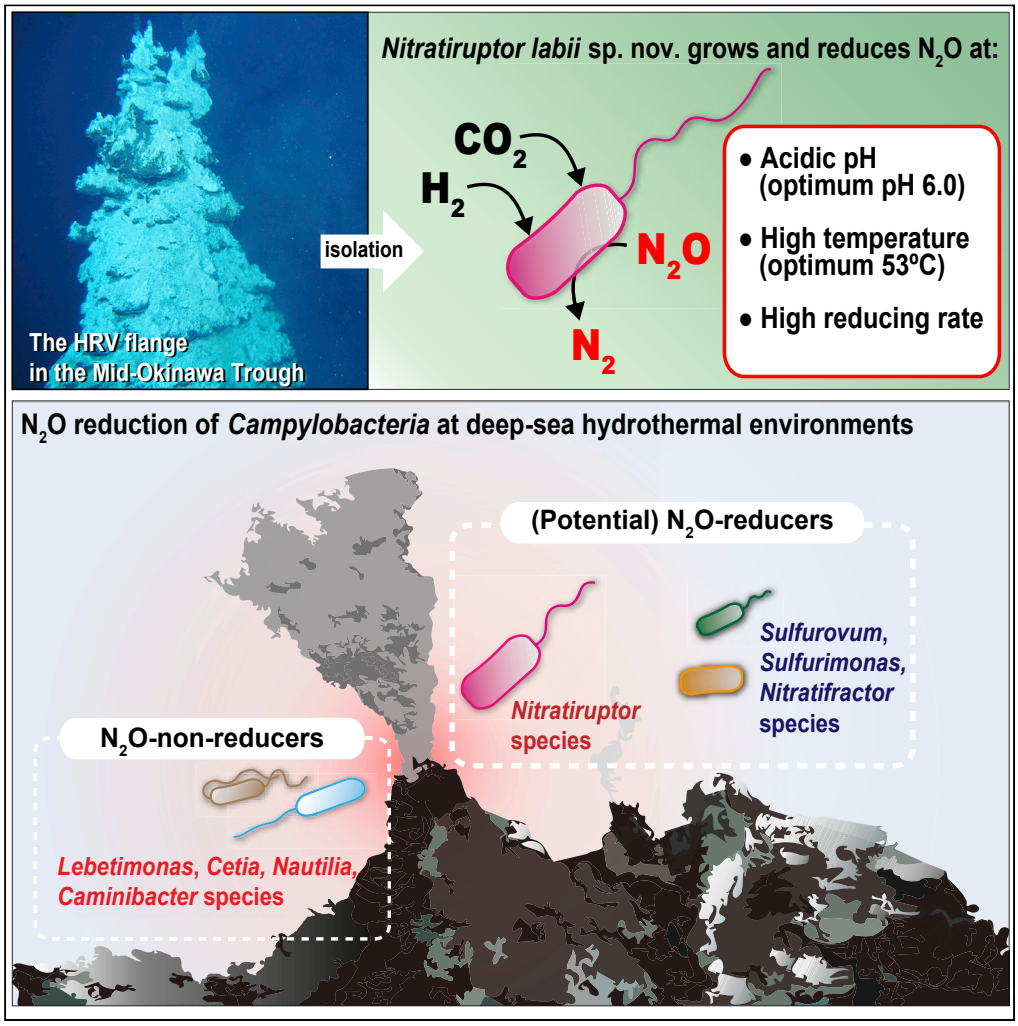
iScience

CellPress
OPEN ACCESS

Article

Biogeochemical Implications of N_2O-Reducing Thermophilic *Campylobacteria* in Deep-Sea Vent Fields, and the Description of *Nitratiruptor labii* sp. nov.



Muneyuki Fukushi, Sayaka Mino, Hirohisa Tanaka, Satoshi Nakagawa, Ken Takai, Tomoo Sawabe

sayaka.mino@fish.hokudai.ac.jp

HIGHLIGHTS

A N_2O-reducing thermophile strain HRV44 was isolated from the deep-sea hydrothermal vent

Strain HRV44^T shows strong exogenous N_2O reduction at pH 6.0 and 53°C

We propose the name *Nitratiruptor labii* sp. nov. for strain HRV44^T

Members of the genus *Nitratiruptor* possibly contribute to the capacity of N_2O mitigation

Fukushi et al., iScience 23, 101462
September 25, 2020
https://doi.org/10.1016/j.isci.2020.101462

iScience

CellPress
OPEN ACCESS

Article

Biogeochemical Implications of N_2O-Reducing Thermophilic *Campylobacteria* in Deep-Sea Vent Fields, and the Description of *Nitratiruptor labii* sp. nov.

Muneyuki Fukushi,[1] Sayaka Mino,[1,4,*] Hirohisa Tanaka,[1] Satoshi Nakagawa,[2,3] Ken Takai,[3] and Tomoo Sawabe[1]

SUMMARY

Nitrous oxide (N_2O) is a potent greenhouse gas and has significantly increased in the atmosphere. Deep-sea hydrothermal fields are representative environments dominated by mesophilic to thermophilic members of the class *Campylobacteria* that possess clade II *nosZ* encoding nitrous oxide reductase. Here, we report a strain HRV44^T representing the first thermophilic campylobacterium capable of growth by H_2 oxidation coupled to N_2O reduction. On the basis of physiological and genomic properties, it is proposed that strain HRV44^T (=JCM 34002 = DSM 111345) represents a novel species of the genus *Nitratiruptor*, *Nitratiruptor labii* sp. nov. The comparison of the N_2O consumption ability of strain HRV44^T with those of additional *Nitratiruptor* and other campylobacterial strains revealed the highest level in strain HRV44^T and suggests the N_2O-respiring metabolism might be the common physiological trait for the genus *Nitratiruptor*. Our findings provide insights into contributions of thermophilic *Campylobacteria* to the N_2O sink in deep-sea hydrothermal environments.

INTRODUCTION

Nitrous oxide (N_2O) is the one of the gaseous nitrogen compounds that is the third most important long-lived greenhouse gas and a major ozone-depleting substance (Crutzen, 1970; Ravishankara et al., 2009; Ciais et al., 2013). Atmospheric N_2O level has increased and its concentration reached 330 ppb in 2018, which is an increase of 23% in comparison with the pre-industrial era (Machida et al., 1995). Construction of a scheme for N_2O mitigation and control of N_2O emissions is therefore an essential and urgent global goal. N_2O is emitted from natural sources (e.g., oceans, forests, and savannas) and anthropogenic sources (e.g., agriculture, biomass burning, power plants, and wastewater treatment plants). The ocean is considered to be the third largest source of N_2O that accounts for average 21% of combined natural and anthropogenic N_2O sources (Ciais et al., 2013). Ongoing environmental changes (e.g., ocean warming, acidification, and eutrophication) may significantly alter the global oceanic N_2O emissions; therefore, a global survey of oceanic N_2O flux must be accurately assessed (Bange et al., 2019). Most of the atmospheric N_2O is produced through the microbial processes of ammonia oxidation coupled with NO_2^- reduction and denitrification, driven by metabolically versatile microorganisms (Bange et al., 2010; Thomson et al., 2012; Hu et al., 2015). Furthermore, N_2O production by dissimilatory nitrate reduction to ammonium (DNRA), nitrite oxidation, anaerobic methane oxidation pathways have also been reported, although their magnitudes for global N_2O budget are unclear (Hallin et al., 2018).

In strong contrast to the manifold N_2O-producing processes, there is only one known N_2O sink in the biosphere: microbial N_2O reduction to harmless N_2 gas. This pathway is catalyzed by N_2O reductase (NosZ) implementing two copper centers (CuA and CuZ), encoded by *nosZ* gene (Zumft and Kroneck, 2007; Pauleta et al., 2013). NosZ encoded by a typical (clade I) *nosZ* gene had been accepted as the only enzyme catalyzing N_2O reduction for a long time (Zumft and Kroneck, 2007). However, an unprecedented *nos* gene cluster with a novel *nosZ* containing an additional c-type heme domain at the C terminus was discovered in *Wolinella succinogenes* (Simon et al., 2004). The novel *nosZ* is currently termed atypical (clade II) *nosZ*, and clade II *nosZ* has been identified in a broad range of microbial taxa extending beyond bacteria to even archaea (Sanford et al., 2012; Jones et al., 2013). The sequences of clade II *nosZ* have been detected in similar abundance to those of clade I *nosZ* in various environments (Sanford et al., 2012; Jones et al., 2013). Recent biochemical approaches for two types of NosZ revealed relatively lower whole-cell half-

[1]Laboratory of Microbiology, Faculty of Fisheries Sciences, Hokkaido University, 3-1-1, Minato-cho, Hakodate 041-8611, Japan

[2]Laboratory of Marine Environmental Microbiology, Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kyoto, Japan

[3]Super-cutting-edge Grand and Advanced Research (SUGAR) Program, Japan Agency for Marine-Earth Science & Technology (JAMSTEC), Yokosuka, Japan

[4]Lead Contact

*Correspondence: sayaka.mino@fish.hokudai.ac.jp

https://doi.org/10.1016/j.isci.2020.101462

saturation constants for N_2O with clade II bacteria compared with those with clade I bacteria (Yoon et al., 2016; Suenaga et al., 2019), suggesting a high affinity for N_2O in clade II *nosZ* organisms. In addition, ecological niche partitioning between two *nosZ* clade microorganisms has been demonstrated by culture-dependent and -independent analyses (Dini-Andreote et al., 2016; Graf et al., 2016; Wittorf et al., 2016; Juhanson et al., 2017). Although these previous studies have emphasized the large contributions of clade II *nosZ* microorganisms to the global N_2O sink, understanding of the genomics, physiology, and ecology of microorganisms with clade II *nosZ* gene is still limited to several model microorganisms.

Members of the class *Campylobacteria* (former class *Epsilonproteobacteria*) are considered as the predominant bacterial group and play a significant role as primary producers in global deep-sea hydrothermal environments (Nakagawa et al., 2005a; Huber et al., 2007; Akerman et al., 2013; Muto et al., 2017). Nitrate respiration is a widespread energy metabolism in chemosynthetic *Campylobacteria*; more than 70% of campylobacterial species from deep-sea hydrothermal environments possess the capability to utilize nitrate as a sole electron acceptor (Sievert and Vetriani, 2012; Vetriani et al., 2014). Culture-dependent studies using several mesophilic and thermophilic isolates have demonstrated their ability to mediate complete nitrate reduction to dinitrogen, suggesting the capability of N_2O as an electron acceptor. In the genomes of campylobacterial isolates from deep-sea hydrothermal environments, clade II *nosZ* genes have been identified (Inagaki et al., 2003, 2004; Nakagawa et al., 2005b; Giovannelli et al., 2016). Recently, the capability of exogeneous N_2O as a sole electron acceptor has been characterized for the first time using an enrichment culture dominated by a novel mesophilic campylobacterial species, which possesses the clade II *nosZ* gene on its genome (Mino et al., 2018). Although diverse *Campylobacteria* with clade II *nosZ* possibly contribute to the nitrogen cycle as N_2O reducers in natural environments (Fortunato and Huber, 2016; Mino et al., 2018), their ability to utilize N_2O has yet to be fully elucidated. In particular, little is known about N_2O-reducing thermophilic *Campylobacteria*.

Here, in this study, a novel thermophilic campylobacterium strain HRV44^T is described that was isolated from a deep-sea hydrothermal vent in the Mid-Okinawa Trough. The genomic and physiological characteristics of strain HRV44^T are further determined, and *Nitratiruptor labii* sp. nov. is proposed as type strain HRV44^T. In addition, we compare the *nosZ* gene cluster and the capability to reduce exogenous N_2O among campylobacterial strains. Our results provide insight into the contribution of thermophiles as N_2O reducers to nitrogen cycles in deep-sea hydrothermal environments.

RESULTS

Isolation and Phylogenetic Analysis Based on 16S rRNA Gene Sequences of N_2O-Reducing Microorganisms

Six isolates (i.e., strains No.9, No.33, No.34, No.37, No.41, and HRV44^T) were obtained from deep-sea hydrothermal samples using HNN medium containing H_2 and N_2O as a sole electron donor and acceptor, respectively, at 55°C. Since the unusual insertion sequence (IS) was found within the 16S rRNA gene sequence of strain HRV44^T, the sequence similarities were calculated after the removal of IS. 16S rRNA gene sequences of strain HRV44^T and other five strains were most closely related to *Nitratiruptor* sp. EPR55-1 (95.2%), which was isolated from a deep-sea hydrothermal vent in the East Pacific Rise (unpublished data), and *Nitratiruptor* sp. SB155-2 (99.5–99.9%) (Nakagawa et al., 2007), respectively. The sequence similarity between HRV44^T and the only formally described *Nitratiruptor* species, *Nitratiruptor tergarcus*, was lower than the threshold for distinct species (98.7%–99.0%) (Stackebrandt and Ebers, 2006). Phylogenetic analysis based on 16S rRNA gene sequences also indicated that all of the six isolates belonged to the genus *Nitratiruptor* (Figure 1).

N_2O Consumption of the Isolates

Comparison of headspace N_2O consumption and cell growth during 72-h cultivation revealed that N_2O consumption rates and growth rates varied markedly among the six isolates. Strain HRV44^T consumed approximately 97% of initial headspace N_2O with a 10-fold increase in the total cell counts after 24 h (Figure 2). Maximum N_2O consumption rate of strain HRV44^T was about 9.9 $\pm$ 1.4 μmol h^{-1} per mL of culture, which was the highest among the six isolates (Figure 2). Higher N_2O consumption and cell growth were observed in HRV44^T than in the other strains (Figure S1). Production of N_2 during the cultivation was also confirmed in all of the strains (Figure S2).

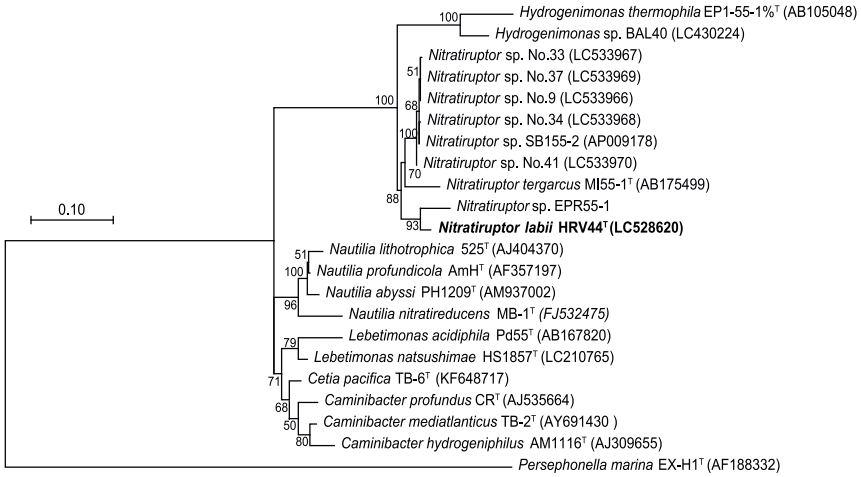


Figure 1. Phylogenetic Relationship of the Isolates Obtained in This Study and Related *Campylobacteria*

The phylogenetic tree was constructed using the maximum-likelihood method based on 1,048 nucleotide positions of 16S rRNA gene sequences. Numbers at nodes represent bootstrap values (%) (1,000 replicates).

Characteristics of Strain HRV44^T

Strain HRV44^T was rod shaped and motile with a polar flagellum (Figure S3). Cells of the strain often formed aggregates. Strain HRV44^T grew at 45°C–60°C, showing optimum growth at 53°C. The strain grew at pH 5.4–6.4, showing optimum growth at pH 6.0. Optimum NaCl concentration for growth of strain HRV44^T was 2.5% (w/v), and growth range was 2.0%–4.0% (w/v) (Figure S4). The doubling time under the optimum condition was 1.17 h. Strain HRV44^T utilized H_2 as a sole electron donor and nitrate, N_2O, elemental sulfur, and oxygen (up to 1.0% (v/v) in the headspace gas) as a sole electron acceptor. The maximum cell yield of strain HRV44^T was approximately 3.6×10^8 cells mL^{-1} in HNN medium. Strain HRV44^T was able to use none of the tested carbon sources other than carbon dioxide. The strain was able to utilize nitrate and ammonium as sole nitrogen sources. Sulfate and elemental sulfur were utilized as sole sulfur sources of the strain (Table 1). When comparing the growth ranges of pH and NaCl among related *Campylobacteria*, those of strain HRV44^T were relatively narrower than those of mesophiles and other thermophiles as well as other *Nitratiruptor* species (Figure S5).

