Obligate aerobes growing optimally under 5 - 21% *v/v* molecular oxygen. Obligate chemolithoautotrophs using thiosulfate, elemental sulfur, sulfide, and tetrathionate as electron donors but not molecular hydrogen, sulfite, thiocyanate, ammonium or nitrite. Heterotrophic growth was not observed in liquid ½ ASW broth supplemented with the following potential carbon sources: diluted lysogeny broth, glyceraldehyde, D-arabinose, D-glucose, D-fructose, D-rhamnose, sucrose, acetate, pyruvate, citrate, 2-oxoglutarate, succinate, malate, oxaloacetate, ethanol, *iso*-propanol, glycerol, D-mannitol, monomethylammonium, dimethylsulfoxide, formate, formaldehyde, or methanol. Nitrogen sources used during growth on thiosulfate were ammonium and L-glutamine but nitrate, nitrite, monomethylammonium, L-cysteine, EDTA, L-serine, glycine, L-aspartate and molecular nitrogen could not be used.

Dominant fatty acids in biomass grown on thiosulfate are palmitoleic acid ($C_{16:1}$), vaccenic acid ($C_{18:1}$), palmitic acid ($C_{16:0}$) and lauric acid ($C_{12:0}$). Dominant respiratory quinone is ubiquinone-8 (UQ-8). Genes encoding the high-affinity *cbb*$_3$-type cytochrome *c* oxidase (EC 7.1.1.9) are present in the genome, which is consistent with isolation site. G+C fraction of genomic DNA is 52.4 mol% (from genome sequence), with a genome size of 2.49 Mbp containing 2,422 genes of which 2,360 are predicted to be protein-coding.

The type strain, HH3^T (=DSM 111593^T; =ATCC TSD-241^T), was isolated from the chemocline of Hospital Hole, an anchialine sinkhole in the Weeki Wachee River (Spring Hill, Florida, USA).

The GenBank accession number for the 16S rRNA gene and whole genome sequences of strain HH3^T are MZ029089 and GCA_013391695.1, respectively. The IMG genome ID for the whole genome sequence of strain HH3^T is 2873448755.

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Conflicts of interest

The authors declare that there are no conflicts of interest to report.

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585 **Table 1.** Chemocline chemistry from Hospital Hole

Parameter	Value ± sd (*n*=3)
temperature (˚C)	24.2 ± 0.0
pH	7.15 ± 0.12
total alkalinity (mg l^{-1})	116 ± 7
salinity (mg l^{-1})	13.3 ± 2.3
dissolved O_2 (µM)	9.68 ± 3.85
sulfide (µM)	0.44 ± 0.65
sulfate (mM)	5.94 ± 0.29
ammonium (µM)	3.06 ± 2.35
nitrite (µM)	1.13 ± 0.87
total nitrogen (mg l^{-1})	1.87 ± 0.85
total phosphorus (mg l^{-1})	0.26 ± 0.07
total organic carbon (mg l^{-1})	0.96 ± 0.31

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Table 2. Comparison of strains HH1^T and HH3^T to members of *Thiomicrorhabdus* and the type species of the genera *Hydrogenovibrio, Galenea,* and *Thiomicrospira*[*]

	HH1^T	**HH3^T**	***Tmr. aquaedulcis* HaS4^T**	***Tmr. arctica* SVAL-E^T**	***Tmr. chilensis* Ch-1^T**	***Tmr. frisia* JB-A2^{T†}**	***Tmr. hydrogeniphila* MAS 2^T**	***Tmr. indica* 13-15A^T**	***Tmr. psychrophila* SVAL-D^T**	***Tmr. sediminis* G1^T**	***Tmr. xiamenensis* G2^T**	**" *Tss. sediminis*" aks77^T**	**" *Tsv. zosterae*" AkT22^T**	***H. marinus* MH-110^T**	***G. microaerophila* P2D^T**	***T. pelophila* DSM 1534^T**
Origin	Sinkhole, USA	Sinkhole, USA	Lake water, Japan	Marine arctic sediments	Coastal shelf, Chile	Deep vent, Northeast Pacific	Coastal seawater, Japan	Deep vent, Indian Ocean	Marine arctic sediments	Marine sediment, China	Marine sediment, China	Brackish lake, Japan	Brackish lake, Japan	Surface seawater, Japan	Shallow vent, Greece	Marine sediment, Netherlands
%16S rRNA gene sequence identity to:																
T. pelophila DSM 1534^T	91.88	91.81	91.88	93.26	92.73	91.96	91.96	92.27	93.19	91.81	92.57	92.34	90.66	93.03	93.03	100
Tmr. frisia JB-A2^T	93.49	94.33	95.64	96.63	96.48	99.30	99.54	95.48	96.63	96.55	95.48	94.79	93.49	94.72	93.72	91.96
H. marinus MH-110^T	94.18	94.95	93.57	94.41	95.48	94.72	95.02	95.48	94.72	95.41	95.41	94.18	93.26	100	93.80	93.03
% average nucleotide identity (ANI) with:																
T. pelophila DSM 1534^T	69.3	69.6	70.7	69.9	69.7	70.5†	N.D.‡	69.6	N.D.	69.9	69.2	69.6	70.2	69.5	N.D.	100
Tmr. frisia JB-A2^T	71.6	70.7	72.7	73.8	71.9	100†	N.D.	72.3	N.D.	73.2	71.1	71.9	71.6	70.9	N.D.	70.5
H. marinus MH-110^T	71.7	71.0	70.3	70.3	70.8	70.9†	N.D.	71.0	N.D.	71.3	70.9	70.5	71.3	100	N.D.	69.5
General properties																
Colony colour	white	white	N.D.	white	white	white/ yellow	white/ cream	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	white	cream/ yellow	white
Heterotrophic	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Carboxysomes	+	+	+‖	-	+	+	N.D.	+‖	N.D.	+‖	+‖	+‖	+‖	+	N.D.	+
G+C fraction (mol%)	N.D.	N.D.	N.D.	42.4	49.9	39.6	39.6	N.D.	42.5	N.D.	N.D.	N.D.	N.D.	44.1	44.9	45.7
In vitro and (*in silico*)	(47.8)	(52.4)	(45.3)	(41.9)	(48.1)	(39.9†)	(N.D.)	(41.6)	(N.D.)	(45.1)	(48.3)	(45.5)	(43.2)	(43.9)	(N.D.)	(44.5)
Maximum specific growth rate on thiosulfate under optimal conditions (h^{-1})	0.29	0.21	N.D.	0.14	0.4	0.45	0.4	0.17	0.2	0.31	0.4	N.D.	N.D.	0.6	0.63	0.3
Cell morphology																
Length (μm)	1.9-2.9	1.5-2.8	1.6-2.5	1.2-1.5	0.8-2.0	1.0-2.7	0.9-1.8	1.0-2.0	1.3-1.7	1.3-1.8	1.1-2.0	1.4-2.8	1.5-3.0	1.0-2.0	0.8-1.3	1.0-2.0
Width (μm)	0.5-0.7	0.6-0.8	0.7-0.9	0.5-0.6	0.3-0.5	0.3-0.5	0.3-0.5	0.4-0.7	0.5-0.6	0.3-0.6	0.5-0.8	0.6-0.9	0.5-1.1	0.2-0.5	0.4-05	0.2-0.3
Shape of cells under optimal and (stress) conditions	rod	rod	rod	rod	rod	Rod	rod	rod	rod	rod	rod	rod	rod	vibrio	rod	vibrio (spiral)
Motility	+	+	+	±	+	±	+	+	+	+	+	+	+	+	+	+
Flagella	N.D.	N.D.	N.D.	1	N.D.	N.D.	1	1	1	+	+	N.D.	N.D.	1	1	1-2

