Genomic and proteomic study of dissimilatory sulfur metabolism

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CONTENTS

Abstract

Sulfur-oxidizing bacteria, sulfate-reducing bacteria and sulfur-disproportionating bacteria utilize inorganic sulfur compounds in a dissimilatory process. These bacteria are important players in the sulfur cycle. Although various bacteria of these functional groups have been isolated, only a few bacteria performing dissimilatory sulfur metabolism have been investigated in detail. In this study, genomic and proteomic analyses were performed using bateria belonging to poorly understood groups of bacteria with dissimilatory sulfur metabolism.

Sulfurifustis variabilis skN76^{T} and *Sulfuricaulis limicola* HA5^{T} are sulfur-oxidizing bacteria belonging to the order *Acidiferrobacterales*, which was established in 2015. In this study, genomic analysis revealed that the important genes involved in dissimilatory sulfur metabolism and carbon fixation are redundant in the genomes of these bacteria.

In addition, an enrichment culture that consisted of almost one species of a novel thiosulfate-disproportionating bacterium belonging to the phylum *Nitrospirae* was established from a biomat of a hot spring. This thiosulfate-disproportinating bacterium was not able to grow with sulfate reduction, and it became clear that the gene sequences related to this thiosulfate-disproportinating bacterium had been previously identified by sequencing in various freshwater environments. The draft genome sequence of the thiosulfate-disproportinating bacterium was obtained by genome sequencing and analysis of the genome reveals that the bacterium possesses the complete set of genes for sulfate reduction.

Some sulfur-disproportionating bacteria belonging to the phyla *Proteobacteria* and *Thermodesulfobacteria* are also incapable of growing with sulfate reduction in spite of possessing all the genes required for sulfate reduction. In order to reveal differences in pathway for sulfur disproportionation and sulfate reduction, comparative genomic analysis was performed using 92 genomes. A relatively specific gene cluster was found in the genomes of

sulfur-disproportionating bacteria without the ability to grow by sulfate reduction, which was not found in the genomes of many sulfate-reducing bacteria. Some proteins encoded by this gene cluster were suggested to be involved in sulfur relay and transport. All proteins encoded by this gene cluster were expressed at high abundance in the cells under growth conditions of thiosulfate and elemental sulfur disproportionation in *Caldimicrobium thiodismutans* TF1^{T} belonging to the phylum *Thermodesulfobacteria.* In addition, all proteins involved in sulfate reduction were also expressed in both culture conditions. These results suggest a possibility that is that proteins encoded by relatively specific gene cluster transfer sulfur substrates to proteins needed to reduce sulfate in sulfur disproportionation.

Chapter 1

General introduction

1. General introduction

Sulfur as a chemical element takes several oxidation states from –II to +VI. In environments, various sulfur compounds are present such as sulfate (+VI), sulfite (+IV), thiosulfate (0, +IV), sulfide (-II) and elemental sulfur (0). There are two processes in the metabolism of living organisms: assimilation and dissimilation. Sulfur is incorporated into amino acids such as cysteine and methionine, vitamins such as thiamine and biotin and iron-sulfur cluster in enzymes by assimilation. Interconversions of these sulfur compounds occur through dissimilatory pathways by prokaryotes including sulfur-oxidizing bacteria, sulfate-reducing bacteria and sulfur-disproportionating bacteria.

Sulfur-oxidizing bacteria generally obtain energy by oxidizing sulfur compounds, electron donors, to sulfate. In contrast, sulfate-reducing bacteria utilize sulfate as an electron acceptor and reduce it to sulfide. Sulfate-reducing bacteria use hydrogen, lactate and other oraganic compounds as an electron donor. Sulfur-disproportionating bacteria grow with inorganic sulfur compounds as a sole energy source. In sulfur disproportionation, sulfide and sulfate are generated from a single inorganic sulfur compound, such as elemental sulfur, thiosulfate or sulfite. Sulfur-disproportionating bacteria are generally closely related to sulfate-reducing bacteria phylogenetically. However, to date, fewer sulfur-disproportionating bacteria have been reported compared to sulfur-oxidizing bacteria and sulfate-reducing bacteria.

Numerous sulfur oxidizers and sulfate reducers have been isolated from a variety of environments. However, to date, there are limited studies on the dissimilatory sulfur metabolic pathway. In this study, dissimilatory sulfur metabolism was investigated by genomic and proteomic analyses of bacteria belonging to groups that are suggested to be important for sulfur cycle in environments.

In chapter 2, the complete genome sequences of *Sulfurifustis variabilis* skN76^{T} and

Sulfuricaulis limicola HA5^T are reported. These sulfur-oxidizing bacteria belong to the family *Acidiferrobacteraceae*, and bacteria belonging to this family are thought to be present abundantly in various freshwater lake sediments and coastal sediments. However, the metabolic pathway of these bacteria was not clear. In order to reveal the metabolic pathway and shed light on why bacteria belonging to this family are abundantly found, characteristics of the genomes were investigated.

In Chapter 3, an enrichment culture containing a novel sulfur-disproportionating bacterium belonging to the phylum *Nitrospirae* was established from a biomat of a hot spring, followed by whole genome sequencing for further analysis. In Chapter 4, a comparative genomic analysis of sulfur-dispropotionating bacteria and sulfate-reducing bacteria was performed in order to identify genes specific to each bacterial type. Additionally, expressed proteins were identified under growth conditions of thiosulfate and elemental sulfur disproportionation in *Caldimicrobium thiodismutans* TF1^T.

Chapter 2

The complete genome sequences of sulfur-oxidizing Gammaproteobacteria *Sulfurifustis variabilis* skN76^{T} and *Sulfuricaulis limicola* HA5^{T}

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2.1. Abstract

Sulfurifustis variabilis and *Sulfuricaulis limicola* are autotrophic sulfur-oxidizing bacteria belonging to the family *Acidiferrobacteraceae* in the order *Acidiferrobacterales*. The type strains of these species, strain skN76^T and strain HA5^T, were isolated from lakes in Japan. Here we describe the complete genome sequences of *Sulfurifustis variabilis* skN76^T and *Sulfuricaulis limicola* HA5^T. The genome of *Sulfurifustis variabilis* skN76^T consists of one circular chromosome with size of 4.0 Mbp including 3864 protein-coding sequences. The genome of *Sulfuricaulis limicola* HA5^T is 2.9 Mbp chromosome with 2763 protein-coding sequences. In both genomes, 46 transfer RNA-coding genes and one ribosomal RNA operon were identified. In the genomes, redundancies of the genes involved in sulfur oxidation and inorganic carbon fixation pathways were observed. This is the first report to show the complete genome sequences of bacteria belonging to the order *Acidiferrobacterales* in the class *Gammaproteobacteria*.

2.2. Introduction

Sulfurifustis variabilis skN76^T and *Sulfuricaulis limicola* HA5^T are gammaproteobacterial sulfur-oxidizing bacteria isolated from sediments of Lake Mizugaki and Lake Harutori, respectively (1, 2). They both belong to the family *Acidiferrobacteraceae* in the order *Acidiferrobacterales*. In this order, only three species have been isolated in pure culture. They are all chemolithoautotrophs and can grow by oxidation of inorganic sulfur compounds. *Sulfurifustis variabilis* and *Sulfuricaulis limicola* are neutrophilic, whereas the other species, *Acidiferrobacter thiooxydans*, is acidophilic (3). Taxonomy of *Acidiferrobacter thiooxydans* has been revised several times, and the family *Acidiferrobacteraceae* and order

Acidiferrobacterales were recently established to accommodate the species (1, 3–5). The members of the family *Acidiferrobacteraceae* have been frequently detected in various environments as gene sequences (2, 3, 6). Here we show the complete genome sequences of *Sulfurifustis variabilis* skN76^T and *Sulfuricaulis limicola* HA5^T as the first genomes of the order *Acidiferrobacterales*.

2.3. Organism information

2.3.1. Classification and features

The cells of *Sulfurifustis variabilis* skN76^T are rodshaped or filamentous form with varying length, and 0.3–0.5 μm in width (Fig. 1a, Table 1). The cells of *Sulfuricaulis limicola* HA5^T are rodshaped, 1.2–6.0 μm in length and 0.3–0.5 μm in width (Fig. 1b, Table 1). They are both Gram-stain-negative. *Sulfurifustis variabilis* and *Sulfuricaulis limicola* belong to the family *Acidiferrobacteraceae* within the class *Gammaproteobacteria* (Fig. 2). They both utilized thiosulfate, tetrathionate and elemental sulfur as electron donors for chemolithoautotrophic growth under aerobic conditions (1, 2).

2.4. Genome sequencing information

2.4.1. Genome project history

Sulfurifustis variabillis skN76^T and *Sulfuricaulis limicola* HA5^T were selected for sequencing as representatives of sulfur-oxidizing bacteria belonging to the order *Acidiferrobacterales*, to reveal characteristics of their genomes. A summary of the project information is shown in Table 2.

2.4.2. Growth conditions and genomic DNA preparation

Sulfurifustis variabilis skN76^T and *Sulfuricaulis limicola* HA5^T were grown with 20 mM thiosulfate as an energy source in a bicarbonate-buffered medium previously described (1), at 45 and 28 °C, respectively. Genomic DNA samples were prepared by using Wizard® genomic DNA purification kit (Promega, Madison, WI, USA) from approximately 0.2 ml (skN76) or 0.1 ml (HA5) of cell pellets. Amounts of the obtained DNA assessed by spectrophotometry were *ca*. 270 μg (skN76) and 90 μg (HA5) respectively, and the UV absorption ratio of 260/ 280 nm was greater than 1.8 in both samples.

2.4.3. Genome sequencing and assembly

The genomic DNA was sheared into approximately 20 kb using g-TUBE (Covaris, Inc., Woburn, MA, USA). The SMRTbell™ templates were prepared from the fragments using SMRTbell™ Template Prep Kit 1.0 (Pacific Biosciences, Menlo Park, CA, USA). The size-selected libraries for sequencing were prepared by using BluePippin (Sage Science, Baverly, MA, USA). The libraries were sequenced on a PacBio RS II instrument (Pacific Biosciences) with P6-C4 chemistry (for *Sulfurifustis variabillis* skN76^T) or P5-C3 chemistry (for *Sulfuricaulis limicola* HA5^T). *De novo* assembly was performed by using RS_HGAP Assembly.3 (for *Sulfurifustis variabillis* skN76^T) or RS_HGAP Assembly.2 (for *Sulfuricaulis limicola* HA5^T), implemented within the SMRT Analysis v2.3 (Pacific Biosciences) software environment. By assembling 79,017 subreads (837,333,548 bp) of *Sulfurifustis variabillis* skN76^T, two contigs with the lengths of *ca*. 4.0 Mbp and *ca*. 5.4 kbp were obtained. The shorter one was identical to a partial sequence of the larger one, and a circular chromosome was manually constructed from the larger contig by finding self-overlapping regions using the *in silico* Molecular Cloning (R) Genomic Edition (In Silico Biology, Inc., Yokohama, Japan) application. As for *Sulfuricaulis limicola* HA5^T, a single contig (*ca*. 2.9 Mbp) was obtained by

assembling 61,565 subreads (409,124,339 bp), and circular chromosome was manually constructed in the same manner.

2.4.4. Genome annotation

The genomes were annotated automatically using the Microbial Genome Annotation Pipeline (7). Further manual annotation of the predicted protein-coding sequences was performed on the basis of BLASTP searches against the NCBI nonredundant database. CDSs were annotated as hypothetical protein-coding genes when they met any of the following four criteria in the top hit of the BLASTP analysis: (i) E-value >1e-8, (ii) length coverage <60 % against query sequence (iii) sequence identity <30 % or (iv) function of the hit was unidentified. The WebMGA server was used to assign the genes to Clusters of Ortholog Groups and Protein family domains (8–11). The Phobius server was used to predict signal peptides and transmembrane helices (12). Clustered Regularly Interspaced Short Palindromic Repeat loci were detected using CRISPRfinder (13).

2.5. Genome properties

The basic statistics of the genomes are shown in Table 3. Both genomes contained 46 tRNA genes and one rRNA operon. The genome size of *Sulfurifustis variabillis* skN76^T was approximately 1.4 times larger than that of *Sulfuricaulis limicola* HA5^T. CRISPR loci were found only in the genome of *Sulfurifustis variabillis* skN76^T (Table 3). The distribution of genes into COGs functional categories is presented in Table 4.

2.6. Insights from the genome sequences

In both the genomes of *Sulfurifustis variabillis* skN76^T and *Sulfuricaulis limicola* HA5^T, genes involved in the sulfur oxidation pathway were identified. The genomes of both strains contain genes of the DSR system related to the oxidation of elemental sulfur to sulfite (14, 15). They contain a *dsr* gene cluster of identical composition, *dsrABEFHCMKLJOPNR* (SVA_1954-1967, SCL_1274-1261). There are some *dsr* genes outside of the gene cluster, *dsrAB* (SVA_0258-0259, SCL_0256-0257), *dsrS* (SVA_2921, SCL_0781) and *dsrC* (SVA_0281, SVA_0284, SVA_0358, SVA_0917, SVA_0969, SVA_1205, SVA_1793, SVA_1949, SVA_2832, SVA_3655; SCL_0275, SCL_0524, SCL_0785, SCL_1279, SCL_1423, SCL_2646).

As genes encoding proteins involved in oxidation of sulfite to sulfate in the cytoplasm, both genomes contain two copies of the *aprAB* genes encoding an adenosine-5'-phosphosulphate reductase (SVA_2607-2608, SVA_3565-3564; SCL_0600-0601, SCL_2474-2473), along with the *sat* gene encoding a sulfate adenylyltransferase (SVA_3563, SCL_2472) and the *aprM* gene (SVA_2609, SCL_0602). In addition, the genome of *Sulfuricaulis limicola* HA5^T contains the *hdrAACB* genes encoding a Hdr (SCL_2523-2520), but that of *Sulfurifustis valiabilis* skN76^T does not. The AprM and Hdr complex are thought to have similar function that interacts with the adenosine-5'-phosphosulphate reductase (16–18). The genomes also contain the *soeABC* genes (SVA_2734, SVA_2736-2737; SCL_0523-0521), encoding a membrane-bound polysulfide reductase-like iron-sulfur molybdoprotein, which is suspected to be involved in sulfite oxidation in the cytoplasm (19). Further, the genome of *Sulfurifustis valiabilis* skN76^T contains the *sorAB* genes (SVA_1391-1390) related to the direct oxidation of sulfite to sulfate in the periplasm (20).

For thiosulfate oxidation, both genomes contain the *soxXYZAB* gene cluster (SVA_2999-3003, SCL_2229-2233). Although sulfide oxidation by these bacteria has not been demonstrated, genes related to sulfide oxidation were identified; the *fccAB* (*soxEF*) genes encoding a flavocytochrome *c*/sulfide dehydrogenase (SVA_0067-0066, SVA_3594-3595;

SCL_0078-0077) and the *sqr* gene encoding a sulfide:quinone oxidoreductase (SVA_1781, SVA_2675, SVA3205).

Sulfurifustis variabillis skN76^T and *Sulfuricaulis limicola* HA5^T are autotrophic bacteria. They both have two copies of the *rbcL* and *rbcS* genes, encoding large and small subunits of ribulose bisphosphate carboxylase/oxygenase (SVA_3460-3459, SVA_3471-3470; SCL_2417-2416, SCL_2425-2424), which is the key enzyme in the Calvin-Benson-Bassham cycle to catalyze inorganic carbon fixation. The two copies of RuBisCO in each genome are phylogenetically distinct, and belong to lineages referred to as green-like form IA and red-like form IC (Fig. 3) (21). In the form IC RuBisCO coded by *rbcL* gene (SVA_3460, SCL_2417), *Sulfurifustis variabillis* skN76^T and *Sulfuricaulis limicola* HA5^T have six-amino-acid inserts at the same position where a similar insert was reported from *Nitrosospira* sp. 40KI (22). There are two other RuBisCO sequences which have six-amino-acid inserts at the same position, and these sequences with inserts formed a monophyletic cluster in the tree of RuBisCO (Fig. 3). In general, RuBisCO of form IA and IC have different properties which are thought to be advantageous to fix inorganic carbon under different concentrations of carbon dioxide and/or oxygen (21). Possession of the genes for these two distinct RuBisCO forms may be beneficial to cope with changing environmental conditions, or to thrive in various types of ecosystems.

2.7. Conclusion

This is the first report on complete genome sequences of bacteria belonging to the order *Acidiferrobacterales*. The genome analysis of *Sulfurifustis variabillis* skN76^T and *Sulfuricaulis limicola* HA5^T revealed that they have similar sets of genes involved in sulfur oxidation pathways. In the both genomes, redundancies of the genes for sulfur oxidation and inorganic carbon fixation were observed, as represented by multiple copies of *dsrAB*, *aprAB*

and *rbcLS*. Such redundancies may provide physiological flexibility to the chemolithotrophic sulfur oxidizers which are fully depending on these functions to obtain energy and carbon source for growth.

2.8. References

1. Kojima H, Shinohara A, Fukui M. *Sulfurifustis variabilis* gen. nov., sp. nov., a sulfur oxidizer isolated from a lake, and proposal of *Acidiferrobacteraceae* fam. nov. and *Acidiferrobacterales* ord. nov. Int J Syst Evol Microbiol. 2015;65:3709-13.
2. Kojima H, Watanabe T, Fukui M. *Sulfuricaulis limicola* gen. nov., sp. nov., a sulfur oxidizer isolated from a lake. Int J Syst Evol Microbiol. 2016;66:266-70.
3. Hallberg KB, Hedrich S, Johnson DB. *Acidiferrobacter thiooxydans*, gen. nov. sp. nov.; an acidophilic, thermo-tolerant, facultatively anaerobic iron- and sulfur-oxidizer of the family *Ectothiorhodospiraceae*. Extremophiles. 2011;15:271-9.
4. Kelly DP, Wood AP. Reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. nov., *Halothiobacillus* gen. nov. and *Thermithiobacillus* gen. nov. Int J Syst Evol Microbiol. 2000;50:511-6.
5. Williams KP, Kelly DP. Proposal for a new class within the phylum *Proteobacteria*, *Acidithiobacillia* classis nov., with the type order *Acidithiobacillales*, and emended description of the class *Gammaproteobacteria*. Int J Syst Evol Microbiol. 2013;63:2901-6.
6. Dyksma S, Bischof K, Fuchs BM, Hoffmann K, Meier D, Meyerdierks A, et al. Ubiquitous *Gammaproteobacteria* dominate dark carbon fixation in coastal sediments. ISME J. 2016;10(8):1–15.
7. Sugawara H, Ohyama A, Mori H, Kurokawa K. Microbial Genome Annotation Pipeline (MiGAP) for diverse users. The 20th International Conference on Genome Informatics (GIW2009) Poster and Software Demonstrations (Yokohama). 2009;S001-1-2.
8. Wu S, Zhu Z, Fu L, Niu B, Li W. WebMGA: a customizable web server for fast metagenomic sequence analysis. BMC Genomics. 2011;12:444.
9. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403-10.

10. Eddy SR. Profile hidden Markov models. Bioinformatics. 1998;14(9):755-63.

11. Finn RD, Mistry J, Tate J, Coggill P, Heger A, Pollington JE, et al. The Pfam protein families database. Nucleic Acids Res. 2010;38:D211-22.

12. Kall L, Krogh A, Sonnhammer ELL. Advantages of combined transmembrane topology and signal peptide prediction-the Phobius web server. Nucleic Acids Res. 2007;35:W429-32.

13. Grissa I, Vergnaud G, Pourcel C. CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats. Nucleic Acids Res. 2007;35:W52-7.

14. Pott AS, Dahl C. Sirohaem sulfite reductase and other proteins encoded by genes at the *dsr* locus of *Chromatium vinosum* are involved in the oxidation of intracellular sulfur. Microbiology. 1998;144:1881-94.

15. Dahl C, Engels S, Pott-Sperling AS, Schulte A, Sander J, Lübbe Y, et al. Novel genes of the *dsr* gene cluster and evidence for close interaction of Dsr proteins during sulfur oxidation in the phototrophic sulfur bacterium *Allochromatium vinosum*. J Bacteriol. 2005;187:1392-404.

16. Parey K, Demmer U, Warkentin E, Wynen A, Ermler U, Dahl C. Structural, biochemical and genetic characterization of dissimilatory ATP sulfurylase from *Allochromatium vinosum*. PLoS One. 2013;8:e7407.

17. Pires RH, Lourenço AI, Morais F, Teixeira M, Xavier AV, Saraiva LM, et al. A novel membrane-bound respiratory complex from *Desulfovibrio desulfuricans* ATCC 27774. Biochim Biophys Acta. 2003;1605:67-82.

18. Meyer B, Kuever J. Molecular analysis of the distribution and phylogeny of dissimilatory adenosine-5'-phosphosulfate reductase-encoding genes (*aprBA*) among sulfur-oxidizing prokaryotes. Microbiology. 2007;153:3478-98.

19. Dahl C, Franz B, Hensen D, Kesselheim A, Zigann R. Sulfite oxidation in the purple sulfur

bacterium *Allochromatium vinosum*: identification of SoeABC as a major player and relevance of SoxYZ in the process. Microbiology. 2013;159:2626-38.

20. Kappler U, Bennett B, Rethmeier J, Schwarz G, Deutzmann R, McEwan AG, et al. Sulfite:cytochrome *c* oxidoreductase from *Thiobacillus novellus* — purification, characterization and molecular biology of a heterodimeric member of the sulfite oxidase family. J Biol Chem. 2000;275:13202-12.
21. Badger MR, Bek EJ. Multiple Rubisco forms in proteobacteria: their functional significance in relation to CO_2 acquisition by the CBB cycle. J Exp Bot. 2008;59:1525-41.
22. Utåker JB, Andersen K, Aakra Å, Moen B, Nes IF. Phylogeny and functional expression of ribulose 1,5-Bisphosphate carboxylase/oxygenase from the autotrophic ammonia-oxidizing bacterium *Nitrosospira* sp. Isolate 40KI. J Bacteriol. 2002;184:468-78.
23. Woese CR, Kandler O, Wheelis ML. Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria, and Eucarya. Proc Natl Acad Sci U S A. 1990;87:4576-9.
24. Garrity GM, Bell JA, Lilburn T. Phylum XIV. *Proteobacteria* phyl. nov. In: Garrity GM, Brenner DJ, Krieg NR, Staley JT, editors. Bergey's Manual of Systematic Bacteriology, Volume 2, Part B. 2nd ed. New York: Springer; 2005. p. 1.
25. Garrity GM, Bell JA, Lilburn T. Class III. *Gammaproteobacteria* class. nov. In: Garrity GM, Brenner DJ, Krieg NR, Staley JT, editors. Bergey's Manual of Systematic Bacteriology, Volume 2, Part B. 2nd ed. New York: Springer; 2005. p. 1.
26. Kojima H, Iwata T, Fukui M. DNA-based analysis of planktonic methanotrophs in a stratified lake. Freshw Biol. 2009;54:1501-9.
27. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30:2725-9.

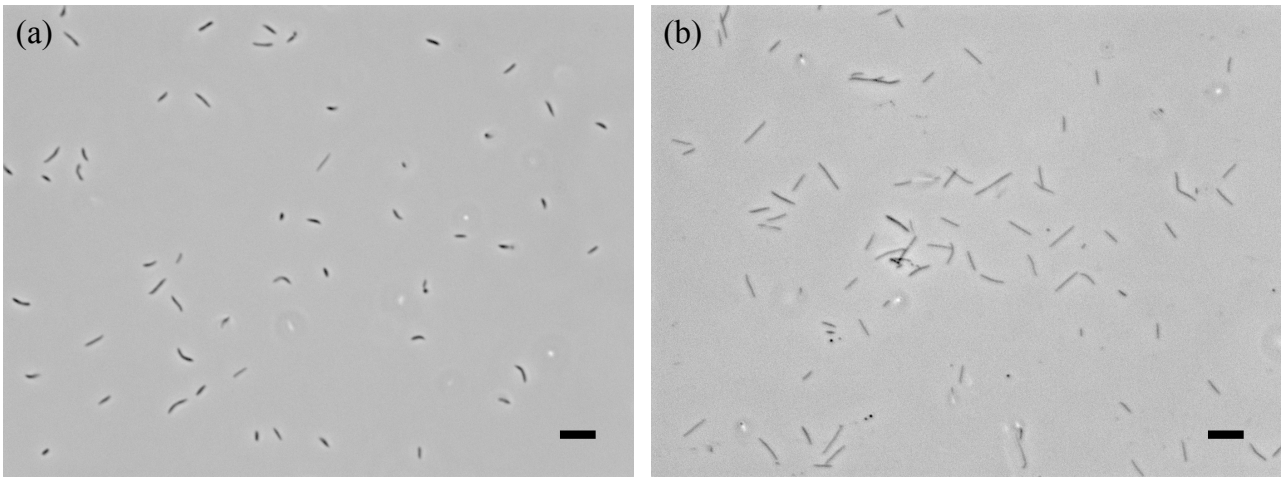


Figure 2-1 Phase-contrast micrographs of *Sulfurifustis variabilis* skN76^{T} (a) and *Sulfuricaulis limicola* HA5^{T} (b), grown with thiosulfate at 45 and 28 °C, respectively. Bars, 5 μm

Table 2-1 Classification and general features of *Sulfurifustis variabilis* skN76^T and *Sulfuricaulis limicola* HA5^T according to MIGS recommendations.