Whole-Genome Sequencing of Strain HRV44^T

We obtained high-quality sequencing reads using PacBio and Illumina sequencing. The *de novo* assembly using both PacBio and Illumina reads resulted in the acquisition of the complete genome of strain HRV44^T, composed of a chromosome and a plasmid. The sizes of chromosome and plasmid are 1,990,315 bp with G + C content of 33.46% and 102,672 bp with G + C content of 33.01%, respectively. The numbers of CDS of chromosome and plasmid were 2,050 and 128, respectively, of which 34.2% and 78.1% were annotated as hypothetical genes, respectively. Three rRNA operons organized by 5S, 16S, and 23S rRNA gene and 41 tRNA genes were identified on the chromosome (Table S1). The three copies of 16S rRNA gene with IS were all completely identical. Several CDSs of plasmid were annotated as genes involved in plasmid replication, DNA replication, and plasmid conjugation. The 41 plasmid CDSs of strain HRV44^T shared 37%–86% identities with plasmid CDSs of *Campylobacter iguaniorum*, isolated from reptiles (Gilbert et al., 2015) (Table S2). No known antibiotic resistance genes were found on the plasmid. A combination of the Oxford Nanopore Technologies (ONT) and Illumina data also yielded two circular units, a large circular chromosome of 2,021,942 bp, and a plasmid of 102,626 bp.

Description of IS and Prediction of the 16S rRNA Secondary Structure of Strain HRV44^T

Insertion sequence located within the V2 region of the 16S rRNA gene sequence of strain HRV44^T was detected, and its size was determined to be 268 bp. There was no ORF in IS. The secondary structure prediction of the 16S rRNA showed IS constructed bifurcated structure at the tip of the stem, and two branched stems had internal and hairpin loops (Figure S6). 16S rRNA secondary structure with IS did not greatly differ from that lacking IS. Identical IS was retrieved from all three copies of 16S rRNA genes.

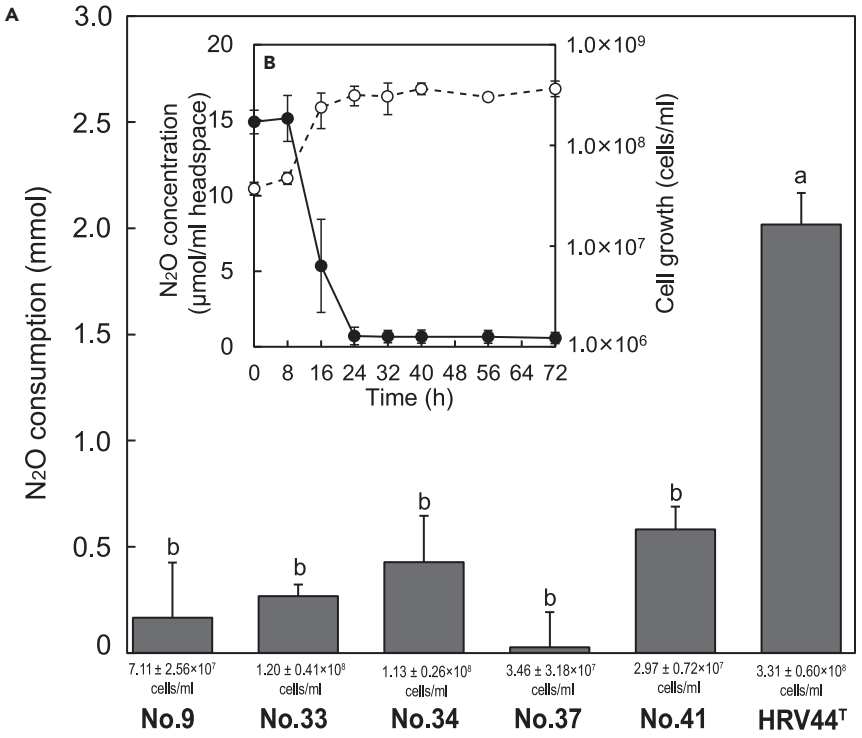


Figure 2. N_2O Consumption of Six Isolates

(A) N_2O consumption for 72-h cultivation of six isolates obtained in this study are shown. Error bars represent standard errors (n = 3). Different small letters above the bars indicate significant differences assessed by Tukey's HSD test ($p < 0.05$). The cell yields of strains are shown below the bars.

(B) Time course changes in headspace N_2O concentration and the growth of strain HRV44^T are shown in filled circles with solid line and open circles with dotted line, respectively.

Calculation of Genome Sequence Similarities and Phylogenomic Analysis

On the basis of the complete genome of strain HRV44^T, *in silico* DNA-DNA hybridization (*in silico* DDH), average nucleotide identity (ANI), and average amino acid identity (AAI) values were calculated against genomes of related species. *In silico* DDH values (Formula 2, recommended) of strain HRV44^T against *Nitratiruptor tergarcus* MI55-1^T, *Nitratiruptor* sp. SB155-2, *Nitratiruptor* sp. EPR55-1, and *Hydrogenimonas thermophila* EP1-55-1%^{T} were 20.2%, 20.4%, 18.7%, and 17.1%, respectively, further distinguishing strain HRV44^T from those species (Wayne et al., 1987). ANI values of the novel strain against *Nitratiruptor tergarcus* MI55-1^T, *Nitratiruptor* sp. SB155-2, *Nitratiruptor* sp. EPR55-1, and *Hydrogenimonas thermophila* EP1-55-1%^{T} were 77.1%, 76.4%, 77.5%, and 72.7%, respectively, well below the threshold for the species circumscription (95.0%–96.0%) (Goris et al., 2007; Richter and Rosselló-Móra, 2009). AAI values of HRV44^T against *Nitratiruptor tergarcus* MI55-1^T, *Nitratiruptor* sp. SB155-2, *Nitratiruptor* sp. EPR55-1, and *Hydrogenimonas thermophila* EP1-55-1%^{T} were 66.3%, 66.2%, 69.9%, and 58.1%, respectively, which fall within the circumscription for genus-level differentiation (Rodriguez-R and Konstantinidis, 2014).

Phylogenomic analysis of HRV44^T and representative *Campylobacteria* strains was performed based on 127 conserved single core protein sequences among the genomes (Figure S7). Strain HRV44^T clustered with the other strains belonging to the genus *Nitratiruptor*. The topology of the phylogenomic tree differed from the 16S rRNA-based tree.

Comparison of *nos* Gene Cluster, Putative Transcription Regulators, and N_2O-Reducing Ability among Related *Campylobacteria* Isolated from Deep-Sea Hydrothermal Fields

We reconstructed *nos* gene clusters from genomes of the clade II *nosZ*-possessing *Campylobacteria* isolated from deep-sea hydrothermal environments. The composition of *nos* gene cluster organized by 11 genes (*nosZ*, *-B*, *-D*, *-G*, *-C1*, *-C2*, *-H*, *-F*, *-Y*, *-L*, and a hypothetical gene) was well conserved in the related

Characteristics	*Nitratiruptor labii* HRV44^T (This Study)	*Nitratiruptor* sp. EPR55-1 (Unpublished Data)	*Nitratiruptor tergarcus* MI55-1^T (Nakagawa et al., 2005b)	*Nitratiruptor* sp. SB155-2 (Nakagawa et al., 2007)
Origin	MOT	EPR	MOT	MOT
Temperature range (℃)	45–60	50–60	40–55	37–65
Optimum temperature (℃)	53	60	55	55
pH range	5.4–6.4	5.4–8.6	5.4–6.9	ND
Optimum pH	6.0	6.6	6.4	ND
NaCl range (% w/v)	2.0–4.0	2.4–3.2	1.5–4.0	ND
Optimum NaCl (% w/v)	2.5	2.4	2.5	ND
Electron donors	H_2	H_2	H_2	H_2, S^{2-}, S^0, $S_2O_3^{2-}$
Electron accepters	NO_3^-, N_2O, S^0, O_2 (up to 1.0%, v/v)	NO_3^-, $S_2O_3^{2-}$, S^0, O_2	NO_3^-, S^0[a], O_2 (up to 0.7%, v/v)	NO_3^-, O_2
Nitrogen sources	NO_3^-, NH_4^+	NH_4^+	NO_3^-, NH_4^+	ND
Carbon sources	CO_2	CO_2	CO_2	ND
DNA G + C content (%)	33.4	37.7	29.6[b]	39.7

Table 1. Comparison of the Major Physiological Characteristics of Strain HRV44^T with Those of Members of the Genus *Nitratiruptor* Isolated from Deep-Sea Hydrothermal Fields

ND, not determined. MOT, Mid-Okinawa Trough; EPR, East Pacific Rise.

[a]S^0 could not serve as a sole electron acceptor to support growth.

[b]The G + C content in mol% of DNA.

campylobacterial members, with an exception of *Sulfurimonas autotrophica*, which lacked one hypothetical gene (Figure 3). Using *Campylobacter jejuni* NssR (Nitrosative stress sensing Regulator; Cj0466) as database search template, two putative transcription regulators of the Crp-Fnr superfamily were found to be encoded in strain HRV44^T (Figure S8) (Kern et al., 2011). Notably, *Nitratiruptor* species encoded a Nss-type regulator upstream of the *nor* gene cluster in reverse orientation altogether, whereas in other mesophilic species the regulators were located upstream the *nap* gene cluster. The three putative Nss-binding sites were found upstream of *nosZ* of strain HRV44^T (Figure S8). In addition, genes necessary for denitrification are clustered together in *Nitratiruptor* species, although mesophiles appeared to form three separate loci, the *nap*, *nir-nor*, and *nos* gene clusters.

Growth under N_2O-respiring condition was observed in all strains belonging to the thermophilic genus *Nitratiruptor*. However, the other mesophilic strains (i.e., *Sulfurimonas autotrophica, Sulfurovum lithotrophicum, Sulfurovum riftiae, Sulfurovum* sp. NBC37-1, *Nitratifractor salsuginis*) showed no growth under the same condition, even though they had the *nosZ* gene on their genomes. Further evaluation of N_2O consumption abilities of the N_2O-respiring strains (i.e., *Nitratiruptor tergarcus*, *Nitratiruptor* sp. SB155-2, *Nitratiruptor* sp. EPR55-1) revealed that 4.3%–80% of initial headspace N_2O was consumed along with an increase in cell numbers (Figure S1).

Multiple Alignment of NosZ Primary Structure and Phylogenetic Analysis Based on Genes within the *nos* Gene Cluster

Multiple alignment of NosZ primary structures that contained strain HRV44^T and other clade I and clade II sequences showed that *nosZ* amino acid sequences of all campylobacterial strains conserved important structural motifs such as seven histidine ligands of CuZ center, two cysteine and three other ligands of CuA center, and the heme c-binding motif (Cys-Xaa-Xaa-Cys-His) at the C terminus (Figure S9). In addition, the five calcium-binding sites were conserved among most campylobacterial strains except for *Sulfurimonas autotrophica*, *Nitratifractor salsuginis*, and *Nitratiruptor* sp. SB155-2.

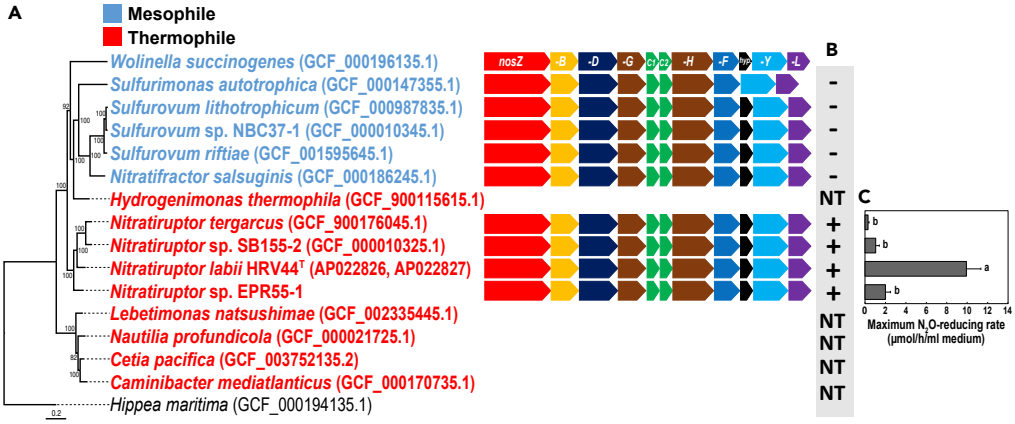


Figure 3. Comparison of *nos* Gene Cluster and of N_2O-Reducing Ability between *Campylobacteria* Isolated from Deep-Sea Hydrothermal Environments

(A) ML tree based on 127 conserved protein sequences with schematic of *nos* gene cluster. Numbers at nodes represent bootstrap values (%) (1,000 replicates). *nosZ* and its accessary genes are colored according to homology across the different species.

(B) Results of N_2O-respiring cultivation test. +; positive, -; negative, NT; not tested.

(C) Maximum N_2O-reducing rate of N_2O-respiring-positive strains. Error bars represent standard errors (n = 3). Different small letters indicate significant differences assessed by Tukey's HSD test ($p < 0.001$).

In order to understand the evolutionary relationship of NosZ and its accessary proteins, phylogenetic analyses were performed based on each gene within *nos* gene cluster. The strains belonging to the genera *Nitratiruptor* and *Sulfurovum* were respectively clustered according to their genus (Figure S10). Mesophilic *Sulfurimonas autotrophica* was often placed in the outermost *Campylobacteria* from deep-sea hydrothermal environments, which is contrary to its phylogenetic position inferred by genome sequences (Figure S7). The tree topologies were relatively conserved among all genes with the exception of *nosC1*, whose topology was consistent with both genome- and 16S rRNA gene-based phylogeny.

DISCUSSION

Physiological and Genomic Characteristics of Strain HRV44^T

The physiological characteristics of strain HRV44^T indicated that the strain is a facultatively anaerobic chemolithoautotroph. 16S rRNA gene sequence analyses indicated that strain HRV44^T was most closely related to *Nitratiruptor* sp. EPR55-1, although its physiological characteristics were more similar to that of *Nitratiruptor tergarcus*. Although it was difficult to determine whether strain HRV44^T belongs to the genus *Nitratiruptor* based solely on 16S rRNA gene sequences, combination of AAI and genome-based phylogeny suggested that strain HRV44^T represents a novel species of the genus *Nitratiruptor*.

Strain HRV44^T showed a relatively narrow growth range when compared with phylogenetically related campylobacterial strains, implying a limited niche of strain HRV44^T. The strain consumed N_2O more than three times as fast as the mesophilic N_2O-reducing campylobacteria (Mino et al., 2018). Although previous studies evaluated microbial N_2O reduction under pH above 7.0 because low pH potentially inhibits NosZ activities in some bacterial species (Bergaust et al., 2012a; Domeignoz-Horta et al., 2016), strain HRV44^T showed high N_2O-reducing activity at around pH 6.0. This study is the first report on a microorganism possessing the N_2O-reducing ability under high temperature (>50°C) and low pH condition. In addition, the doubling time of strain HRV44^T under the N_2O-respiring condition was shorter than those of *Nitratiruptor tergarcus* (2.0 h), *Nitratiruptor* sp. EPR55-1 (3.5 h) under their optimum conditions. Thus, strain HRV44^T might adapt its metabolism that facilitates highly efficient utilization of N_2O in limited habitats. We could not directly compare the N_2O-reducing rate of strain HRV44^T with those of other clade I and clade II bacteria since initial N_2O concentration and culture conditions varied between studies. Additionally, cell numbers used for calculating cell-specific N_2O consumption rate are possibly retrieved from different growth phase of growing cells among studies, which might lead to the misevaluation of N_2O-reducing ability of clade I and clade II microorganisms. Evaluation of N_2O-reducing rate at single-cell level and enzymatic characterizations of NosZ may allow for a fair comparison with other N_2O-reducing microorganisms.