Growth conditions																
pH optimum	7.4	7.5	6.6-7.4	7.3-8.0	7.0	6.5	6.0	7.0	7.5-8.5	7.5	6.5	7.0-7.9	6.7–7.8	6.5	5.5	7.0
pH minimum	6.5	6	6.2	6.5	5.3	4.2	5.0	4.5	6.5	6	5	5.8	5.8	N.D.	4.5	5.9
pH maximum	8.5	8	8.8	9.0	8.5	8.5	8.0	9.0	9.0	9	8	8.5	8.0	N.D.	8.0	6.0
Temperature optimum (°C)	32.8	32.0	22.0	11.5-13.2	32-27	32-35	30.0	28.0	14.6-15.4	30	28	22	22	37	35	28-30
Temperature minimum (°C)	15.0	15.0	0.0	-2.0	3.5	3.5	2.0	10.0	-2.0	10	4	5	5	N.D.	20	3.5
Temperature maximum (°C)	35.0	35.0	25.0	20.8	42	39	40.0	45.0	20.8	40	45	32	37	N.D.	50	42
NaCl optimum (mM)	410	80	150-250	250	470	470	270	680	250	510	340	344	344	500	514	470
NaCl minimum (mM)	80	80	0	40	100	100	30	85	40	85	85	0	0	N.D.	171	40
NaCl maximum (mM)	689	517	450	1,240	1,240	1,240	1,380	1,700	1,240	1530	1530	1,030	862	N.D.	856	1,240
Physiology																
Tetrathionate as an energy source	+	+	+	+	+	+	+	+	+	+	+	-	-	+	N.D.	+
Elemental sulfur as an energy source	+	+	+	N.D.	+	N.D.	+	+	N.D.	+	+	-	-	+	-	N.D.
Auxotrophic for vitamin B_{12}	-	-	-	-	-	-	-	-	-	N.D.	N.D.	-	-	-	N.D.	+
Production of elemental sulfur when growing on thiosulfate at neutrality	+[§]	+/-[§]	N.D.	+	+	±	+	N.D.	+	N.D.	N.D.	N.D.	N.D.	-	+	+
Molecular hydrogen as an energy source	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-
Diazotrophy	-	-	-	N.D.	N.D.	N.D.	-	-	N.D.	N.D.	N.D.	N.D.	N.D.	-	N.D.	N.D.
Dominant fatty acids	$C_{16:1}$ $C_{18:1}$ $C_{16:0}$ $C_{10:0\ 3\text{-}OH}$	$C_{16:1}$ $C_{18:1}$ $C_{16:0}$ $C_{12:0}$	$C_{16:1}$ $C_{18:1}$ $C_{16:0}$ $C_{18:0}$	$C_{16:1}$ $C_{18:1}$ $C_{16:0}$ $C_{14:1}$	$C_{16:1}$ $C_{18:1}$ $C_{16:0}$ $C_{18:0}$	N.D.	$C_{16:1}$ $C_{18:0}$ $C_{16:0}$ $C_{12:0}$	$C_{16:1}$ $C_{18:1}$ $C_{16:0}$ $C_{18:0}$	$C_{16:1}$ $C_{18:0}$ $C_{16:0}$ $C_{12:1}$	$C_{16:1}$ $C_{16:0}$ $C_{18:0}$	$C_{16:1}$ $C_{16:0}$ $C_{18:0}$	$C_{16:1}$ $C_{18:1}$ $C_{10:0\ 3\text{-}OH}$ $C_{16:0}$	$C_{16:1}$ $C_{18:1}$ $C_{16:0}$ $C_{10:0\ 3\text{-}OH}$	$C_{16:1}$ $C_{16:0}$ $C_{18:0}$	$C_{16:1}$ $C_{16:0}$ $C_{18:1}$ $C_{18:0}$	N.D.
Dominant respiratory quinone	UQ-8	UQ-8	N.D	UQ-8	UQ-8	UQ-8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	UQ-8	UQ-8	UQ-8
[NiFe]-hydrogenase genes	+	-	-	-	-	-[†]	N.D.	-	N.D.	-	-	-	-	+	N.D.	-
RuBisCO Forms																
Form IAc	+	+	+	-	+	+[†]	N.D.	+	N.D.	++	+	++	+	+	N.D.	+
Form IAq	+	+	-	+	+	+[†]	N.D.	-	N.D.	-	+	-	+	+	N.D.	-
Form II	+	+	+	+	+	+[†]	N.D.	+	N.D.	+	+	-	+	+	N.D.	+

[*]Data from strains HH1 and HH3 are novel; data for the other species are from [1-3, 5-7, 10-12, 19, 51-55]

[†]Data for *Tmr. frisia* are given for type strain *Tmr. frisia* JB-A2[T], excepting the indicated genomic data which are from *Tmr. frisia* Kp2. The 16S rRNA gene sequences of these two strains have 99.3% identity

[‡]N.D. = no data available

[⁋]A carboxysome locus is apparent in the genome sequence data

599 §Elemental sulfur production was inferred from the powdery white appearance of the colonies
600
601

Table 3. Cellular fatty acid composition of members of *Thiomicrorhabdus*, "*Thiosulfativibrio"* and "*Thiosulfatimonas"* based on fatty acid methyl ester analysis as detailed in the text.