		Sulfurifustis variabilis skN76^T		*Sulfuricaulis limicola* HA5^T	
MIGS ID	Property	Term	Evidence code[a]	Term	Evidence code[a]
	Classification	Domain *Bacteria*	TAS [23]	Domain *Bacteria*	TAS [23]
		Phylum *Proteobacteria*	TAS [24]	Phylum *Proteobacteria*	TAS [24]
		Class *Gammaproteobacteria*	TAS [25]	Class *Gammaproteobacteria*	TAS [25]
		Order *Acidiferrobacterales*	TAS [1]	Order *Acidiferrobacterales*	TAS [1]
		Family *Acidiferrobacteraceae*	TAS [1]	Family *Acidiferrobacteraceae*	TAS [1]
		Genus *Sulfurifustis*	TAS [1]	Genus *Sulfuricaulis*	TAS [2]
		Species *Sulfurifustis variabilis*	TAS [1]	Species *Sulfuricaulis limicola*	TAS [2]
		Type strain skN76		Type strain HA5	
	Gram stain	negative	TAS [1]	negative	TAS [2]
	Cell shape	rod or filaments	TAS [1]	rod	TAS [2]
	Motility	motile	TAS [1]	not reported	
	Sporulation	not reported		not reported	
	Temperature range	28-46 °C	TAS [1]	8-37 °C	TAS [2]
	Optimum temperature	42-45 °C	TAS [1]	28-32 °C	TAS [2]
	pH range; Optimum	6.3-8.9; 6.8-8.2	TAS [1]	6.1-9.2; unknown	TAS [2]
	Carbon source	bicarbonate	TAS [1]	bicarbonate	TAS [2]
MIGS-6	Habitat	Sediment of a lake	TAS [1]	Sediment of a lake	TAS [2]
MIGS-6.3	Salinity	< 2.6 % NaCl (w/v)	TAS [1]	< 1.2 % NaCl (w/v)	TAS [2]
MIGS-22	Oxygen requirement	aerobic	TAS [1]	aerobic	TAS [2]
MIGS-15	Biotic relationship	free-living	TAS [1]	free-living	TAS [2]
MIGS-14	Pathogenicity	non-pathogen	NAS	non-pathogen	NAS
MIGS-4	Geographic location	Lake Mizugaki, Japan	TAS [1]	Lake Harutori, Japan	TAS [2]
MIGS-5	Sample collection	November 30, 2010	NAS	April 26, 2012	NAS
MIGS-4.1	Latitude	35°51.5′ N	TAS [26]	42°58.4′ N	NAS
MIGS-4.2	Longitude	138°30.0′ E	TAS [26]	144°23.9′ E	NAS
MIGS-4.4	Altitude	not reported		not reported	

[a] Evidence codes - IDA: Inferred from Direct Assay; TAS: Traceable Author Statement (i.e., a direct report exists in the literature); NAS: Non-traceable Author Statement (i.e., not directly observed for the living, isolated sample, but based on a generally accepted property for the species, or anecdotal evidence). These evidence codes are from the Gene Ontology project [cite this reference]

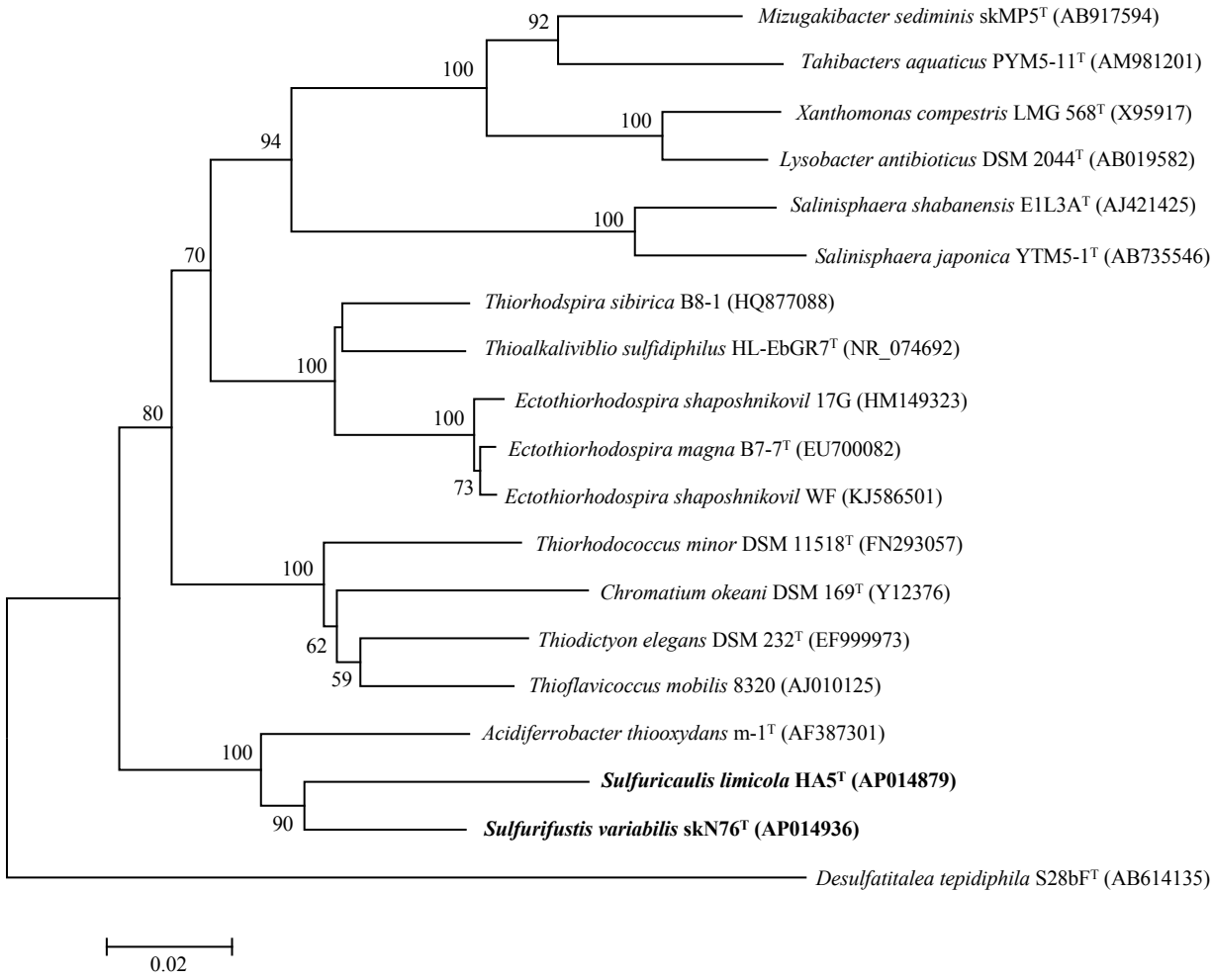


Figure 2-1 Phylogenetic tree showing the relationships of *Sulfurifustis variabilis* skN76^T and *Sulfuricaulis limicola* HA5^T with other members of the class *Gammaproteobacteria* based on 16S rRNA gene sequences aligned by using CLUSTAL W. *Desulfatitalea tepidiphila* S28bF^T was used as an outgroup. This tree was reconstructed using 1412 sites with the neighbor-joining method by using MEGA6 (27). Percentage values of 1000 bootstrap resamplings are shown at nodes; values below 50 % were not shown.

Table2-2 Project information.

MIGS ID	Property	*Sulfurifustis variabilis* skN76^{T}	*Sulfuricaulis limicola* HA5^{T}
		Term	Term
MIGS 31	Finishing quality	Completed	Completed
MIGS-28	Libraries used	15 - 20 kb SMRTbell™ library	10 - 20 kb SMRTbell™ library
MIGS 29	Sequencing platforms	PacBio RS II	PacBio RS II
MIGS 31.2	Fold coverage	x 210	x 142
MIGS 30	Assemblers	RS_HGAP Assembly.2	RS_HGAP Assembly.3
MIGS 32	Gene calling method	Microbial Genome Annotation Pipeline	Microbial Genome Annotation Pipeline
	Locus Tag	SVA	SCL
	Genbank ID	AP014936	AP014879
	GenBank Date of Release	July 29, 2016	July 29, 2016
	BIOPROJECT	PRJDB4108	PRJDB3927
MIGS 13	Source Material Identifier	DSM 100313	DSM 100373
	Project relevance	Enviromental	Enviromental

Table2-3 Genome statistics of *Sulfurifustis variabilis* skN76^{T} and *Sulfuricaulis limicola* HA5^{T}.

Attribute	*Sulfurifustis variabilis* skN76^{T}		*Sulfuricaulis limicola* HA5^{T}	
	Value	% of Total	Value	% of Total
Genome size (bp)	3,958,814	100.00	2,864,672	100.00
DNA coding (bp)	3,565,567	90.06	2,567,493	89.63
DNA G+C (bp)	2,670,566	67.46	1,759,557	61.42
DNA scaffolds	1	100.00	1	100.00
Total genes	3913	100.00	2812	100.00
Protein coding genes	3864	98.75	2763	98.26
RNA genes	49	1.25	49	1.74
Pseudo genes	unknown		unknown	
Genes in internal clusters	unknown		unknown	
Genes with function prediction	2930	75.83	2036	73.69
Genes assigned to COGs	2921	75.60	2165	78.36
Genes with Pfam domains	2970	76.86	2208	79.91
Genes with signal peptides	893	23.11	562	20.34
Genes with transmembrane helices	845	21.87	622	22.51
CRISPR repeats	6		0	

Table2-4 Number of genes associated with general COG functional categories.

Code	*Sulfurifustis variabilis* skN76^T		*Sulfuricaulis limicola* HA5^T		Description
	Value	%age	Value	%age	
J	164	4.24	159	5.75	Translation, ribosomal structure and biogenesis
A	5	0.13	2	0.07	RNA processing and modification
K	191	4.94	130	4.71	Transcription
L	154	3.99	117	4.23	Replication, recombination and repair
B	1	0.03	1	0.04	Chromatin structure and dynamics
D	36	0.93	31	1.12	Cell cycle control, Cell division, chromosome partitioning
V	43	1.11	29	1.05	Defense mechanisms
T	283	7.32	218	7.89	Signal transduction mechanisms
M	265	6.86	210	7.60	Cell wall/membrane biogenesis
N	66	1.71	64	2.32	Cell motility
U	123	3.18	98	3.55	Intracellular trafficking and secretion
O	185	4.79	142	5.14	Posttranslational modification, protein turnover, chaperones
C	265	6.86	192	6.95	Energy production and conversion
G	148	3.83	101	3.66	Carbohydrate transport and metabolism
E	201	5.20	150	5.43	Amino acid transport and metabolism
F	63	1.63	59	2.14	Nucleotide transport and metabolism
H	167	4.32	129	4.67	Coenzyme transport and metabolism
I	90	2.33	65	2.35	Lipid transport and metabolism
P	189	4.89	127	4.60	Inorganic ion transport and metabolism
Q	56	1.45	35	1.27	Secondary metabolites biosynthesis, transport and catabolism
R	394	10.20	247	8.94	General function prediction only
S	346	8.95	230	8.32	Function unknown
-	943	24.40	598	21.64	Not in COGs

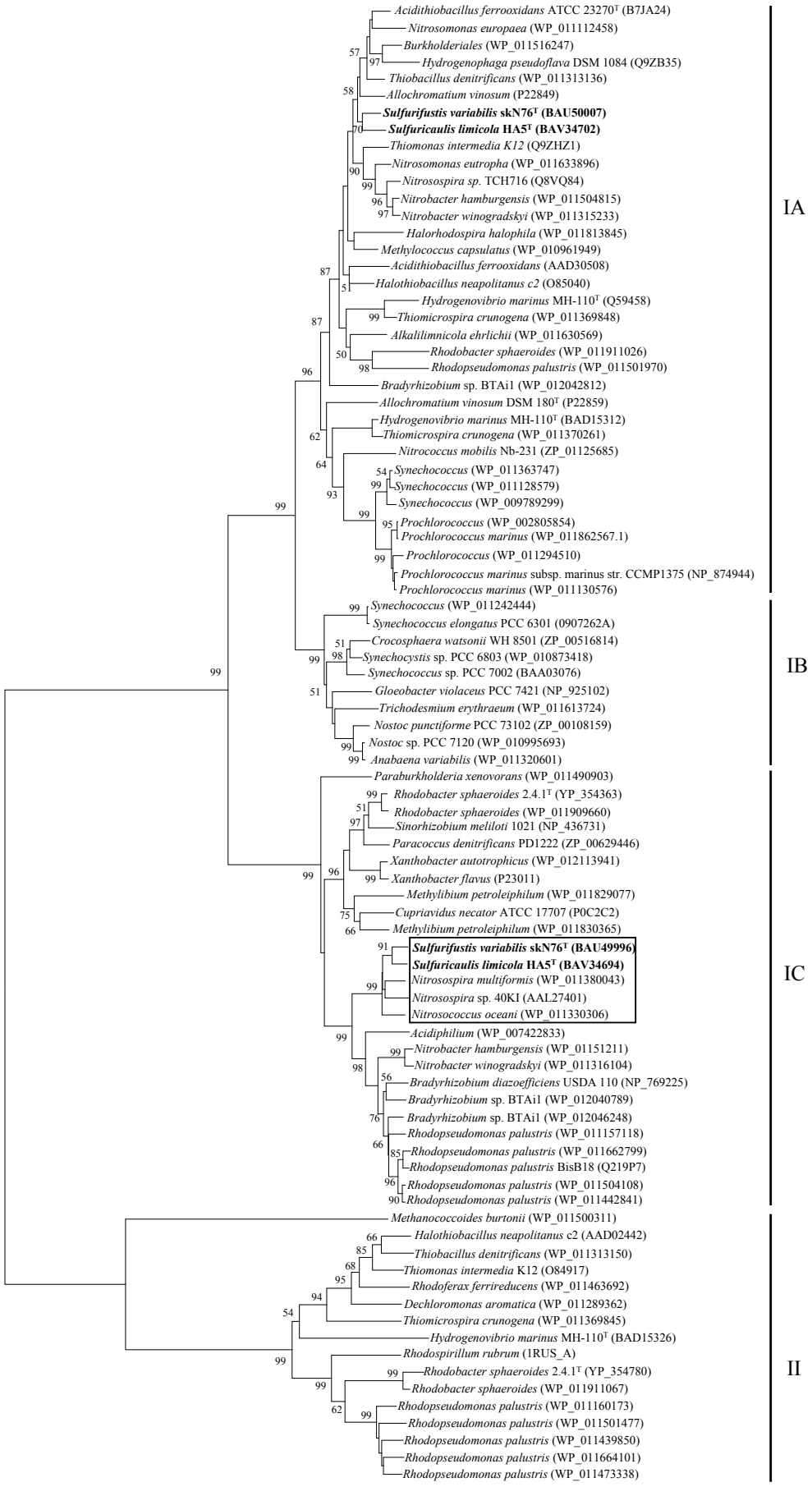

Acidithiobacillus ferrooxidans ATCC 23270T (B7JA24)
Nitrosomonas europaea (WP_011112458)
Burkholderiales (WP_011516247)
Hydrogenophaga pseudoflava DSM 1084 (Q9ZB35)
Thiobacillus denitrificans (WP_011313136)
Allochromatium vinosum (P22849)
Sulfurifustis variabilis skN76T (BAU50007)
Sulfuricaulis limicola HA5T (BAV34702)
Thiomonas intermedia K12 (Q9ZHZ1)
Nitrosomonas eutropha (WP_011633896)
Nitrosospira sp. TCH716 (Q8VQ84)
Nitrobacter hamburgensis (WP_011504815)
Nitrobacter winogradskyi (WP_011315233)
Halorhodospira halophila (WP_011813845)
Methylococcus capsulatus (WP_010961949)
Acidithiobacillus ferrooxidans (AAD30508)
Halothiobacillus neapolitanus c2 (O85040)
Hydrogenovibrio marinus MH-110T (Q59458)
Thiomicrospira crunogena (WP_011369848)
Alkalilimnicola ehrlichii (WP_011630569)
Rhodobacter sphaeroides (WP_011911026)
Rhodopseudomonas palustris (WP_011501970)
Bradyrhizobium sp. BTAi1 (WP_012042812)
Allochromatium vinosum DSM 180T (P22859)
Hydrogenovibrio marinus MH-110T (BAD15312)
Thiomicrospira crunogena (WP_011370261)
Nitrococcus mobilis Nb-231 (ZP_01125685)
Synechococcus (WP_011363747)
Synechococcus (WP_011128579)
Synechococcus (WP_009789299)
Prochlorococcus (WP_002805854)
Prochlorococcus marinus (WP_011862567.1)
Prochlorococcus (WP_011294510)
Prochlorococcus marinus subsp. marinus str. CCMP1375 (NP_874944)
Prochlorococcus marinus (WP_011130576)
IA
Synechococcus (WP_011242444)
Synechococcus elongatus PCC 6301 (0907262A)
Crocosphaera watsonii WH 8501 (ZP_00516814)
Synechocystis sp. PCC 6803 (WP_010873418)
Synechococcus sp. PCC 7002 (BAA03076)
Gloeobacter violaceus PCC 7421 (NP_925102)
Trichodesmium erythraeum (WP_011613724)
Nostoc punctiforme PCC 73102 (ZP_00108159)
Nostoc sp. PCC 7120 (WP_010995693)
Anabaena variabilis (WP_011320601)
IB
Paraburkholderia xenovorans (WP_011490903)
Rhodobacter sphaeroides 2.4.1T (YP_354363)
Rhodobacter sphaeroides (WP_011909660)
Sinorhizobium meliloti 1021 (NP_436731)
Paracoccus denitrificans PD1222 (ZP_00629446)
Xanthobacter autotrophicus (WP_012113941)
Xanthobacter flavus (P23011)
Methylibium petroleiphilum (WP_011829077)
Cupriavidus necator ATCC 17707 (P0C2C2)
Methylibium petroleiphilum (WP_011830365)
Sulfurifustis variabilis skN76T (BAU49996)
Sulfuricaulis limicola HA5T (BAV34694)
Nitrosospira multiformis (WP_011380043)
Nitrosospira sp. 40KI (AAL27401)
Nitrosococcus oceani (WP_011330306)
Acidiphilium (WP_007422833)
Nitrobacter hamburgensis (WP_01151211)
Nitrobacter winogradskyi (WP_011316104)
Bradyrhizobium diazoefficiens USDA 110 (NP_769225)
Bradyrhizobium sp. BTAi1 (WP_012040789)
Bradyrhizobium sp. BTAi1 (WP_012046248)
Rhodopseudomonas palustris (WP_011157118)
Rhodopseudomonas palustris (WP_011662799)
Rhodopseudomonas palustris BisB18 (Q219P7)
Rhodopseudomonas palustris (WP_011504108)
Rhodopseudomonas palustris (WP_011442841)
IC
Methanococcoides burtonii (WP_011500311)
Halothiobacillus neapolitanus c2 (AAD02442)
Thiobacillus denitrificans (WP_011313150)
Thiomonas intermedia K12 (O84917)
Rhodoferax ferrireducens (WP_011463692)
Dechloromonas aromatica (WP_011289362)
Thiomicrospira crunogena (WP_011369845)
Hydrogenovibrio marinus MH-110T (BAD15326)
Rhodospirillum rubrum (1RUS_A)
Rhodobacter sphaeroides 2.4.1T (YP_354780)
Rhodobacter sphaeroides (WP_011911067)
Rhodopseudomonas palustris (WP_011160173)
Rhodopseudomonas palustris (WP_011501477)
Rhodopseudomonas palustris (WP_011439850)
Rhodopseudomonas palustris (WP_011664101)
Rhodopseudomonas palustris (WP_011473338)
II
0.1

Figure 2-3 Neighbor-joining tree showing the phylogenetic positions of RuBisCO amino acid sequences coded in the genomes of *Sulfurifustis variabilis* skN76^{T} and *Sulfuricaulis limicola* HA5^{T}. The sequences aligned by using CLUSTAL W. This tree was reconstructed using 421 sites with MEGA6 (27). Percentage values of 1000 bootstrap resamplings are shown at nodes; values below 50 % were not shown. The sequences shown in box have six-amino-acid inserts at the same position.

Chapter 3

Enrichment culture and genomic analysis of a novel sulfur-disproportionating bacterium

3.1. Introduction

Sulfur-disproportionating bacteria utilize inorganic sulfur compounds as a sole energy source. In sulfur disproportionation, sulfide and sulfate are generated from a single inorganic sulfur compound such as thiosulfate, sulfite and elemental sulfur. Some sulfur-disproportionating bacteria are also able to grow by sulfate reduction and others are able to grow by sulfur oxidation. The others are specialized to disproportionate inorganic sulfur compounds. Most of sulfur-disproportionating bacteria are phylogenetically closely related to sulfate-reducing bacteria (1, 2). Some sulfate-reducing bacteria are also able to grow by sulfur disproportionation and others disproportionate sulfur compounds without growth (1, 2). Majority of known sulfur-disproportionating bacteria belong to the class *Deltaproteobacteria* (1–4). Some sulfur-disproportinating bacteria belong to the phyla *Thermodesulfobacteria* and *Firmicutes* (4–6). However, reports on sulfur disproportionating-bacteria have been less than reports on sulfate-reducing bacteria. In addition, sulfur-disproportionating bacteria appear to be phylogenetically more restricted and are less widespread than sulfate-reducing bacteria. In the phylum *Nitrospirae*, sulfate-reducing bacteria belonging to the genus *Thermodesulfovibrio* are well known (7, 8). In addition, sulfur-oxidizing bacteria are also thought to belong to the phylum *Nitrospirae* (9). However, sulfur-disproportionating bacteria belonging to the phylum *Nitrospirae* have not been reported.

Sulfate-reducing bacteria generally possess common genes for sulfate reduction: the *sat* gene encoding a sulfate adenylyltransferase, the *aprAB* genes encoding an adenosine 5' phosphosulfate reductase, the *dsrABC* and *dsrMK(JOP)* genes encoding a dissimilatory sulfite reductase and the *qmoABC* genes encoding a protein interacting with AprAB for electron transfer (10, 11). Although some sulfur-disproportionating bacteria are incapable of growth by sulfate reduction, their genomes contain the complete set of genes for sulfate reduction (12–15).

Many sulfur-disproportionating bacteria are autotrophic and possess genes for carbon fixation via the Wood-Ljungdahl pathway (12–14, 16). The genes for dissimilatory sulfate reduction and carbon fixation are similar in the genome of sulfur-disproportionating bacteria.

In this chapter, the physiological and phylogenetic characteristics of a novel sulfur-disproportinoating bacterium belonging to the phylum *Nitropirae* are described. In addition, the bacterium has been further characterized by means of genome sequencing.

3.2. Materials and methods

3.2.1. Cultivation

Enrichment culture was derived from a microbial mat sample of a hot spring obtained from Jozankei hot spring in Hokkaido, Japan. Enrichment culture was grown under growth condition of thiosulfate disproportionation in bicarbonate-buffered low-salt medium described below with 20 mM $Na_2S_2O_3$ as a sole energy source and 20 mM poorly crystalline Fe(III) oxide as a sulfide trap at 45 °C. Poorly crystalline Fe(III) oxide was prepared as follows: $FeCl_3$ solution was neutralizated with NaOH solution and washed with water for desalinization (17). In addition to $Na_2S_2O_3$ and poorly crystalline Fe(III) oxide, the culture medium contains the following components per liter: 200 mg $MgCl_2$-$6H_2O$, 100 mg $CaCl_2$-$2H_2O$, 100 mg NH_4Cl, 100 mg KH_2PO_4, 100 mg KCl, 30 mL of 1 M $NaHCO_3$ solution and 1 mL of trace element solution, 1 mL of selenite-tungstate solution, 1 mL of vitamin mixture solution, 1 mL vitamin B_{12} solution and 1 mL thiamine solution. The pH of the culture medium was adjusted to around 7.0-7.2. Those solutions were prepared as described by Widdel and Bak (18). Headspace of culture bottles was filled with N_2/CO_2 (80:20, v/v).

3.2.2. Community analysis

After several subculturing of enrichment culture, genomic DNA was extracted from the enrichment culture using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA). The genomic DNA was purified using the NucleoSpin® gDNA Clean up kit (MACHEREY NAGEL, Düran, Germany). The 16S V3-V4 region was amplified with forward and reverse primer which were added to Illumina adapter overhang nucleotide sequence (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′, 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC -3′) from extracted DNA. After purification of PCR products, secondary PCR was performed in order to attach the Illumina sequencing index adaptors using forward and reverse primers. Sequence library from purified amplicon was sequenced by the Illumina MiSeq using the paired-end method. Reads from the sequence library were collected based on index sequence. Trimming of Illumina adapter sequences from sequences of reads were performed using cutadapt version 1.1 (19). Low quality regions were removed using Trimmomatic version 0.32 when scanning started from 5'end and average quality score fell below 20 in a window of 20 bp (20). Paired reads were connected to a single sequence using fastq-join version 1.1.2-537 when both sequences were longer than 50 bp and the sequence of the 3'end overlapped by at least six bases and the bases of mismatch were lower than eight percent (21). Further sequence cleanup was performed using Cleanup program in QIME version 1.8.0 as following conditions (22): (i) Sequence reagions with ambiguous bases were trimmed; (ii) Sequences with an average quality score that fell below 25 within a 50 bp window were trimmed; (iii) Reads were removed when the length of a homopolymer is longer than 6 bp; (iv) Reads were removed when the length was shorter than 200 bp or longer than 1000 bp; (v) Sequences of forward and reverse primers were trimmed (a 1% maximum mismatch). Operational taxonomic units (OTUs) were grouped from reads at 97% similarity by UCLUST method using QIME. Taxonomy information of OTU was obtained using QIME annotation program at a similarity threshold of 0.9. 16S representative

sequences of the OTUs were checked for Chimera sequences using QIME ChimeraSlayer program.

3.2.3. Genome sequencing

The genomic DNA that was used for the community analysis was also used for genome sequencing. The genomic DNA obtained from enrichment culture was purified with the AMPure XP reagents (Beckman Coulter, Inc., Tokyo, Japan) and sheared into approximately 20 kb using the g-TUBE (Covaris, Inc., Wolburn, MA, USA). The SMRTbell™ template was prepared from fragments using the SMRTbell™ Template Prep kit 1.0 (Pacific Biosciences, Menlo Park, CA, USA), and this template was size selected using BluePippin (Sage Science, Baverly, MA, USA). The size-selected template was annealed with sequence primers using the SMRTbell™ Template Prep kit 1.0, and that was bound to DNA polymerase using the DNA/Polymerase Binding Kit P6 v2 (Pacific Biosciences). That template complex was attached with MagBead using the MagBead Kit v2 (Pacific Biosciences). The template was sequenced on PacBio RS II instrument (Pacific Biosciences) using DNA sequencing Reagent 4.0 (Pacific Biosciences). *De novo* assembly was performed with the "RS_HGAP_Assembly.3" protocol included in the application of SMRT Analysis version 2.3.0. In addition, completeness and contamination of the obtained genome were evaluated using CheckM (23).