Two assembly approaches using PacBio, ONT, and Illumina reads clearly showed the presence of a plasmid of strain HRV44^T. Although there are few studies on plasmids isolated from (hyper)thermophiles living in deep-sea hydrothermal vents (Lossouarn et al., 2015), there is only a report of the presence of plasmid in *Campylobacteria* from deep-sea hydrothermal environments. The difference of G + C content between the chromosome and the plasmid in strain HRV44^T was smaller than that in other deep-sea thermophiles (Table S3), suggesting the long-term persistence of the plasmid (Lawrence and Ochman, 1998; Rocha and Danchin, 2002), but its role in host physiology is still unclear. In addition, several plasmid CDSs including *tra* genes showed homologies to genomes of *Campylobacter iguaniorum* (Gilbert et al., 2015) as well as of other terrestrial and deep-sea hydrothermal vent campylobacterial species. Genomic traits imply the evolutionary link between terrestrial nonpathogenic *Campylobacteria* and their deep-sea chemolithoautotrophic relatives as suggested in previous studies (Nakagawa et al., 2007; Pérez-Rodríguez et al., 2015). Further efforts in describing novel strains with acquisition of complete genome sequences and genome comparison might provide insights into the ecological role of plasmids in deep-sea hydrothermal environments and contribute to the understanding of evolutionary links among *Campylobacteria*.

IS within the 16S rRNA Gene of Strain HRV44^T

Insertion sequences within 23S and 16S rRNA genes have been detected in some bacterial genera to date (Linton et al., 1994; Rainey et al., 1996; Selenska-Pobell and Döring, 1998; Pabbaraju et al., 2000) and are known to be excised during rRNA maturation processes (Pronk and Sanderson, 2001; Salman et al., 2012) by self-splicing caused by catalytic RNA (Salman et al., 2012) or RNase-III-mediated mechanism (Evguenieva-Hackenberg and Klug, 2000). Despite strain HRV44^T having ISs within all three copies of the 16S rRNA genes, it shows robust growth under HNN medium, reaching 3.69×10^8 cells/mL after 24 h. Thus, ISs might be selectively neutral when present within 16S rRNA, as they are apparently not detrimental to growth as observed in *Escherichia* and *Salmonella* strains (Mattatall and Sanderson, 1998), but we could not evaluate the growth of ISs-deficient strain HRV44^T here. Additionally, no ORFs found within ISs supports the rarity of ISs encoding ORFs in bacteria outside the several taxa (Brown et al., 2015). Although little is known about the biological role and evolutionary history of ISs in deep-sea hydrothermal environments, some positive influences of ISs have been reported in both eukaryotic and prokaryotic cells (Hsu et al., 1994; Cheng and Deutscher, 2003; Parenteau et al., 2019; Morgan et al., 2019). Insertion sequences therefore might be advantageous genomic traits for strain HRV44^T. The taxonomic diversity of organisms possessing IS within rRNA genes is fundamental information toward understanding its evolutional and physiological functions; however, such microbes might be overlooked using meta 16S rRNA sequencing, a general method for the evaluation of bacterial and archaeal diversities (Brown et al., 2015). Metagenomic and cultivation approaches are likely to contribute toward revealing its ecophysiological advantages, coupled with enhanced success in discovery of microorganisms possessing ISs.

N_2O-Reducing Ability among *Campylobacteria*

All *Nitratiruptor* spp. strains tested here grew under the N_2O-respiring condition, suggesting that N_2O-respiring metabolism is one of the common physiological traits in the genus *Nitratiruptor*. In addition to the highly conserved primary structure of the NosZ, organization of the *nos* gene cluster was remarkably conserved among *nosZ*-possessing *Campylobacteria* including the genus *Nitratiruptor*, as described in previous studies (Sanford et al., 2012; Mino et al., 2018). Phylogenetic analyses based on each gene within the *nos* gene cluster imply that *nosC1* encoding the mono-heme cytochrome c and other genes have undergone respective evolutionary histories. These results from comparative genomics were not strong enough to explain the difference in N_2O-reducing abilities demonstrated here. It is of interest that the significant difference in the rate of N_2O reduction was observed among strains presenting similar genetic characteristics. *W. succinogenes*, a model microorganism for clade II NosZ (Kern and Simon, 2009), is known to employ Nss-type transcriptional regulatory proteins (Kern and Simon, 2016) to mediate the up-regulation of NosZ. Members of *Campylobacteria* we studied are found to encode regulators of the Crp-Fnr superfamily on their genomes as well. However, their positions were different from *W. succinogenes* and highly diverse in genus. Further transcriptomic analyses might be useful in providing more insights into the mechanisms producing the difference in the rate of N_2O reduction.

Incompatibility between genetic and physiological traits has been reported in nitrate reduction metabolism in campylobacterial species such as *Sulfurimonas autotrophica, Nautilia profundicola*, and *Hydrogenimonas* sp. BAL40 (Inagaki et al., 2003; Smith et al., 2008; Mino et al., 2018). In this study, mesophiles, i.e., *Sulfurimonas autotrophica, Sulfurovum lithotrophicum, Sulfurovum riftiae*, and *Sulfurovum* sp. NBC37-1, did not show N_2O-respiring ability despite possessing the *nos* gene cluster on their genome.

N_2O-respiring metabolism among *Campylobacteria* is possibly influenced by environmental factors as clade I denitrifying bacteria regulate their N_2O reductase in response to the presence of nitrate, nitrite, NO, and/or oxygen (Bergaust et al., 2012b; Bueno et al., 2012).

Biogeochemical Features of Strain HRV44^T and N_2O Emission from Deep-Sea Hydrothermal Environments

Denitrification has been recognized as a modular pathway that is mediated by microorganisms both possessing and lacking *nosZ* genes (Zumft, 1997; Jones et al., 2008; Graf et al., 2014). In addition to taxonomically diverse denitrifiers (Fortunato and Huber, 2016; Pjevac et al., 2018), microorganisms with other nitrogen metabolisms can contribute to N_2O emission and mitigation in deep-sea hydrothermal environments. For example, thermophilic *Campylobacteria* such as *Hydrogenimonas thermophila*, *Nautilia profundicola*, *Caminibacter mediatlanticus*, (Voordeckers et al., 2005), *Cetia pacifica* (Grosche et al., 2015), and *Lebetimonas natsushimae* (Nagata et al., 2017) can indirectly relate to the denitrification process as DNRA microorganisms, causing decrease in nitrate, the first substrate of denitrification. The ability of strain HRV44^T to utilize not only nitrate but N_2O might be one of the niche partitioning strategies for environmental adaptation to deep-sea hydrothermal environments where many chemolithoautotrophs utilize nitrate as electron acceptors.

N_2O emission from the environments is a gross result of the balance between its production and consumption. Although redox gradient around deep-sea hydrothermal vent is conductive to microbial denitrification and nitrification, its contribution to N_2O level at deep sea is quite low (Bange and Andreae, 1999). Our results imply members of the genus *Nitratiruptor* can significantly contribute to the capacity of N_2O mitigation of the environments and the magnitude of contribution on the total N_2O sink might differ at species or strain levels and be influenced by environmental factors. In addition, not all denitrifying microorganisms might account for the N_2O emission as members of *Nitratiruptor* can utilize exogenous N_2O in this study. In deep-sea hydrothermal environments, denitrifying mesophiles and nitrifying microorganisms likely appeared to be the biological sources of excess N_2O observed in previous biogeochemical studies (Lilley et al., 1982; Kawagucci et al., 2010). Further comprehensive omics and physiological analysis of deep-sea hydrothermal vent microorganisms will lead us to evaluate the accurate N_2O flux in these environments and their contribution to mediate climate change.

Description of *Nitratiruptor labii* sp. nov.

Nitratiruptor labii (la.bi'i. L. neut. gen. n. *labii*, flange structure from which the strain was isolated).

Weak motile cells with polar flagellum, approximately 2.0 μm long and 0.5 μm wide were frequently observed. Aggregated cells were often observed. The temperature range for growth is 45°C–60°C (optimum 53°C). The pH range was 5.4–6.4 (optimum 6.0). The NaCl requirement is 2.0%–4.0% (w/v) (optimum 2.5% (w/v)). Strictly chemolithoautotrophic growth occurs with molecular hydrogen as a sole electron donor and with nitrate, nitrous oxide, oxygen (up to 1.0%), elemental sulfur as a sole electron accepter. Nitrous oxide was reduced with construction of the pink colored pericle-like structure at gas liquid interface in HNN medium. Elemental sulfur or sulfate was used as a sole sulfur source. Nitrate or ammonium was utilized as a sole nitrogen source. The size of the genome, including megaplasmid and the calculated G + C content was 2,092,987 bp and 33.4%, respectively.

The type strain, HRV44^T (=JCM 34002 = DSM 111345), was isolated from a deep-sea hydrothermal flange structure at the Iheya North hydrothermal field in the Mid-Okinawa Trough, Japan.

Limitations of the Study

This study reports the first evidence of *Nitratiruptor labii* sp. nov, isolated from the deep-sea hydrothermal vent in the Mid-Okinawa Trough, as a N_2O-reducing thermophile coupling with H_2 as an electron donor, and describes N_2O-reducing ability of different campylobacterial strains from deep-sea hydrothermal environments. It should be noted that N_2O-reducing rate at single-cell level has not been elucidated in this study because HRV44^T forms the pericle-like structure under N_2O-reducing condition, that might affect to the ability to reduce N_2O. Furthermore, no data on *in situ* N_2O-reducing rate of campylobacterial members was available. These limitations could be addressed by the use of comprehensive analysis including enzymatic analysis of NosZ, *in situ* transcriptome and measurement of N_2O reduction in deep-sea hydrothermal environments.

Resource Availability

Lead Contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Sayaka Mino (sayaka.mino@fish.hokudai.ac.jp).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

This project has been deposited at DDBJ/EMBL/GenBank under the BioProject PRJDB9307. Sequences for complete genome and 16S rRNA gene of strain HRV44^T are available with DDBJ/EMBL/GenBank AP022826, AP0228267, and LC528620, respectively. The 16S rRNA sequences of strains No.9, No.33, No.34, No.37, No.41 are available with DDBJ/EMBL/GenBank LC533966 to LC533970, respectively.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101462.

ACKNOWLEDGMENTS

We gratefully acknowledge the R/V *Natsushima*, R/V *Kaiyo*, and ROV *Hyper dolphin* operation teams for helping us to obtain deep-sea hydrothermal vent samples from the Mid-Okinawa Trough. We thank Prof. Masahira Hattori and Dr. Wataru Suda (University of Tokyo, Kashiwa, Japan) for assistance with sequencing of metagenome by Illumina. This work was partially supported by the JSPS KAKENHI (No. 15H05991 and 17K15301) and the Takahashi Industrial and Economic Research Foundation.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: M.F., S.M., and T.S. Performed the experiments: M.F. and H.T. Analyzed the data: M.F., S.M., and H.T. Contributed reagents/materials/analysis tools: S.M., S.N., K.T., and T.S. Wrote the paper: M.F., S.M., S.N., K.T., and T.S.

DECLARATION OF INTERESTS

The authors have no competing interests.

Received: March 29, 2020
Revised: May 21, 2020
Accepted: August 12, 2020
Published: September 25, 2020

REFERENCES

Akerman, N.H., Butterfield, D.A., and Huber, J.A. (2013). Phylogenetic diversity and functional gene patterns of sulfur-oxidizing subseafloor *Epsilonproteobacteria* in diffuse hydrothermal vent fluids. Front. Microbiol. *4*, 1–14.

Bange, H.W., and Andreae, M.O. (1999). Nitrous oxide in the deep waters of the world's oceans. Global Biogeochem. Cycles *13*, 1127–1135.

Bange, H.W., Freing, A., Kock, A., and Löscher, C.R. (2010). Marine pathways to nitrous oxide. In Nitrous Oxide and Climate Change, K. Smith, ed. (Routledge), pp. 36–62.

Bange, H.W., Arevalo-Martinez, D.L., de la Paz, M., Farias, L., Kaiser, J., Kock, A., Law, C.S., Rees, A.P., Rehder, G., Tortell, et al.. (2019). A harmonized nitrous oxide (N_2O) ocean observation network for the 21st century. Front. Mar. Sci. *6*, 157.

Bergaust, L., Mao, Y., Bakken, L.R., and Frostegård, A. (2012a). Denitrification response patterns during the transition to anoxic respiration and posttranscriptional effects of suboptimal pH on nitrous oxide reductase in *Paracoccus denitrificans*. Appl. Environ. Microbiol. *76*, 6387–6396.

Bergaust, L., van Spanning, R.J.M., Frostegård, Å., and Bakken, L.R. (2012b). Expression of nitrous oxide reductase in *Paracoccus denitrificans* is regulated by oxygen and nitric oxide through FnrP and NNR. Microbiology *158*, 826–834.

Brown, C.T., Hug, L.A., Thomas, B.C., Sharon, I., Castelle, C.J., Singh, A., Wilkins, M.J., Wrighton, K.C., Williams, K.H., and Banfield, J.F. (2015). Unusual biology across a group comprising more than 15% of domain Bacteria. Nature *523*, 208–211.

Bueno, E., Mesa, S., Bedmar, E.J., Richardson, D.J., and Delgado, M.J. (2012). Bacterial adaptation of respiration from oxic to microoxic and anoxic conditions: redox control. Antioxid. Redox Signal. *16*, 819–852.

Cheng, Z.F., and Deutscher, M.P. (2003). Quality control of ribosomal RNA mediated by polynucleotide phosphorylase and RNase R. Proc. Natl. Acad. Sci. U S A *100*, 6388–6393.

Ciais, P., Sabine, C., Bala, G., Bopp, L., Brovkin, V., Canadell, J., Chhabra, A., DeFries, R., Galloway, J., Heimann, M., et al. (2013). Carbon and other biogeochemical cycles. In Climate Change 2013: The Physical Science Basis: Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, T.F. Stocker, D. Qin, G.K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex, and P.M. Midgley, eds. (Cambridge University Press), pp. 465–570.

Crutzen, P.J. (1970). The influence of nitrogen oxides on the atmospheric ozone content. Q. J. R. Meteorol. Soc. *96*, 320–325.

Dini-Andreote, F., Brossi, M.J., van Elsas, J.D., and Salles, J.F. (2016). Reconstructing the genetic potential of the microbially-mediated nitrogen cycle in a salt marsh ecosystem. Front. Microbiol. *7*, 1–13.

Domeignoz-Horta, L.A., Spor, A., Bru, D., Philippot, L., Putz, M., Breuil, M.C., and Hallin, S. (2016). Non-denitrifying nitrous oxide-reducing bacteria -an effective N_2O sink in soil. Soil Biol. Biochem. *103*, 376–379.

Evguenieva-Hackenberg, E., and Klug, G. (2000). RNase III processing of intervening sequences found in helix 9 of 23S rRNA in the alpha subclass of *Proteobacteria*. J. Bacteriol. *182*, 4719–4729.

Fortunato, C.S., and Huber, J.A. (2016). Coupled RNA-SIP and metatranscriptomics of active chemolithoautotrophic communities at a deep-sea hydrothermal vent. ISME J. *10*, 1925–1938.

Giovannelli, D., Chung, M., Staley, J., Starovoytov, V., Bris, N., and Vetriani, C. (2016). *Sulfurovum riftiae* sp. nov., a mesophilic, thiosulfate-oxidizing, nitrate-reducing chemolithoautotrophic epsilonproteobacterium isolated from the tube of the deep-sea hydrothermal vent polychaete *Riftia pachyptila*. Int. J. Syst. Evol. Microbiol. *66*, 2697–2701.

Gilbert, M.J., Kik, M., Miller, W.G., Duim, B., and Wagenaar, J.A. (2015). *Campylobacter iguaniorum* sp. nov., isolated from reptiles. Int. J. Syst. Evol. Microbiol. *65*, 975–982.

Goris, J., Konstantinidis, K.T., Klappenbach, J.A., Coenye, T., Vandamme, P., and Tiedje, J.M. (2007). DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. Int. J. Syst. Evol. Microbiol. *57*, 81–91.

Graf, D.R.H., Jones, C.M., and Hallin, S. (2014). Intergenomic comparisons highlight modularity of the denitrification pathway and underpin the importance of community structure for N_2O emissions. PLoS One *9*, 1–20.

Graf, D.R.H., Zhao, M., Jones, C.M., and Hallin, S. (2016). Soil type overrides plant effect on genetic and enzymatic N_2O production potential in arable soils. Soil Biol. Biochem. *100*, 125–128.

Grosche, A., Sekaran, H., Pérez-Rodríguez, I., Starovoytov, V., and Vetriani, C. (2015). *Cetia pacifica* gen. nov., sp. nov., a chemolithoautotrophic, thermophilic, nitrate-ammonifying bacterium from a deep-sea hydrothermal vent. Int. J. Syst. Evol. Microbiol. *65*, 1144–1150.