Fatty acid	HH1^T	HH3^T	*Tmr. aquaedulcis* HaS4^T	*Tmr. arctica* SVAL-E^T	*Tmr. chilensis* Ch-1^T	*Tmr. hydrogeniphila* MAS 2^T	*Tmr. indica* 13-15A^T	*Tmr. psychrophila* SVAL-D^T	*Tmr. sediminis* G1^T	*Tmr. xiamenensis* G2^T	*"Tsv. zosterae"* AkT22^T	*"Tss. sediminis"* aks77^T
Saturated fatty acids												
$C_{9:0}$	0.1	-	-	-	-	-	-	-	-	-	-	-
$C_{10:0}$	1.7	0.3	-	-	-	-	1.3	-	-	-	0.1	1.4
$C_{11:0}$	-	0.1	-	-	-	-	-	-	4.1	-	0.1	0.1
$C_{12:0}$	0.6	2.5	2.6	2.4	-	5.3	1.0	1.6	-	3.5	4.6	2.4
$C_{14:0}$	1.6	0.4	0.3	0.8	-	1.8	0.1	0.5	-	-	2.0	0.2
$C_{16:0}$	21.7	19.3	16.1	12.7	18.9	23.0	20.0	9.7	29.0	20.6	13.0	10.7
$C_{17:0}$	0.9	1.4	0.7	-	-	-	-	-	-	-	0.7	0.5
$C_{18:0}$	1.3	2.3	3.7	0.8	3.5	2.5	3.7	32.0	12.0	4.3	1.3	1.0
Unsaturated fatty acids												
$C_{12:1}$	-	-	-	3.2	3.4	0.6	-	4.5	-	-	-	-
$C_{14:1}$	-	-	-	11.6	-	-	-	5.0	-	-	-	-
$C_{15:1}$	-	0.1	-	-	-	-	-	-	-	-	-	-
$C_{16:1}$*	40.3	43.2	45.7	39.1	43.4	46.3	45.0	40.0	33.9	34.0	47.1	51.9
$C_{17:1}$	0.6	0.9	0.5	-	-	-	-	-	-	-	0.9	0.6
$C_{18:1}$†	25.1	24.9	29.6	26.5	27.8	15.4	22.5	3.2	21.2	18.1	27.3	19.4
$C_{20:1}$	-	0.1	0.2	-	-	-	-	-	-	-	-	-
Hydroxylated fatty acids												
$C_{3:0}$ 3-OH	-	-	-	-	-	-	-	-	-	-	-	0.1
$C_{8:0}$ 3-OH	-	-	-	-	-	-	-	-	-	3.3	-	-
$C_{10:0}$ 3-OH	2.8	1.9	0.6	0.4	1.7	2.5	5.0	0.7	-	8.4	2.0	11.4
$C_{11:0}$ 3-OH	0.1	-	-	-	-	-	-	-	-	-	-	-
$C_{12:0}$ 3-OH	1.2	-	0.1	-	-	-	-	-	-	-	-	-
$C_{12:1}$ 3-OH	0.1	-	-	-	-	-	-	-	-	-	0.3	-
$C_{13:1}$ 3-OH‡	0.2	-	-	-	-	-	-	-	-	-	-	-
$C_{14:1}$ 3-OH	-	-	-	-	-	-	-	-	-	-	-	-
$C_{14:1}$ 3-OH	-	-	-	1.6	2.1	-	-	2.2	-	-	-	-
Summed feature 2‖	1.3	0.5	0.1	-	-	-	-	-	-	-	0.2	0.2

*Includes summed feature 3 ($C_{16:1}$ ω6c and ω7c; *iso*-$C_{15:0}$ 2-OH)

†Includes summed feature 8 ($C_{18:1}$ ω6c and ω7c)

‡Includes summed feature 1 ($C_{13:0}$ 3-OH, *iso*-$C_{15:1}$ I/H)

⁋Includes $C_{14:0}$ 3-OH and *iso*-$C_{16:1}$

610 **Table 4.** 16S rRNA (*rrs*) gene identities (%) for HH1^T and HH3^T *versus* type strans of species of *Thiomicrorhabdus* spp.
611 and allied genera. Accession numbers in parentheses refer to the IMG/ER database locus tags with the exception of *Tmr.*
612 *frisia*, *Tmr. hydrogeniphila* and *Tmr. psychrophila*, for which they refer to the GenBank database.

	HH1^T (Ga0438909_048_380_1921)	**HH3^T** (Ga0438910_20_354_1897)
HH1^T (Ga0438909_048_380_1921)	100	95.25
Tmr. frisia JB-A2^T (AF013974)	93.49	94.33
Tmr. aquaedulcis HaS4^T (Ga0397736_1734)	92.73	95.56
Tmr. arctica DSM 13458^T (F612DRAFT_2093)	93.26	94.56
Tmr. chilensis DSM 12352^T (B076DRAFT_0255)	93.42	94.87
Tmr. hydrogeniphila MAS2^T (LC010781)	93.03	94.18
Tmr. indica 13-15A^T (Ga0398173_1980)	94.79	94.26
Tmr. psychrophila SVAL-D^T (AJ404732)	93.11	94.41
Tmr. sediminis G1^T (Ga0451571_01_1896662_1898205)	93.72	94.95
Tmr. xiamenensis G2^T (Ga0451572_01_332655_334198)	94.87	93.64
Galenea microaerophila P2D^T (NR_126238)	92.57	93.42
"Tss. sediminis" aks77^T (Ga0443151_01_2511058_2512601)	93.26	94.26
"Tsv. zosterae" AkT22^T (Ga0442965_01_724513_726058)	92.50	93.87
Hydrogenovibrio spp.	92.72 – 94.49	93.34 – 94.95
Thiomicrospira spp.	91.81 – 92.11	91.73 – 92.04

613

Table 5. Whole-genome comparison parameters, namey digital DNA-DNA hybridization (dDDH) percentages, average nucleotide identities (ANI), and alignment fractions (AF) for strains HH1^{T} and HH3^{T} compared to type strains of species of *Thiomicrorhabdus, Hydrogenovibrio,* and *Thiomicrospira.* The type species of each genus is emboldened.