3.2.4. Physiology test

After genomic DNA was extracted from the enrichment culture, two sets of ten-fold serial dilutions of the enrichment culture were performed in order to purify for the dominant species as much as possible. Cultivation test for substrate usage was performed at 55 °C with the following substrate combinations: sulfate (20 mM) as an electron acceptor with formate (10 mM), lactate (20 mM) or hydrogen; thiosulfate (20 mM) as an electron acceptor with formate

(10 mM), lactate (20 mM), pyruvate (20 mM), propionate (10 mM), ethanol (10 mM), glucose (10mM) or hydrogen; poorly crystalline Fe(III) oxide (20 mM) as an electron acceptor with hydrogen; nitrate (20 mM) as an electron acceptor with pyruvate (20 mM), hydrogen or thiosulfate as an electron donor (20 mM); lactate (40 mM) and pyruvate (40 mM) as a sole energy source for fermentation, respectively. Hydrogen utilization was tested by filling mixture gas of about two times volume of headspace ($H_2/N_2/CO_2$, 50:40:10). Thiosulfate disproportionation was hardly detected without the sulfide trap. Therefore, culture under growth condition with thiosulfate (20 mM) without poorly crystalline Fe(III) oxide (20 mM) was utilized as negative control in cultivation test. After several subculturing under growth condition with thiosulfate disproportionation, DNA was extracted from the enrichment culture. 16S rRNA gene was amplified from that by PCR using primer 341F that has a GC-clamp (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCTACGGGAGGCAG CAG-3′) and 907R primer (5′-CCGTCAATTCCTTTRAGTTT-3′) (24). The 16S rRNA gene sequence matched the sequence of dominant species obtained from community analysis.

3.2.5. Alignments and phylogenetic analysis

Reference sequences were obtained from NCBI. The 16S rRNA gene sequences of novel sulfur-disproportionating bacteria were obtained from genome sequences of that. Sequences were aligned using ClustalW alignment tool (http://www.genome.jp/tools/clustalw/). A phylogenetic tree of 16S rRNA genes was constructed with Maximum-likelihood method by using MEGA7 (25).

3.2.6. Genome annotation

The genome derived from an enrichment culture was automatically annotated using RAST server (26). Additionally, protein coding sequences (CDSs) which were predicted by

automatic annotation were manually annotated with basis on BLASTP search as described previously (27). In the genome of 45J, short putative CDSs were removed when the length of the CDSs were shorter than 400 bp and when more than half of the length of the CDSs overlapped with other CDSs.

The candidates for genes involved in sulfur metabolism, carbon metabolism and nitrogen metabolism were identified by performing a BLASTP search using amino acid sequences from representative genes as a query (Table 3-1). The genes were identified as candidates when the amino acid sequences from genes satisfied the following three criteria of BLASTP searches as query of the above proteins: (i) *E*-value ≤1e -8, (ii) length coverage ≥60% against query sequence and (iii) sequence identity ≥30%. The identified candidates were annotated from BLASTP search against "Non-redundant protein sequences".

3.3. Results and discussion

3.3.1. Microbial community and physiological characteristics

In order to obtain a novel sulfur-disproportionating bacterium, enrichment culture was established from a microbial mat sample obtained from a hot spring. Microbial community in the enrichment culture was investigated based on amplicon sequence of the variable region of 16S rRNA gene sequence. When OTUs were grouped at 97% similarity, 99% of the total reads (*ca*. 28,000 reads) were assembled into single OTU (this OTU is called 45J) (Table 3-2). 45J belongs to the phylum *Nitrospirae*. None of bacteria in the enrichment culture grew by sulfate reduction with formate, lactate nor hydrogen and nitrate reduction with pyruvate, hydrogen nor thiosulfate. Growth under conditions by thiosulfate reduction with following electron donor were not or minimally observed, as it was at a similar level to the negative control: formate, lactate, pyruvate, propionate, ethanol, glucose or hydrogen. These results indicate that 45J is a

thiosulfate-disproportionating bacterium unable to grow by sulfate reduction. Although growth was not observed with poorly crystalline Fe(III) oxide and hydrogen, poorly crystalline Fe(III) oxide became dark color, indicating that 45J reduces Fe(III) oxide. However, more experiments are needed to further understand this observation. Growth with fermentation by pyruvate and lactate was not observed. Cells of bacteria in enrichment culture were motile and curved rod-shaped (Fig. 3-1).

3.3.2. Genome sequencing

A draft genome sequence consisting of two contigs (*ca*. 2.4 Mbp and 43 kbp) was assembled from 39,741 subreads (420,633,872 bp) obtained from PacBio sequencing (Table 3-3). Two 16S rRNA genes were found in the genome. Both 16S rRNA gene sequences contain insertion near the 5′ ends. The sequences of 16S rRNA genes in the genome correspond to the sequences of 45J. Completeness and contamination of the draft genome sequence were evaluated to 100% and 0.91%, respectively. Therefore, the genome provided sufficient information for near-complete coverage of the genes present in 45J in order to comprehensively analyze its metabolism.

3.3.3. Phylogenetic characteristics

Phylogenetic position of 45J based on 16S rRNA gene sequence belongs to the phylum *Nitrospirae* and was located outside of cultured bacteria such as the genus *Thermodesulfovibrio* (Fig. 3-2). The 16S rRNA gene sequences in the genome exhibited low identity to the sequence of closely cultured bacterium, *Thermodesulfovibrio hydrogeniphilus* Hbr5^T (88%) and *Thermodesulfovibrio yellowstonii* YP87^T (88%). The 16S rRNA genes of 45J were related to uncultured magnetotactic bacteria such as *Candidatus* Magnetoovum mohavensis LO-1 and uncultured bacterium clone HSMV 1 (Fig. 3-2, Fig. 3-3). Many clones

were phylogenetically related to the 16S rRNA gene sequences of 45J from freshwater environments such as hot spring mat (HM114293.1), ground water (AB924430.1), and lake sediment (LC124500.1) (Fig. 3-3). In addition, clones related to that were derived from endosphere of rice roots (JN697708.1) and polluted soil (EU037906.1) (Fig. 3-3). Genus *Thermodesulfovibrio* belonging to the phylum *Nitrospirae* is known as a group of sulfate-reducing bacteria (7, 8). Sulfur-oxidizing bacteria belonging to the phylum *Nitrospirae* are suggested to be present in environments by phylogenetic analysis based on AprBA sequences (9). The AprBA sequences of the phylum *Nitrospirae* were frequently detected in freshwater environments (9). 16S rRNA sequences related to 45J were found from various freshwater environments. Therefore, sulfur-disproportionating bacteria belonging to the phylum *Nitrospirae* may be important for sulfur cycle in freshwater ecosystem.

3.3.4. Insight of the genome sequence

The genome of 45J contains the complete set of genes to reduce sulfate to sulfide: the *sat* gene (A45J_1341), the *aprAB* genes (A45J_1343-1342), the *qmoABC* genes (A45J_1344-1346), the *dsrAB* (A45J_1167-1168), the *dsrC* genes (A45J_1172) and the *dsrMKJOP* (A45J_1174-1178). Some sulfur-disproportionating bacteria are also unable to grow by sulfate reduction in spite of relative to sulfate-reducing bacteria such as *Desulfocapsa sulfexigens* SB164P1^T, *Desulfurivibrio alkaliphilus* AHT2^T and *Dissulfuribacter thermophiles* S69^T belonging to the class *Deltaproteobacteria* and *Thermosulfurimonas dismutans* S95^T, *Caldimicrobium thiodismutans* TF1^T and *Thermosulfuriphilus ammonigenes* ST65^T belonging to the family *Theremodesulfobacteriacea* (3–6, 28, 29). Most of these bacteria possess the complete genes necessary for sulfate reduction (12–15). In *Desulfurivibrio alkaliphilus* AHT2^T, all genes for sulfate reduction are involved in sulfur disproportionation (30). Therefore, these genes are thought to be involved in sulfur disproportionation of 45J. However, the reason why

45J is incapable of growth with sulfate reduction is still unknown. The genome of 45J contains a proton-translocating pyrophosphatase (A45J_1347). The genome contains all the genes for carbon fixation via the Wood-Ljungdahl pathway: a formate dehydrogenase encoded by the genes (A45J_1159, A45J_2441), the *fhs* gene encoding a formyl-tetrahydrofolate synthase (A45J_2443), the *folD* gene encoding a bifunctional methylene-tetrahydrofolate dehydrogenase/formyl-tetrahydrofolate cyclohydrolase (A45J_0531, A45J_0807), the *metF* gene encoding a methylene-tetrahydrofolate reductase (A45J_0529), the *acsD* gene encoding a corrinoid iron-sulfur protein small subunit (A45J_0559), the *acsE* gene encoding a methyltransferase (A45J_0560), the *acsA* gene encoding a carbon monoxide dehydrogenase (A45J_0556), the *acsB* gene encoding an acetyl-CoA synthase (A45J_0557), the *acsC* gene encoding a corrinoid iron-sulfur protein large subunit (A45J_0558) (31, 32). The genome of 45J contains the small and large subunits of a hydrogenase and a cytochrome *b* (A45J_1969, A45J_1971-1972).

Although 45J is unable to grow by nitrate reduction with hydrogen, pyruvate or thiosulfate, the genome of 45J contains the *napAGH* genes encoding a nitrate reductase (A145J_0202, A45J_0200-0199). In addition, the genome of 45J contains the gene encoding an octaheme nitrite reductase involved in reduction of nitrite to ammonium (A45J_0204). Some sulfur disproportionaters, *Desulfurivibrio alkaliphilus* AHT2^T and *Dissulfuribacter thermophiles* S69^T and *Thermosulfurimonas dismutans* S95^T, are able to grow by dissimilatory nitrate reduction with oxidation of inorganic sulfur compounds (15, 30). Dominant species in bioreactor received ammonium, nitrate, methane and sulfide were thought to be nitrate reducer, and that was phylogenetically distantly related to the genus *Thermodesulfovibrio* (33). Therefore, one possibility is that 45J grow by nitrate reduction with oxidation of inorganic sulfur compounds.

Interestingly, two gene clusters in the genome of 45J were predicted to the *dsrEFH*

homologous genes (A45J_0297-0294, A45J_1181-1183). One *dsrE* gene was separated into two genes (A45J_0296-0297). The gene sequence of A45J_0297 contains a TGA codon as an end codon. TGA codon is also known to be translated as selenocysteine in some cases. It is thought that *Desulfovibrio vulgaris* possess a selenocysteine-containing DsrE-like protein (34). The TGA codon of A45J_0297 was located to the Cys78 of Alvin_1253 which is DsrE of DsrEFH involved in sulfur relay in *Allochromatium vinosum* (Fig. 3-4a) (35). Therefore, this TGA codon of DsrE (A45J_0297) is thought to be selenocysteine. DsrE sequences of modified A45J_0297 and A45J_1181 has low identity to Alvin_1253 (24% and 26%, respectively). It is known that the Cy20 of DsrH (Alvin_1255) of *Allochromatium vinosum* is needed for sulfur relay. However, this cysteine was not found in the DsrH proteins (A45J_0294, A45J_1183) (Fig. 3-4b) (35). The *tusBCD* genes in the genome of *Escherichia coli* are also homologous to the *dsrEFH* genes and TusBCD was related to modification of tRNA into thiouridine at the wobble position (36). Therefore, the function of *dsrEFG*-like genes of 45J is currently unclear. However, nucleophilic reactivity of selenocysteine is higher than that of cysteine, indicating that the reaction properties of the DsrEFG-like proteins (A45J_0297-0294) are thought to be different from DsrEFH proteins or TusBCD proteins (37).

The genes that make up the Sox system involved in thiosulfate oxidation were not found. The genes corresponding to the *phsA* gene encoding a thiosulfate reductase or the *psrA* gene encoding a polysulfide reductase was not found in the genome of 45J. In *Dethiobacter alkaliphilus* AHT1^{T} also does not possess the *phsA* gene (16). In thiosulfate disproportionation, thiosulfate is thought to reduce to sulfite and sulfide during the first step. However, it is unclear how thiosulfate is reduced in 45J.

3.4. Conclusion

The genome of a novel thiosulfate-disproportionating bacterium 45J belonging to the phylum *Nitrospirae* was obtained from enrichment culture. The genome of 45J shares similar gene sets for sulfate reduction and carbon fixation with other sulfur-disproportionating bacteria of different phyla. The genome of 45J does not contain the *phsA* gene involved in thiosulfate reduction. Therefore, thiosulfate-disproportonating pathway of 45J may be similar in large to other sulfur-disproportionating bacteria, but except for its initial step.

3.5. Reference

1. Krämer M, Cypionka H. Sulfate formation via ATP sulfurylase in thiosulfate- and sulfite-disproportionatig bacteria. Arch Microbiol. 1989;151(3):232–7.
2. Finster K. Microbiological disproportionation of inorganic sulfur compounds. J Sulfur Chem. 2008;29(3–4):281–92.
3. Finster K, Liesack W, Thamdrup B. Elemental sulfur and thiosulfate disproportionation by *Desulfocapsa sulfoexigens* sp. nov., a new anaerobic bacterium isolated from marine surface sediment. Appl Environ Microbiol. 1998;64(1):119–25.
4. Poser A, Lohmayer R, Vogt C, Knoeller K, Planer-Friedrich B, Sorokin D, et al. Disproportionation of elemental sulfur by haloalkaliphilic bacteria from soda lakes. Extremophiles. 2013;17(6):1003–12.
5. Slobodkin AI, Reysenbach AL, Slobodkina GB, Baslerov RV, Kostrikina NA, Wagner ID, et al. *Thermosulfurimonas dismutans* gen. nov., sp. nov., an extremely thermophilic sulfur-disproportionating bacterium from a deep-sea hydrothermal vent. Int J Syst Evol Microbiol. 2012;62(11):2565–71.
6. Kojima H, Umezawa K, Fukui M. *Caldimicrobium thiodismutans* sp. nov., a sulfur-disproportionating bacterium isolated from a hot spring, and emended description of the genus *Caldimicrobium*. Int J Syst Evol Microbiol. 2016;66(4):1828–31.
7. Henry EA, Devereux R, Maki JS, Gilmour CC, Woese CR, Mandelco L, et al. Characterization of a new thermophilic sulfate-reducing bacterium — *Thermodesulfovibrio yellowstonii*, gen. nov. and sp. nov.: its phylogenetic relationship to *Thermodesulfobacterium commune* and their origins deep within the bacterial domain. Arch Microbiol. 1994;161(1):62–9.
8. Sekiguchi Y, Muramatsu M, Imachi H, Narihiro T, Ohashi A, Harada H, et al. *Thermodesulfovibrio aggregans* sp. nov. and *Thermodesulfovibrio thiophilus* sp. nov.,

anaerobic,thermophilic, sulfate-reducing bacteria isolated from thermophilic methanogenic sludge, and emended description of the genus *Thermodesulfovibrio*. Int J Syst Evol Microbiol. 2008;58(11):2541–8.

9. Watanabe T, Kojima H, Fukui M. Identity of major sulfur-cycle prokaryotes in freshwater lake ecosystems revealed by a comprehensive phylogenetic study of the dissimilatory adenylylsulfate reductase. Sci Rep. 2016;6:36262.
10. Bradley AS, Leavitt WD, Johnston DT. Revisiting the dissimilatory sulfate reduction pathway. Geobiology. 2011;9(5):446–57.
11. Pereira IAC, Ramos AR, Grein F, Marques MC, da Silva SM, Venceslau SS. A comparative genomic analysis of energy metabolism in sulfate reducing bacteria and archaea. Front Microbiol. 2011;2:69.
12. Finster KW, Kjeldsen KU, Kube M, Reinhardt R, Mussmann M, Amann R, et al. Complete genome sequence of *Desulfocapsa sulfexigens*, a marine deltaproteobacterium specialized in disproportionating inorganic sulfur compounds. Stand Genomic Sci. 2013;8(1):58–68.
13. Melton ED, Sorokin DY, Overmars L, Chertkov O, Clum A, Pillay M, et al. Complete genome sequence of *Desulfurivibrio alkaliphilus* strain AHT2^{T}, a haloalkaliphilic sulfidogen from Egyptian hypersaline alkaline lakes. Stand Genomic Sci. 2016;11(1):67.
14. Mardanov A, Beletsky A, Kadnikov V, Slobodkin A, Ravin N. Genome analysis of *Thermosulfurimonas dismutans*, the first thermophilic sulfur-disproportionating bacterium of the phylum *Thermodesulfobacteria*. Front Microbiol. 2016;7:950.
15. Slobodkina GB, Mardanov AV., Ravin NV., Frolova AA, Chernyh NA, Bonch-Osmolovskaya EA, et al. Respiratory ammonification of nitrate coupled to anaerobic oxidation of elemental sulfur in deep-sea autotrophic thermophilic bacteria. Front Microbiol. 2017;8:87.

16. Melton ED, Sorokin DY, Overmars L, Lapidus AL, Pillay M, Ivanova N, et al. Draft genome sequence of *Dethiobacter alkaliphilus* strain AHT1^{T}, a gram-positive sulfidogenic polyextremophile. Stand Genomic Sci. 2017;12:57.

17. Lovley DR. Dissimilatory Fe(III)- and Mn(IV)-reducing prokaryotes. In: Rosenberg E, Delong EF, Lory S, Stackebrandt E, Thompson F, editors. The Prokaryotes, 4th ed. Berlin, Heidelberg: Springer; 2013. p. 287-308.

18. Widdel F, Bak F. Gram-negative mesophilic sulfate-reducing bacteria. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH, editors. The Prokaryotes, 2nd ed, vol. 4. New York: Springer; 1992. p. 3352-78.

19. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal. 2011;17(1):10-2.

20. Bolger AM, Lohse M, Usadel B. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30(15):2114–20.

21. Aronesty E. ea-utils : Command-line tools for processing biological sequencing data. 2011.

22. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7(5):335–6.

23. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: assessing the quality of microbial genomes recovered from. Genome Res. 2015;25(7):1043-55.

24. Muyzer G, Teske A, Wirsen CO, Jannasch HW. Phylogenetic relationships of Thiomicrospira species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch Microbiol. 1995;164(3):165-72.

25. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis

Version 7.0 for Bigger Datasets. Mol Biol Evol. 2016;33(7):1870–4.

26. Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, et al. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res. 2014;42:D206–14.

27. Kojima H, Ogura Y, Yamamoto N, Togashi T, Mori H, Watanabe T, et al. Ecophysiology of *Thioploca ingrica* as revealed by the complete genome sequence supplemented with proteomic evidence. ISME J. 2015;9(5):1166–76.

28. Slobodkin AI, Reysenbach AL, Slobodkina GB, Kolganova TV., Kostrikina NA, Bonch-Osmolovskaya EA. *Dissulfuribacter thermophilus* gen. nov., sp. nov., a thermophilic, autotrophic, sulfur-disproportionating, deeply branching deltaproteobacterium from a deep-sea hydrothermal vent. Int J Syst Evol Microbiol. 2013(6);63:1967–71.

29. Slobodkina GB, Reysenbach AL, Kolganova TV., Novikov AA, Bonch-Osmolovskaya EA, Slobodkin AI. *Thermosulfuriphilus ammonigenes* gen. Nov., sp. nov., a thermophilic, chemolithoautotrophic bacterium capable of respiratory ammonification of nitrate with elemental sulfur. Int J Syst Evol Microbiol. 2017;67(9):3474–9.

30. Thorup C, Schramm A, Findlay AJ, Finster KW, Schreiber L. Disguised as a sulfate reducer: Growth of the deltaproteobacterium *Desulfurivibrio alkaliphilus* by sulfide oxidation with nitrate. mBio. 2017;8(4):e00671-17.

31. Pierce E, Xie G, Barabote RD, Saunders E, Han CS, Detter JC, et al. The complete genome sequence of *Moorella thermoacetica* (f. *Clostridium thermoaceticum*). Environ Microbiol. 2008;10(10):2550–73.

32. Ragsdale SW, Pierce E. Acetogenesis and the Wood-Ljungdahl pathway of CO_2 fixation. Biochim Biophys Acta - Proteins Proteomics. 2008;1784(12):1873–98.

33. Arshad A, Dalcin Martins P, Frank J, Jetten MSM, Op den Camp HJM, Welte CU.

Mimicking microbial interactions under nitrate-reducing conditions in an anoxic bioreactor: enrichment of novel Nitrospirae bacteria distantly related to *Thermodesulfovibrio*. Environ Microbiol. 2017;19(12):4965-77

34. Zhang Y, Gladyshev VN. An algorithm for identification of bacterial selenocysteine insertion sequence elements and selenoprotein genes. Bioinformatics. 2005;21(11):2580–9.

35. Stockdreher Y, Venceslau SS, Josten M, Sahl HG, Pereira IAC, Dahl C. Cytoplasmic sulfurtransferases in the purple sulfur bacterium *Allochromatium vinosum*: Evidence for sulfur transfer from DsrEFH to DsrC. PLoS One. 2012;7(7):e40785.

36. Ikeuchi Y, Shigi N, Kato J, Nishimura A, Suzuki T. Mechanistic insights into sulfur relay by multiple sulfur mediators involved in thiouridine biosynthesis at tRNA wobble positions. Mol Cell. 2006;21(1):97–108.

37. Arnér ES. Selenoproteins-What unique properties can arise with selenocysteine in place of cysteine?. Exp Cell Res. 2010;316(8):1296-1303.

Table 3-1 List showing the accession numbers to the sequences that was used as a query for gene annotation of gene in the genomes of 45J.

accesion number	gene name	product	Species
EAT05667.1	*acsA*	Carbon-monoxide dehydrogenase, catalytic subunit	delta proteobacterium MLMS-1
EAT05665.1	*acsB*	CO dehydrogenase/acetyl-CoA synthase complex beta subunit	delta proteobacterium MLMS-1
EAT05664.1	*acsC*	CO dehydrogenase/acetyl-CoA synthase delta subunit:Putative Fe-S cluster	delta proteobacterium MLMS-1
ABC19512.1	*acsD*	acetyl-CoA decarbonylase/synthase delta subunit	*Moorella thermoacetica* ATCC 39073
ADO12092.1	*acsE*	5-methyltetrahydrofolate corrinoid iron-sulfur protein methyltransferase	*Clostridium carboxidivorans* P7^T
OAQ20318.1	*aprA*	Adenylylsulfate reductase alpha-subunit	*Thermosulfurimonas dismutans* S95^T
OAQ20319.1	*aprB*	Adenylylsulfate reductase beta-subunit	*Thermosulfurimonas dismutans* S95^T
OAQ20198.1	*dsrA*	Dissimilatory sulfite reductase alpha subunit	*Thermosulfurimonas dismutans* S95^T
OAQ20199.1	*dsrB*	Dissimilatory sulfite reductase beta subunit	*Thermosulfurimonas dismutans* S95^T
OAQ20264.1	*dsrC*	Dissimilatory sulfite reductase, subunit gamma	*Thermosulfurimonas dismutans* S95^T
OAQ21324.1	*dsrJ*	Sulfite reduction-associated complex DsrMKJOP multiheme protein DsrJ	*Thermosulfurimonas dismutans* S95^T
OAQ21325.1	*dsrK*	Sulfite reduction-associated complex DsrMKJOP protein DsrK	*Thermosulfurimonas dismutans* S95^T
OAQ21326.1	*dsrM*	Sulfite reduction-associated complex DsrMKJOP protein DsrM	*Thermosulfurimonas dismutans* S95^T
OAQ21323.1	*dsrO*	Sulfite reduction-associated complex DsrMKJOP iron-sulfur protein DsrO	*Thermosulfurimonas dismutans* S95^T
OAQ21322.1	*dsrP*	Sulfite reduction-associated complex DsrMKJOP protein DsrP	*Thermosulfurimonas dismutans* S95^T
EAT05670.1	*fhs*	Formate--tetrahydrofolate ligase	delta proteobacterium MLMS-1
EAT05668.1	*folD*	Methenyltetrahydrofolate cyclohydrolase	delta proteobacterium MLMS-1
EAT05662.1	*fdh*	Molybdopterin oxidoreductase:Molydopterin dinucleotide-binding region	delta proteobacterium MLMS-1
EAT05663.1	*fdh*	Ferredoxin:FAD-dependent pyridine nucleotide-disulphide oxidoreductase:4Fe-4S ferredoxin, iron-sulfur binding:FAD dependent oxidoreductase:Molybdopterin oxidoreductase Fe4S4 region	delta proteobacterium MLMS-1
ABC19505.1	*metF*	Methylenetetrahydrofolate reductase	*Moorella thermoacetica* ATCC 39073
OAQ21381.1	*napA*	Periplasmic nitrate reductase, large catalytic subunit NapA	*Thermosulfurimonas dismutans* S95^T
OAQ21379.1	*napG*	Periplasmic nitrate reductase, subunit NapG	*Thermosulfurimonas dismutans* S95^T
OAQ21378.1	*napH*	Periplasmic nitrate reductase, subunit NapH	*Thermosulfurimonas dismutans* S95^T
CAI56199.2		octaheme nitrate reductase	*Thioalkalivibrio nitratireducens* DSM 14787^T
OAQ20317.1	*qmoA*	Adenylylsulfate reductase-associated electron transfer protein QmoA	*Thermosulfurimonas dismutans* S95^T
OAQ20316.1	*qmoB*	Adenylylsulfate reductase-associated electron transfer protein QmoB	*Thermosulfurimonas dismutans* S95^T
OAQ20315.1	*qmoC*	Adenylylsulfate reductase-associated electron transfer protein QmoC	*Thermosulfurimonas dismutans* S95^T
BAV34749.1	*sat*	sulfate adenylyltransferase	*Sulfuricaulis limicola* HA5^T
OAQ20990.1		[Ni/Fe] hydrogenase, small subunit	*Thermosulfurimonas dismutans* S95^T
OAQ20991.1		[Ni/Fe] hydrogenase, large subunit	*Thermosulfurimonas dismutans* S95^T
OAQ20992.1		[Ni/Fe] hydrogenase, cytochrome b subunit	*Thermosulfurimonas dismutans* S95^T
ACL05062.1		V-type H(+)-translocating pyrophosphatase	*Desulfatibacillum alkenivorans* AK-01

Table 3-2 Result of community analysis of the enrichment culture.