Hallin, S., Philippot, L., Löffler, F.E., Sanford, R.A., and Jones, C.M. (2018). Genomics and ecology of novel N_2O-reducing microorganisms. Trends Microbiol. *26*, 43–55.

Hsu, D., Shih, L.M., and Zee, Y.C. (1994). Degradation of rRNA in *Salmonella* strains: a novel mechanism to regulate the concentrations of rRNA and ribosomes. J. Bacteriol. *176*, 4761–4765.

Hu, H.W., Chen, D., and He, J.Z. (2015). Microbial regulation of terrestrial nitrous oxide formation: understanding the biological pathways for prediction of emission rates. FEMS Microbiol. Rev. *39*, 729–749.

Huber, J.A., Mark Welch, D.B., Morrison, H.G., Huse, S.M., Neal, P.R., Butterfield, D.A., and Sogin, M.L. (2007). Microbial population structures in the deep marine biosphere. Science *318*, 97–100.

Inagaki, F., Takai, K., Kobayashi, H., Nealson, K.H., and Horikoshi, K. (2003). *Sulfurimonas autotrophica* gen. nov., sp. nov., a novel sulfur-oxidizing ϵ-proteobacterium isolated from hydrothermal sediments in the Mid-Okinawa Trough. Int. J. Syst. Evol. Microbiol. *53*, 1801–1805.

Inagaki, F., Takai, K., Nealson, K.H., and Horikoshi, K. (2004). *Sulfurovum lithotrophicum* gen. nov., sp. nov., a novel sulfur-oxidizing chemolithoautotroph within the ϵ-*Proteobacteria* isolated from Okinawa Trough hydrothermal sediments. Int. J. Syst. Evol. Microbiol. *54*, 1477–1482.

Jones, C.M., Stres, B., Rosenquist, M., and Hallin, S. (2008). Phylogenetic analysis of nitrite, nitric oxide, and nitrous oxide respiratory enzymes reveal a complex evolutionary history for denitrification. Mol. Biol. Evol. *25*, 1955–1966.

Jones, C.M., Graf, D.R.H., Bru, D., Philippot, L., and Hallin, S. (2013). The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. ISME J. *7*, 417–426.

Juhanson, J., Hallin, S., Söderström, M., Stenberg, M., and Jones, C.M. (2017). Spatial and phyloecological analyses of *nosZ* genes underscore niche differentiation amongst terrestrial N_2O reducing communities. Soil Biol. Biochem. *115*, 82–91.

Kawagucci, S., Shirai, K., Lan, T.F., Takahata, N., Tsunogai, U., Sano, Y., and Gamo, T. (2010). Gas geochemical characteristics of hydrothermal plumes at the HAKUREI and JADE vent sites, the Izena Cauldron, Okinawa Trough. Geochem. J. *44*, 507–518.

Kern, M., and Simon, J. (2009). Electron transport chains and bioenergetics of respiratory nitrogen metabolism in *Wolinella succinogenes* and other Epsilonproteobacteria. Biochim. Biophys. Acta Bioenerg. *1787*, 646–656.

Kern, M., Winkler, C., and Simon, J. (2011). Respiratory nitrogen metabolism and nitrosative stress defence in ϵ-proteobacteria: the role of NssR-type transcription regulators. Biochem. Soc. Trans. *39*, 299–302.

Kern, M., and Simon, J. (2016). Three transcription regulators of the Nss family mediate the adaptive response induced by nitrate, nitric oxide or nitrous oxide in *Wolinella succinogenes*. Environ. Microbiol. *18*, 2899–2912.

Lawrence, J.G., and Ochman, H. (1998). Molecular archaeology of the *Escherichia coli* genome. Proc. Natl. Acad. Sci. U S A *95*, 9413–9417.

Lilley, M.D., de Angelis, M.A., and Gordon, L.I. (1982). CH_4, H_2, CO and N_2O in submarine hydrothermal vent waters. Nature *300*, 48–50.

Linton, D., Ciewley, J.P., Burnens, A., Owen, R.J., and Stanley, J. (1994). An intervening sequence (IVS) in the 16S rRNA gene of the eubacterium *Helicobacter canis*. Nucleic Acids Res. *22*, 1954–1958.

Lossouarn, J., Dupont, S., Gorlas, A., Mercier, C., Bienvenu, N., Marguet, E., Forterre, P., and Geslin, C. (2015). An abyssal mobilome: viruses, plasmids and vesicles from deep-sea hydrothermal vents. Res. Microbiol. *166*, 742–752.

Machida, T., Nakazawa, T., Fujii, Y., Aoki, S., and Watanabe, O. (1995). Increase in the atmospheric nitrous oxide concentration during the last 250 years. Geophys. Res. Lett. *22*, 2921–2924.

Mattatall, N.R., and Sanderson, K.E. (1998). RNase III deficient *Salmonella typhimurium* LT2 contains intervening sequences (IVSs) in its 23S rRNA. FEMS Microbiol. Lett. *159*, 179–185.

Mino, S., Yoneyama, N., Nakagawa, S., Takai, K., and Sawabe, T. (2018). Enrichment and genomic characterization of a N_2O-reducing chemolithoautotroph from a deep-sea hydrothermal vent. Front. Bioeng. Biotechnol. *6*, 1–8.

Morgan, J.T., Fink, G.R., and Bartel, D.P. (2019). Excised linear introns regulate growth in yeast. Nature *565*, 606–611.

Muto, H., Takaki, Y., Hirai, M., Mino, S., Sawayama, S., Takai, K., and Nakagawa, S. (2017). A simple and efficient RNA extraction method from deep-sea hydrothermal vent chimney structures. Microbes Environ. *32*, 330–335.

Nagata, R., Takaki, Y., Tame, A., Nunoura, T., Muto, H., Mino, S., Sawayama, S., Takai, K., and Nakagawa, S. (2017). *Lebetimonas natsushimae* sp. nov., a novel strictly anaerobic, moderately thermophilic chemoautotroph isolated from a deep-sea hydrothermal vent polychaete nest in the Mid-Okinawa Trough. Syst. Appl. Microbiol. *40*, 352–356.

Nakagawa, S., Takai, K., Inagaki, F., Hirayama, H., Nunoura, T., Horikoshi, K., and Sako, Y. (2005a). Distribution, phylogenetic diversity and physiological characteristics of epsilon-*Proteobacteria* in a deep-sea hydrothermal field. Environ. Microbiol. *7*, 1619–1632.

Nakagawa, S., Takai, K., Inagaki, F., Horikoshi, K., and Sako, Y. (2005b). *Nitratiruptor tergarcus* gen. nov., sp. nov. and *Nitratifractor salsuginis* gen. nov., sp. nov., nitrate-reducing chemolithoautotrophs of the ϵ-*Proteobacteria* isolated from a deep-sea hydrothermal system in the Mid-Okinawa Trough. Int. J. Syst. Evol. Microbiol. *55*, 925–933.

Nakagawa, S., Takaki, Y., Shimamura, S., Reysenbach, A., Takai, K., and Horikoshi, K. (2007). Deep-sea vent ε-proteobacterial genomes provide insights into emergence of pathogens. Proc. Natl. Acad. Sci. U S A *104*, 12146–12150.

Pabbaraju, K., Miller, W.L., and Sanderson, K.E. (2000). Distribution of intervening sequences in the genes for 23S rRNA and rRNA fragmentation among strains of the *Salmonella* Reference Collection B (SARB) and SARC sets. J. Bacteriol. *182*, 1923–1929.

Parenteau, J., Maignon, L., Berthoumieux, M., Catala, M., Gagnon, V., and Abou Elela, S. (2019). Introns are mediators of cell response to starvation. Nature *565*, 612–617.

Pauleta, S.R., Dell'Acqua, S., and Moura, I. (2013). Nitrous oxide reductase. Coord. Chem. Rev. *257*, 332–349.

Pérez-Rodríguez, I., Bolognini, M., Ricci, J., Bini, E., and Vetriani, C. (2015). From deep-sea volcanoes to human pathogens: a conserved quorum-sensing signal in *Epsilonproteobacteria*. ISME J. *9*, 1222–1234.

Pjevac, P., Meier, D.V., Markert, S., Hentschker, C., Schweder, T., Becher, D., Gruber-Vodicka, H.R., Richter, M., Bach, W., Amann, R., et al. (2018). Metaproteogenomic profiling of microbial communities colonizing actively venting hydrothermal chimneys. Front. Microbiol. *9*, 680.

Pronk, L.M., and Sanderson, K.E. (2001). Intervening sequences in *rrl* genes and fragmentation of 23S rRNA in genera of the family *Enterobacteriaceae*. J. Bacteriol. *183*, 5782–5787.

Rainey, F.A., Ward-Rainey, N.L., Janssen, P.H., Hippe, H., and Stackebrandt, E. (1996). *Clostridium paradoxum* DSM 7308^T contains multiple 16S rRNA genes with heterogeneous intervening sequences. Microbiology *142*, 2087–2095.

Ravishankara, A.R., Daniel, J.S., and Portmann, R.W. (2009). Nitrous oxide (N_2O): the dominant ozone-depleting substance emitted in the 21st century. Science *326*, 123–125.

Richter, M., and Rosselló-Móra, R. (2009). Shifting the genomic gold standard for the prokaryotic species definition. Proc. Natl. Acad. Sci. U S A *106*, 19126–19131.

Rocha, E.P.C., and Danchin, A. (2002). Base composition bias might result from competition for metabolic resources. Trends Genet. *18*, 291–294.

Rodriguez-R, L.M., and Konstantinidis, K.T. (2014). Bypassing cultivation to identify bacterial species. Microbe *9*, 111–118.

Salman, V., Amann, R., Shub, D.A., and Schulz-Vogt, H.N. (2012). Multiple self-splicing introns in the 16S rRNA genes of giant sulfur bacteria. Proc. Natl. Acad. Sci. U S A *109*, 4203–4208.

Sanford, R.A., Wagner, D.D., Wu, Q., Chee-Sanford, J.C., Thomas, S.H., Cruz-García, C., Rodríguez, G., Massol-Deyá, A., Krishnani, K.K., Ritalahti, K.M., et al. (2012). Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. Proc. Natl. Acad. Sci. U S A *109*, 19709–19714.

Selenska-Pobell, S., and Döring, H. (1998). Sequences around the fragmentation sites of the large subunit ribosomal RNA in the family *Rhizobiaceae*. 23S-like rRNAs in *Rhizobiaceae*. Antonie van Leeuwenhoek. Int. J. Gen. Mol. Microbiol. *73*, 55–67.

Sievert, S.M., and Vetriani, C. (2012). Chemoautotrophy at deep-sea vents, past, present, and future. Oceanography *25*, 218–233.

Simon, J., Einsle, O., Kroneck, P.M.H., and Zumft, W.G. (2004). The unprecedented *nos* gene cluster of *Wolinella succinogenes* encodes a novel respiratory electron transfer pathway to cytochrome *c* nitrous oxide reductase. FEBS Lett. *569*, 7–12.

Smith, J.L., Campbell, B.J., Hanson, T.E., Zhang, C.L., and Cary, S.C. (2008). *Nautilia profundicola* sp. nov., a thermophilic, sulfur-reducing epsilonproteobacterium from deep-sea hydrothermal vents. Int. J. Syst. Evol. Microbiol. *58*, 1598–1602.

Stackebrandt, E., and Ebers, J. (2006). Taxonomic parameter revised: tarnishes gols standards. Microbiol. Today *33*, 152–155.

Suenaga, T., Hori, T., Riya, S., Hosomi, M., Smets, B.F., and Terada, A. (2019). Enrichment, isolation, and characterization of high-affinity N_2O-reducing bacteria in a gas-permeable membrane reactor. Environ. Sci. Technol. *53*, 12101–12112.

Thomson, A.J., Giannopoulos, G., Pretty, J., Baggs, E.M., and Richardson, D.J. (2012). Biological sources and sinks of nitrous oxide and strategies to mitigate emissions. Philos. Trans. R. Soc. B Biol. Sci. *367*, 1157–1168.

Vetriani, C., Voordeckers, J.W., Crespo-Medina, M., O'Brien, C.E., Giovannelli, D., and Lutz, R.A. (2014). Deep-sea hydrothermal vent *Epsilonproteobacteria* encode a conserved and widespread nitrate reduction pathway (Nap). ISME J. *8*, 1510–1521.

Voordeckers, J.W., Starovoytov, V., and Vetriani, C. (2005). *Caminibacter mediatlanticus* sp. nov., a thermophilic, chemolithoautotrophic, nitrate-ammonifying bacterium isolated from a deep-sea hydrothermal vent on the Mid-Atlantic Ridge. Int. J. Syst. Evol. Microbiol. *55*, 773–779.

Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I., Moore, L.H., Moore, W.E.C., Murray, R.G.E., Stackebrandt, E., et al. (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. *37*, 463–464.

Wittorf, L., Bonilla-Rosso, G., Jones, C.M., Bäckman, O., Hulth, S., and Hallin, S. (2016). Habitat partitioning of marine benthic denitrifier communities in response to oxygen availability. Environ. Microbiol. Rep. *8*, 486–492.

Yoon, S., Nissen, S., Park, D., Sanford, R.A., and Löffler, E. (2016). Nitrous oxide reduction kinetics distinguish bacteria harboring clade I NosZ from those harboring clade II NosZ. Appl. Environ. Microbiol. *82*, 3793–3800.

Zumft, W.G. (1997). Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. *61*, 533–616.

Zumft, W.G., and Kroneck, P.M.H. (2007). Respiratory transformation of nitrous oxide (N_2O) to dinitrogen by *Bacteria* and *Archaea*. Adv. Microb. Physiol. *52*, 107–227.

iScience, Volume *23*

Supplemental Information

Biogeochemical Implications of N_2O-Reducing Thermophilic *Campylobacteria* in Deep-Sea Vent Fields, and the Description of *Nitratiruptor labii* sp. nov.

Muneyuki Fukushi, Sayaka Mino, Hirohisa Tanaka, Satoshi Nakagawa, Ken Takai, and Tomoo Sawabe

Transparent Methods

Sample collection, enrichment and isolation

Hydrothermal samples i.e., chimney and flange structures, and polychaeta nests, were collected from the deep-sea hydrothermal system at the Iheya North Original field in the Mid-Okinawa Trough, Japan using R/V *Natsushima*, R/V *Kaiyo* and ROV *Hyper dolphin* during the scientific cruises KY14-01 and NT15-13. Each sample was slurried with sterilized seawater in the presence or absence of 0.05% (w/v) neutralized sodium sulfide in 100 ml glass bottles (Schott Glaswerke, Mainz, Germany). Bottles were then tightly sealed with butyl rubber caps under a gas phase of 100% N_2 (0.2MPa). Samples were stored at 4 °C until use.

For enrichment, serial dilution cultures were performed using 15 ml test tubes containing 3 ml HNN medium (Mino et al., 2018) at 55°C. HNN medium contained 1 g $NaHCO_3$ per liter of MJ synthetic sea water (Sako et al., 1996). Concentrated solution of $NaHCO_3$ was added to the MJ synthetic sea water before gas bubbling of 100% N_2O. The tubes were tightly sealed with butyl rubber stoppers. Then, the tubes were sterilized with autoclaves and pressured with H_2/CO_2 mixed gas (80:20). Gas phase of the tubes was composed of $N_2O/H_2/CO_2$ (33:54:13) (300 kPa). The pH of HNN medium was pH6.2. The pure cultures were obtained from subsamples using the dilution-to-extinction technique (Baross, 1995) with HNN medium at 55°C. The purity of cultures was confirmed by sequencing of the 16S rRNA gene using several primers (Lane, 1991).

Phylogenetic analysis based on 16S rRNA gene sequence

Genomic DNA of the six isolates was extracted from the cells grown in HNN medium with Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) according to the *manufacturer's protocol.* The amplicons of 16S rRNA genes of the isolates were obtained by PCR using primers Eubac 27F and 1492R (Lane, 1991). The sequences of amplicons were determined directly in both strands using the dideoxynucleotide chain-termination method. The raw data of sequences were trimmed, assembled and outputted in the multi-FASTA format using ChromasPro ver. 2.1.6 (Technelysium Pty Ltd, South Brisbane, Australia). The similarity analysis of 16S rRNA gene sequences was conducted using the BLAST search algorithm.