Organism 1	Organism 2	dDDH	ANI1→2	ANI2→1	AF1→2	AF2→1
HH1^{T}	HH3T ^{T}	21.0	73.8	73.8	40.0	45.2
	Tmr. aquaedulcis HaS4^{T}	22.1	71.3	71.3	34.1	35.4
	Tmr. arctica SVAL-E^{T}	20.7	71.1	71.1	39.7	41.7
	Tmr. chilensis Ch-1^{T}	20.8	72.7	72.7	43.6	46.3
	***Tmr. frisia* Kp2***	20.5	71.6	71.6	43.3	41.8
	Tmr. indica 13-15A^{T}	23.2	72.2	72.2	39.7	36.9
	Tmr. sediminis G1^{T}	20.7	73.6	73.6	39.9	44.1
	Tmr. xiamenensis G2^{T}	21.9	75.3	75.2	48.4	49.1
	***"Tsv. zosterae"* AkT22^{T}**	20.0	70.4	70.4	35.3	33.9
	***"Tss. sediminis"* aks77^{T}**	20.6	72.7	72.7	41.4	40.2
HH1^{T}	*H. crunogenus* XCL-2†	21.5	70.6	70.7	33.6	39.3
	H. halophilus HL 5^{T}	22.2	70.5	70.5	28.6	31.2
	H. kuenenii JB-A1^{T}	21.3	70.7	70.7	36.2	37.9
	***H. marinus* MH-110^{T}**	23.2	71.6	71.7	37.5	37.0
HH1^{T}	*T. aerophila* AL 3^{T}	18.5	69.1	69.1	25.0	28.8
	T. cyclica ALM 1^{T}	20.2	69.4	69.4	22.5	28.8
	T. microaerophilia ASL8-2^{T}	18.9	69.4	69.3	26.4	22.1
	***T. pelophila* DSM 1534^{T}**	19.1	69.3	69.3	29.4	35.2
	T. thyasirae TG-2^{T}	18.4	69.3	69.3	29.8	33.5
HH3^{T}	*Tmr. aquaedulcis* HaS4^{T}	20.0	72.1	72.1	39.0	39.3
	Tmr. arctica SVAL-E^{T}	19.1	70.6	70.6	42.5	43.3
	Tmr. chilensis Ch-1^{T}	19.3	75.0	75.0	59.1	60.8
	***Tmr. frisia* Kp2***	19.8	70.7	70.7	46.5	43.5
	Tmr. indica 13-15A^{T}	20.2	70.8	70.8	35.9	32.4
	Tmr. sediminis G1^{T}	20.1	73.0	73.0	42.2	45.2
	Tmr. xiamenensis G2^{T}	20.7	73.7	73.7	42.2	41.6
	***"Tsv. zosterae"* AkT22^{T}**	19.9	70.6	70.6	37.8	35.2
	***"Tss. sediminis"* aks77^{T}**	20.6	71.9	71.9	39.5	39.5
HH3^{T}	*H. crunogenus* XCL-2†	19.8	70.3	70.3	39.0	40.3
	H. halophilus HL 5^{T}	18.2	71.9	71.9	34.0	35.9
	H. kuenenii JB-A1^{T}	20.1	70.2	70.2	36.1	36.6

	***H. marinus* MH-110^{T}**	20.8	71.0	71.0	36.5	34.8
HH3^{T}	*T. aerophila* AL 3^{T}	18.5	69.6	69.6	26.6	29.7
	T. cyclica ALM 1^{T}	18.6	69.7	69.7	25.2	31.3
	T. microaerophilia ASL8-2^{T}	17.4	69.9	69.9	30.7	24.9
	***T. pelophila* DSM 1534^{T}**	18.9	69.6	69.6	33.1	38.5
	T. thyasirae TG-2^{T}	18.3	69.9	69.9	32.8	32.8

[*]The genome of *Tmr. frisia* JB-A2^{T}, the type species for genus *Thiomicrorhabdus,* has not been sequenced. ANI and AF values were computed using the genome of *Tmr. frisia* Kp2, whose 16S sequence is 99.3% identical to *Tmr. frisia* JB-A2^{T}

[†]The genome of *H. crunogenus* TH-55^{T}, the type strain for this species, has not been sequenced. ANI and AF values were computed using the genome of *H. crunogenus* XCL-2, whose 16S sequence is 99.9% identical to TH-55^{T}

Figure legends

Fig. 1. Growth habit on solid media and ultrastructure of strains HH1^T and HH3^T. Colonies of strain HH1^T (A) and HH3^T (B) on solid ½ TASW supplemented with phenol red (0.0005% w/v). Transmission electron micrographs (14,000× magnification, bars indicate 2 µm) of strain HH1^T (C) and HH3^T (D) when cultivated in chemostats under optimal [NaCl] and pH, under dissolved inorganic carbon limitation (dilution rate = 0.05 h^{-1}) at 20˚C. Growth of strains HH1^T (E) and HH3^T (F) when stabbed into ½ TASW slush agar deeps supplemented with phenol red (0.0005% w/v).