OTU ID	Chimera	Phylum	Class	Read count	rate (%)
denovo3023*	**.**	***Nitrospirae***	***Nitrospira***	**27896**	**99.391**
denovo2591	.	*Nitrospirae*	*Nitrospira*	6	0.021
denovo2267	.	*Nitrospirae*	*Nitrospira*	3	0.011
denovo4444	.	*Nitrospirae*	*Nitrospira*	2	0.007
denovo7000	.	*Nitrospirae*	*Nitrospira*	2	0.007
denovo242	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo264	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo782	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo889	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo922	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo1245	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo1555	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo2100	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo2195	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo2347	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo2415	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo2471	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo2511	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo2518	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo2572	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo2589	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo3230	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo3638	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo3875	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo3885	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo4306	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo4365	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo4628	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo4688	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo4763	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo5014	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo5290	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo5919	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo5978	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo6010	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo6014	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo6109	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo6361	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo6694	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo6829	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo6926	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo6991	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo7109	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo7119	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo7134	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo7252	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo7363	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo7498	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo7723	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo8034	.	*Nitrospirae*	*Nitrospira*	1	0.004
denovo3677	.	*Bacteroidetes*	*Bacteroidia*	1	0.004
denovo7740	.	*Proteobacteria*	*Gammaproteobacteria*	48	0.171
denovo73	.	*Proteobacteria*	*Gammaproteobacteria*	3	0.011
denovo428	.	*Firmicutes*	*Clostridia*	3	0.011
denovo4410	.	*Firmicutes*	*Clostridia*	2	0.007
denovo1215	.	*Firmicutes*	*Clostridia*	1	0.004
denovo4960	.	*Proteobacteria*	*Betaproteobacteria*	3	0.011
denovo7035	.	*Spirochaetes*	*Spirochaetes*	46	0.164
denovo6395	.	*Proteobacteria*	*Deltaproteobacteria*	5	0.018
denovo3077	.	*Proteobacteria*	*Deltaproteobacteria*	1	0.004
denovo505	chimera	*Nitrospirae*	*Nitrospira*	1	-
denovo1146	chimera	*Spirochaetes*	*Spirochaetes*	6	-
denovo6374	chimera	*Spirochaetes*	*Spirochaetes*	2	-

*The OTU assigned to denovo3023 was referred to as 45J in the thesis.

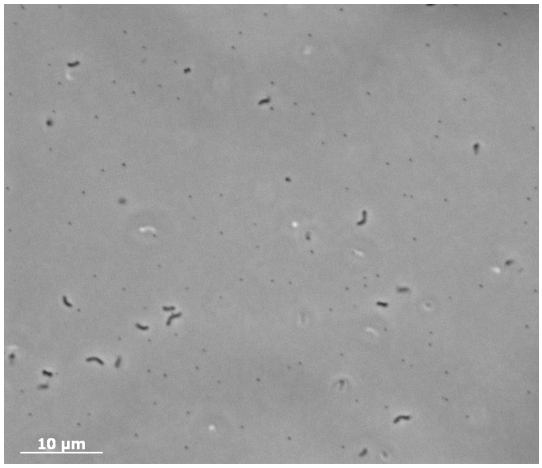


Figure 3-1 Phase-contrast micrograph of bacteria in enrichment culture.

Table 3-3 Genome statistics of 45J.

Attribute	Long contig	Short contig
Genome size (bp)	2,445,501	43,863
G+C content (%)	38.8	41.4
CDSs	2619	61
5S rRNA gene	1	0
16S rRNA genes	2	0
23S rRNA genes	2	0
tRNA genes	46	0

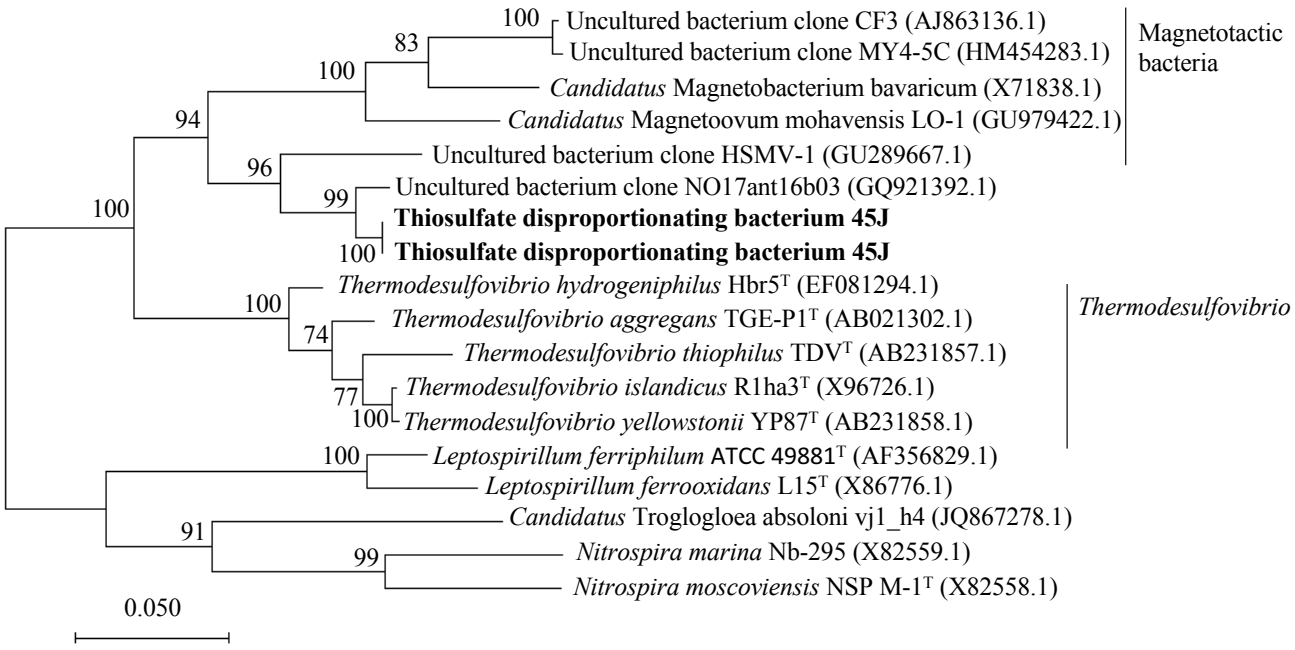


Figure 3-2 The phylogenetic position of 16S rRNA genes sequences of 45J within the members of the phylum *Nitrospirae* and closely related uncultured bacterium clones. This tree was reconstructed using 1308 sites with maximum-likelihood method. Number represent percent value of 100 bootstrap resamplings; values below 50% were not shown.

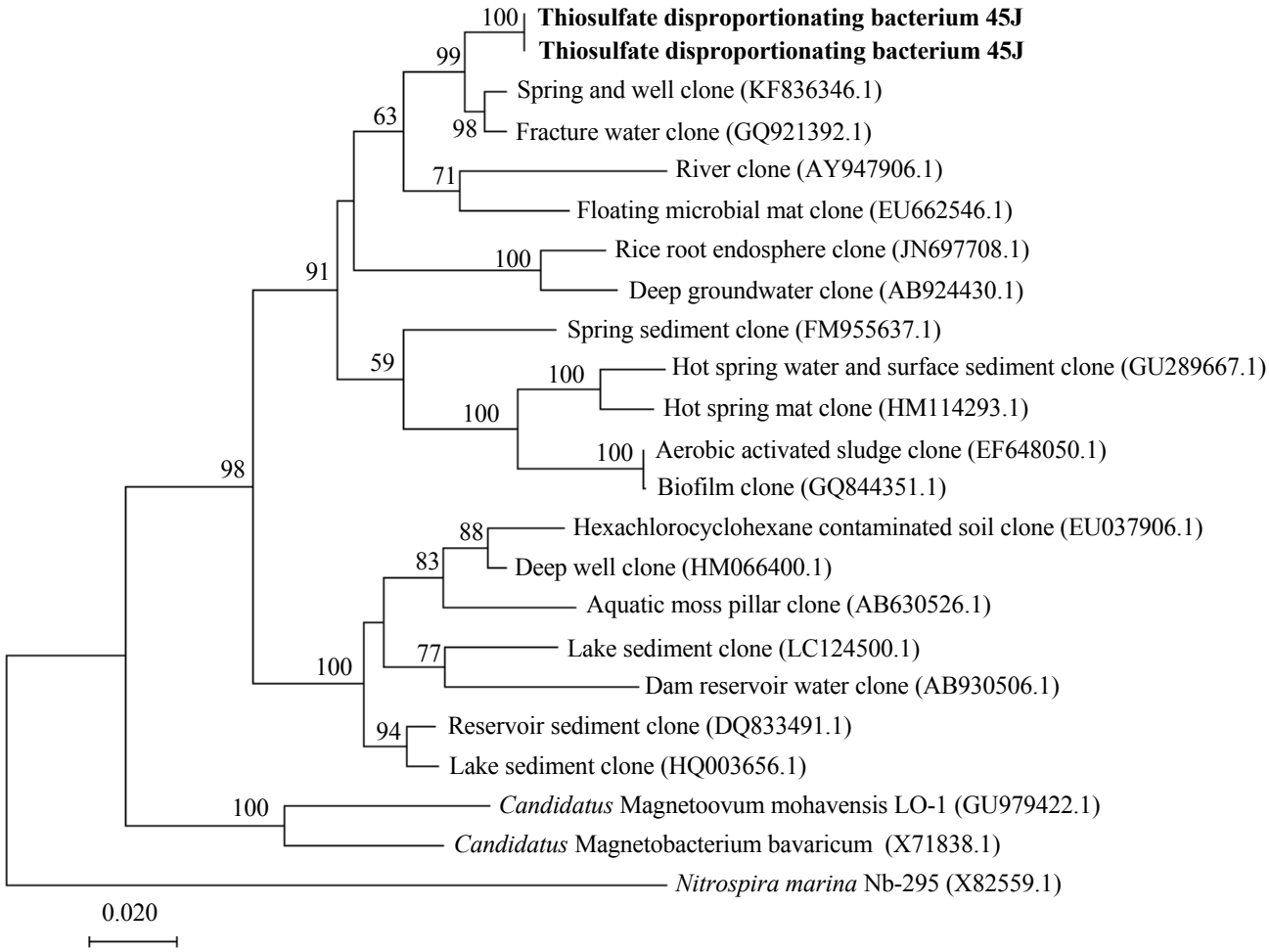
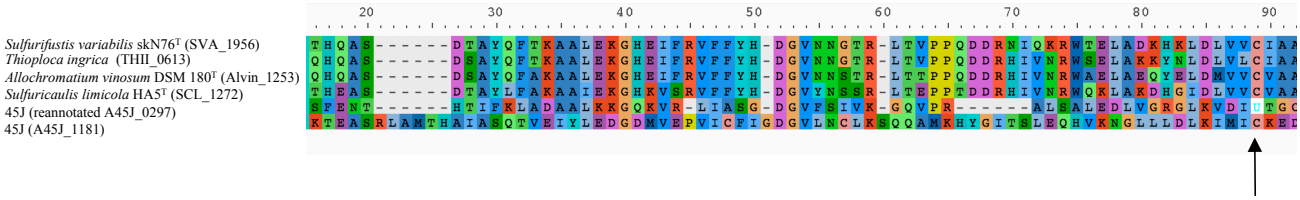


Figure 3-3 Maximum-likelihood phylogenetic tree showing the phylogenetic position of 16S rRNA genes sequences in the genome of 45J and closely related uncultured bacterium clones within the members of phylum *Nitrospirae*. This tree was reconstructed using 1288 sites. The numbers represent the percent value of 100 bootstrap resamplings; values below 50% are not shown.

(a)



(b)

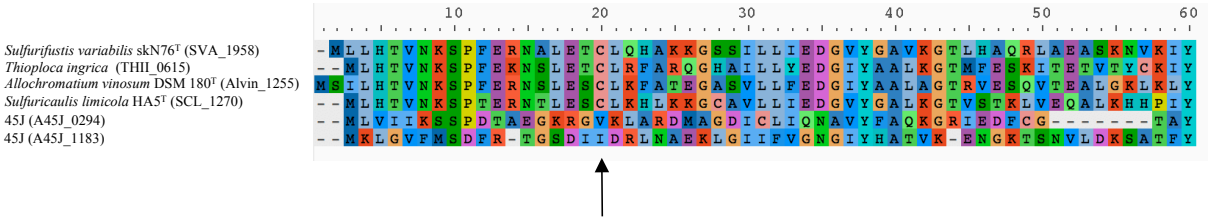


Figure 3-4 Alignment of DsrE-like protein (a) and DsrH-like protein (b) with DsrE and DsrH of DsrEFH complex, respectively. Arrow indicates the cysteine residue of Cys78 of DsrE (a) and Cys20 of DsrH (b), respectively.

Chapter 4

Comparative genomic analysis of sulfate-reducing bacteria and sulfur-disproportionating bacteria and proteomic analysis of a sulfur-diproportionating bacterium

4.1. Introduction

Most of sulfur-disproportionating bacteria are phylogenetically related to sulfate-reducing bacteria (1, 2). Some sulfur-disproportionating bacteria which disproportionate thiosulfate and/or elemental sulfur are incapable of growth with sulfate reduction. Such bacteria will be refered to as ISR-SDB in this chapter. Only six genomes of ISR-SDB belonging to four different phyla have been described in detail. *Desulfocapsa sulfexigens* SB164P1^T, *Desulfurivibrio alkaliphilus* AHT2^T and *Dissulfuribacter thermophiles* S69^T belong to the class *Deltaproteobacteria* in the phylum *Proteobacteria* (3–8). *Dethiobacter alkaliphilus* AHT1^T belongs to the phylum *Firmicutes* (7, 9). 45J belongs to the phylum *Nitrospirae* (Chapter 4). *Thermosulfurimonas dismutans* S95^T belongs to the phylum *Thermodesulfobacteria* (10, 11). In the phylum *Thermodesulfobacteria*, *Caldimicrobium thiodismutans* TF1^T and *Thermosulfuriphilus ammonigenes* ST65^T are also known as ISR-SDB, although the genome data of these bacteria are minimally described (12, 13). In sulfate-reducing pathway, sulfate is reduced to adenosine-5'-phosphosulfate (APS) by a sulfate adenylyltrasnferase encoded by the *sat* gene. APS is reduced to sulfite by APS reductase encoded by the *aprAB* genes. Sulfite is reduced to a trisulfide formation of the protein DsrC by dissimilatory sulfite reductase encoded by the *dsrAB* genes (14). Finally, DsrC trisulfide is thought to be reduced by DsrMK(JOP) (14). ISR-SDB, except for *Dethiobacter alkaliphilus* AHT1^T, possess the complete genes for sulfate reduction. Some sulfur-oxidizing bacteria also possess those genes, where sulfur is oxidized through these enzymes working in reverse reactions (15).

Four species of ISR-SDB are able to grow by oxidation of inorganic sulfur compounds with nitrate reduction: *Desulfurivibrio alkaliphilus* AHT2^T, *Dissulfuribacter thermophiles* S69^T, *Thermosulfurimonas dismutans* S95^T and *Thermosulfuriphilus ammonigenes* ST65^T (5, 13, 16). In *Desulfurivibrio alkaliphilus* AHT2^T, transcriptomic analysis

revealed that the genes for sulfate reduction were involved in sulfur disproportionation and sulfide oxidation in combination with nitrate reduction (16). In the sulfur-disproportionating pathway, the direction of enzyme reactions of an APS reductase and a sulfate adenylyltransferase are thought to be different from sulfate reduction (2, 16, 17). Genes that are specifically involved in the sulfate-reducing pathway, but not sulfur-disproportionating pathway, have not been identified. In addition, genes that are specific to sulfur-disproportionating pathway are also not identified.

In this study, we investigated whether there are specific genes to ISR-SDB with the Dsr system through comparative genomic analysis using 92 genomes including the genomes of *Caldimicrobium thiodismutans* TF1^T and 45J. In order to investigate whether these genes are related to sulfur disproportionation, expressed proteins were identified under growth conditions of thiosulfate and elemental sulfur disproportionation in *Caldimicrobium thiodismutans* TF1^T.

4.2. Materials and methods

4.2.1. Genome sequencing of TF1

Sequence library (350 bp insert) was prepared using the TruSeq Nano DNA LT Sample Prep Kit (Illumina, San Diego, CA, USA) as described in the standard protocol. Sequencing with 100 bp paired-end reads was performed on Illumina Hiseq (Illumina). *De novo* assembly was performed using Velvet version 1.2.08 (18). After genome assembly, gaps in contigs were closed and contigs were connected to other contigs by PCR and Sanger sequencing.

4.2.2. Genome annotation

Automatic annotation of the genome of *Caldimicrobium thiodismutans* TF1^T was

performed by Microbial Genome Annotation Pipeline (19). In addition, manual annotation of predicted protein coding sequences (CDSs) was performed by the basis on BLASTP search. The CDSs were annotated as hypothetical proteins when CDSs satisfied any of three criteria of BLASTP search: (i) *E*-value >1e -8, (ii) length coverage <60% against query sequence and (iii) sequence identity <30%. In order to find the genes for sulfur, carbon and nitrate metabolism, BLASTP search was performed using representative genes as query against the CDSs in the genome of *Caldimicrobium thiodismutans* TF1^T (Table 4-1). The candidates were determined by satisfying the following three criteria of BLASTP searches: (i) *E*-value ≤1e -8, (ii) length coverage ≥60% against query sequence and (iii) sequence identity ≥30%. The identified candidates were annotated from BLASTP search against "Non-redundant protein sequences". Additionally, amino acid sequences from genes in the genome of *Caldimicrobium thiodismutans* TF1^T were searched using Batch Web CD Search Tool performed with default setting (20). Proteins including a domain of RHOD superfamily (cl00125) were determined as candidates for the Rhodanese protein. The candidates were annotated based on BLASTP search against "Non-redundant protein sequences".

4.2.3. Comparative genomic analysis

For comparative genomic analysis, a total of 92 genomes were selected from bacteria which reduce and/or disproportionate inorganic sulfur compounds (Table 4-2, Table 4-3). They belong to the phylum *Thermodesulfobacteria*, the class *Deltaproteobacteria*, the class *Clostridia*, the class *Nitrospira* and the class *Synergistia* (Table 4-2, Table 4-3). Genomes except for 45J were obtained from NCBI. Protein-coding sequences of 92 genomes were classified into homologous genes (orthogroup) using orthofinder (21).

4.2.4. Prediction of gene function

In order to predict functions of genes, functional domains of genes were found using "Conserved Domain Search Service" (20). Localization of protein in the cells was predicted using tool of psortb v3.0 (22).

4.2.5. Alignment and phylogenetic analyses

Reference sequences were obtained from NCBI. The sequences of proteins were aligned using ClustalW alignment tool (http://www.genome.jp/tools/clustalw/) and then alignment was modified manually as needed. A phylogenetic tree was reconstructed with Maximum-likelihood method by using MEGA6 (23).

4.2.6. Proteomic analysis

Cells of *Caldimicrobium thiodismutans* $TF1^T$ were cultured under anaerobic conditions (N_2/CO_2, 80:20 by vol.) in a bicarbonate-buffered medium described previously, modifying vitamin solution, with 20 mM thiosulfate or elemental sulfur (0.8 g/L) (12). The culture medium was comprised as follows per liter: 200 mg $MgCl_2$-$6H_2O$, 100 mg $CaCl_2$-$2H_2O$, 100 mg NH_4Cl, 100 mg KH_2PO_4, 100 mg KCl, 30 mL of 1 M $NaHCO_3$ solution and 1 mL of trace element solution, 1 mL of selenite-tungstate solution and 1 mL of vitamin solution. Vitamin solution is comprised as follows per liter: 2 mg biotin, 2 mg folic acid, 10 mg pyridoxine-HCl, 5 mg lipoic acid and 0.1 mg cyanocobalamine. The pH of culture medium was adjusted from 7.0 to 7.2. Additionally, a dialysis tubing containing poorly crystalline Fe(III) oxide was utilized as a sulfide trap. Poorly crystalline Fe(III) oxide was added into dialysis tubings (6000 to 8000 Da molecular-mass cutoff Spectra/Por 1, 6.4-mm diameters; Spectrum Labs, Rancho Dominguez, CA, USA) to obtain final concentrations of 20 mmol per 1 L of cultured medium.

Samples of cells were harvested by centrifugation and washed in phosphate-buffered

saline. Three samples were grown for 108 hr with disproportionation of thiosulfate and two samples were grown for 240 hr with disproportionation of elemental sulfur. The concentrations of sulfate and thiosulfate were measured by an ion chromatograph (ICS-1500, column: IonPacAS12A, Dionex, Sunnyvale, CA, USA) in order to observe growth. Samples were suspended using ReadyPrep protein extraction kit (total protein) (Bio-Rad Laboratories, Hercules, CA, USA). Suspended samples were sonicated on ice 10 times for 10 s each at 30 s intervals, and lysates were centrifuged by 16,000 x g for 30 min at 20 ˚C. Acetone precipitation of supernatants were performed. Pellets were redissolved in lysis solution (8 M urea in 50 mM NH_4HCO_3). The protein concentration of the supernatant was determined using Bio-Rad protein assay kit (Bio-Rad Laboratories). 20 μg of total protein sampoles were trichloroacetic acid (TCA) precipitated and washed by ice-cold acetone for three times followed by centrifugation for 5 min at 16,000 x g. Pellets were resuspended in 1M urea in 25 mM ammonium bicarbonate buffer. Protein samples were then reduced for 30 min at 50 ˚C in 5 mM DTT, followed by alkylation with 15 mM iodoacetamide for 30 min at room temperature in the dark. Samples were then digested by adding proteomics grade trypsin (Roche, Germany) at a 1:100 trypsin/protein ratio for 10 hrs at 37 ˚C. ZipTip-C18 column clean up was performed (Merk, NJ, USA), then eluted into 0.1 % formic acid solution. Mass spectrum was obtained by using Easy nLC1000 hooked up with Q-excutive plus orbitrap mass spectrometer (Thermo Fisher Scientific, IL, USA), and operated by Xcalibur software (ver. 3.1, Thermo Fisher Scientific). The peptides were separated on a C18 capillary tip column (NTCC-360/75-3-125, Nikkyo Techno, Japan) by linear gradient from 5 to 30 % in 120 min in 0.1 % formic acid in acetonitrile. Full scan mass spectra were obtained in the Orbitrap with scan range of 300.0 to 2,000.0 *m*/*z* with a resolution of 70,000.

Proteins were identified from the acquired MS/MS spectra using Proteome Discoverer 2.0 (Thermo Fisher Scientific) with CDSs of *Caldimicrobium thiodismutans* $TF1^T$ obtained in

this study. The peptide mass tolerance was set at 10 ppm, and fragment mass tolerance was set at 0.8 Da. The peptide charge was set at +2, +3 and +4. The accuracy and sensitivity of peptide identification was attempted by automatic decoy function and percolator function built in the Proteome Discoverer software. Proteins including detected peptides that are unique in the CDSs of *Caldimicrobium thiodismutans* TF1^T were determined as expressed proteins.

4.3. Results and discussion

4.3.1. Genome property of *Caldimicrobium thiodismutans* TF1^T

In the Illumina sequencing, a total of 21 contigs were obtained by assembly using 11,027,898 reads. All gaps were closed by PCR and Sanger sequencing. The sequences of 11 contigs corresponded to the sequences of other longer contigs. Single chromosome (*ca*. 1.8 Mbp) was reconstructed from other contigs except for 1 contig by PCR and Sanger sequencing. The sequence of residual 1 contig was very short length (362 bp) and highly similar to the sequence of the chromosome (99%). Therefore, the sequence of this contig was considered as a sequencing error. Finally, a single chromosome of *Caldimicrobium thiodismutans* TF1^T was obtained.

4.3.2. Insight of genome sequence of *Caldimicrobium thiodismutans* TF1^T

Table 4-3 shows statistics of the genome of *Caldimicrobium thiodismutans* TF1^T. The genome of *Caldimicrobium thiodismutans* TF1^T contains the complete gene set to reduce sulfate to sulfide as well as other sulfur disproportionaters: the *sat* gene (THC_1309), the *aprAB* genes (THC_1312, THC_1311), the *qmoABC* genes (THC_1313-1315), the *dsrABD* and the *dsrC* genes (THC_0449-051, THC_0323) and the *dsrMKJOP* (THC_0590-0594).

It was reported that most of ISR-SDB possess the all the genes necessary for CO_2

fixation via Wood-Ljungdahl pathway (3, 4, 9, 11). The genome of *Caldimicrobium thiodismutans* TF1^T also contains all genes for CO_2 fixation via Wood-Ljungdahl pathway. Wood-Ljungdahl pathway consists of Eastern and Western branch (24). In the Eastern branch, the genes are involved in conversion of carbon dioxide to methyl-tetrahydrofolate: a formate dehydrogenase encoded by the genes (THC_1766-1767), the *fhs* gene encoding a formyl-tetrahydrofolate synthase (THC_1752), the *folD* gene encoding a bifunctional methylene-tetrahydrofolate dehydrogenase/formyl-tetrahydrofolate cyclohydrolase (THC_1753) and the *metF* and *acsD* genes encoding a methylene-tetrahydrofolate reductase and a corrinoid iron-sulfur protein small subunit (THC_1761 as the *metF/acsD* fusion gene). In the Western branch, the genes are involved in reduction of carbon dioxide to carbon monoxide and generation of acetyl-CoA from methyl-tetrahydrofolate and carbon monoxide: the *acsA* gene encoding a carbon monoxide dehydrogenase (THC_1754), the *acsB* gene encoding an acetyl-CoA synthase (THC_1762), the *acsC* gene encoding a corrinoid iron-sulfur protein large subunit (THC_1763), the metF/acsD gene (THC_1761) and the *acsE* gene encoding a methyltransferase (THC_1765). The formate dehydrogenase alpha and beta subunits (Moth_2312, Moth_2314) of *Moorella thermoacetica* is thought to reduce CO_2 to formate with the oxidation of NADPH in Wood-Ljungdahl pathway (25). The sequences of the formate dehydrogenase beta subunit (Moth_2314) and N-terminal of formate dehydrogenase (THC_1766) contain GltD domain. Additionally, this domain was found in formate dehydrogenases of other sulfur-disproportionating bacteria: *Desulfocapsa sulfexigens* SB164P1^T, *Desulfurivibrio alkaliphilus* AHT2^T, *Thermosulfurimonas dismutans* S95^T, *Dissulfuribacter thermophiles* S69^T, but not 45J and *Dethiobacter alkaliphilus* AHT1^T. Most of those genes were separated into two genes such as THC_1766 and THC_1767, because the sequences of those genes contain a TGA codon as the end codon. When the TGA codons of the sequences of the above genes were translated to selenocysteine, the selenocysteine was found to

be located at same position when aligned with formate dehydrogenase sequences (Moth_2312) of *Moorella thermoacetica* (Fig. 4-3), which is thought to contain selenocysteine (24, 25). Therefore, TGA codon of those formate dehydrogenase may be thought to be translated as selenocysteine.