To determine the phylogenetic position of the isolates, 16S rRNA gene sequences of related campylobacterial species were retrieved from the Silva database (Quast et al., 2013) and were aligned using the Silva Incremental Aligner (SINA) v1.2.11 (Pruesse et al., 2012). The phylogenetic tree based on 1,048 bp of 16S rRNA gene sequences was constructed using the maximum-likelihood method with MEGA version X (Kumar et al., 2018) with TN93+G+I model. Bootstrap analysis was carried out using 1,000 replications.

Identification of insertion sequence and prediction of the 16S rRNA second structure of strain HRV44^T

An insertion sequence (IS) in the 16S rRNA gene of strain HRV44^T was predicted by BLAST search. IS was determined via multiple sequence alignment of 16S rRNA gene from related species, i.e. *Nitratiruptor tergarcus* (AB175499), *Nitratifractor salsuginis* (AB175500), *Sulfurimonas autotrophica* (CP002205), *Sulfurovum*

lithotrophicum (CP01130), *Wolinella succinogenes* (AF463534), and *Persephonella marina* (AF188332), using SINA v1.2.11 (Pruesse et al., 2012) with default parameters.

To investigate the effect of IS on the 16S rRNA secondary structure, the secondary structure was predicted using the Mfold web server (Zuker, 2003). In addition, open reading flame (ORF) within IS was searched using the NCBI ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) with the following parameters; Minimal ORF length: 75 nt (default), Genetic code: Bacterial, Archaeal and Plant Plastid, ORF start codon to use: "ATG" and alternative initiation codons.

Measurement of N_2O-reducing ability of the isolates

The six isolates were precultured in HNN medium for 72 h at 55°C. Then, 2 mL of preculture was inoculated in 70 mL glass vials each containing 20 mL medium under $N_2O/H_2/CO_2$ (33:54:13) gas phase (300 kPa). The vials were incubated with mixing at 900 rpm at 55℃ for 3 days. The N_2O concentration of headspace of the cultivation vial and cell growth were measured for over 3 days using gas chromatography (GC-2014; Shimadzu, Kyoto, Japan) with the SHINCARBON ST 50/80 (2 m × 3 mmϕ) column (Shinwa Chemical Industries, Kyoto, Japan) and direct cell counts after staining with 4', 6-diamidino-2-phenylindole (DAPI) (Porter and Feig, 1980), respectively.

Characterization of strain HRV44 ^{T}

The cell morphology of strain HRV44 ^{T} was observed using a ZEISS Axiophot microscope (Carl Zeiss Co., Oberkochen, Germany). For transmission electron microscopy, cells grown in HNN medium at 55ºC in the late exponential phase were

66 stained with EM Stainer (Nisshin EM Co.,Ltd., Tokyo, Japan). Micrographs were
67 obtained using a JEM-1011 transmission electron microscope (JEOL, Tokyo, Japan).
68 Growth of the novel isolate was measured using direct cell counts after staining
69 with DAPI (Porter and Feig, 1980). To determine optimum temperatures, pH and NaCl
70 requirements, the cells were grown in 15 ml test tubes (Asahi Glass Co., Ltd. Tokyo,
71 Japan) containing 3 ml HNN medium in various conditions. When optimum pH was being
72 determined, the pH of the HNN medium was adjusted to various values with 10 mM
73 acetate/acetic acid buffer (pH 4.0-5.0), MES (pH 5.0-6.0), PIPES (pH 6.5), HEPES (pH
74 7.0) and Tris (pH 8.0-9.5) at room temperature. The pH of the medium was readjusted
75 with HCl or NaOH after sterilization.
76 Strain HRV44^T was tested for the ability to grow on couples of a single electron
77 donor and acceptor. MJ synthetic seawater with the addition of 1 g $NaHCO_3$ per liter was
78 used as a basal medium. H_2/CO_2 (80:20) gas phase (300 kPa) was used in an attempt to
79 examine growth on hydrogen as an electron donor. Electron acceptors were provided at
80 final concentrations of 0.1% (w/v) ($NaNO_3$ and $Na_2S_2O_3 \cdot 5H_2O$), 33% (v/v) (N_2O), 0.01–
81 0.1% (w/v) (Na_2SO_3 and $NaNO_2$), 1% (w/v) (elemental sulfur) or 0.1–10% (v/v) (O_2). To
82 determine alternative electron donors other than H_2, each of $Na_2S_2O_3 \cdot 5H_2O$ (0.1%, w/v)
83 and elemental sulfur (1%, w/v) was examined in combination with $NaNO_3$ (0.1%, w/v),
84 N_2O (33%, v/v), Na_2SO_3 (0.01–0.1%, w/v), $NaNO_2$ (0.01–0.1%, w/v), elemental sulfur
85 (1%, w/v) or O_2 (0.1–1.0%, v/v) as an electron acceptor under a gas phase of N_2/CO_2
86 (80:20) (300 kPa).

Heterotrophic growth of strain HRV44^T was tested in HNN medium without $NaHCO_3$ under gas phase of H_2/N_2O (67:33) (300 kPa), containing each of the following potential carbon sources: 0.1% (w/v) yeast extract (Difco), 0.1% (w/v) peptone (Difco), 0.1% (w/v) tryptone, 0.1% (w/v) casamino acids, 0.1% (w/v) D (+) -glucose, galactose, 0.1% (w/v) sucrose, 0.1% (w/v) fructose, 0.1% (w/v) lactose, 0.1% (w/v) maltose, 0.1% (w/v) starch, 10 mM formate, 10 mM acetate, 10 mM glycerol, 10 mM citrate, 10 mM tartrate, 10 mM malate, 10 mM propionate, 10 mM lactate, 10 mM pyruvate, 0.05% (v/v) methanol, 0.1% (v/v) ethanol and 0.1% (v/v) 2-propanol. In addition, to assess the utilization of these organic compounds as an alternative energy source, the substrates were added to HNN medium without hydrogen under $N_2O/N_2/CO_2$ (33:54:13) gas phase (300 kPa).

To determine the nitrogen source for growth of the isolate, 0.1% (w/v) $NaNO_3$, 0.1% (w/v) $NaNO_2$, 0.025% (w/v) NH_4Cl were tested in HNN medium lacking all nitrogen sources under $H_2/CO_2/O_2$ (80:19.5:0.5) gas phase (300 kPa). In addition, utilization of N_2 and N_2O as the nitrogen source was examined under $H_2/N_2/CO_2/O_2$ (60:20:19.5:0.5) and $N_2O/N_2/CO_2$ (33:54:13) gas phase (300 kPa), respectively. To examine the sulfur source for growth of the isolates, each of the following potential sulfur sources was tested at final concentrations of 0.34% (w/v) SO_4^{2-}, 0.1% (w/v) Na_2SO_3, 0.1% (w/v) $Na_2S_2O_3 \cdot 5H_2O$, and 1.0% (w/v) elemental sulfur in HNN medium lacking all sulfur sources.

Whole genome sequencing of strain HRV44^T

For genome sequencing, genomic DNA of strain HRV44^T was extracted from the cells grown in HNN medium using the NucleoSpin Soil DNA kit (Macherey-Nagel, Düren, Germany) according to the *manufacturer's protocol.* Paired-end library was generated using the Nextera library preparation method, and was sequenced with the Illumina MiSeq platform (2x300 bp). Adaptor sequences were trimmed with the Platanus_trim function (Kajitani et al., 2014). PacBio sequencing was performed at The Center of Medical Innovation and Translational Research, Graduate School of Medicine, Osaka University. The library was prepared using the SMRTbell template prep kit 1.0 and the DNA polymerase binding kit P6 version 2, and was sequenced on a single SMRTR® cell with the PacBio® RS II System (Pacific Biosciences, Menlo Park, CA, USA). The ONT sequencing library was prepared using the Rapid Barcoding Sequence kit (Oxford Nanopore Technologies, Oxford, UK) following the standard *manufacturer's protocol*. ONT sequencing was conducted by loading the prepared library into the FlowCell (FLO-MIN106) on a MinION device (Oxford Nanopore Technologies, Oxford, UK) and a 48-hour sequencing run with MinKNOW 1.15.4 software was performed. ONT reads were basecalled using Guppy v1.1 (Oxford Nanopore Technologies) under the following settings: --qscore_filtering and --calib_detect. Deepbinner (Wick et al., 2018) was used for binning of basecalled reads.

PacBio and ONT reads are respectively assembled using Canu ver. 1.6 (Koren et al., 2017) with setting 2.1 M genome size parameter. Circlator ver. 1.5.1 (Hunt et al., 2015) was used to circularize the genome assembly. The circular genomes obtained from the PacBio and ONT reads were further corrected for potential errors with Pilon ver. 1.22

130 (Walker et al., 2014) using the Illumina reads as previously described (Mino et al., 2018).
131 Whole genome sequences were annotated with RAST Server v. 2.0 (Aziz et al., 2008),
132 RNAmmer 1.2 Server (Lagesen et al., 2007) and tRNAscan-SE v. 2.0 (Lowe and Chan,
133 2016). A total of 128 CDSs on plasmid was further used for blastp homology search. The
134 best hits with E-value less than e-10 were considered in this study.
135
136 **Calculation of genome sequence similarities**
137 In attempt to determine the taxonomic positioning of strain HRV44^T, genome-
138 based taxonomic values were calculated. The *in silico* DNA-DNA hybridization (*in silico*
139 DDH) values of strain HRV44^T against relative species such as *Nitratiruptor tergarcus*
140 MI55-1^T (Nakagawa et al., 2005), *Nitratiruptor* sp. SB155-2 (Nakagawa et al., 2007),
141 *Nitratiruptor* sp. EPR55-1 (unpublished data) and *Hydrogenimonas thermophila* EP1-55-
142 1%^{T} (Takai et al., 2004) were calculated using the Genome to-Genome Distance
143 Calculator 2.1 (GGDC) (Meier-Kolthoff et al., 2013) within the recommended parameters.
144 Average nucleotide identity (ANI) and average amino acid identity (AAI) values were
145 calculated using ANI and AAI calculators (Rodriguez-R and Konstantinidis, 2014),
146 respectively. The genome sequences of related species were retrieved from the NCBI
147 RefSeq and GenBank.
148 **Phylogenomic analysis**
149 The phylogenomic tree of strain HRV44^T and its related campylobacterial
150 species was constructed based on 127 conserved proteins. In order to obtain the amino
151 acid sequences of conserved proteins, pan-genome analysis was performed using Anvi'o

v.5.5.0 (Eren et al., 2015). The best-fit model for phylogenomic analysis was determined using ModelTest-NG v. 0.1.6 (Darriba et al., 2019). The phylogenomic tree was constructed using RAxML-NG v.0.9.0 (Kozlov et al., 2019) using the maximum-likelihood method with the LG+I+G4+F model. Bootstrap analysis was carried out using 1,000 replications.

Comparison of *nos* gene cluster, NosZ primary structure, transcription regulator, and N_2O-reducing ability among *Campylobacteria* isolated from deep-sea hydrothermal field

A total of 14 complete or draft genome sequences of *Campylobacteria* isolated from deep-sea hydrothermal environments were used for comparison of *nos* gene cluster. Sequences of functionally characterized NosZ protein and its accessory genes in *nos* gene cluster from *W. succinogenes* DSMZ1740^T (GCF_000196135.1) were used as reference sequences. In order to confirm the conservation of important structural motifs of NosZ, multiple alignment of NosZ amino acid sequences was generated from *nosZ* sequences of HRV44^T, related campylobacterial species, and other bacteria harboring experimentally characterized NosZ using MAFFT v. 7.0 (Katoh et al., 2017). The potential transcription regulators were searched using *Campylobacter jejuni* NssR as data base search template by BLAST search with an E-value cutoff of e-10 and Nss-binding sites upstream of *nosZ* were predicted using *W. succinogenes* consensus binding sequences TTGA-N_6-TCAA.

Nine strains possessing the *nos* gene cluster, HRV44^T, *Nitratiruptor tergarcus* MI55-1^T (Nakagawa et al., 2005), *Nitratiruptor* sp. SB155-2 (Nakagawa et al.,

2007), *Nitratiruptor* sp. EPR55-1 (unpublished data), *Nitratifractor salsuginis* E9I37-1^T (Nakagawa et al., 2005), *Sulfurovum lithotrophicum* 42BKT^T (Inagaki et al., 2004), *Sulfurovum riftiae* 1812E^T (Giovannelli et al., 2016), *Sulfurovum* sp. NBC37-1 (Nakagawa et al., 2007), *Sulfurimonas autotrophica* OK10^T (Inagaki et al., 2003), were tested for N_2O-respiring ability using test tubes. For strains possessing N_2O-respiring abilities, further evaluation of N_2O consumption rate and cell growth in 70 mL glass vials was performed using a gas chromatograph and total cell counts. In these experiments, HNN medium was used except for *Sulfurimonas autotrophica, Sulfurovum lithotrophicum* and *Sulfurovum riftiae,* which are not capable of using hydrogen as a sole electron acceptor. For these three strains, thiosulfate or elemental sulfur was substituted for hydrogen as a sole electron donor under N_2/CO_2 (80:20) gas phase (300 kPa).

Phylogenetic analysis based on genes within a *nos* gene cluster

In attempt to understand comprehensive phylogenetic relationships of the *nos* gene cluster, phylogenetic analyses based on each gene within *nos* gene cluster (*nosZ*, -*B*, -*D*, -*G*, -*C1*, -*C2*, -*H*, -*F*, -*hyp.,* -*Y* and -*L*) were performed. Each *nos* gene sequence except the hypothesis gene from the related *Campylobacteria* was aligned and each phylogenetic tree was constructed as mentioned above. Bootstrap analyses for tree based on each gene sequence was carried out using 1,000 replications.

Supplemental Figures

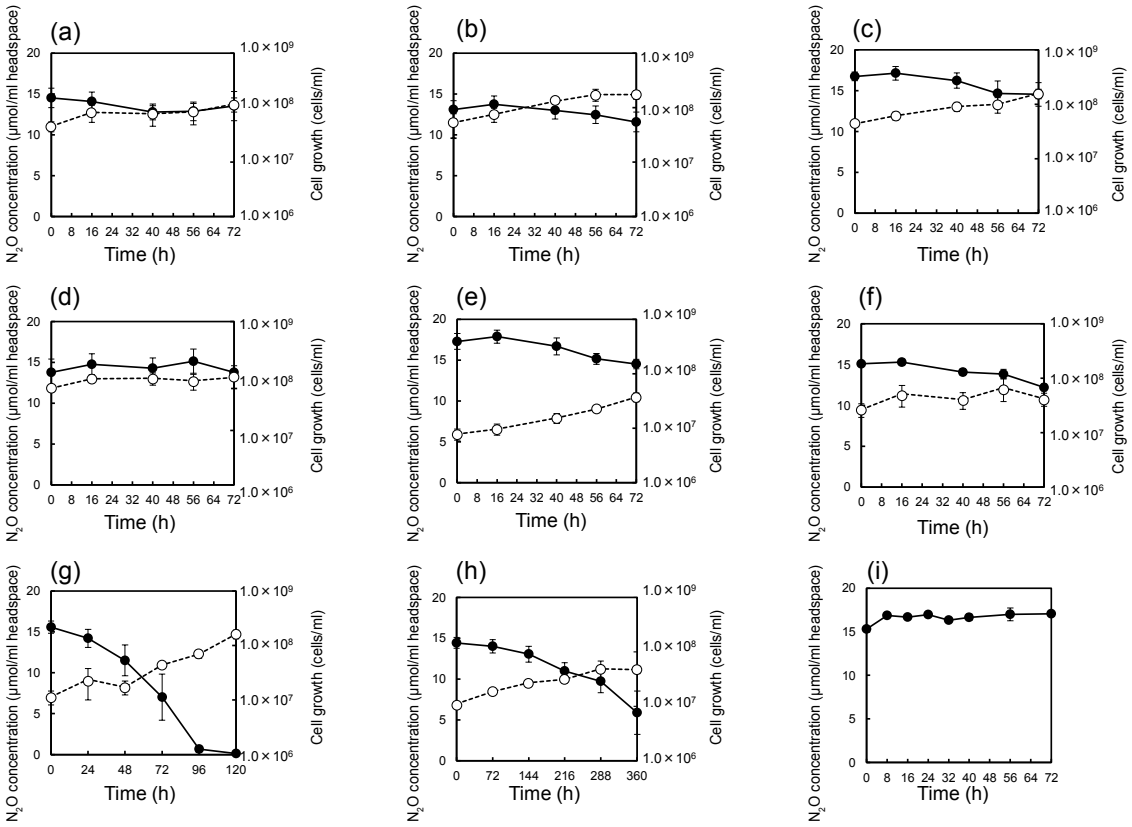


Figure S1. Time-course concentration of headspace N_2O and cell growth of N_2O-reducing *Campylobacteria*. The N_2O concentration and total cell growth are shown in filled circle with solid line and open circle with dotted line, respectively. Data points and error bars represent means and standard errors (n=3), respectively. Strains: *Nitratiruptor* sp. No.9 (a), *Nitratiruptor* sp. No.33 (b), *Nitratiruptor* sp. No.34 (c), *Nitratiruptor* sp. No.37 (d), *Nitratiruptor* sp. No.41 (e), *Nitratiruptor* sp. SB155-2 (f), *Nitratiruptor* sp. EPR55-1 (g), *Nitratiruptor tergarcus* MI55-1^T (h), Control (i). Related to Figure 2 and 3.