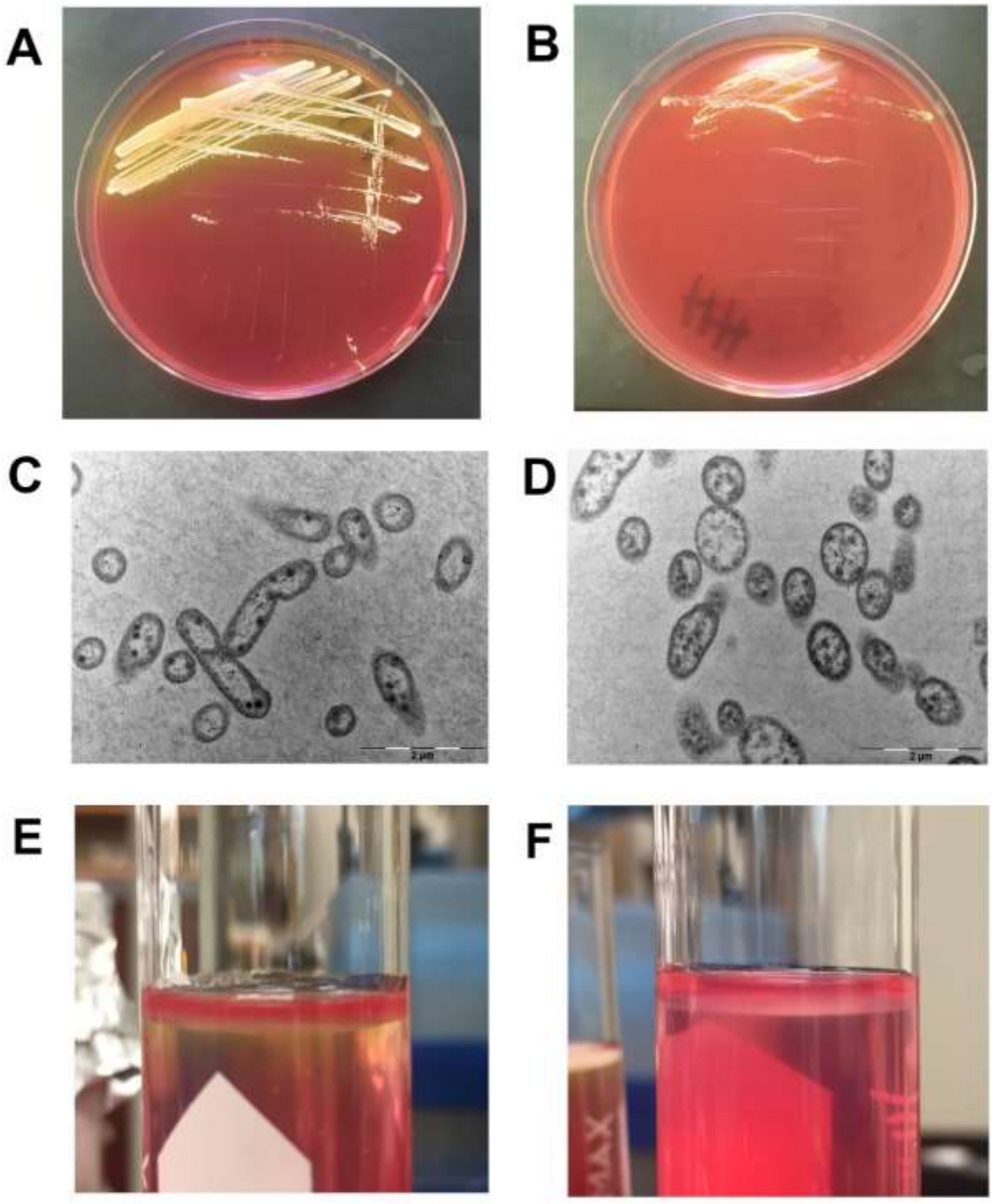
Fig. 2. Determination of optimal growth conditions for strains HH1^T (solid squares; A, C, D, E) and HH3^T (open circles; B, C, D, E). Curves in graphs depicting growth response to temperature, pH, and NaCl concentration have been fitted to the data with 3rd-order polynomial equations to determine optima. For A, B, and D, CO_2 fixed was measured 24 hours after inoculation, after the cultures had reached stationary phase. For C and E, apparent proton production rates were calculated from the time necessary to lower pH from 8 to 6.8. For both strains, no pH drop was observed after 40 hours of incubation at 40˚C. Error bars, which in some cases are obscured by the symbols used to plot the data, indicate standard deviations.

Fig. 3. Maximum-likelihood tree showing the position of HH1^T and HH3^T relative to *Thiomicrorhabdus*, *Galenea*, "*Thiosulfatimonas*", "*Thiosulfativibrio*", *Thiomicrospira* and *Hydrogenovibrio* isolates, on the basis of the 16S rRNA (*rrs*) gene. Compressed taxa *Hydrogenovibrio* and *Thiomicrospira* use the sequences given in [1]. Sequences were curated from the GenBank and IMG/ER databases favoring the complete gene over PCR amplicons and aligned using the MUSCLE algorithm [56] in MEGA X [57] per [1]. The aligned data were model-tested in MEGA X on the basis of the lowest corrected Akaike information criterion (AIC_C, [58]; [59], per [60]). The outgroup is the same gene from *Thiothrix nivea* JP2^T. Type species of each genus are emboldened. Numbers in parentheses refer to genome accession numbers in the GenBank (short) and IMG/ER (long/containing underscore characters). The tree was constructed in MEGA X with partial deletion of gaps (95 % cut-off) and the final analysis used 1,384 nt. The model of Kimura (1980) was used with a discrete gamma distribution (5 categories, gamma parameter = 0.2206) with 37.21 % of sites evolutionarily invariant. Tree shown had the highest log-likelihood (-7,494.87). Branch lengths are proportional to the number of substitutions, the bar representing 0.10 substitutions per site. Bootstrap values at nodes are on the basis of 5,000 replications (values < 70 % are omitted for clarity).