The genome of *Caldimicrobium thiodismutans* TF1^T contains the *napAGH* genes encoding a nitrate reductase (THC_1378, THC_1380-1381). For nitrite reduction to ammonium, the genome of *Caldimicrobium thiodismutans* TF1^T contains the gene a octaheme nitrite reductase (this gene was called *onr* gene in this chapter) (THC_1376) and the *otr* gene a octaheme tetrathionate reductase (THC_0525).

4.3.3. Statistics of comparative genomic analysis

The six genomes of ISR-SDB contain all genes for sulfate reduction: *Desulfocapsa sulfexigens* SB164P1^T, *Desulfurivibrio alkaliphilus* AHT2^T, *Thermosulfurimonas dismutans* S95^T, *Dissulfuribacter thermophiles* S69^T, *Caldimicrobium thiodismutans* TF1^T and 45J (3–5, 11). *Dethiobacter alkaliphilus* AHT1^T which is also ISR-SDB possesses the *aprAB* genes but does not possess the Dsr related genes, the *sat* gene or the *qmoABC* genes (9). In order to identify genes that are specific to ISR-SDB with the Dsr system or specific to sulfate-reducing bacteria, the presence or absence of each gene assigned to homologous genes in the genomes of ISR-SDB was compared to sulfate-reducing bacteria. In the 92 genomes including 69 genomes of sulfate-reducing bacteria and 6 genomes of ISR-SDB with Dsr system, there are 304,075 protein coding sequences. By comparative genomic analysis, 283,684 genes were classified in orthogroups and 20,391 genes were unassigned. Genes that are common in the genomes of sulfate-reducing bacteria but are not found in the genomes of all ISR-SDB with Dsr system were not found.

4.3.4. Relatively specific genes in the genomes of ISR-SDB

Table 4-5 shows orthogroups that were evaluated to lowest rate of possessing assigned genes in genomes of sulfate-reducing bacteria within orthogroups assigned genes contained in all six genomes of ISR-SDB with Dsr system. Four genes assigned to OG00003485, OG0002281, OG0002186 and OG0001592 in those orthogroups are components of similar gene clusters in different genomes of ISR-SDB with Dsr system (Fig. 4-1, Table 4-5). In that gene cluster of 45J, the protein (A45J_1802) encoded by gene assigned to OG0001592 is annotated to YeE/YedE family protein. The sequence of YeE/YedE family protein (A45J_1802) contains the domain of sulfur transporter (PF04143) family, and proteins of this family are thought to transport sulfur containing compounds (26). The *tusA* genes encoding a TusA protein, which is known to be related to sulfur relay, were found in all gene clusters of ISR-SDB with Dsr system except for 45J. DsrE is also known to be involved in sulfur relay. The Cys residues of DsrE like proteins encoded by genes assigned to OG0002281 were conserved with Cys78 of DsrE of DsrEFH complex in *Allochromatinum vinosum*. In *Allochromatinum vinosum*, DsrEFH complex is known to transfer sulfur to DsrC, and the residue of Cys78 of DsrE is needed to combine with sulfide (27). Therefore, relatively specific gene cluster of ISR-SDB is thought to be involved in sulfur relay and transport. Functions of the genes assigned to OG0003485 and OG0002186 were unknown. Genes assigned to OG0003485 were not found in the genomes of sulfate-reducing bacteria that are not able to grow by sulfur disproportionation except for *Dsulfobulbus mediterraneus* 86FS1^T (28). Some sulfate-reducing bacteria are known to be able to disproportionate inorganic sulfur compounds without growth (Table 4-3). Therefore, it is unclear whether *Dsulfobulbus mediterraneus* 86FS1^T is able to disproportionate inorganic sulfurcompounds. The genes assigned to OG0001930 and OG0002138 are also relatively specific genes to ISR-SDB with Dsr system and are encoding a cytochrome *c* and a DUF3373 domain containing protein, respectively.

4.3.5. Genes related to dissimilatory nitrate reduction in the genomes of ISR-SDB

Some ISR-SDB are able to grow by nitrate reduction with oxidation of sulfur compounds. In order to reveal commonalities and differences of genes related to dissimilatory nitrate metabolism of ISR-SDB belonging to four different phyla, *Proteobacteria*, *Thermodesulfobacteria*, *Firmicutes* and *Nitrospirae*, the genes in the genomes of these were compared. Most of sulfur-disproportionating bacteria possess the *napA* gene related to reduce nitrate to nitrite (Table 4-7). The *nrfA* gene encoding a nitrite reductase, the *onr* gene and the *otr* gene are known to be involved in nitrite reduction to ammonium, respectively (29–32). The genomes of *Desulfurivibrio alkaliphilus* AHT2^T and 45J contain the *nrfA* gene (DaAHT2_0065, A45J_0204) (16). The genomes of *Thermosulfurimonas dismutans* S95^T and *Dissulfuribacter thermophiles* S69^T contain *otr* genes (TDIS_1881, DBT_1161) (5). Although it was unclear whether *Desulfocapsa sulfexigens* SB164P1^T and *Dethiobacter alkaliphilus* AHT1^T possess the genes related to nitrite reduction, comparative genomic analysis reveal that those genes were found in the genomes of both bacteria. The genome of *Dethiobacter alkaliphilus* AHT1^T contains the *nrfA* gene and/or the *onr* gene (DealDRAFT_RS04350, DealDRAFT_RS09375, DealDRAFT_RS14145) assigned to OG0001389 that was assigned by the above genes (THC_1376, DaAHT2_0065, A45J_0204). The genome of *Desulfocapsa sulfexigens* SB164P1^T contains the *otr* gene (UWK_02550) that was assigned by the above genes (THC_0525, TDIS_1881, DBT_1161). Therefore, ISR-SDB with Dsr system possess all genes for reduction of nitrate to ammonium (Table 4-7). In *Desulfurivibrio alkaliphilus* AHT2^T, all proteins for sulfate reduction such as DsrAB, AprAB and Sat are involved in oxidization of sulfide to sulfate and are thought to react in the reverse direction compared to sulfate reduction (16). ISR-SDB with Dsr system possess all genes for sulfate reduction (Table 4-7). One possibility is that some ISR-SDB that have not been known to grow with sulfur oxidation are able to grow by

dissimilatory nitrate reduction with oxidation of sulfur compounds.

4.3.6. Proteomic analysis

The expressed proteins were identified under growth conditions of thiosulfate and elemental sulfur disproportionation in *Caldimicrobium thiodismutans* TF1^T (Table 4-8). A total of 947, 1025 and 894 proteins were detected from three samples under growth conditions of thiosulfate disproportionation and a total of 877 and 988 proteins were detected from two samples under growth conditions of elemental sulfur disproportionation. A total of 633 proteins were detected in all samples. Although 52 genes encoding ribosomal proteins which formed large and small complexes were found in the genome of *Caldimicrobium thiodismutans* TF1^T, only 26 ribosomal proteins were detected in all samples and 5 were not detected in any samples. Therefore, the detected proteins are thought not to consist of whole proteome in the cells, but are likely present at relatively high abundance in the samples. Protein abundance was evaluated based on a peak area. All proteins for sulfate reduction were detected in all samples (Table 4-9); DsrAB (THC_0449-0450), DsrC (THC_0323), DsrMKJOP (THC_0590-0594), AprAB (THC_1311-1312), QmoABC (THC_1313 1315) and Sat (THC_1309). The proteins (THC_1428-1432) encoded by genes in the relatively specific gene cluster were expressed at high abundance in all samples (Table 4-9). In addition, THC_1435 and THC_1436 encoded by genes that are located near the gene cluster were also expressed at high abundance in all samples. However, the functions of THC_1435 and THC_1436 are unknown. Several proteins that may be related to sulfur relay were detected in all samples; DsrE (THC_0958), Rhodanese-like domain-containing proteins (THC_0726 and THC_1155-1156) and TusA (THC_1429).

The cytochrome *c* genes (THC1325-1326) are related specific genes, which were assigned to OG0001929, in sulfur-disproportionating bacteria (Table 4-9). These cytochrome *c*

(THC_1325-1326) proteins were detected at a low level or not detected in samples (Table 4-9). However, the proteins (THC_1322-1324) encoded by genes next to the cytochrome *c* gene (THC_1325) were detected in all samples (Table 4-9). A polysulfide reductase and a 4Fe-4S ferredoxin containing protein were encoded by genes (THC_1323-1324), which may be related to electron transfer in sulfur disproportionation. Although the protein (THC_1322) was not annotated, the proteomic result and gene location indicates that this protein (THC_1322) may be related to electron transfer or may interact with the above-mentioned electron transfer proteins. As electron flow is thought to be affected by the direction of the enzyme reaction, electron flow in sulfur disproportionation is thought to be different from that in sulfate reduction. Therefore, one possibility is that these proteins (THC_1322-1326) are important for erectron transfer in sulfur disproportionation.

In addition, THC_0289 encoded by gene assigned to OG0002138, which is relatively specific to ISR-SDB, was expressed at high abundance (Table 4-9). Although the function of DUF3373 domain containing protein (THC_0289) is still unknown, the localization of this protein was predicted to be at the outer membrane.

The SorAB (THC_0088-0087) proteins were not detected or detected at low amounts in the samples (Table 4-9). In *Desulfocapsa sulfexigens* SB164P1^T, sulfite oxidoreductase is thought to be a key protein at disproportionation of thiosulfate and elemental sulfur (17). However, it has not been reported that the *sorAB* genes were found in the other sulfur-disproportionating bacteria including *Desulfocapsa sulfexigens* SB164P1^T. Additionally, the SorAB proteins were not detected in most of samples in *Caldimicrobium thiodismutans* TF1^T (Table 4-9), indicating that SorAB proteins do not have a major role in thiosulfate and elemental sulfur disproportionation in *Caldimicrobium thiodismutans* TF1^T. Therefore, other genes may be involved in sulfite oxidation to sulfate directly. PhsA/PsrA (THC_0921) was detected within top 100 in all samples. PhsA/PsrA of *Caldimicrobium thiodismutans* TF1^T is

suggested to reduce thiosulfate and/or polysulfide in thiosulfate and elemental sulfur disproportionation.

Interestingly, the protein (THC_1310) encoded by the gene located next to the *sat* gene was present at relatively high abundance within top 10 in all samples. Homologous genes were found in the genomes of sulfur- and/or sulfate-reducing prokaryotes and sulfur-disproportionating bacteria using by BLASTP search. These prokaryotes except *Desulfonatronospira thiodismutans* and uncultured bacteria are thermophilic prokaryotes. The genes were located adjacent to the *sat* gene in these genomes, and the Sat sequence of these prokaryotes formed a monophyletic cluster by phylogenetic analysis (Fig 4-4). The genome of *Archaeoglobus fulgidus* as sulfate reducing archaea contains this gene as ORF2 gene (33), and the transcription level of this gene was high under the growth condition of thiosulfate and sulfate reduction (34). Sat was reported to be a homodimer in *Archaeoglobus fulgidus* (33). Although the function of the protein (THC_1310) is unclear, these data suggest that the protein is involved in the reaction of sulfate adenylyltransferase indirectly in the thermophilic condition.

It is confirmed that the proteins involved in Wood-Ljungdahl pathway for carbon fixation were detected in all samples (THC_1752-1754, THC_1761-1763, THC_1766-1767) (Table 4-10).

4.3.7. Hypothesis of sulfur-disproportionating pathway

The relatively specific gene cluster was found in the genomes of ISR-SDB. This gene cluster contains four characteristic genes; the *dsrE like* gene (OG0002281) and the gene (OG0001592) encoding YeeE/YedE family protein and two uncharacterized genes (OG0002186, OG0003483) (Fig. 4-1, Table 4-6). This gene cluster is suggested to be involved in sulfur relay in sulfur disproportionation. This gene cluster was not found in the genome of *Dethiobacter*

alkaliphilus AHT1^T, which is sulfur disproportionater without the Dsr system. The genomes of a variety of prokaryotes contain gene clusters including the *yedE*, the *sirA like* genes and genes related to selenocysteine metabolism, such as the *selD* gene encoding a selenide, water dikinase and the gene encoding a Sec lyase (35). The function of those gene clusters was thought to be related to selenocysteine metabolism (35). The sequence of the *sirA* gene was similar to the sequence of the *tusA* gene in general. However, the *selD* gene encoding a selenide, water dikinase (THC_1500) was not found near this gene cluster (THC_1428-1432) in the genome of *Caldimicrobium thiodismutans* TF1^T. In addition, all proteins encoded by genes (THC_1428-1432) in this gene cluster were expressed at high levels under growth conditions of thiosulfate and elemental sulfur disproportionation in *Caldimicrobium thiodismutans* TF1^T. Therefore, these proteins (THC_1428-1432) are suggested to be involved in thiosulfate and elemental sulfur disproportionation but not selenocysteine metabolism. All sequences of DsrE like proteins encoded by genes in the gene cluster contain cysteine residues conserved at Cys78 in the sequence of DsrE (Alvin_1253) (Fig. 4-2). In *Allochromiatium vinosum*, DsrE of DsrEFH is known to participate in dissimilatory sulfur-oxidizing pathway by transfer of sulfuide as substrate to DsrC and Cys78 of DsrE is needed to transfer of it (27). This cysteine residue is conserved to the sequence of DsrE3A protein which is related to transfer thiosulfate to TusA in *Metallosphaera cuprina* (36). Therefore, DsrE like protein encoded by genes of the gene cluster in ISR-SDB is thought to transfer inorganic sulfur compounds in sulfur disproportionation.

In *Desulfurivibrio alkaliphilus* AHT2^T, all proteins for sulfate reduction such as DsrAB, AprAB and Sat are involved in oxidization of sulfide to sulfate and are thought to react in the reverse direction (16). In *Allochromatium vinosum*, several proteins related to sulfur relay participate in sulfur oxidizing pathway such as DsrEFH, TusA and rhodanese (27, 37, 38). By genomic analysis of *Dissulfuribacter thermophiles* S69^T, the genes (DBT_0017-0019) in the gene cluster are thought to related to elemental sulfur oxidation by sulfur relay with DsrC

protein (5). The results in this chapter support this hypothesis because that gene cluster was not found in most of sulfate reducing bacteria.

In *Caldimicrobium thiodismutans* TF1^T, it was confirmed that all proteins for sulfate reduction were expressed under growth conditions of thiosulfate and elemental sulfur disproportionation (Table 4-9). In sulfur disproportionation, reactions of APS reductase and sulfate transferase were thought to be reverse direction against sulfate reduction. In sulfur disproportionation, it is possible that this gene cluster is related to sulfur relay with Dsr system. Reaction of elemental sulfur disproportionation is an endothermal reaction. It is needed for growth with elemental sulfur disproportionation that sulfide generated from disproportionation is removed. One possibility is that the proteins encoded by genes of this gene cluster were involved in excretion of sulfide from cytoplasm to the outside via sulfur relay and sulfur transport.

In general, reaction of enzyme such as AprAB, Sat and DsrAB are reversible. However, sulfur transfer was occurred from DsrEFH to DsrC but reverse reaction of that was not occurred *in vitro* in *Allochromatium vinosum* (27). It is thought that DsrC persulfurated by transfer from DsrE persulfurated is oxidized to DsrC trisulfide by DsrMKJOP, and DsrC trisulfide was oxidized to sulfite by DsrAB (14, 15). Therefore, irreversible production of DsrC persulfurated by sulfur relay may be one of the key factors to determine the direction of reaction with the Dsr system. One possibility is that sulfur transfer occurs from the DsrE like protein to DsrC in a sulfur-disproportionting pathway as well as sulfur oxidation of *Allochromatium vinosum*. This possibility may explain the reason why some sulfur-disproportionating bacteria are incapable of growth with sulfate reduction in spite of possessing the complete genes for sulfate reduction. It is possible that sulfur relay optimizes direction of enzyme reaction of Dsr system. Sulfate reduction may be inhibited as a sequel to accumulation of DsrC persulfurated by reductive reaction of DsrAB. However, this hypothesis

has important problem that sulfide is not produce by Dsr system. In any case, result of comparative genomic analysis and proteomic analysis in this study strongly suggest that those proteins related to sulfur relay participate in sulfur-disproportionating pathway. However, more research such as genetics, biochemistry and isotopic analysis are necessary to reveal actual sulfur-disproportionating pathway.

Most of sulfur disproportionaters possess the complete gene set necessary for the fixation of carbon dioxide via Wood-Ljungdahl pathway (3, 4, 9, 11). Interestingly, formate dehydrogenases of several sulfur-disproportionating bacteria contain GltD domain (Fig. 4-3). This domain is involved in electron transfer with NADPH. This domain is also contained in the sequence of DsrL in *Allochromatium vinosum* (39, 40). The *dsrL* gene is located in the *dsr* gene cluster in *Allochromatium vinosum* and DsrL is known to be necessary for sulfur oxidation (40). One possibility is that DsrL is involved in electron transfer in Dsr system. Therefore, it is possible that sulfur-disporoportionating pathway is linked to carbon fixation. Some sulfate-reducing bacteria are able to disproportionate inorganic sulfur compounds without growth. Although the reason is still unclear why sulfur disproportionation is not supported to be growth, linking sulfur-disproportionation pathway with carbon fixation may be advantage for growth with sulfur disproportionation.

4.4. Conclusion

Sulfur-disproportionating bacteria belonging to four different phyla possess many genes in common for dissimilatory sulfur and nitrogen metabolism and carbon fixation. The genomes of ISR-SDB with Dsr system contain a relatively specific gene cluster, which was not found in the most of sulfate-reducing bacteria. The gene cluster contains the genes related to sulfur relay as well as a transporter. Proteins encoded by this gene cluster were expressed at

high abundance in the cells under growth conditions of thiosulfate and elemental sulfur disproportionation in *Caldimicrobium thiodismutans* TF1^{T}. This gene cluster may determine the direction of reaction of Dsr system in sulfur-disproportionating pathway.

4.5. Reference

1. Finster K. Microbiological disproportionation of inorganic sulfur compounds. J Sulfur Chem. 2008;29(3–4):281–92.
2. Krämer M, Cypionka H. Sulfate formation via ATP sulfurylase in thiosulfate- and sulfite-disproportionating bacteria. Arch Microbiol. 1989;151(3):232–7.
3. Finster KW, Kjeldsen KU, Kube M, Reinhardt R, Mussmann M, Amann R, et al. Complete genome sequence of *Desulfocapsa sulfexigens*, a marine deltaproteobacterium specialized in disproportionating inorganic sulfur compounds. Stand Genomic Sci. 2013;8(1):58–68.
4. Melton ED, Sorokin DY, Overmars L, Chertkov O, Clum A, Pillay M, et al. Complete genome sequence of *Desulfurivibrio alkaliphilus* strain AHT2^{T}, a haloalkaliphilic sulfidogen from Egyptian hypersaline alkaline lakes. Stand Genomic Sci. 2016;11(1):67.
5. Slobodkina GB, Mardanov AV., Ravin NV., Frolova AA, Chernyh NA, Bonch-Osmolovskaya EA, et al. Respiratory ammonification of nitrate coupled to anaerobic oxidation of elemental sulfur in deep-sea autotrophic thermophilic bacteria. Front Microbiol. 2017;8:87.
6. Finster K, Liesack W, Thamdrup B. Elemental sulfur and thiosulfate disproportionation by *Desulfocapsa sulfoexigens* sp. nov., a new anaerobic bacterium isolated from marine surface sediment. Appl Environ Microbiol. 1998;64(1):119–25.
7. Poser A, Lohmayer R, Vogt C, Knoeller K, Planer-Friedrich B, Sorokin D, et al. Disproportionation of elemental sulfur by haloalkaliphilic bacteria from soda lakes. Extremophiles. 2013;17(6):1003–12.
8. Slobodkin AI, Reysenbach AL, Slobodkina GB, Kolganova TV., Kostrikina NA, Bonch-Osmolovskaya EA. *Dissulfuribacter thermophilus* gen. nov., sp. nov., a thermophilic, autotrophic, sulfur-disproportionating, deeply branching

deltaproteobacterium from a deep-sea hydrothermal vent. Int J Syst Evol Microbiol. 2013;63(6):1967–71.

9. Melton ED, Sorokin DY, Overmars L, Lapidus AL, Pillay M, Ivanova N, et al. Draft genome sequence of *Dethiobacter alkaliphilus* strain AHT1^{T}, a gram-positive sulfidogenic polyextremophile. Stand Genomic Sci. 2017;12:57.

10. Slobodkin AI, Reysenbach AL, Slobodkina GB, Baslerov RV, Kostrikina NA, Wagner ID, et al. *Thermosulfurimonas dismutans* gen. nov., sp. nov., an extremely thermophilic sulfur-disproportionating bacterium from a deep-sea hydrothermal vent. Int J Syst Evol Microbiol. 2012;62(11):2565–71.

11. Mardanov A, Beletsky A, Kadnikov V, Slobodkin A, Ravin N. Genome analysis of *Thermosulfurimonas dismutans*, the first thermophilic sulfur-disproportionating bacterium of the phylum *Thermodesulfobacteria*. Front Microbiol. 2016;7:950.

12. Kojima H, Umezawa K, Fukui M. *Caldimicrobium thiodismutans* sp. nov., a sulfur-disproportionating bacterium isolated from a hot spring, and emended description of the genus *Caldimicrobium*. Int J Syst Evol Microbiol. 2016;66(4):1828–31.

13. Slobodkina GB, Reysenbach AL, Kolganova TV., Novikov AA, Bonch-Osmolovskaya EA, Slobodkin AI. *Thermosulfuriphilus ammonigenes* gen. nov., sp. nov., a thermophilic, chemolithoautotrophic bacterium capable of respiratory ammonification of nitrate with elemental sulfur. Int J Syst Evol Microbiol. 2017;67(9):3474–9.

14. Santos AA, Venceslau SS, Grein F, Leavitt WD, Dahl C, Johnston DT, et al. A protein trisulfide couples dissimilatory sulfate reduction to energy conservation. Science. 2015;350(6267):1541–5.

15. Dahl C. Sulfur Metabolism in Phototrophic Bacteria. In: Hallenbeck P, editor. Modern Topics in the Phototrophic Prokaryotes — Metabolism, Bioenergetics, and Omics. Cham: Springer; 2017. p. 27-66.

16. Thorup C, Schramm A, Findlay AJ, Finster KW, Schreiber L. Disguised as a sulfate reducer: Growth of the deltaproteobacterium *Desulfurivibrio alkaliphilus* by sulfide oxidation with nitrate. mBio. 2017;8(4):e00671-17.

17. Frederiksen T-M, Finster K. Sulfite-oxido-reductase is involved in the oxidation of sulfite in *Desulfocapsa sulfoexigens* during disproportionation of thiosulfate and elemental sulfur. Biodegradation. 2003;14(3):189–98.

18. Zerbino DR, Birney E. Velvet: Algorithms for de novo short read assembly using de Bruijn graphs. Genome Res. 2008;18(5):821–9.

19. Sugawara H, Mori H, Kurokawa K. Microbial Genome Annotation Pipeline (MiGAP) for diverse users. The 20th International Conference on Genome Informatics (GIW2009) Poster and Software Demonstrations (Yokohama). 2009;S001-1–2.

20. Marchler-Bauer A, Bo Y, Han L, He J, Lanczycki CJ, Lu S, et al. CDD/SPARCLE: Functional classification of proteins via subfamily domain architectures. Nucleic Acids Res. 2017;45:D200–3.

21. Emms DM, Kelly S. OrthoFinder: solving fundamental biases in whole genome comparisons dramatically improves orthogroup inference accuracy. Genome Biol. 2015;16(1):157.

22. Yu NY, Wagner JR, Laird MR, Melli G, Rey S, Lo R, et al. PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. Bioinformatics. 2010;26(13):1608–15.

23. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013;30(12):2725–9.

24. Ragsdale SW, Pierce E. Acetogenesis and the Wood-Ljungdahl pathway of CO_2 fixation. Biochim Biophys Acta - Proteins Proteomics. 2008;1784(12):1873–98.

25. Pierce E, Xie G, Barabote RD, Saunders E, Han CS, Detter JC, et al. The complete

genome sequence of *Moorella thermoacetica* (f. *Clostridium thermoaceticum*). Environ Microbiol. 2008;10(10):2550–73.

26. Gristwood T, McNeil MB, Clulow JS, Salmond GPC, Fineran PC. PigS and PigP regulate prodigiosin biosynthesis in *Serratia* via differential control of divergent operons, which include predicted transporters of sulfur-containing molecules. J Bacteriol. 2011;193(5):1076–85.

27. Stockdreher Y, Venceslau SS, Josten M, Sahl HG, Pereira IAC, Dahl C. Cytoplasmic sulfurtransferases in the purple sulfur bacterium *Allochromatium vinosum*: Evidence for sulfur transfer from DsrEFH to DsrC. PLoS One. 2012;7(7):e40785.