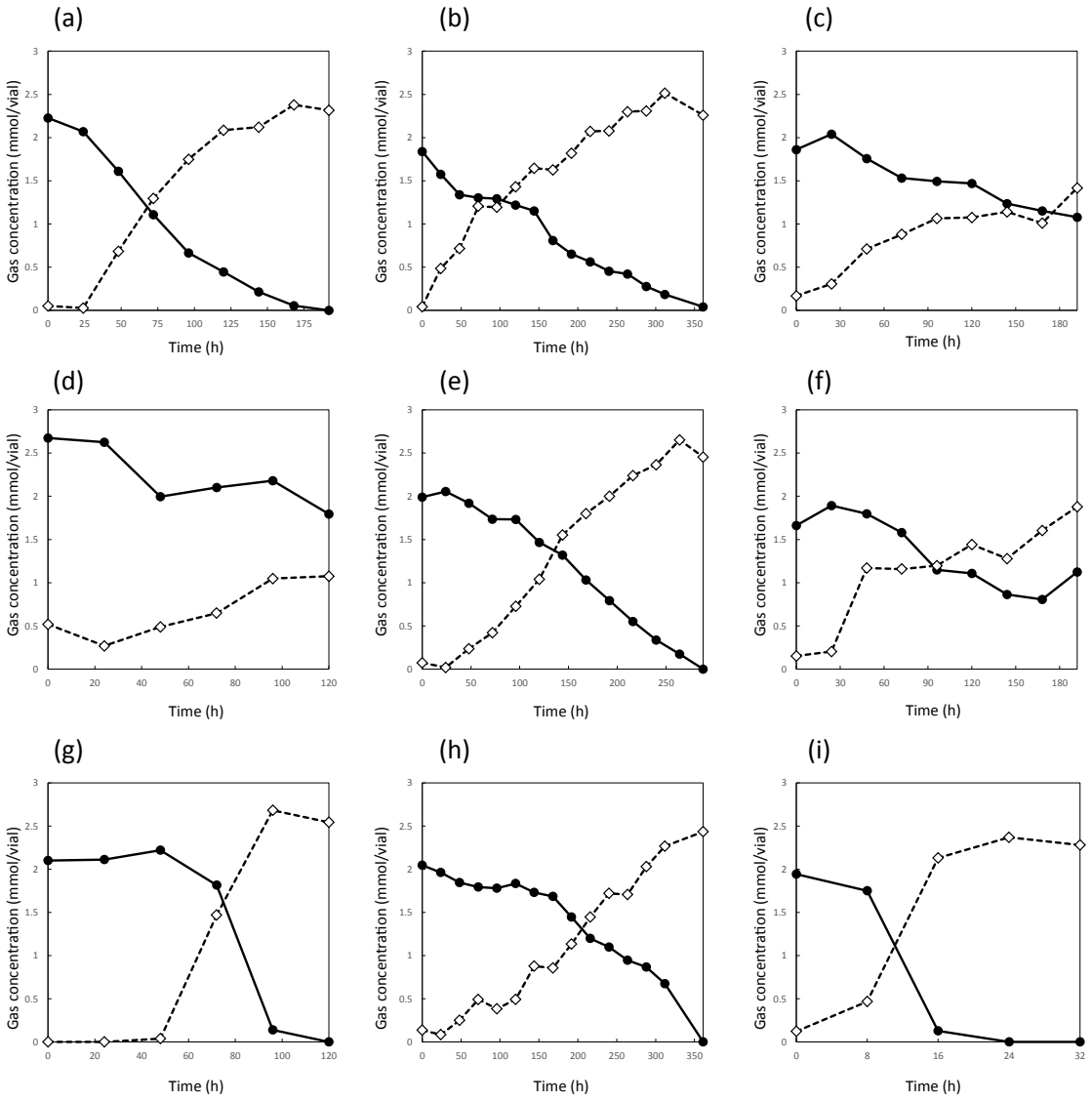


Figure S2. Time-course N_2 production during N_2O reduction of strains belonging to the genus *Nitratiruptor*. The headspace N_2O and N_2 concentrations are shown in filled circle with solid line and diamond with dotted line, respectively. Strains: *Nitratiruptor* sp. No.9 (a), *Nitratiruptor* sp. No.33 (b), *Nitratiruptor* sp. No.34 (c), *Nitratiruptor* sp. No.37 (d), *Nitratiruptor* sp. No.41 (e), *Nitratiruptor* sp. SB155-2 (f), *Nitratiruptor* sp. EPR55-1 (g), *Nitratiruptor tergarcus* MI55-1^T (h), *Nitratiruptor* sp. HRV44^T (i). Related Figure 2 and 3.

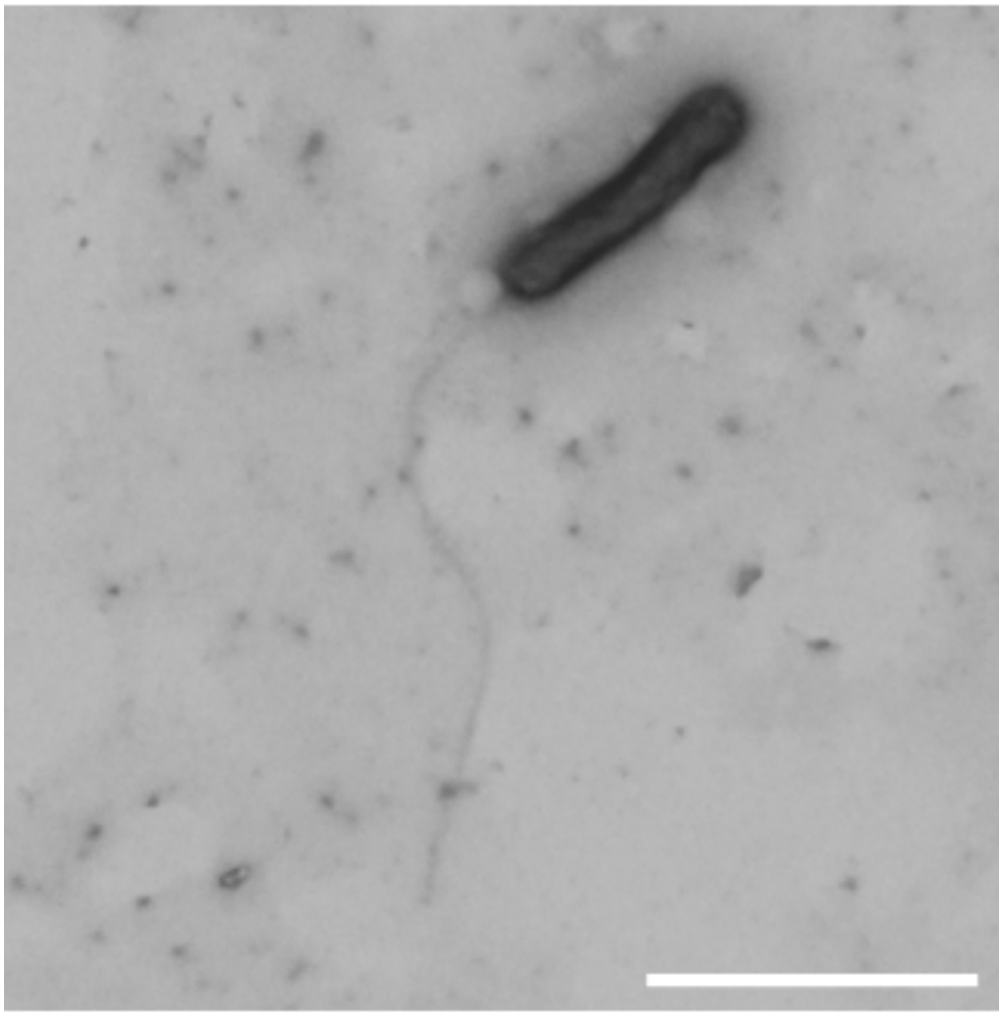


Figure S3. Transmission electron micrograph of negatively stained cell of strain HRV44^{T}.

Scale bar represents 2.0 μm. Related to Figure 1 and Table 1.

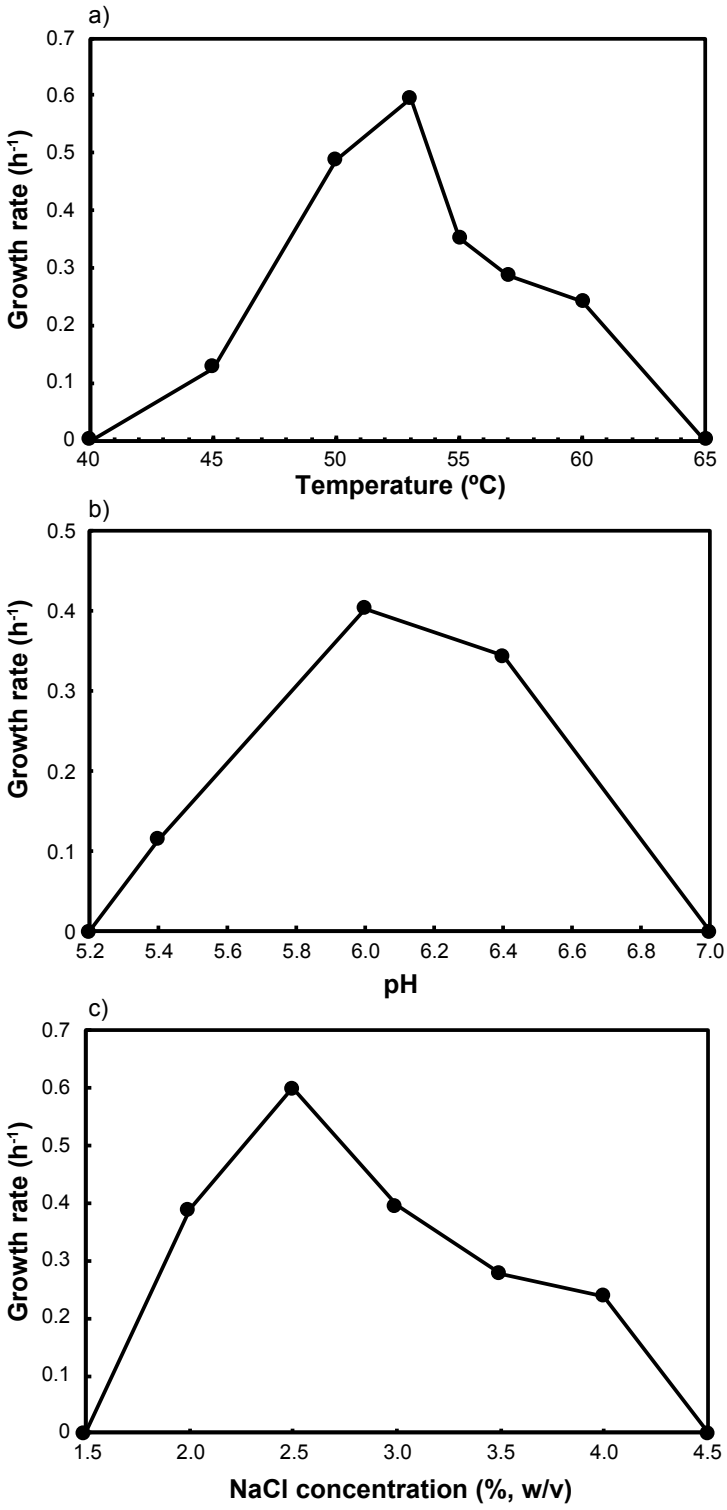


Figure S4. The effect of temperature (a), pH (b), and NaCl concentration (c) on growth of strain HRV44^{T}. Related to Table 1.

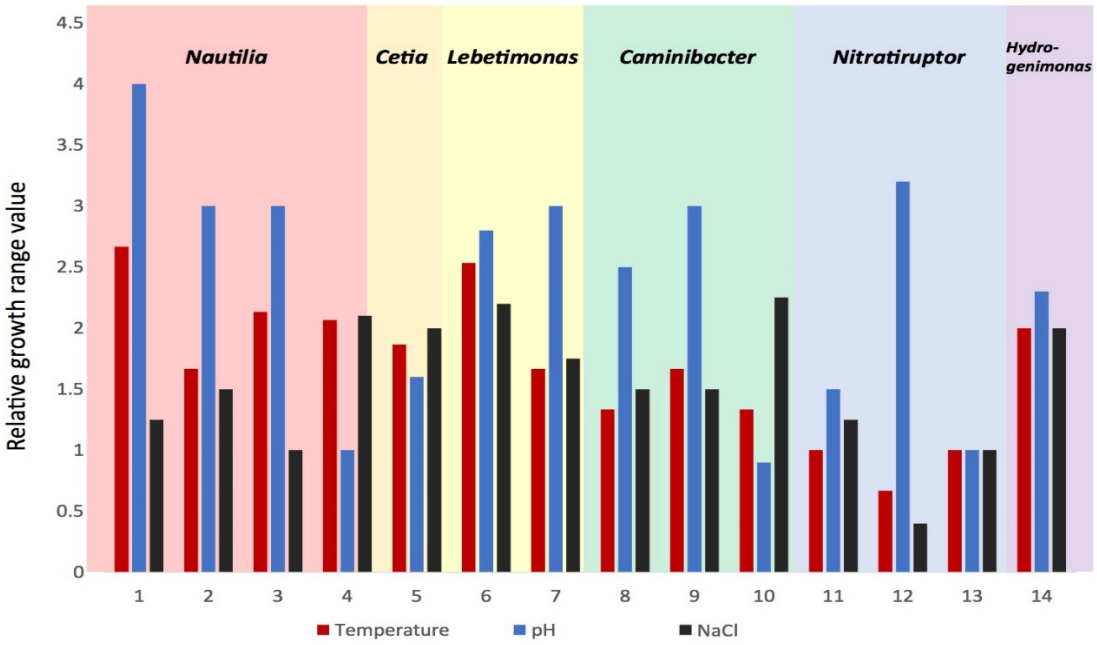


Figure S5. Comparison of growth range for temperature, pH and NaCl concentration among representative deep-sea thermophilic *Campylobacteria*. Red, blue and gray bars respectively represent growth ranges of temperature, pH and NaCl concentration of the thermophiles. The relative growth range values of thermophiles were calculated using the growth ranges of HRV44^T as a base of 1.0. Strains: 1, *Nautilia nitratireducens* MB-1^T (Pérez-Rodríguez et al., 2010); 2, *Nautilia profundicola* AmH^T (Smith et al., 2008); 3, *Nautilia abyssi* PH1209^T (Alain et al., 2009); 4, *Nautilia lithotrophica* 525^T (Miroshnichenko et al., 2002); 5, *Lebetimonas natsushimae* HS1857^T (Nagata *et al*., 2017); 6, *Lebetimonas acidiphila* Pd55^T (Takai et al., 2005); 7, *Cetia pacifica* TB-6^T (Grosche *et al*., 2015); 8, *Caminibacter hydrogeniphilus* AM1116^T (Alain et al., 2002); 9, *Caminibacter mediatlanticus* TB-2^T (Voordeckers et al., 2005); 10, *Caminibacter profundus* CR^T (Miroshnichenko et al., 2004); 11, *Nitratiruptor tergarcus* MI55-1^T

234 (Nakagawa *et al*., 2005); 12, *Nitratiruptor* sp. EPR55-1 (unpublished data); 13,

235 *Nitratiruptor labii* HRV44^T (in this study); 14, *Hydrogenimonas thermophila* EP1-55-

236 1%^{T} (Takai *et al*., 2004). Related to Table 1.