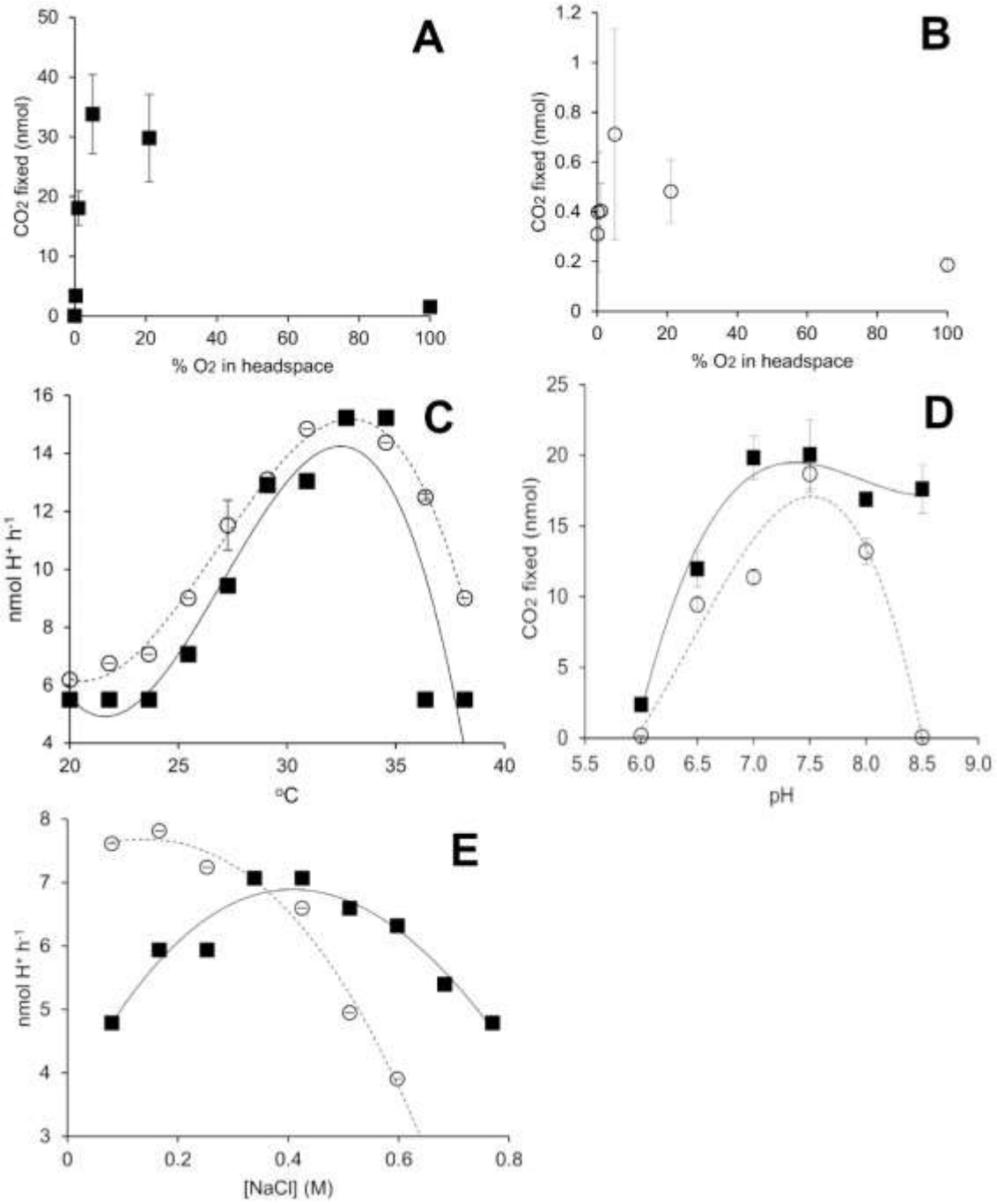
Fig. 4. Maximum-likelihood tree of *Thiomicrorhabdus*, *Thiomicrospira*, "*Thiosulfatimonas*", "*Thiosulfativibrio*" and *Hydrogenovibrio* isolates for which genome

sequences are available, on the basis of the 53 concatenated ribosomal protein gene sequences translated *in silico* into amino acyl sequences, pertaining to *rpsA*-*rpsU*, *rplA*-*rplF*, *rplL*-*rplX*, and *rpmA*-*rpmJ*. Omissions of sequences with detected problems (internal stop codons, partial sequences *etc*) were made, *viz*. *Tms. pelophila* DSM 1534^{T} (*rpmF*), *Tms. thyasirae* DSM 5322^{T} (*rpsA*), *Tmr. aquaedulcis* HaS4^{T} (*rpsR*, *rplD*, *rplE*, *rplO*, *rplR*) and strain HH3 (*rpmE*). Gene concatamer sequences were downloaded *en bloc* from the ribosomal multilocus sequence typing (rMLST) database (http://pubmlst.org/rmlst) and were translated *in silico* before aligning using the MUSCLE algorithm [56] in MEGA X [57] per [1]. The aligned data were model-tested in MEGA X on the basis of the lowest corrected Akaike information criterion (AIC_C, [58, 59] per [60]). The outgroup is the equivalent concatamer from *Thiothrix nivea* DSM 5205^{T}. Type species of each genus are emboldened. *Thiomicrorhabdus frisia* Kp2 is used *in lieu* of the type strain of the type species of *Thiomicrorhabdus* (*Tmr. frisia* JB-A2^{T}), for which the genome has not been sequenced. Numbers in parentheses refer to genome accession numbers in the rMLST database. The tree was constructed in MEGA X with partial deletion of gaps (95 % cut-off) and the final analysis used 6,751 aa. The model of Le and Gascuel [61] was used with a discrete gamma distribution (5 categories, gamma parameter = 0.5695) with 22.52 % of sites evolutionarily invariant. Tree shown had the highest log-likelihood (-82,736.29). Branch lengths are proportional to the number of substitutions, the bar representing 0.10 substitutions per site. Bootstrap values at nodes are on the basis of 5,000 replications (values < 70 % are omitted for clarity).

Fig. 5. Pairwise comparisons of genome-derived parameters from type strain members of family *Piscirickettsiaceae* to A) *Thiomicrorhabdus frisia* Kp2*,* B) *Hydrogenovibrio marinus* DSM 11271^{T}*,* and C) *Thiomicrospira pelophila* DSM 1534^{T}*,* which are type strains of the type species of their respective genera, excepting *Tmr. frisia* Kp2 (see Fig. 4 legend). Symbols on the plots indicate the averages of the values from comparing the genomes (average of genome 1 vs. genome 2, and genome 2 vs. genome 1), and error bars indicate the individual values (genome 1 vs. genome 2, and genome 2 vs. genome 1). Boundary values for alignment fractions (AF) and average nucleotide identities (ANI) suggested for genera *Thiomicrorhabdus, Hydrogenovibrio,* and *Thiomicrospira* [50] are demarcated with dotted lines.