28. Sass A, Rütters H, Cypionka H, Sass H. *Desulfobulbus mediterraneus* sp. nov., a sulfate-reducing bacterium growing on mono- and disaccharides. Arch Microbiol. 2002;177(6):468–74.

29. Atkinson SJ, Mowat CG, Reid GA, Chapman SK. An octaheme *c*-type cytochrome from *Shewanella oneidensis* can reduce nitrite and hydroxylamine. FEBS Lett. 2007;581(20):3805–8.

30. Tikhonova TV, Trofimov AA, Popov VO. Octaheme nitrite reductases: structure and properties. Biochem (Mosc) Suppl Ser A Membr Cell Biol. 2012;77(10):1129–38.

31. Tikhonova T, Tikhonov A, Trofimov A, Polyakov K, Boyko K, Cherkashin E, et al. Comparative structural and functional analysis of two octaheme nitrite reductases from closely related *Thioalkalivibrio* species. FEBS J. 2012;279(21):4052–61.

32. Simon J, Kern M, Hermann B, Einsle O, Butt JN. Physiological function and catalytic versatility of bacterial multihaem cytochromes *c* involved in nitrogen and sulfur cycling. Biochem Soc Trans. 2011;39(6):1864–70.

33. Sperling D, Kappler U, Wynen A, Dahl C, Trüper HG. Dissimilatory ATP sulfurylase from the hyperthermophilic sulfate reducer *Archaeoglobus fulgidus* belongs to the group

of homo-oligomeric ATP sulfurylases. FEMS Microbiol Lett. 1998;162(2):257–64.

34. Hocking WP, Stokke R, Roalkvam I, Steen IH. Identification of key components in the energy metabolism of the hyperthermophilic sulfate-reducing archaeon *Archaeoglobus fulgidus* by transcriptome analyses. Front Microbiol. 2014;5:95.

35. Lin J, Peng T, Jiang L, Ni J-Z, Liu Q, Chen L, et al. Comparative genomics reveals new candidate genes involved in selenium metabolism in prokaryotes. Genome Biol Evol. 2015;7(3):664–76.

36. Liu LJ, Stockdreher Y, Koch T, Sun ST, Fan Z, Josten M, et al. Thiosulfate transfer mediated by DsrE/TusA homologs from acidothermophilic sulfur-oxidizing archaeon *Metallosphaera cuprina*. J Biol Chem. 2014;289(39):26949–59.

37. Stockdreher Y, Sturm M, Josten M, Sahl HG, Dobler N, Zigann R, et al. New proteins involved in sulfur trafficking in the cytoplasm of *Allochromatium vinosum*. J Biol Chem. 2014;289(18):12390–403.

38. Dahl C. Cytoplasmic sulfur trafficking in sulfur-oxidizing prokaryotes. IUBMB Life. 2015;67(4):268–74.

39. Dahl C, Engels S, Pott-Sperling AS, Schulte A, Sander J, Lübbe Y, et al. Novel genes of the *dsr* gene cluster and evidence for close interaction of Dsr proteins during sulfur oxidation in the phototrophic sulfur bacterium *Allochromatium vinosum*. J Bacteriol. 2005 Feb;187(4):1392–404.

40. Lübbe YJ, Youn HS, Timkovich R, Dahl C. Siro(haem)amide in *Allochromatium vinosum* and relevance of DsrL and DsrN, a homolog of cobyrinic acid *a*,*c*-diamide synthase, for sulphur oxidation. FEMS Microbiol Lett. 2006;261(2):194–202.

41. Nielsen MB, Kjeldsen KU, Ingvorsen K. *Desulfitibacter alkalitolerans* gen. nov., sp. nov., an anaerobic, alkalitolerant, sulfite-reducing bacterium isolated from a district heating plant. Int J Syst Evol Microbiol. 2006;56(12):2831–6.

42. Niggemyer A, Spring S, Stackebrandt E, Rosenzweig RF. Isolation and characterization of a novel As(V)-reducing bacterium: implications for arsenic mobilization and the genus *Desulfitobacterium*. Appl Environ Microbiol. 2001;67(12):5568–80.

43. Lie TJ, Godchaux W, Leadbetter ER. Sulfonates as terminal electron acceptors for growth of sulfite-reducing bacteria (*Desulfitobacterium* spp.) and sulfate-reducing bacteria: effects of inhibitors of sulfidogenesis. Appl Env Microbiol. 1999;65(10):4611–7

44. Ramamoorthy S, Sass H, Langner H, Schumann P, Kroppenstedt RM, Spring S, et al. *Desulfosporosinus lacus* sp. nov., a sulfate-reducing bacterium isolated from pristine freshwater lake sediments. Int J Syst Evol Microbiol. 2006;56(12):2729–36.

45. Campbell L, Postgate J. Classification of the spore-forming sulfate-reducing bacteria. Bacteriol Rev. 1965;29(3):359–63.

46. Liu Y, Karnauchow TM, Jarrell KF, Balkwill DL, Drake GR, Ringelberg D, et al. Description of two new thermophilic *Desulfotomaculum* spp., *Desulfotomaculum putei* sp. nov., from a deep terrestrial subsurface, and *Desulfotomaculum luciae* sp. nov., from a hot spring. Int J Syst Bacteriol. 1997;47(3):615–21.

47. Pikuta E, Lysenko A, Suzina N, Osipov G, Kuznetsov B, Tourova T, et al. *Desulfotomaculum alkaliphilum* sp. nov., a new alkaliphilic, moderately thermophilic, sulfate-reducing bacterium. Int J Syst Evol Microbiol. 2000;50(1):25–33.

48. Kaksonen AH, Spring S, Schumann P, Kroppenstedt RM, Puhakka JA. *Desulfurispora thermophila* gen. nov., sp. nov., a thermophilic, spore-forming sulfate-refucer isolated from a sulfidogenic fluidized-bed reactor. Int J Syst Evol Microbiol. 2007;57(5):1089–94.

49. Van Eldere JR, De Pauw G, Eyssen HJ. Steroid sulfatase activity in a *Peptococcus niger* strain from the human intestinal microflora. Appl Environ Microbiol. 1987;53(7):1655–60.

50. Sorokin DY, Tourova TP, Mussmann M, Muyzer G. *Dethiobacter alkaliphilus* gen. nov. sp. nov., and *Desulfurivibrio alkaliphilus* gen. nov. sp. nov.: two novel representatives of

reductive sulfur cycle from soda lakes. Extremophiles. 2008;12(3):431–9.

51. Frank YA, Kadnikov V V., Lukina AP, Banks D, Beletsky A V., Mardanov A V., et al. Characterization and genome analysis of the first facultatively alkaliphilic *Thermodesulfovibrio* isolated from the deep terrestrial subsurface. Front Microbiol. 2016;7:2000.

52. Sekiguchi Y, Muramatsu M, Imachi H, Narihiro T, Ohashi A, Harada H, et al. *Thermodesulfovibrio aggregans* sp. nov. and *Thermodesulfovibrio thiophilus* sp. nov., anaerobic, thermophilic, sulfate-reducing bacteria isolated from thermophilic methanogenic sludge, and emended description of the genus *Thermodesulfovibrio*. Int J Syst Evol Microbiol. 2008;58(11):2541–8.

53. Sonne-Hansen J, Ahring BK. *Thermodesulfobacterium hveragerdense* sp. nov., and *Thermodesulfovibrio islandicus* sp. nov., two thermophilic sulfate reducing bacteria isolated from a Icelandic hot spring. Syst Appl Microbiol. 1999;22(4):559–64.

54. Henry EA, Devereux R, Maki JS, Gilmour CC, Woese CR, Mandelco L, et al. Characterization of a new thermophilic sulfate-reducing bacterium. Arch Microbiol. 1994;161(1):62–9.

55. Kuever J, Rainey FA, Widdel F. Genus I. *Desulfarculus* gen. nov. In: Brenner DJ, Krieg NR, Staley JT, Garrity GM, editors. Bergey's Manual of Systematic Bacteriology, Volume 2, Part C. 2nd ed. New York: Springer; 2005. p. 1004-5.

56. An TT, Picardal FW. *Desulfocarbo indianensis* gen. nov., sp. nov., a benzoate-oxidizing, sulfate-reducing bacterium isolated from water extracted from a coal bed. Int J Syst Evol Microbiol. 2014;64(8):2907–14.

57. Davidova IA, Wawrik B, Callaghan A V., Duncan K, Marks CR, Suflita JM. *Dethiosulfatarculus sandiegensis* gen. nov., sp. nov., isolated from a methanogenic paraffindegrading enrichment culture and emended description of the family

Desulfarculaceae. Int J Syst Evol Microbiol. 2016;66(3):1242–8.

58. Descamps ECT, Monteil CL, Menguy N, Ginet N, Pignol D, Bazylinski DA, et al. *Desulfamplus magnetovallimortis* gen. nov., sp. nov., a magnetotactic bacterium from a brackish desert spring able to biomineralize greigite and magnetite, that represents a novel lineage in the *Desulfobacteraceae*. Syst Appl Microbiol. 2017;40(5):280–9.

59. Cravo-Laureau C, Matheron R, Cayol JL, Joulian C, Hirschler-Réa A. *Desulfatibacillum aliphaticivorans* gen. nov., sp. nov., an n-alkane- and n-alkene-degrading, sulfate-reducing bacterium. Int J Syst Evol Microbiol. 2004;54(1):77–83.

60. Schnell S, Bak F, Pfennig N. Anaerobic degradation of aniline and dihydroxybenzenes by newly isolated sulfate-reducing bacteria and description of *Desulfobacterium anilini*. Arch Microbiol. 1989;152(6):556–63.

61. Balk M, Altinbaş M, Rijpstra WIC, Damsté JSS, Stams AJM. *Desulfatirhabdium butyrativorans* gen. nov., sp. nov., a butyrate-oxidizing, sulfate-reducing bacterium isolated from an anaerobic bioreactor. Int J Syst Evol Microbiol. 2008;58(1):110–5.

62. Higashioka Y, Kojima H, Watanabe M, Fukui M. *Desulfatitalea tepidiphila* gen. nov., sp. nov., a sulfate-reducing bacterium isolated from tidal flat sediment. Int J Syst Evol Microbiol. 2013;63(2):761–5.

63. Widdel F. New types of acetate-oxidizing, sulfate-reducing *Desulfobacter* species, *D. hydrogenophilus* sp. nov., *D. latus* sp. nov., and *D. curvatus* sp. nov. Arch Microbiol. 1987;148(4):286–91.

64. Lien T, Beeder J. *Desulfobacter vibrioformis* sp. nov., a sulfate reducer from a water-oil separation system. Int J Syst Bacteriol. 1997;47(4):1124–8.

65. Brysch K, Schneider C, Fuchs G, Widdel F. Lithoautotrophic growth of sulfate-reducing bacteria, and description of *Desulfobacterium autotrophicum* gen. nov., sp. nov. Arch Microbiol. 1987;148(4):264–74.

66. Bak F, Widdel F. Anaerobic degradation of phenol and phenol derivatives by *Desulfobacterium phenolicum* sp. nov. Arch Microbiol. 1986;146(2):177–80.

67. Kuever J, Rainey FA, Widdel F. Genus VI. *Desulfococcus*. In: Brenner DJ, Krieg NR, Staley JT, Garrity GM, editors. Bergey's Manual of Systematic Bacteriology, Volume 2, Part C. 2nd ed. New York: Springer; 2005. p. 972-4.

68. Ahn YB, Kerkhof LJ, Häggblom MM. *Desulfoluna spongiiphila* sp. nov., a dehalogenating bacterium in the *Desulfobacteraceae* from the marine sponge *Aplysina aerophoba*. Int J Syst Evol Microbiol. 2009;59(9):2133–9.

69. Gavin NR, Patel BKC. *Desulforegula conservatrix* gen. nov., sp. nov., a long-chain fatty acid-oxidizing, sulfate-reducing bacterium isolated from sediments of a freshwater lake. Int J Syst Evol Microbiol. 2001;51(5):1911–6.

70. Galushko AS, Rozanova EP. *Desulfobacterium cetonicum* sp. nov.: a sulfate-reducing bacterium which oxidizes fatty acids and ketones. Microbiol (English translation of Mikrobiologiya (Russia)). 1991;60(6):742–6.

71. Finster K, Liesack W, Tindall BJ. *Desulfospira joergensenii*, gen. nov., sp. nov., a new sulfate-reducing bacterium isolated from marine surface sediment. Syst Appl Microbiol. 1997;20(2):201–8.

72. Schink B, Thiemann V, Laue H, Friedrich MW. *Desulfotignum phosphitoxidans* sp. nov., a new marine sulfate reducer that oxidizes phosphite to phosphate. Arch Microbiol. 2002;177(5):381–91.

73. Widdel F, Pfennig N. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids II. Incomplete oxidation of propionate by *Desulfobulbus propionicus* gen. nov., sp. nov. Arch Microbiol. 1982;131(4):360–5.

74. Lovley DR, Phillips EJ. Novel processes for anaerobic sulfate production from elemental sulfur by sulfate-reducing bacteria. Appl Environ Microbiol. 1994;60(7):2394–9.

75. Frederiksen TM, Finster K. The transformation of inorganic sulfur compounds and the assimilation of organic and inorganic carbon by the sulfur disproportionating bacterium *Desulfocapsa sulfoexigens*. Antonie van Leeuwenhoek. 2004;85(2):141–9.

76. Friedrich M, Springer N, Ludwig W, Schink B. Phylogenetic positions of *Desulfofustis glycolicus* gen. nov., sp. nov., and *Syntrophobotulus glycolicus* gen. nov., sp. nov., two new strict anaerobes growing with glycolic acid. Int J Syst Bacteriol. 1996;46(4):1065–9.

77. Suzuki D, Ueki A, Amaishi A, Ueki K. *Desulfopila aestuarii* gen. nov., sp. nov., a Gram-negative, rod-like, sulfate-reducing bacterium isolated from an estuarine sediment in Japan. Int J Syst Evol Microbiol. 2007;57(3):520–6.

78. Lie TJ, Clawson ML, Godchaux W, Leadbetter ER. Sulfidogenesis from 2-aminoethanesulfonate (taurine) fermentation by a morphologically unusual sulfate-reducing bacterium, *Desulforhopalus singaporensis* sp. nov. Appl Environ Microbiol. 1999;65(8):3328–34.

79. Knobtauch C, Sahm K, Jørgensen BB. Psychrophilic sulfate-reducing bacteria isolated from permanently cold arctic marine sediments: description of *Desulfofrigus oceanense* gen. nov., sp. nov., *Desulfofrigus fragile* sp. nov., *Desulfofaba gelida* gen. nov., sp. nov., *Desulfotalea psychrophila* gen. nov., sp. nov. and *Desulfotalea arctica* sp. nov. Int J Syst Bacteriol. 1999;49(4):1631–43.

80. Ollivier B, Hatchikian CE, Prensier G, Guezennec J, Garcia J. *Desulfohalobium retbaense* gen. nov. sp. nov. a halophilic sulfate-reducing bacterium from sediments of a hypersaline lake in Senegal. Int J Syst Evol Microbiol. 1991;41(1):74–81.

81. Sorokin DY, Tourova TP, Henstra AM, Stams AJM, Galinski EA, Muyzer G. Sulfidogenesis under extremely haloalkaline conditions by *Desulfonatronospira thiodismutans* gen. nov., sp. nov., and *Desulfonatronospira delicata* sp. nov., - a novel lineage of *Deltaproteobacteria* from hypersaline soda lakes. Microbiology.

2008;154(5):1444–53.

82. Zhilina TN, Zavarzin GA, Rainey FA, Pikuta EN, Osipov GA, Kostrikina NA. *Desulfonatronovibrio hydrogenovorans* gen. nov., sp. nov., an alkaliphilic, sulfate-reducing bacterium. Int J Syst Bacteriol. 1997;47(1):144–9.

83. Sorokin DY, Tourova TP, Kolganova T V., Detkova EN, Galinski EA, Muyzer G. Culturable diversity of lithotrophic haloalkaliphilic sulfate-reducing bacteria in soda lakes and the description of *Desulfonatronum thioautotrophicum* sp. nov., *Desulfonatronum thiosulfatophilum* sp. nov., *Desulfonatronovibrio thiodismutans* sp. nov., and *Desulfonatronovibrio magnus* sp. nov. Extremophiles. 2011;15(3):391–401.

84. Audiffrin C, Cayol JL, Joulian C, Casalot L, Thomas P, Garcia JL, et al. *Desulfonauticus submarinus* gen. nov., sp. nov., a novel sulfate-reducing bacterium isolated from a deep-sea hydrothermal vent. Int J Syst Evol Microbiol. 2003;53(5):1585–90.

85. Belyakova E V, Rozanova EP, Borzenkov IA, Turova TP, Pusheva MA, Lysenko AM, et al. The new facultatively chemolithoautotrophic, moderately halophilic, sulfate-reducing bacterium *Desulfovermiculus halophilus* gen. nov., sp. nov., isolated from an oil field. Microbiol (English translation of Mikrobiologiya (Russia)). 2006;75(2):161–71.

86. Miller JDA, Saleh AM. A sulphate-reducing bacterium containing cytochrome c_3 but lacking desulfoviridin. J Gen Microbiol. 1964;37(3):419–23.

87. Biebl H, Pfennig. Growth of sulfate-reducing bacteria with sulfur as electron acceptor. Arch Microbiol. 1977;112(1):115–7.

88. Watanabe M, Kojima H, Fukui M. *Desulfoplanes formicivorans* gen. nov., sp. nov., a novel sulfate-reducing bacterium isolated from a blackish meromictic lake, and emended description of the family *Desulfomicrobiaceae*. Int J Syst Evol Microbiol. 2015;65(6):1902–7.

89. Pikuta E V., Hoover RB, Bej AK, Marsic D, Whitman WB, Cleland D, et al.

Desulfonatronum thiodismutans sp. nov., a novel alkaliphilic, sulfate-reducing bacterium capable of lithoautotrophic growth. Int J Syst Evol Microbiol. 2003;53(5):1327–32.

90. Klouche N, Basso O, Lascourreges JF, Cayol JL, Thomas P, Fauque G, et al. *Desulfocurvus vexinensis* gen. nov., sp. nov., a sulfate-reducing bacterium isolated from a deep subsurface aquifer. Int J Syst Evol Microbiol. 2009;59(12):3100–4.

91. Warthmann R, Vasconcelos C, Sass H, McKenzie JA. *Desulfovibrio brasiliensis* sp. nov., a moderate halophilic sulfate-reducing bacterium from Lagoa Vermelha (Brazil) mediating dolomite formation. Extremophiles. 2005;9(3):255–61.

92. Sass H, Berchtold M, Branke J, König H, Cypionka H, Babenzien HD. Psychrotolerant sulfate-reducing bacteria from an oxic freshwater sediment, description of *Desulfovibrio cuneatus* sp. nov. and *Desulfovibrio litoralis* sp. nov. Syst Appl Microbiol. 1998;21(2):212–9.

93. Magot M, Caumette P, Desperrier JM, Matheron R, Dauga C. *Desulfovibrio longus* sp. nov., a sulfate-reducing bacterium isolated from an oil-producing well. Int J Syst Bacteriol. 1992;42(3):398–403.

94. Baena S, Fardeau ML, Labat M, Ollivier B, Garcia JL, Patel BK. *Desulfovibrio aminophilus* sp. nov., a novel amino acid degrading and sulfate reducing bacterium from an anaerobic dairy wastewater lagoon. Syst Appl Microbiol. 1998;21(4):498–504.

95. Hernandez-Eugenio G, Fardeau ML, Patel BKC, Macarie H, Garcia JL, Ollivier B. *Desulfovibrio mexicanus* sp. nov., a sulfate-reducing bacterium isolated from an upflow anaerobic sludge blanket (UASB) reactor treating cheese wastewaters. Anaerobe. 2000;6(5):305–12.

96. Krekeler D, Sigalevich P, Teske A, Cypionka H, Cohen Y. A sulfate-reducing bacterium from the oxic layer of a microbial mat from Solar Lake (Sinai), *Desulfovibrio oxyclinae* sp. nov. Arch Microbiol. 1997;167(6):369–75.

97. Devereux R, He SH, Doyle CL, Orkland S, Stahl DA, LeGall J, et al. Diversity and origin of *Desulfovibrio* species: phylogenetic definition of a family. J Bacteriol. 1990;172(7):3609–19.

98. Postgate JR, Campbell LL. Classification of *Desulfovibrio* species, the nonsporulating sulfate-reducing bacteria. Bacteriol Rev. 1966;30(4):732–8.

99. Kuever J, Rainey FA, Widdel F. Genus I. *Desulfovibrio*. In: Brenner DJ, Krieg NR, Staley JT, Garrity GM, editors. Bergey's Manual of Systematic Bacteriology, Volume 2, Part C. 2nd ed. New York: Springer; 2005. p. 926-38.

100. Takii S, Hanada S, Hase Y, Tamaki H, Uyeno Y, Sekiguchi Y, et al. *Desulfovibrio marinisediminis* sp. nov., a novel sulfate-reducing bacterium isolated from coastal marine sediment via enrichment with Casamino acids. Int J Syst Evol Microbiol. 2008;58(10):2433–8.

101. Cao J, Gayet N, Zeng X, Shao Z, Jebbar M, Alain K. *Pseudodesulfovibrio indicus* gen. nov., sp. nov., a piezophilic sulfate-reducing bacterium from the Indian ocean and reclassification of four species of the genus *Desulfovibrio*. Int J Syst Evol Microbiol. 2016;66(10):3904–11.

102. Bonch-Osmolovskaya EA, Sokolova TG, Kostrikina NA, Zavarzin GA. *Desulfurella acetivorans* gen. nov. and sp. nov. - a new thermophilic sulfur-reducing eubacterium. Arch Microbiol. 1990;153(2):151–5.

103. Florentino AP, Brienza C, Stams AJM, Sánchez-Andrea I. *Desulfurella amilsii* sp. nov., a novel acidotolerant sulfur-respiring bacterium isolated from acidic river sediments. Int J Syst Evol Microbiol. 2016;66(3):1249–53.

104. Miroshnichenko ML, Rainey FA, Rhode M, Bonch-Osmolovskaya EA. *Hippea maritima* gen. nov., sp. nov., a new genus of thermophilic, sulfur-reducing bacterium from submarine hot vents. Int J Syst Bacteriol. 1999;49(3):1033–8.

105. Pfennig N, Biebl H. *Desulfuromonas acetoxidans* gen. nov. and sp. nov., a new anaerobic, sulfur-reducing, acetate-oxidizing bacterium. Arch Microbiol. 1976;110(1):3–12.

106. Finster K, Bak F. Complete oxidation of propionate, valerate, succinate, and other organic compounds by newly isolated types of marine, anaerobic, mesophilic, gram-negative, sulfur-reducing eubacteria. Appl Environ Microbiol. 1993;59(5):1452–60.

107. Dehning I, Schink B. *Malonomonas rubra* gen. nov. sp. nov., a microaerotolerant anaerobic bacterium growing by decarboxylation of malonate. Arch Microbiol. 1989;151(5):427–33.

108. Vandieken V, Mussmann M, Niemann H, Jørgensen BB. *Desulfuromonas svalbardensis* sp. nov. and *Desulfuromusa ferrireducens* sp. nov., psychrophilic, Fe(III)-reducing bacteria isolated from Arctic sediments, Svalbard. Int J Syst Evol Microbiol. 2006;56(5):1133–9.

109. Schink B. Fermentation of 2,3-butanediol by *Pelobacter carbinolicus* sp. nov. and *Pelobacter propionicus* sp. nov., and evidence for propionate formation from C_2 compounds. Arch Microbiol. 1984;137(1):33–41.

110. Lovley DR, Phillips EJP, Lonergan DJ, Widman PK. Fe(III) and S^0 Reduction by *Pelobacter carbinolicus*. Appl Environ Microbiol. 1995;61(6):2132–8.

111. Greene AC, Patel BKC, Yacob S. *Geoalkalibacter subterraneus* sp. nov., an anaerobic Fe(III)- and Mn(IV)-reducing bacterium from a petroleum reservoir, and emended descriptions of the family *Desulfuromonadaceae* and the genus *Geoalkalibacter*. Int J Syst Evol Microbiol. 2009;59(4):781–5.

112. Caccavo FJR, Lonergan DJ, Lovley DR, Davis M, Stolz JF, Mcinerney MJ. *Geobacter sulfurreducens* sp. nov., a hydrogen-and acetate- oxidizing dissimilatory metal-reducing microorganism. Appl Environ Microbiol. 1994;60(10):3752–9

113. Holmes DE, Nicoll JJ, Bond DR, Lovley DR. Potential role of a novel psychrotolerant member of the family *Geobacteraceae*, *Geopsychrobacter electrodiphilus* gen. nov., sp. nov., in electricity production by a marine sediment fuel cell. App Env Microbiol.

2004;70(10):6023–30.

114. Oude Elferink SJ, Akkermans-van Vliet WM, Bogte JJ, Stams AJ. *Desulfobacca acetoxidans* gen. nov., sp. nov., a novel acetate-degrading sulfate reducer isolated from sulfidogenic granular sludge. Int J Syst Bacteriol. 1999;49(2):345–50.

115. DeWeerd KA, Mandelco L, Tanner RS, Woese CR, Suflita JM. *Desulfomonile tiedjei* gen. nov. and sp. nov., a novel anaerobic, dehalogenating, sulfate-reducing bacterium. Arch Microbiol. 1990;154(1):23–30.

116. Mohn WW, Tiedje JM. Catabolic thiosulfate disproportionation and carbon dioxide reduction in strain DCB-1, a reductively dechlorinating anaerobe. 1990;172(4):2065–70.

117. Rees GN, Grassia GS, Sheehy AJ, Dwivedi PP, Patel BKC. *Desulfacinum infernurn* gem nov., sp. nov., a thermophilic sulfate-reducing bacterium from a petroleum reservoir. Int J Syst Bacteriol. 1995;45(1):85–9.

118. Harmsen HJ, Van Kuijk BL, Plugge CM, Akkermans AD, De Vos WM, Stams AJ. *Syntrophobacter fumaroxidans* sp. nov., a syntrophic propionate-degrading sulfate-reducing bacterium. Int J Syst Bacteriol. 1998;48(4):1383–7.