237

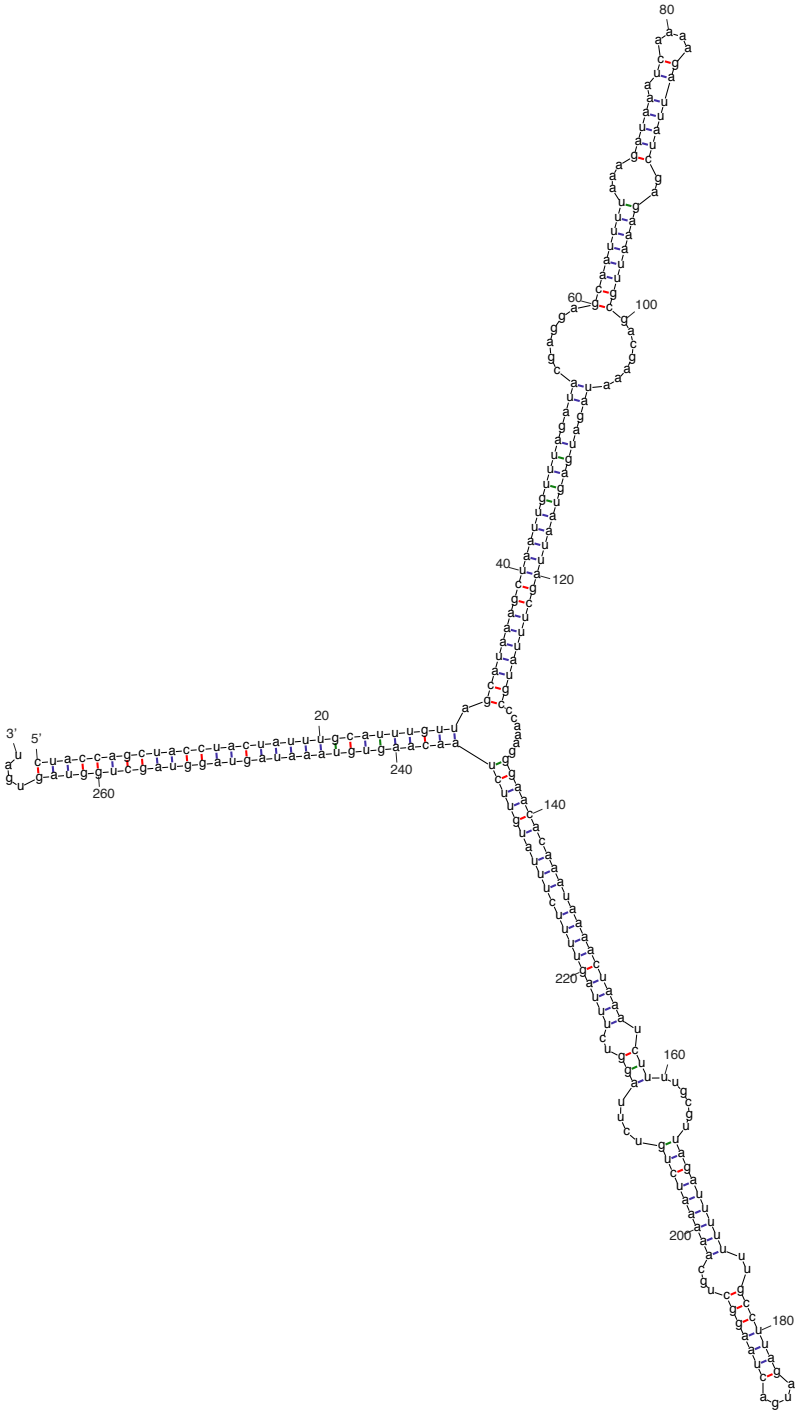
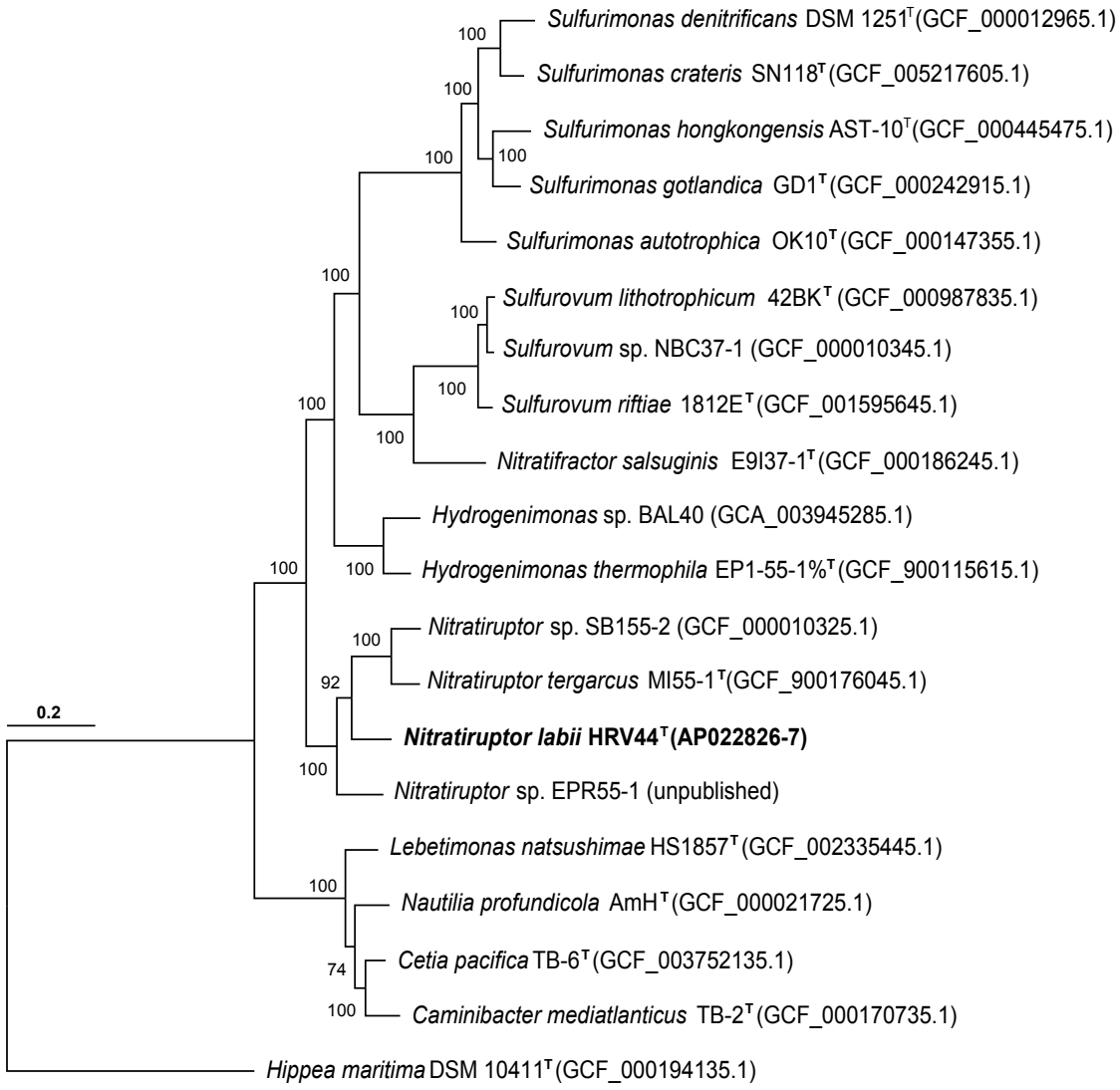


Figure S6. Predicted secondary structure of IS (268 bp) within the 16S rRNA gene of strain HRV44^{T}. Related Figure 1.



241

242 Figure S7. Phylogenomic relationship of representative *Campylobacteria* closely related
243 to strain HRV44^T. The phylogenomic tree was constructed using the maximum-likelihood
244 method based on 127 conserved protein amino acid sequences. Numbers at nodes
245 represent bootstrap values (%) (1,000 replicates). Related to Figure 1.

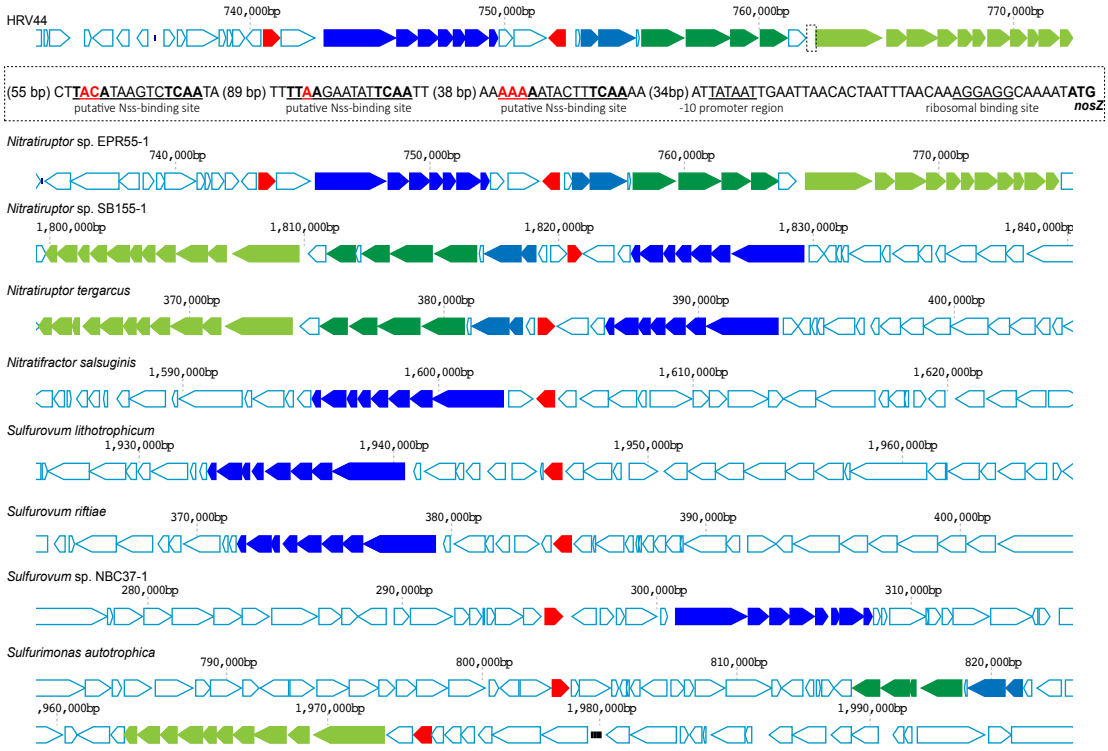


Figure S8. Localization of putative Nss-type transcription regulators in *nosZ*-possessing *Campylobacteria* isolated from deep-sea hydrothermal environments. The transcriptional regulators, *nap*, *nir*, *nor*, and *nos* gene clusters are colored in red, blue, green, light blue, and light green, respectively. Putative three Nss-binding sites upstream of *nosZ* in strain HRV44^T are underlined. Mismatches with the *Wolinella succinogenes* consensus binding sequence TTGA-N_6-TCAA are highlighted with red. Related Figure 3.

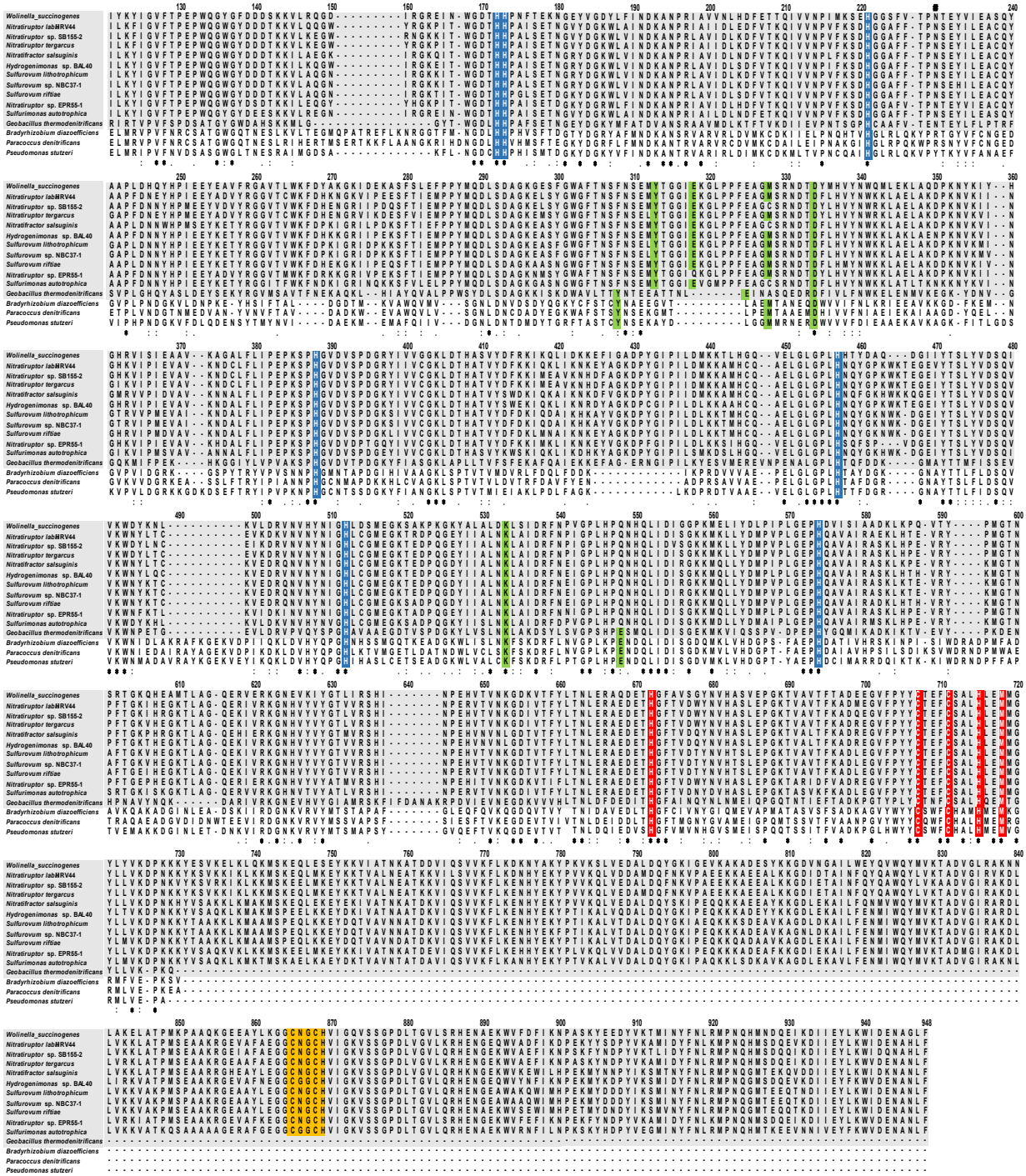


Figure S9. Primary structure alignment of NosZ from *Nitratiruptor* sp. HRV44^{T} and other bacteria possessing clade I or clade II NosZ. The conserved ligands of CuZ center, CuA center, calcium binding sites and heme c-binding motif are highlighted in blue, red, green and yellow, respectively. Related Figure 3.