710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B
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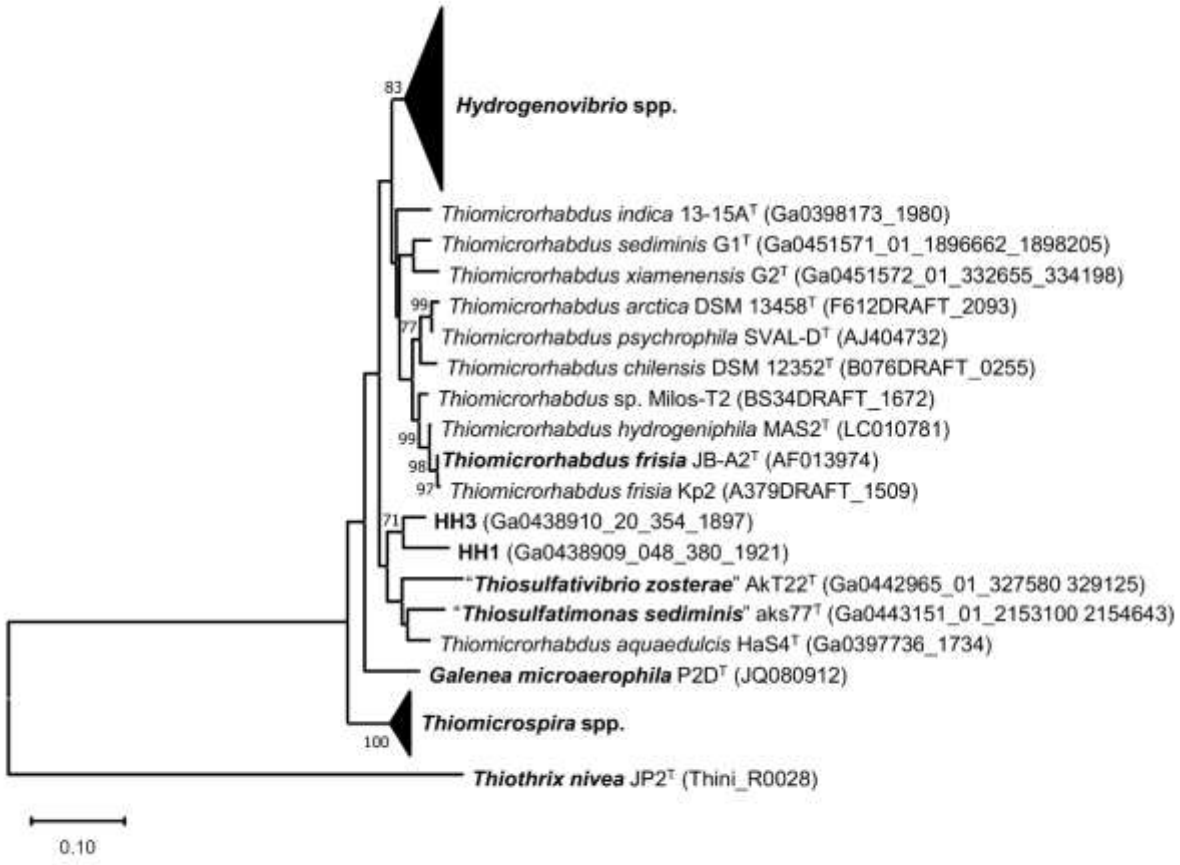
% O2 in headspace
C
nmol H+ h-1
°C
D
CO2 fixed (nmol)
pH
E
nmol H+ h-1
[NaCl] (M)

711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Hydrogenovibrio spp.
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Thiomicrorhabdus sediminis G1^T (Ga0451571_01_1896662_1898205)
Thiomicrorhabdus xiamenensis G2^T (Ga0451572_01_332655_334198)
99
Thiomicrorhabdus arctica DSM 13458^T (F612DRAFT_2093)
77
Thiomicrorhabdus psychrophila SVAL-D^T (AJ404732)
Thiomicrorhabdus chilensis DSM 12352^T (B076DRAFT_0255)
Thiomicrorhabdus sp. Milos-T2 (BS34DRAFT_1672)
99
Thiomicrorhabdus hydrogeniphila MAS2^T (LC010781)
98
Thiomicrorhabdus frisia JB-A2^T (AF013974)
97
Thiomicrorhabdus frisia Kp2 (A379DRAFT_1509)
71
HH3 (Ga0438910_20_354_1897)
HH1 (Ga0438909_048_380_1921)
"Thiosulfativibrio zosterae" AkT22^T (Ga0442965_01_327580 329125)
"Thiosulfatimonas sediminis" aks77^T (Ga0443151_01_2153100 2154643)
Thiomicrorhabdus aquaedulcis HaS4^T (Ga0397736_1734)
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Thiomicrospira spp.
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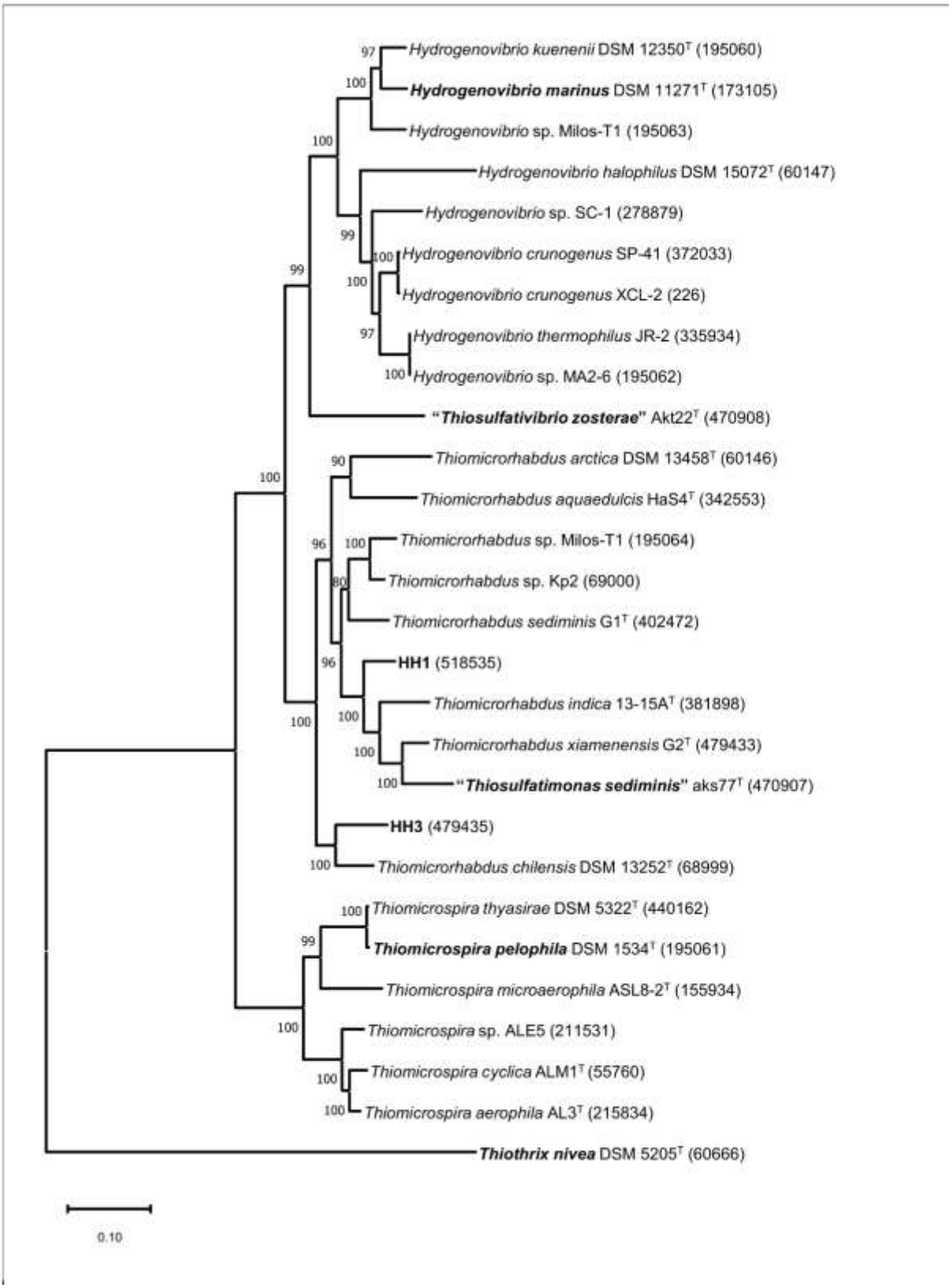
Thiothrix nivea JP2^T (Thini_R0028)
0.10