119. Beeder J, Torsvik T, Lien T. *Thermodesulforhabdus norvegicus* gen. nov., sp. nov., a novel thermophilic sulfate-reducing bacterium from oil field water. Arch Microbiol. 1995;164(5):331–6.

120. Hoeft SE, Kulp TR, Stolz JF, Hollibaugh JT, Oremland RS. Dissimilatory arsenate reduction with sulfide as the electron donor: experiments with Mono Lake water and isolation of strain MLMS-1, a chemoautotrophic arsenate-respirer. Appl Env Microbiol. 2004;70(5):2741–7.

121. Planer-Friedrich B, Härtig C, Lohmayer R, Suess E, McCann SH, Oremland R. Anaerobic chemolithotrophic growth of the haloalkaliphilic bacterium strain MLMS-1 by disproportionation of monothioarsenate. Environ Sci Technol. 2015;49(11):6554–63.

122. Magot M, Ravot G, Campaignolle X, Ollivier B, Patel BKC, Fardeau M, et al. *Dethiosulfovibrio peptidovorans* gen. nov., sp. nov., a new anaerobic, slightly halophilic, thiosulfate-reducing bacterium from corroding offshore oil wells. 1997;47(3):818–24.

123. Alain K, Postec A, Grinsard E, Lesongeur F, Prieur D, Godfroy A. *Thermodesulfatator atlanticus* sp. nov., a thermophilic, chemolithoautotrophic, sulfate-reducing bacterium isolated from a Mid-Atlantic Ridge hydrothermal vent. Int J Syst Evol Microbiol. 2010;60(1):33–8.

124. Moussard H, L'Haridon S, Tindall BJ, Banta A, Schumann P, Stackebrandt E, et al. *Thermodesulfatator indicus* gen. nov., sp. nov., a novel thermophilic chemolithoautotrophic sulfate-reducing bacterium isolated from the Central Indian Ridge. Int J Syst Evol Microbiol. 2004;54(1):227–33.

125. Lai Q, Cao J, Dupont S, Shao Z, Jebbar M, Alain K. *Thermodesulfatator autotrophicus* sp. nov., a thermophilic sulfate-reducing bacterium from the Indian Ocean. Int J Syst Evol Microbiol. 2016;66(10):3978–82.

126. Zeikus JG, Dawson MA, Thompson TE, Ingvorsen K, Hatchikian EC. Microbial ecology of volcanic sulphidogenesis: isolation and characterization of *Thermodesulfobacterium commune* gen. nov. and sp. nov. J Gen Microbiol. 1983;129(4):1159–69.

127. Hamilton-Brehm SD, Gibson RA, Green SJ, Hopmans EC, Schouten S, van der Meer MTJ, et al. *Thermodesulfobacterium geofontis* sp. nov., a hyperthermophilic, sulfate-reducing bacterium isolated from Obsidian Pool, Yellowstone National Park. Extremophiles. 2013;17(2):251–63.

128. Jeanthon C, L'Haridon S, Cueff V, Banta A, Reysenbach AL, Prieur D. *Thermodesulfobacterium hydrogeniphilum* sp. nov., a thermophilic, chemolithoautotrophic, sulfate-reducing bacterium isolated from a deep-sea hydrothermal vent at Guaymas Basin, and emendation of the genus *Thermodesulfobacterium*. Int J Syst Evol Microbiol.

2002;52(3):765–72.

129. Hatchikan EC, Ollivier B. Genus I. *Thermodesulfobacterium*. In: Boone DR, Castenholz RW, Garrity GM, editors. Bergey's Manual of Systematic Bacteriology, Volume 1. 2nd ed. New York: Springer; 2001. p. 390-3.

Table 4-1 List showing the sequences which using as query for annotation of gene in the genomes of *Caldimicrobium thiodismutans* TF1^T.

accesion number	gene name	product	Species
EAT05667.1	*acsA*	Carbon-monoxide dehydrogenase, catalytic subunit	delta proteobacterium MLMS-1
EAT05665.1	*acsB*	CO dehydrogenase/acetyl-CoA synthase complex beta subunit	delta proteobacterium MLMS-1
EAT05664.1	*acsC*	CO dehydrogenase/acetyl-CoA synthase delta subunit:Putative Fe-S cluster	delta proteobacterium MLMS-1
EAT05666.1	*acsD/mthfr*	Methylenetetrahydrofolate reductase	delta proteobacterium MLMS-1
ADO12092.1	*acsE*	5-methyltetrahydrofolate corrinoid iron-sulfur protein methyltransferase	*Clostridium carboxidivorans* P7
OAQ20318.1	*aprA*	Adenylylsulfate reductase alpha-subunit	*Thermosulfurimonas dismutans* S95^T
OAQ20319.1	*aprB*	Adenylylsulfate reductase beta-subunit	*Thermosulfurimonas dismutans* S95^T
OAQ20198.1	*dsrA*	Dissimilatory sulfite reductase alpha subunit	*Thermosulfurimonas dismutans* S95^T
OAQ20199.1	*dsrB*	Dissimilatory sulfite reductase beta subunit	*Thermosulfurimonas dismutans* S95^T
OAQ20264.1	*dsrC*	Dissimilatory sulfite reductase, subunit gamma	*Thermosulfurimonas dismutans* S95^T
OAQ20200.1	*dsrD*	Dissimilatory sulfite reductase clustered protein DsrD	*Thermosulfurimonas dismutans* S95^T
OAQ21324.1	*dsrJ*	Sulfite reduction-associated complex DsrMKJOP multiheme protein DsrJ	*Thermosulfurimonas dismutans* S95^T
OAQ21325.1	*dsrK*	Sulfite reduction-associated complex DsrMKJOP protein DsrK	*Thermosulfurimonas dismutans* S95^T
OAQ21326.1	*dsrM*	Sulfite reduction-associated complex DsrMKJOP protein DsrM	*Thermosulfurimonas dismutans* S95^T
OAQ21323.1	*dsrO*	Sulfite reduction-associated complex DsrMKJOP iron-sulfur protein DsrO	*Thermosulfurimonas dismutans* S95^T
OAQ21322.1	*dsrP*	Sulfite reduction-associated complex DsrMKJOP protein DsrP	*Thermosulfurimonas dismutans* S95^T
EAT05670.1	*fhs*	Formate--tetrahydrofolate ligase	delta proteobacterium MLMS-1
EAT05668.1	*folD*	Methenyltetrahydrofolate cyclohydrolase	delta proteobacterium MLMS-1
EAT05662.1	formate dehydrogenase	Molybdopterin oxidoreductase:Molydopterin dinucleotide-binding region	delta proteobacterium MLMS-1
EAT05663.1	formate dehydrogenase	Ferredoxin:FAD-dependent pyridine nucleotide-disulphide oxidoreductase:4Fe-4S ferredoxin, iron-sulfur binding:FAD dependent oxidoreductase:Molybdopterin oxidoreductase Fe4S4 region	delta proteobacterium MLMS-1
OAQ21381.1	*napA*	Periplasmic nitrate reductase, large catalytic subunit NapA	*Thermosulfurimonas dismutans* S95^T
OAQ21379.1	*napG*	Periplasmic nitrate reductase, subunit NapG	*Thermosulfurimonas dismutans* S95^T
OAQ21378.1	*napH*	Periplasmic nitrate reductase, subunit NapH	*Thermosulfurimonas dismutans* S95^T
CAI56199.2	*onr**	octaheme nitrate reductase	*Thioalkalivibrio nitratireducens* DSM 14787
OAQ20062.1	*otr*	octaheme tetrathionate reductase	*Thermosulfurimonas dismutans* S95^T
ADH85126.1	*phsA/psrA*	molybdopterin oxidoreductase	*Desulfurivibrio alkaliphilus* AHT2^T
OAQ20317.1	*qmoA*	Adenylylsulfate reductase-associated electron transfer protein QmoA	*Thermosulfurimonas dismutans* S95^T
OAQ20316.1	*qmoB*	Adenylylsulfate reductase-associated electron transfer protein QmoB	*Thermosulfurimonas dismutans* S95^T
OAQ20315.1	*qmoC*	Adenylylsulfate reductase-associated electron transfer protein QmoC	*Thermosulfurimonas dismutans* S95^T
OAQ20321.1	*sat*	Sulfate adenylyltransferase, dissimilatory-type	*Thermosulfurimonas dismutans* S95^T
AAF64400.1	*sorA*	sulfite:cytochrome c oxidoreductase subunit A	*Starkeya novella* DSM 506
AAF64401.1	*sorB*	sulfite:cytochrome c oxidoreductase subunit B	*Starkeya novella* DSM 506
OAQ20686.1	*nrfD*	Polysulfide reductase, membrane subunit NrfD subunit	*Thermosulfurimonas dismutans* S95^T
OAQ20687.1	*nrfC*	Cytochrome c nitrite reductase, 4Fe-4S ferredoxin subunit NrfC	*Thermosulfurimonas dismutans* S95^T
OAQ20688.1		Cytochrome c family protein	*Thermosulfurimonas dismutans* S95^T
KLU67643.1	*dsrE*	DsrE/DsrF-like family protein	*Desulfosporosinus acididurans* M1^T
OCC15837.1	*tusA*	Sulfurtransferase TusA	*Dissulfuribacter thermophilus* S69^T

Table 4-2 Lineage and physiological property of the bacteria used for comparative genomic analysis. Most of genomes using comparative genomics were divided into three groups based on sulfur metabolism of bacteria: sulfate-reducing bacteria, ISR-SDB with Dsr system. Within the sulfate reducing bacteria, bacteria that are incapable growing with sulfur disproportionation were made into groups.

		Sufate reduction	±	+	+	-
Class	Family	Sulfur disproportionation	±	±	-	+*
Clostridia	*Peptococcaceae*		8	5	3	0
Clostridia	*Syntrophomonadaceae*		1	0	0	0
Nitrospira	*Nitrospiraceae*		6	5	0	1
Deltaproteobacteria	*Desulfarculaceae*		3	2	0	0
Deltaproteobacteria	*Desulfobacteraceae*		15	15	7	0
Deltaproteobacteria	*Desulfobulbaceae*		8	6	2	2
Deltaproteobacteria	*Desulfohalobiaceae*		6	6	0	0
Deltaproteobacteria	*Desulfomicrobiaceae*		2	2	0	0
Deltaproteobacteria	*Desulfonatronaceae*		3	3	0	0
Deltaproteobacteria	*Desulfovibrionaceae*		12	12	4	0
Deltaproteobacteria	*Desulfurellaceae*		3	0	0	0
Deltaproteobacteria	*Desulfuromonadaceae*		4	0	0	0
Deltaproteobacteria	*Geobacteraceae*		3	0	0	0
Deltaproteobacteria	unclassified Deltaproteobacteria		2	0	0	1
Deltaproteobacteria	*Syntrophaceae*		2	2	0	0
Deltaproteobacteria	*Syntrophobacteraceae*		3	3	0	0
Synergistia	*Synergistaceae*		1	0	0	0
Thermodesulfobacteria	*Thermodesulfobacteriaceae*		10	8	0	2
		Total	92	69	16	6

* Bacteria able to grow by disproportionation of thiosulfate or sulfite, but not by sulfate reduction with the Dsr system.

Table 4-3 Physiological property of the individual bacterium used for comparative genomic analysis.

			Sulfur compounds used for disproportionation			Sulfur compounds used for reduction					
Class	Family	Organism	$S_2O_3^{2-}$	SO_3^{2-}	S^0	SO_4^{2-}	$S_2O_3^{2-}$	SO_3^{2-}	S^0	Reference	Accession.version (Genome)
Clostridia	*Peptococcaceae*	*Desulfitibacter alkalitolerans*	nr	nr	nr	-	+	+	+	41	NZ_JHVU00000000.1
Clostridia	*Peptococcaceae*	*Desulfitobacterium hafniense*	nr	nr	nr	-	+	+	+	42,43	NC_011830.1
Clostridia	*Peptococcaceae*	*Desulfosporosinus lacus*	N	N	N	+	+	+	-	44	NZ_FQXJ00000000.1
Clostridia	*Peptococcaceae*	*Desulfotomaculum ruminis*	nr	nr	nr	+	nr	nr	nr	45	CP002780.1
Clostridia	*Peptococcaceae*	*Desulfotomaculum nigrificans*	(D)	N	nr	+	+	-	nr	2,45,46	NZ_AEVP00000000.2
Clostridia	*Peptococcaceae*	*Desulfotomaculum alkaliphilum*	N	N	nr	+	+	+	-	47	NZ_JONT00000000.1
Clostridia	*Peptococcaceae*	*Desulfurispora thermophila*	nr	nr	nr	+	+	+	+	48	NZ_AQWN00000000.1
Clostridia	*Peptococcaceae*	*Peptococcus niger*	nr	nr	nr	nr	nr	+	nr	49	NZ_FNAF00000000.1
Clostridia	*Syntrophomonadaceae*	*Dethiobacter alkaliphilus*	nr	nr	G	-	+	nr	+	7,50	NZ_ACJM00000000.1
Nitrospira	*Nitrospiraceae*	*Thermodesulfovibrio* sp. N1	nr	nr	nr	+	+	+	-	51	MAVV00000000.1
Nitrospira	*Nitrospiraceae*	*Thermodesulfovibrio aggregans*	nr	nr	nr	+	+	-	-	52	NZ_BCNO00000000.1
Nitrospira	*Nitrospiraceae*	*Thermodesulfovibrio islandicus*	nr	nr	nr	+	+	-	-	53	NZ_AXWU00000000.1
Nitrospira	*Nitrospiraceae*	*Thermodesulfovibrio thiophilus*	nr	nr	nr	+	+	+	-	52	NZ_AUIU00000000.1
Nitrospira	*Nitrospiraceae*	*Thermodesulfovibrio yellowstonii*	nr	nr	nr	+	+	+	-	54	NC_011296.1
Nitrospira	*Nitrospiraceae*	45J	G	nr	nr	-	-	nr	nr	Chapter 3	
Deltaproteobacteria	*Desulfarculaceae*	*Desulfarculus baarsii*	nr	nr	nr	+	+	+	-	55	NC_014365.1
Deltaproteobacteria	*Desulfarculaceae*	*Desulfocarbo indianensis*	nr	nr	nr	+	+	-	-	56	NZ_LBMP00000000.1
Deltaproteobacteria	*Desulfarculaceae*	*Dethiosulfatarculus sandiegensis*	nr	nr	nr	-	+	-	nr	57	NZ_AZAC00000000.1
Deltaproteobacteria	*Desulfobacteraceae*	*Desulfamplus magnetovallimortis*	nr	nr	nr	+	+	-	-	58	NZ_FWEV00000000.1
Deltaproteobacteria	*Desulfobacteraceae*	*Desulfatibacillum aliphaticivorans*	N	N	nr	+	+	+	-	59	NZ_AUCT00000000.1
Deltaproteobacteria	*Desulfobacteraceae*	*Desulfatiglans anilini*	D	nr	nr	+	nr	+	-	60	NZ_AULM00000000.1
Deltaproteobacteria	*Desulfobacteraceae*	*Desulfatirhabdium butyrativorans*	N	N	nr	+	+	-	-	61	NZ_AUCU00000000.1
Deltaproteobacteria	*Desulfobacteraceae*	*Desulfatitalea tepidiphila*	nr	nr	nr	+	+	nr	nr	62	NZ_BCAG00000000.1
Deltaproteobacteria	*Desulfobacteraceae*	*Desulfobacter curvatus*	D	N	nr	+	+	+	-	2,63	NZ_AREY00000000.1
Deltaproteobacteria	*Desulfobacteraceae*	*Desulfobacter vibrioformis*	N	N	nr	+	+	+	nr	64	NZ_JQKJ00000000.1
Deltaproteobacteria	*Desulfobacteraceae*	*Desulfobacterium autotrophicum*	nr	nr	nr	+	+	-	-	65	NC_012108.1/NC_012109.1
Deltaproteobacteria	*Desulfobacteraceae*	*Desulfobacula phenolica*	nr	nr	nr	+	+	-	-	66	NZ_FNLL00000000.1
Deltaproteobacteria	*Desulfobacteraceae*	*Desulfococcus multivorans*	D	N	nr	+	+	+	-	2,67	CP015381.1
Deltaproteobacteria	*Desulfobacteraceae*	*Desulfoluna spongiiphila*	nr	nr	nr	+	+	+	nr	68	NZ_FMUX00000000.1
Deltaproteobacteria	*Desulfobacteraceae*	*Desulforegula conservatrix*	nr	nr	nr	+	-	-	-	69	NZ_AUEY00000000.1
Deltaproteobacteria	*Desulfobacteraceae*	*Desulfosarcina cetonica*	nr	nr	nr	+	nr	+	+	70	NZ_BBCC00000000.1
Deltaproteobacteria	*Desulfobacteraceae*	*Desulfospira joergensenii*	nr	nr	nr	+	+	+	+	71	NZ_ATUG00000000.1
Deltaproteobacteria	*Desulfobacteraceae*	*Desulfotignum phosphitoxidans*	N	nr	nr	+	+	+	-	72	NZ_APJX00000000.1
Deltaproteobacteria	*Desulfobulbaceae*	*Desulfobulbus propionicus*	D	N	(G)	+	+	+	-	2,73,74	NC_014972.1
Deltaproteobacteria	*Desulfobulbaceae*	*Desulfobulbus mediterraneus*	N[1]	N[1]	nr	+	+	+	-	28	NZ_AUCW00000000.1
Deltaproteobacteria	*Desulfobulbaceae*	*Desulfocapsa sulfexigens*	G	G	G	-	+	+	+	6,75	CP003985.1/CP003986.1

Table 4-3 Physiological property of the individual bacterium used for comparative genomic analysis — cont'd.

			Sulfur compounds used for disproportionation			Sulfur compounds used for reduction					
Class	Family	Organism	$S_2O_3^{2-}$	SO_3^{2-}	S^0	SO_4^{2-}	$S_2O_3^{2-}$	SO_3^{2-}	S^0	Reference	Accession.version (Genome)
Deltaproteobacteria	*Desulfobulbaceae*	*Desulfofustis glycolicus*	nr	nr	G	+	-	+	+	1,76	NZ_FQXS00000000.1
Deltaproteobacteria	*Desulfobulbaceae*	*Desulfopila aestuarii*	nr	nr	nr	+	+	+	nr	77	NZ_FRFE00000000.1
Deltaproteobacteria	*Desulfobulbaceae*	*Desulforhopalus singaporensis*	N	G	nr	+	+	+	nr	78	NZ_FNJI00000000.1
Deltaproteobacteria	*Desulfobulbaceae*	*Desulfotalea psychrophila*	N	nr	N	+	+	+	-	79	CR522870.1/CR522871.1/CR522872.1
Deltaproteobacteria	*Desulfobulbaceae*	*Desulfurivibrio alkaliphilus*	nr	nr	G	-	+	nr	+	7,50	CP001940.1
Deltaproteobacteria	*Desulfohalobiaceae*	*Desulfohalobium retbaense*	nr	nr	nr	+	+	+	+	80	CP001734.1/ CP001735.1
Deltaproteobacteria	*Desulfohalobiaceae*	*Desulfonatronospira thiodismutans*	G	G	nr	+	+	+	+	81	NZ_ACJN00000000.2
Deltaproteobacteria	*Desulfohalobiaceae*	*Desulfonatronovibrio hydrogenovorans*	D^2	nr	nr	+	+	+	-	82	NZ_JMKT00000000.1
Deltaproteobacteria	*Desulfohalobiaceae*	*Desulfonatronovibrio magnus*	G	G	nr	+	+	+	+	83	NZ_JYNP00000000.1
Deltaproteobacteria	*Desulfohalobiaceae*	*Desulfonauticus submarinus*	nr	nr	nr	+	+	+	+	84	NZ_FNIN00000000.1
Deltaproteobacteria	*Desulfohalobiaceae*	*Desulfovermiculus halophilus*	nr	nr	nr	+	+	+	+	85	NZ_JIAK00000000.1
Deltaproteobacteria	*Desulfomicrobiaceae*	*Desulfomicrobium norvegicum*	nr	nr	nr	+	+	+	+	86,87	NZ_FOTO00000000.1
Deltaproteobacteria	*Desulfomicrobiaceae*	*Desulfoplanes formicivorans*	nr	nr	nr	+	+	+	nr	88	BDFE00000000.1
Deltaproteobacteria	*Desulfonatronaceae*	*Desulfonatronum thioautotrophicum*	G	G	nr	+	+	+	+	83	NZ_JYNO00000000.1
Deltaproteobacteria	*Desulfonatronaceae*	*Desulfonatronum thiodismutans*	G	G	nr	+	+	+	-	89	NZ_JPIK00000000.1
Deltaproteobacteria	*Desulfonatronaceae*	*Desulfonatronum thiosulfatophilum*	G	G	nr	+	+	+	+	83	NZ_FMXO00000000.1
Deltaproteobacteria	*Desulfovibrionaceae*	*Desulfocurvus vexinensis*	nr	nr	nr	+	+	+	-	90	NZ_JAEX00000000.1
Deltaproteobacteria	*Desulfovibrionaceae*	*Desulfovibrio brasiliensis*	G	nr	nr	+	+	+	+	91	NZ_BBCB00000000.1
Deltaproteobacteria	*Desulfovibrionaceae*	*Desulfovibrio cuneatus*	N	G	nr	+	+	+	(+)	92	NZ_AUCY00000000.1
Deltaproteobacteria	*Desulfovibrionaceae*	*Desulfovibrio longus*	N	G	nr	+	+	+	+	93	NZ_ATVA00000000.1
Deltaproteobacteria	*Desulfovibrionaceae*	*Desulfovibrio aminophilus*	D^2	D^2	nr	+	+	+	-	94	NZ_AUMA00000000.1
Deltaproteobacteria	*Desulfovibrionaceae*	*Desulfovibrio mexicanus*	D^2	D^2	nr	+	+	+	+	95	NZ_FZOC00000000.1
Deltaproteobacteria	*Desulfovibrionaceae*	*Desulfovibrio oxyclinae*	G	G	nr	+	+	+	+	96	NZ_AQXE00000000.1
Deltaproteobacteria	*Desulfovibrionaceae*	*Desulfovibrio desulfuricans* subsp. *desulfuricans* str. ATCC 27774	N	N	nr	+	nr	nr	nr	2,97	NC_011883.1
Deltaproteobacteria	*Desulfovibrionaceae*	*Desulfovibrio litoralis*	N	N	nr	+	+	+	+	92	NZ_FRDI00000000.1
Deltaproteobacteria	*Desulfovibrionaceae*	*Desulfovibrio vulgaris* str. Hildenborough	N	N	nr	+	+	+	-	2,98,99	AE017285.1/AE017286.1
Deltaproteobacteria	*Desulfovibrionaceae*	*Halodesulfovibrio marinisediminis*	N	nr	nr	+	+	+	-	100	NZ_FSRG00000000.1
Deltaproteobacteria	*Desulfovibrionaceae*	*Pseudodesulfovibrio indicus*	nr	nr	nr	+	+	+	-	101	NZ_CP014206.1
Deltaproteobacteria	*Desulfurellaceae*	*Desulfurella acetivorans*	nr	nr	G	-	-	-	+	102,103	NZ_CP007051.1/NZ_CP007052.1
Deltaproteobacteria	*Desulfurellaceae*	*Desulfurella amilsii*	nr	nr	G	-	+	-	+	103	NZ_MDSU00000000.1
Deltaproteobacteria	*Desulfurellaceae*	*Hippea maritima*	nr	nr	nr	-	-	-	+	104	NC_015318.1
Deltaproteobacteria	*Desulfuromonadaceae*	*Desulfuromonas acetoxidans*	N	N	nr	-	-	-	+	2,105	NZ_AAEW00000000.2
Deltaproteobacteria	*Desulfuromonadaceae*	*Desulfuromusa kysingii*	nr	nr	nr	-	-	-	+	106	NZ_FNQN00000000.1
Deltaproteobacteria	*Desulfuromonadaceae*	*Malonomonas rubra*	nr	nr	nr	-	-	-	+	107,108	NZ_FQZT00000000.1
Deltaproteobacteria	*Desulfuromonadaceae*	*Pelobacter carbinolicus*	nr	nr	nr	-	-	-	+	109,110	NC_007498.2

Table 4-3 Physiological property of the individual bacterium used for comparative genomic analysis — cont'd.