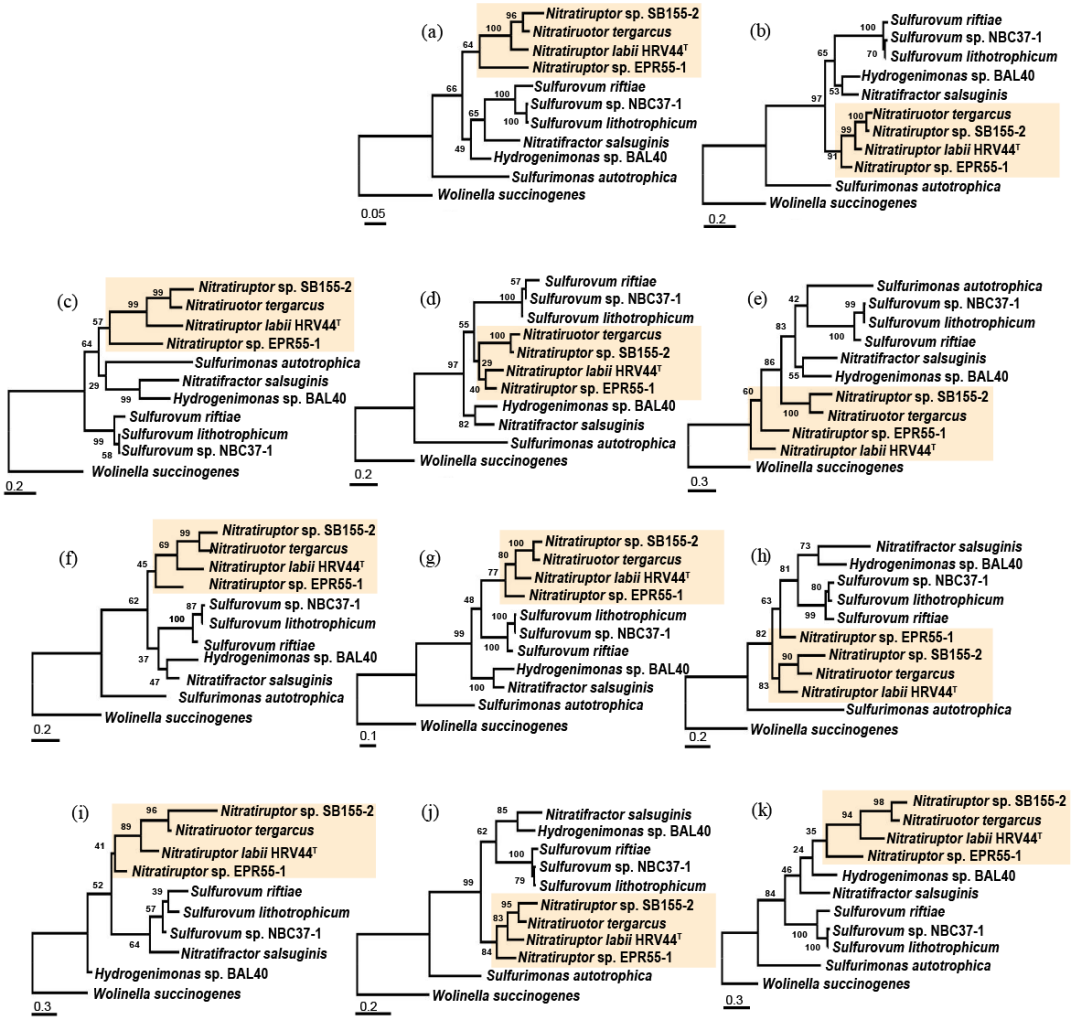


Figure S10. Phylogenetic trees based on each gene within the *nos* gene cluster of related *Campylobacteria*. (a)-(k) represent the phylogenies based on amino acid sequences of *nosZ*, *-B*, *-D*, *-G*, *-C1*, *-C2*, *-H*, *-F*, *-hyp.*, *-Y* and *-L* genes, respectively. *Nitratiruptor* strains are highlighted by yellow. Numbers at nodes represent bootstrap values (%) (1,000 replicates). Related to Figure 3.

Supplemental Tables

Table S1. General genomic characteristics among the genus *Nitratiruptor*. The accession numbers were described under the strain names. Related to Table 1.

	Nitratiruptor labii HRV44^T			*Nitratiruptor tergarcus* (GCF_900176045.1)	*Nitratiruptor* sp. SB155-2 (GCF_000010325.1)	*Nitratiruptor* sp. EPR55-1 (unpublished)
	Chromosome	Plasmid	Total	Draft genome	Chromosome	Chromosome
Size (bp)	1,990,315	102,672	2,092,987	1,929,433	1,877,931	1,807,889
GC content (%)	33.46	33.01	33.4	29.6†	39.7	37.7
No. of cording sequences	2050	128	2178	2020	1952	1797
No. of rRNA	9	0	9	9	9	9
No. of tRNA	41	0	41	39	45	41
No. of hypothetical genes	701	100	801	689	568	565
Hypothetical genes (%)	34.2	78.1	36.8	34.1	29.1	31.4

†The G + C content in mol% of DNA.

Table S3. Comparison of the differences of G+C content between chromosome and plasmid among deep-sea hydrothermal vent microorganisms. The accession numbers of genome sequences used for the comparison are represented in parentheses. Related to Table 1.

	Nitratiruptor labii HRV44^T (AP022826 and AP022827)	*Cetia pacifica* TB-6T (GCF_005083985.2)	*Persephonella marina* EX-H1^T (GCF_000021565.1)	*Deferribacter desulfuricans* SSM1^T (GCF_000010985.1)	*Oceanithermus profundus* 506^T (GCF_000183745.1)	*Marinitoga piezophila* KA3^T (GCF_000255135.1)	*Thermovibrio ammonificans* HB-1^T (GCF_000185805.1)
Chromosome G+C content (%)	33.46	34.3	37.16	31.12	70.02	29.16	52.11
Plasmid G+C content (%)	33.01	29.7	34.35	24.46	66.24	26.52	52.46
Difference in G+C content (%)	0.45	4.6	2.81	6.66	3.78	2.24	-0.35

References

Alain, K., Querellou, J., Lesongeur, F., Pignet, P., Crassous, P., Raguénès, G., Cueff, V. and Cambon-Bonavita, M.-A. (2002). *Caminibacter hydrogeniphilus* gen. nov., sp. nov., a novel thermophilic, hydrogen-oxidizing bacterium isolated from an East Pacific Rise hydrothermal vent. Int. J. Syst. Evol. Microbiol. 52, 1317–1323.

Alain, K., Callac, N., Guégan, M., Lesongeur, F., Crassous, P., Cambon-Bonavita, M., Querellou, J. and Prieur, D. (2009). *Nautilia abyssi* sp. nov., a thermophilic, chemolithoautotrophic, sulfur-reducing bacterium isolated from an East Pacific Rise hydrothermal vent. Int. J. Syst. Evol. Microbiol. 59, 1310–1315.

Aziz, R.K., Bartels, D., Best, A., DeJongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., *et al*. (2008). The RAST Server: rapid annotations using subsystems technology. BMC Genomics 9, 75.

Baross, J.A. (1995). Isolation, growth, and maintenance of hyperthermophiles. In Archaea: a Laboratory Manual. Thermophiles, F.T. Robb, ed. (Cold Spring Harbor Laboratory), pp. 15–23.

Darriba, D., Posada, D., Kozlov, A.M., Stamatakis, A., Morel, B., and Flouri, T. (2019). ModelTest-NG: a new and scalable tool for the selection of DNA and protein evolutionary models. Mol. Biol. Evol. 1–4.

Eren, A.M., Esen, O.C., Quince, C., Vineis, J.H., Morrison, H.G., Sogin, M.L., and Delmont, T.O. (2015). Anvi'o: an advanced analysis and visualization platformfor 'omics data. PeerJ 3, e1319.

Giovannelli, D., Chung, M., Staley, J., Starovoytov, V., Bris, N. Le, and Vetriani, C. (2016). *Sulfurovum riftiae* sp. nov., a mesophilic, thiosulfate-oxidizing, nitrate-reducing chemolithoautotrophic epsilonproteobacterium isolated from the tube of the deep-sea hydrothermal vent polychaete *Riftia pachyptila*. Int. J. Syst. Evol. Microbiol. 66, 2697–2701.

Grosche, A., Sekaran, H., Pérez-Rodríguez, I., Starovoytov, V., and Vetriani, C. (2015). *Cetia pacifica* gen. nov., sp. nov., a chemolithoautotrophic, thermophilic, nitrate-ammonifying bacterium from a deep-sea hydrothermal vent. Int. J. Syst. Evol. Microbiol. 65, 1144–1150.

Hunt, M., Silva, N. De, Otto, T.D., Parkhill, J., Keane, J.A., and Harris, S.R. (2015). Circlator: automated circularization of genome assemblies using long sequencing reads. Genome Biol. 16, 1–16.

Inagaki, F., Takai, K., Kobayashi, H., Nealson, K.H., and Horikoshi, K. (2003). *Sulfurimonas autrotrophica* gen. nov., sp. nov., a novel sulfur-oxidizing ε-proteobacterium isolated from hydrothermal sediments in the Mid-Okinawa Trough. Int. J. Syst. Evol. Microbiol. 53, 1801–1805.

Inagaki, F., Takai, K., Nealson, K.H., and Horikoshi, K. (2004). *Sulfurovum lithotrophicum* gen. nov., sp. nov., a novel sulfur-oxidizing chemolithoautotroph within the *ε-Proteobacteria* isolated from Okinawa Trough hydrothermal sediments. Int. J. Syst. Evol. Microbiol. 54, 1477–1482.

Kajitani, R., Toshimoto, K., Noguchi, H., Toyoda, A., Ogura, Y., Okuno, M., Yabana, M., Harada, M., Nagayasu, E., Maruyama, H., *et al*. (2014). Efficient de novo assembly of highly heterozygous genomes from whole-genome shotgun short reads. Genome Res. 24, 1384–1395.

Katoh, K., Rozewicki, J., and Yamada, K.D. (2017). MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Brief. Bioinform. 20, 1160–1166.

Koren, S., Walenz, P. B., Bwrlin, K., Miller, R. J., Bergman, H. N., and Phillippy, M. A. (2017). Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. Genome Res., 27, 722–736.

Kozlov, A.M., Darriba, D., Flouri, T., Morel, B., Stamatakis, A., and Wren, J. (2019). RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. Bioinformatics 35, 4453–4455.

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35, 1547–1549.

Lagesen, K., Hallin, P., Rødland, E.A., Stærfeldt, H.H., Rognes, T., and Ussery, D.W. (2007). RNAmmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Res. 35, 3100–3108.

Lane, D.J. (1991). 16S/23S rRNA sequencing. In Nucleic Acid Techniques in Bacterial Systematic, E. Stackebrandt and M. Goodfellow, ed. (John Wiley and Sons), pp. 115-175.

341 Lowe, T.M. and Chan, P.P. (2016). tRNAscan-SE On-line: integrating search and context
342 for analysis of transfer RNA genes. Nucleic Acids Res. 44, W54–W57.
343 Meier-Kolthoff, J.P., Auch, A.F., Klenk, H.P., and Göker, M. (2013). Genome sequence-
344 based species delimitation with confidence intervals and improved distance functions.
345 BMC Bioinf. 14, 60.
346 Miroshnichenko, M.L., Kostrikina, N.A., L'Haridon, S., Jeanthon, C., Hippe,
347 H.,Stackebrandt, E. and Bonch-Osmolovskaya, E.A. (2002). *Nautilia lithotrophica*
348 gen. nov., sp. nov., a thermophilic sulfur-reducing ε-proteobacterium isolated from a
349 deep-sea hydrothermal vent. Int. J. Syst. Evol. Microbiol. 52, 1299–1304.
350 Miroshnichenko, M.L., L'Haridon, S., Schumann, P., Spring, S., Bonch-Osmolovskaya,
351 E.A., Jeanthon, C. and Stackebrandt, E. (2004). *Caminibacter profundus* sp. nov., a
352 novel thermophile of *Nautiliales* ord. nov. within the class '*Epsilonproteobacteria*',
353 isolated from a deep-sea hydrothermal vent. Int. J. Syst. Evol. Microbiol. 54, 41–45.
354 Mino, S., Yoneyama, N., Nakagawa, S., Takai, K., and Sawabe, T. (2018). Enrichment
355 and genomic characterization of a N_2O-reducing chemolithoautotroph from a deep-sea
356 hydrothermal vent. Front. Bioeng. Biotechnol. 6, 1–8.
357 Nagata, R., Takaki, Y., Tame, A., Nunoura, T., Muto, H., Mino, S., Sawayama, S., Takai,
358 K., and Nakagawa, S. (2017). *Lebetimonas natsushimae* sp. nov., a novel strictly
359 anaerobic, moderately thermophilic chemoautotroph isolated from a deep-sea
360 hydrothermal vent polychaete nest in the Mid-Okinawa Trough. Syst. Appl. Microbiol.
361 40, 352–356.
362 Nakagawa, S., Takai, K., Inagaki, F., Horikoshi, K., and Sako, Y. (2005). *Nitratiruptor*
363 *tergarcus* gen. nov., sp. nov. and *Nitratifractor salsuginis* gen. nov., sp. nov., nitrate-
364 reducing chemolithoautotrophs of the *ε-Proteobacteria* isolated from a deep-sea
365 hydrothermal system in the Mid-Okinawa Trough. Int. J. Syst. Evol. Microbiol. 55,
366 925–933.
367 Nakagawa, S., Takaki, Y., Shimamura, S., Reysenbach, A., Takai, K., and Horikoshi, K.
368 (2007). Deep-sea vent ε-proteobacterial genomes provide insights into emergence of
369 pathogens. 104, 12146–12150.
370 Pérez-Rodríguez, I., Ricci, J., Voordeckers, J.W., Starovoytov, V., and Vetriani, C.
371 (2010). *Nautilia nitratireducens* sp. nov., a thermophilic, anaerobic, chemosynthetic,
372 nitrate-ammonifying bacterium isolated from a deep-sea hydrothermal vent. Int. J.
373 Syst. Evol. Microbiol. 60, 1182–1186.

374 Porter, K. G. and Feig, Y. S. (1980). The use of DAPI for identifying aquatic microfloral.
375 Limnol. Oceanogr. 25, 943–948.
376 Pruesse, E., Peplies, J., and Glöckner, F.O. (2012). SINA: accurate high-throughput
377 multiple sequence alignment of ribosomal RNA genes. Bioinformatics 28, 1823–1829.
378 Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and
379 Glöckner, F.O. (2013). The SILVA ribosomal RNA gene database project: improved
380 data processing and web-based tools. Nucleic Acids Res. 41, 590–596.
381 Rodriguez-R, L.M. and Konstantinidis, K.T. (2014). Bypassing cultivation to identify
382 bacterial species. Microbe 9, 111–118.
383 Sako, Y., Takai, K., Ishida, Y., Uchida, A., and Katayama, Y. (1996). *Rhodothermus*
384 *obamensis* sp. nov., a modern lineage of extremely thermophilic marine bacteria. Int.
385 J. Syst. Bacteriol. 46, 1099-1104.
386 Smith, J.L., Campbell, B.J., Hanson, T.E., Zhang, C.L., and Cary, S.C. (2008). *Nautilia*
387 *profundicola* sp. nov., a thermophilic, sulfur-reducing epsilonproteobacterium from
388 deep-sea hydrothermal vents. Int. J. Syst. Evol. Microbiol. 58, 1598–1602.
389 Takai, K., Nealson, K.H., and Horikoshi, K. (2004). *Hydrogenimonas thermophila* gen.
390 nov., sp. nov., a novel thermophilic, hydrogen-oxidizing chemolithoautotroph within
391 the *ε-Proteobacteria*, isolated from a black smoker in a Central Indian Ridge
392 hydrothermal field. Int. J. Syst. Evol. Microbiol. 54, 25–32.
393 Takai, K., Hirayama, H., Nakagawa, T., Suzuki, Y., Nealson, K.H. and Horikoshi, K.
394 (2005). *Lebetimonas acidiphila* gen. nov., sp. nov., a novel thermophilic, acidophilic,
395 hydrogen-oxidizing chemolithoautotroph within the '*Epsilonproteobacteria*', isolated
396 from a deep-sea hydrothermal fumarole in the Mariana Arc. Int. J. Syst. Evol.
397 Microbiol. 55, 183–189.
398 Voordeckers, J.W., Starovoytov, V., and Vetriani, C. (2005). *Caminibacter*
399 *mediatlanticus* sp. nov., a thermophilic, chemolithoautotrophic, nitrate-ammonifying
400 bacterium isolated from a deep-sea hydrothermal vent on the Mid-Atlantic Ridge. Int.
401 J. Syst. Evol. Microbiol. 55, 773–779.
402 Walker, B.J., Abeel, T., Shea, T., Priest, M., Abouelliel, A., Sakthikumar, S., Cuomo,
403 C.A., Zeng, Q., Wortman, J., Young, S.K., *et al*. (2014). Pilon: an integrated tool for
404 comprehensive microbial variant detection and genome assembly improvement. PLoS
405 One 9, 1–15.

406 Wick, R.R., Judd, L.M., and Holt, K.E. (2018). Deepbinner: demultiplexing barcoded
407 Oxford Nanopore reads with deep convolutional neural networks. PLoS Comput. Biol.
408 14, 1–11.
409 Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction.
410 Nucleic Acids Res. 31, 3406–3415.
411
412