712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Hydrogenovibrio sp. SC-1 (278879)
Hydrogenovibrio crunogenus SP-41 (372033)
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Hydrogenovibrio thermophilus JR-2 (335934)
Hydrogenovibrio sp. MA2-6 (195062)
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Thiomicrorhabdus aquaedulcis HaS4T (342553)
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Thiomicrorhabdus sp. Kp2 (69000)
Thiomicrorhabdus sediminis G1T (402472)
HH1 (518535)
Thiomicrorhabdus indica 13-15AT (381898)
Thiomicrorhabdus xiamenensis G2T (479433)
"Thiosulfatimonas sediminis" aks77T (470907)
HH3 (479435)
Thiomicrorhabdus chilensis DSM 13252T (68999)
Thiomicrospira thyasirae DSM 5322T (440162)
Thiomicrospira pelophila DSM 1534T (195061)
Thiomicrospira microaerophila ASL8-2T (155934)
Thiomicrospira sp. ALE5 (211531)
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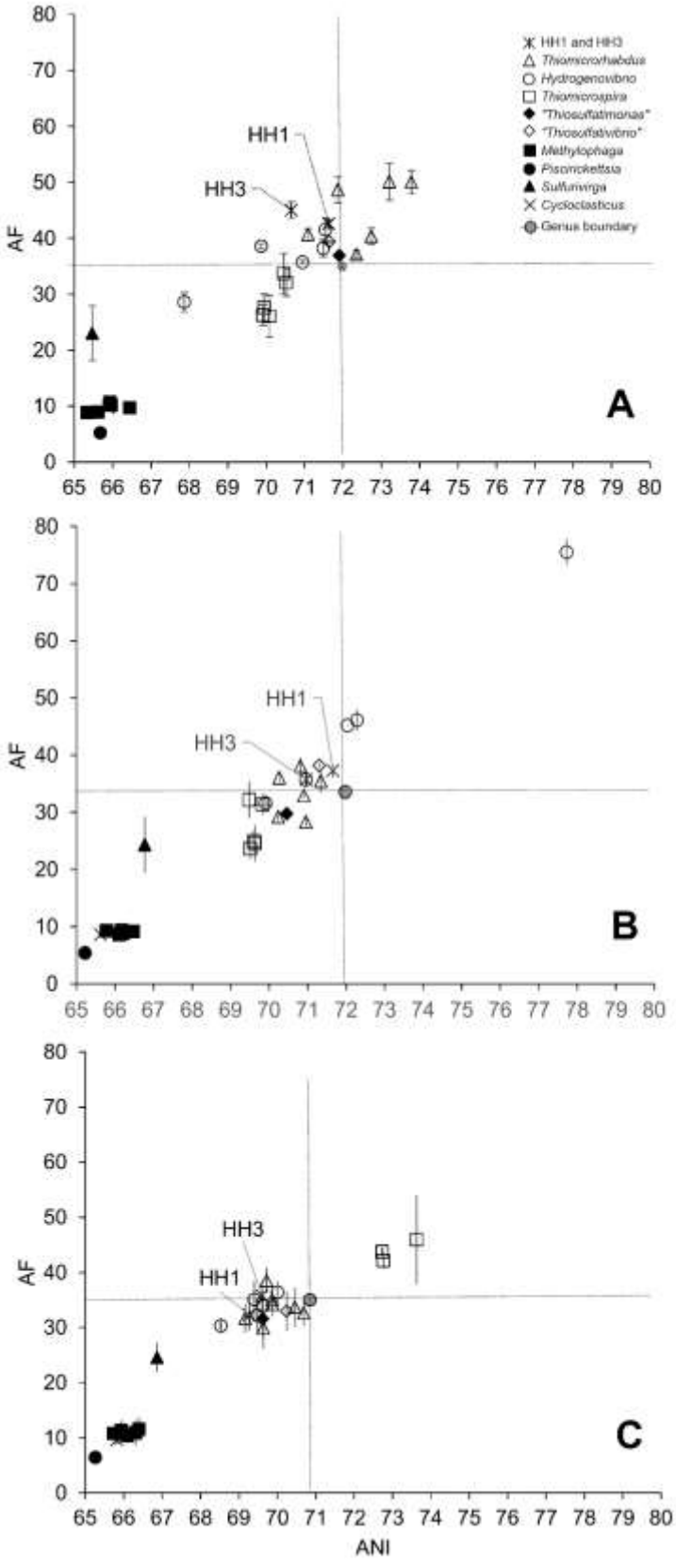
HH1 and HH3
Thiomicrorhabdus
Hydrogenovibrio
Thiomicrospira
"Thiosulfatimonas"
"Thiosulfativibrio"
Methylophaga
Piscirickettsia
Sulfurivirga
Cycloclasticus
Genus boundary
HH1
HH3
AF
ANI
A
B
C

714