			Sulfur compounds used for disproportionation			Sulfur compounds used for reduction					
Class	Family	Organism	$S_2O_3^{2-}$	SO_3^{2-}	S^0	SO_4^{2-}	$S_2O_3^{2-}$	SO_3^{2-}	S^0	Reference	Accession.version (Genome)
Deltaproteobacteria	*Geobacteraceae*	*Geoalkalibacter subterraneus*	nr	nr	nr	-	-	-	+	111	NZ_CP010311.1/NZ_CP010312.1
Deltaproteobacteria	*Geobacteraceae*	*Geobacter sulfurreducens*	nr	nr	nr	-	-	-	+	112	NC_002939.5
Deltaproteobacteria	*Geobacteraceae*	*Geopsychrobacter electrodiphilus*	nr	nr	nr	-	-	-	+	113	NZ_ARWE00000000.1
Deltaproteobacteria	unclassified *Deltaproteobacteria*	*Dissulfuribacter thermophilus*	G	G	G	-	nr	nr	nr	8	MAGO00000000.1
Deltaproteobacteria	*Syntrophaceae*	*Desulfobacca acetoxidans*	nr	nr	nr	+	+	+	-	114	NC_015388.1
Deltaproteobacteria	*Syntrophaceae*	*Desulfomonile tiedjei*	G	nr	nr	+	+	+	-	115,116	NC_018025.1/NC_018026.1
Deltaproteobacteria	*Syntrophobacteraceae*	*Desulfacinum infernum*	nr	nr	nr	+	+	+	-	117	NZ_FQVB00000000.1
Deltaproteobacteria	*Syntrophobacteraceae*	*Syntrophobacter fumaroxidans*	nr	nr	nr	+	+	nr	nr	118	NC_008554.1
Deltaproteobacteria	*Syntrophobacteraceae*	*Thermodesulforhabdus norvegica*	nr	nr	nr	+	-	+	-	119	NZ_FOUU00000000.1
Deltaproteobacteria	unclassified *Deltaproteobacteria*	delta proteobacterium MLMS-1[3]	nr	nr	nr	-	nr	nr	nr	120,121	NZ_AAQF00000000.1
Synergistia	*Synergistaceae*	*Dethiosulfovibrio peptidovorans*	N	nr	N	-	+	nr	+	122	NZ_ABTR00000000.2
Thermodesulfobacteria	*Thermodesulfobacteriaceae*	*Caldimicrobium thiodismutans*	G	G	G	-	-	nr	nr	12	AP014945.1
Thermodesulfobacteria	*Thermodesulfobacteriaceae*	*Thermodesulfatator atlanticus*	nr	nr	nr	+	-	-	-	123	NZ_ATXH00000000.1
Thermodesulfobacteria	*Thermodesulfobacteriaceae*	*Thermodesulfatator indicus*	nr	nr	nr	+	-	-	-	124	NC_015681.1
Thermodesulfobacteria	*Thermodesulfobacteriaceae*	*Thermodesulfatator autotrophicus*	nr	nr	nr	+	-	-	-	125	NZ_LSFI00000000.1
Thermodesulfobacteria	*Thermodesulfobacteriaceae*	*Thermodesulfobacterium commune*	nr	nr	nr	+	+	nr	nr	126	NZ_CP008796.1
Thermodesulfobacteria	*Thermodesulfobacteriaceae*	*Thermodesulfobacterium geofontis*	nr	nr	nr	+	+	-	+	127	NC_015682.1
Thermodesulfobacteria	*Thermodesulfobacteriaceae*	*Thermodesulfobacterium hveragerdense*	nr	nr	nr	+	+	+	-	53	NZ_AUIT00000000.1
Thermodesulfobacteria	*Thermodesulfobacteriaceae*	*Thermodesulfobacterium hydrogeniphilum*	nr	nr	nr	+	-	-	-	128	NZ_JQKW00000000.1
Thermodesulfobacteria	*Thermodesulfobacteriaceae*	*Thermodesulfobacterium thermophilum*	nr	nr	nr	+	+	+	nr	129	NZ_ATXI00000000.1
Thermodesulfobacteria	*Thermodesulfobacteriaceae*	*Thermosulfurimonas dismutans*	G	G	G	-	nr	nr	nr	10	LWLG00000000.1

G: disproportionation and growth, (G): disproportionation and weak growth, D: disproportionation without growth, (D): weak disproportionation without growth, +: reduction, (+): weak reduction, -: not reduction, nr: not reported

1: Growth by disproportionation was not observed. However, ability of disproportionation was not determined.

2: Although disproportionateion was observed, growth was not mentioned.

3: disproportionation of monothioarsenate

Table 4-4 Genome statistics of *Caldimicrobium thiodismutans* TF1^{T}.

Attribute	
Genome size (bp)	1,814,952
G+C content (%)	38.3
CDSs	1774
5S rRNA gene	1
16S rRNA genes	1
23S rRNA genes	1
tRNA genes	46

Table 4-5 Characteristic genes in the genomes of ISR-SDB with Dsr system. The number in the table shows the ratio of the number of bacteria that possess genes assigned to orthogroups.

	Number of genomes	92	69	16	6	
	Sulfur disproportionation	±	±	-	+*	
Orthogroup	Sufate reduction	±	+	+	-	term
OG0003485		0.14	0.09	0.06	1.00	*hyp1*
OG0001929		0.24	0.12	0.06	1.00	
OG0002137		0.28	0.20	0.19	1.00	
OG0002281		0.27	0.25	0.13	1.00	*dsrE-like*
OG0002186		0.28	0.26	0.13	1.00	*hyp2*
OG0001592		0.40	0.32	0.19	1.00	*yedE-like*
OG0001934		0.37	0.36	0.19	1.00	
OG0001376		0.40	0.39	0.38	1.00	
OG0001304		0.45	0.42	0.50	1.00	
OG0001452		0.58	0.48	0.69	1.00	

*: Bacteria able to grow by disproportionation of thiosulfate or sulfite, but not by sulfate reduction with the Dsr system

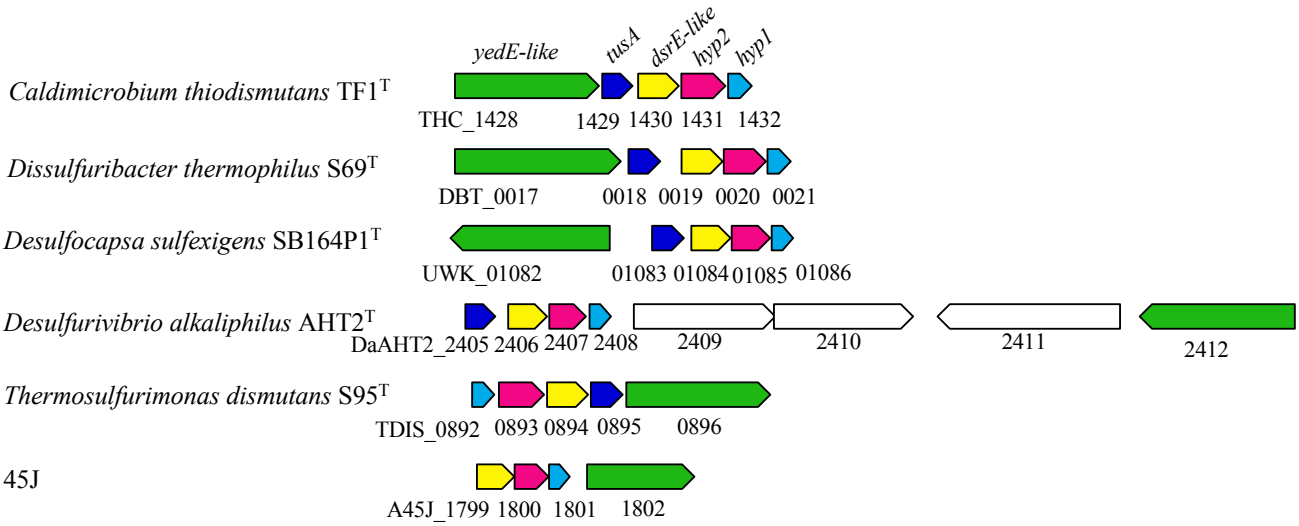


Figure 4-1 Schematic comparison of the relatively specific gene cluster in the genome of ISR-SDB with Dsr system.

Table 4-6 Annoatation of genes in the gene cluster.

	gene	*yedE-like*	*tusA*	*dsrE-like*	*hyp2*	*hyp1*
	Orthogroup	OG0001592	OG0000132	OG0002281	OG0002186	OG0003485
C.thiodismutans TF1^T		hypothetical protein	SirA-like domain-containing protein	hypothetical protein	hypothetical protein	hypothetical protein
D.sulfexigens SB164P1^T		YeeE/YedE family protein (DUF395)	putative redox protein, regulator of disulfide bond formation	putative peroxiredoxin	hypothetical protein	hypothetical protein
D.alkaliphilus AHT2^T		protein of unknown function DUF395 YeeE/YedE	SirA family protein	conserved hypothetical protein	hypothetical protein	hypothetical protein
T.dismutans S95^T		Sulfur transport protein	Sulfurtransferase TusA	hypothetical protein	hypothetical protein	hypothetical protein
45J		YeeE/YedE family protein	-	hypothetical protein	hypothetical protein	hypothetical protein
D.thermophilus S69^T (in genbank file)		hypothetical protein	Sulfurtransferase TusA	hypothetical protein	hypothetical protein	hypothetical protein
D.thermophilus S69^T (in article 5)		a transmembrane putative transport system permease	TusA	DsrE		

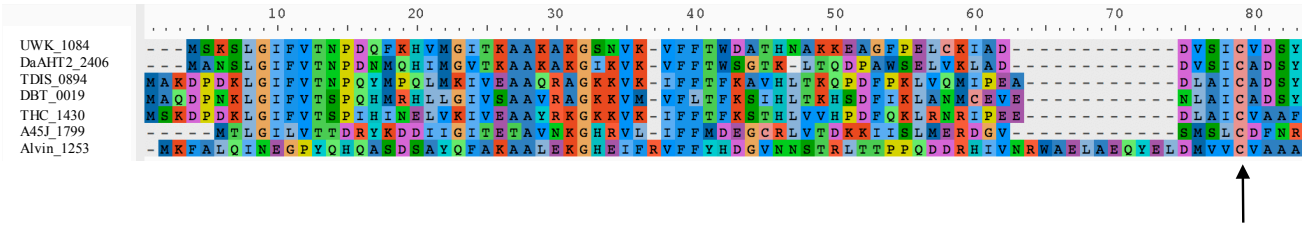


Figure 4-2 Alignment of DsrE-like protein and DsrE (Alvin_1253) protein of DsrEFH. The arrow indicates the position of conserved cysteine residues.

Table 4-7 Presence of genes related to sulfate reduction and nitrate reduction.

	sulfate reduction						nitrate reduction			
Species	*sat*	*aprAB*	*dsrAB*	*dsrC*	*qmoABC*	*dsrMKJOP*	*napA*	*nrfA*	*otr*	reference
45J	+	+	+	+	+	+	+	+	n.f	Chapter 3
Caldimicrobium thiodismutans TF1^T	+	+	+	+	+	+	+	+	+	Chapter 4
Thermosulfurimonas dismutans S95^T	+	+	+	+	+	+	+	n.f	+	5, 11
Desulfocapsa sulfexigens SB164P1^T	+	+	+	+	+	+[a]	+	n.f[b]	+[b]	3, 16
Desulfurivibrio alkaliphilus AHT2^T	+	+	+	+	+	+	+	+	n.f[b]	4, 16
Dissufuribacter thermophilus S69^T	+	+	+	+	+	+	+	n.f	+	5
Dethiobacter alkaliphilus AHT1^T	+	n.f	n.f	n.f	n.f	n.f	u	+[b]	n.f[b]	9

+: present, n.f: not found, u: unknown, a: *dsrO* gene is absent, b: this study

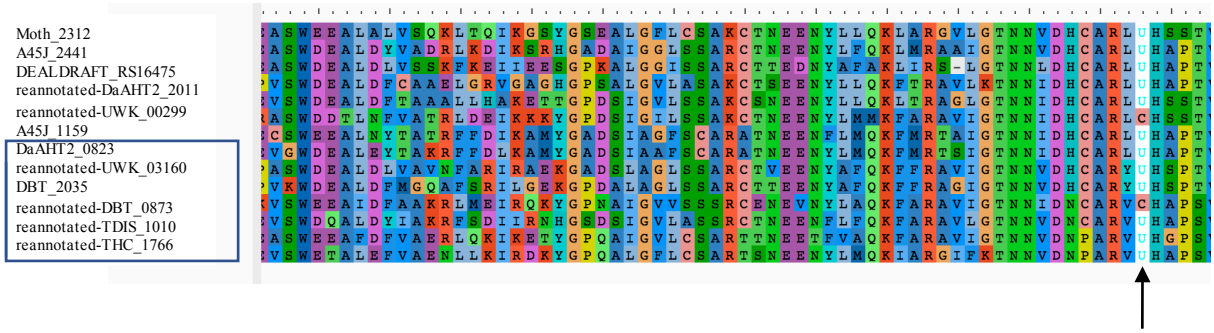


Figure 4-3 Alignments of amino acids sequence of formate dehydrogenases. The arrow indicates the positions of selenocysteine and cysteine rediues which were conserved. The sequences indicated within the box contain GltD domain. Several proteins containing TGA codon as end codon are reannotated by substitution of end codon represented by TGA codon for selenocysteine (DaAHT2_2011, UWK_00299, UWK_03160, DBT_0873, THC_1766). In addition, start codon of TDIS_1010 in the genome of *Thermosulfurimonas dismutans* S95^{T} was reannotated. TDIS_1010 was also reannotated by TGA codon as selenocysteine.

Table 4-8 Concentration of thiosulfate and sulfate under growth condition of thiosulfate (A) and elemental sulfur (B) disproportionation of *Caldimicrobium thiodismutans* TF1^{T}. *Caldimicrobium thiodismutans* TF1^{T} grew by thiosulfate (A) and elemental sulfur (B) disproportionation in the poorly crystalline Fe(III) oxide.

A

	$[S_2O_3^{2-}]$ (mM)	$[SO_4^{2-}]$ (mM)
Sample	0-108hr	0-108hr
T1	18.1-4.8	1.1-13.6
T2	18.3-4.5	1.1-12.6
T3	18.0-7.0	1.1-11.0

B

	$[SO_4^{2-}]$ (mM)
Sample	0-240hr
E1	0.1-2.1
E2	0.1-2.3

Table 4-9 Ranking of the relative abundancies of the proteins that may be involved in the sulfur metabolism in *Caldimicrobium thiodismutans* TF1^{T}. The number that are less than 150 are indicated in red, and the numbers that are less than 50 are highlighted in bold.

		Thiosulfate			Elemental sulfur	
Locus tag	Name	T1	T2	T3	E1	E2
THC_0087	SorB	876	-	480	-	-
THC_0088	SorA	-	-	-	-	899
THC_0289	Hypothetical protein	64	69	**46**	**11**	**32**
THC_0323	DsrC	107	167	252	105	128
THC_0449	DsrA	**5**	**4**	**16**	**8**	**5**
THC_0450	DsrB	**7**	**5**	**8**	**6**	**2**
THC_0451	DsrD	**34**	**31**	**28**	**34**	**32**
THC_0525	Otr	**38**	**25**	**36**	58	**38**
THC_0590	DsrM	139	53	102	73	62
THC_0591	DsrK	**39**	**41**	81	**41**	**40**
THC_0592	DsrJ	246	164	562	221	260
THC_0593	DsrO	**30**	**30**	122	**41**	**20**
THC_0594	DsrP	155	60	173	152	109
THC_0726	Rhodanese-like domain-containing protein	295	281	252	152	275
THC_0920	4Fe-4S ferredoxin	79	83	310	276	155
THC_0921	Phs/PsrA	57	**37**	99	85	72
THC_0958	DsrE	60	78	**10**	65	163
THC_0997	Rhodanese-like domain-containing protein	120	83	96	53	53
THC_1155	Rhodanese-like domain-containing protein	60	69	162	117	139
THC_1156	Rhodanese-like domain-containing protein	69	104	127	105	177
THC_1309	Sat	**16**	**3**	**15**	**16**	**6**
THC_1310	Hypothetical protein	**3**	**9**	**1**	**3**	**3**
THC_1311	AprB	**2**	**5**	**11**	**1**	**7**
THC_1312	AprA	**1**	**1**	**2**	**2**	**1**
THC_1313	QmoA	**17**	**16**	55	**21**	**18**
THC_1314	QmoB	**12**	**12**	**6**	**13**	**12**
THC_1315	QmoC	69	**31**	75	56	**35**
THC_1322	Hypothetical protein	26	48	109	23	26
THC_1323	Polysulfide reductase	69	60	119	75	77
THC_1324	4Fe-4S ferredoxin containing protein	79	83	346	160	53
THC_1325	Cytochrome *c*	-	-	291	413	317
THC_1326	Cytochrome *c*	946	1022	-	605	832
THC_1428	YedE-like	79	78	119	**50**	**48**
THC_1429	TusA	**15**	**13**	**23**	**15**	**14**
THC_1430	DsrE-like	**44**	53	**9**	**29**	**46**
THC_1431	Hypothetical protein	**49**	64	**25**	**27**	**48**
THC_1432	Hypothetical protein	64	83	**21**	**25**	79
THC_1435	Hypothetical protein	**26**	**39**	**14**	**34**	**29**
THC_1436	Hypothetical protein	**25**	53	**17**	**29**	**35**
THC_1534	TusA	-	-	709	-	-

- : not detected

Table 4-10 Ranking of the proteins involved in carbon fixation via Wood-Ljungdahl pathway in *Caldimicrobim thiodismutans* TF1^{T}. The numbers that are less than 150 are indicated in red, and the numbers that are less than 50 are highlighted in bold.

		Thiosulfate			Elemental sulfur	
Locus tag	Name	T1	T2	T3	E1	E2
THC_1752	Fhs	**39**	**31**	94	52	**40**
THC_1753	FolD	86	108	310	143	122
THC_1754	AcsA	343	208	557	428	317
THC_1761	MetF/AcsD	135	151	102	191	174
THC_1762	AcsB	114	118	199	235	204
THC_1763	AcsC	155	127	273	181	121
THC_1765	AcsE	86	97	**39**	69	101
THC_1766	Formate dehydrogenase	456	322	447	393	436
THC_1767	Formate dehydrogenase	810	695	740	808	507

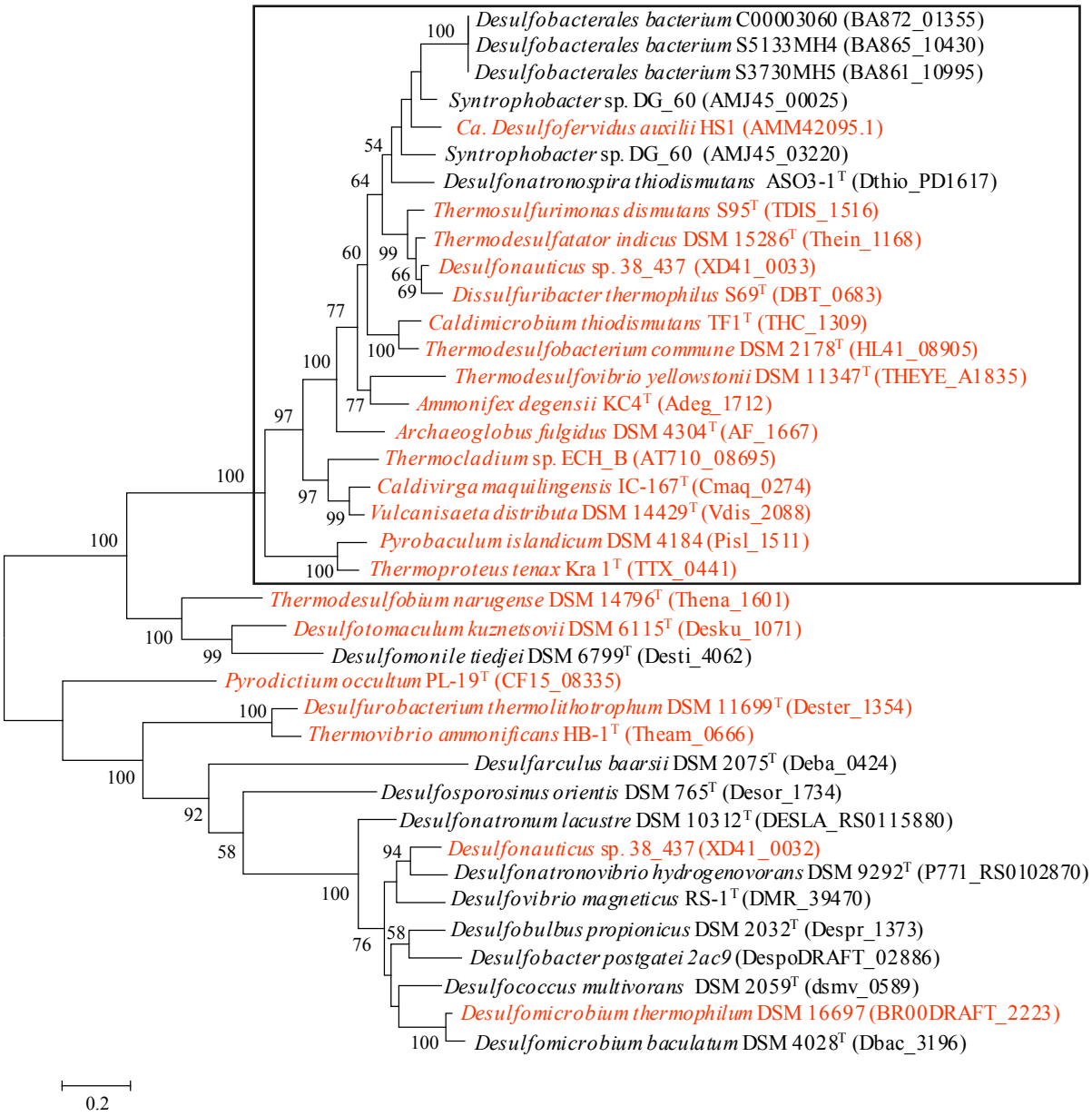


Figure 4-4 Phylogenetic relationship of Sat proteins. This phylogenetic tree was reconstructed using 348 sites. Percentage values of 100 bootstrap resamplings are shown at nodes; values below 50 % were not shown. The sequences of thermophilic prokaryotes are written in red. The sequences shown in box are encoded the *sat* genes located next to homologous gene (THC_1310).

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日本語要旨

硫黄酸化細菌、硫酸還元細菌および硫黄不均化細菌は、無機硫黄化合物を異化的に代謝することで生育に必要なエネルギーを獲得する。硫黄酸化細菌は硫黄化合物を硫酸イオンに酸化し、硫酸還元細菌は硫酸イオンを硫化水素に還元する。これらの機能群による硫黄化合物の酸化・還元の際には、他元素の化合物が電子受容体あるいは電子供与体として用いられる。これに対し、硫黄不均化細菌は単一の硫黄化合物のみからエネルギーを獲得して生育することができる。チオ硫酸や単体硫黄の不均化反応では、最終産物として硫化水素と硫酸イオンが生じる。硫黄不均化細菌の中には、硫黄酸化によっても生育できるもの、硫酸還元によっても生育できるもの、硫黄不均化のみによって生育するものが知られている。硫酸還元菌による硫酸イオンから硫化水素への還元に直接関わる酵素群はほぼ解明されており、その遺伝子セットはほとんどの硫黄不均化細菌にも保存されている。これらの酵素群は可逆的に作用することが知られており、一部の硫黄酸化細菌は硫黄酸化のためにこれを利用している。一方で、これらの酵素群の硫黄不均化における役割は未だ解明されていない。本研究では、知見の乏しい系統に属する微生物を中心としたゲノム解析とプロテオーム解析を通して、異化的硫黄代謝機構を解明することを目的とした。

*Acidiferrobacterales*目は、硫黄酸化細菌を含む新たな目として2015年に提唱された。この目に属する細菌は遺伝子解析により様々な環境から広く検出されており、その検出頻度の高さから生態学的な重要性も示唆されている。その一方で、純粋培養に基づいて記載されているのはわずか3属3種のみであり、異化的硫黄代謝機構は明らかとなっていない。本研究では、本目に属する硫黄酸化細菌である*Sulfuricaulis limicola* HA5^{T}株および*Sulfurifustis variabilis* skN76^{T}株のゲノム解析を行った。その結果、両株とも硫酸還元菌と共通する酵素群を介した硫黄酸化を行うことが明らかとなった。また、それら遺伝子の一部と炭酸固定に関わる遺伝子を重複して有しており、それぞれが系統的に明確に異なっていた。

既知の硫黄不均化細菌のほとんどは硫酸還元細菌と近縁であるが、硫酸還元細菌に比べて限られた系統からしか見出されていない。*Nitrospira*門では、硫酸還元細菌の存在が知られている一方で、硫黄不均化細菌は報告されていない。この門には硫黄酸化細菌が含まれることも示唆されており、環境中における硫黄循環に大きな影響を与えていると考えられている。本研究では、*Nitrospira*門に属する硫黄不均化細菌の集積培養系を確立した。集積培養は、温泉のバイオマットをチオ硫酸不均化細菌用の培地に接種して45℃で培養することによって行った。数回の継代後に集積培養系からDNAを抽出し、細菌群集構造を解析した。PCRを介した16S rRNA遺伝子配列解析の結果、得られた集積培養系は*Nitrospira*門に属するほぼ1種のみから構成されることが明らかとなった。集積培養系から植え継ぐことによって生理学試験を行ったところ、硫酸還元による生育は認められなかった。これらの結果から、集積培養系の優占種は硫酸還元できない硫黄不均化細菌であると結論づけた。また、集積培養系から抽出したDNAについて直接塩基配列を決定することにより、優占種のドラフトゲノムを得ることに成功した。得られたゲノムは約2.4Mbpと約44kbpの2本のコンティグからなり、保存性の高い遺伝子を全て有していた。これらのことから、得られたゲノムは本細菌の代謝経路を推定するのに十分な完成度を持ったものであると考えられる。ゲノム上の16S rRNA遺伝子に基づく系統解析の結果、本細菌は既知の培養株から独立した系統に位置しており、最近縁種との配列相同性は88%に留まった。その一方で、本細菌と近縁な遺伝子配列は温泉、湖底堆積物や地下水などから検出されていることが明らかとなった。*Nitrospira*門に属する硫黄不均化細菌は環境中に広く分布し、硫黄循環において重要な役割を持っている可能性がある。

硫酸還元もしくは硫黄不均化に特異的に関与する遺伝子を探索するため、比較ゲノム解析を行った。約90種の比較ゲノム解析の結果、硫黄不均化細菌が有しておらず硫酸還元細菌が共通して有する遺伝子は見つからなかった。一方で、硫酸還元を行わない硫黄不均化細菌に特徴的な遺伝子群が見つかった。この遺伝子群には、硫黄の輸送と受渡しを行うタンパク質の遺伝子が含まれていた。以上のことから、硫黄不均化と硫酸還元は同じ酵素群

に触媒されるもののその一部で反応が逆向きになっていること、その反応の方向性は硫黄の受け渡しと輸送に関わるタンパク質によって決定づけられている可能性が示された。また、3つの異なる門に属する硫黄不均化細菌は、炭素固定や硝酸還元に関わる遺伝子の多くが共通していた。

Caldimicrobium thiodismutans TF1^{T} 株は、*Thermodesulfobacteria* 門に属する好熱性細菌であり、硫黄不均化によってのみ生育する。この株を用いて、チオ硫酸不均化条件および単体硫黄不均化条件で発現するタンパク質を特定するためのプロテオーム解析を行った。その結果、不均化反応の基質によるタンパク質発現パターンの明確な違いは認められなかった。一方、上述の硫黄不均化細菌に特徴的な遺伝子群にコードされたタンパク質はどちらの条件においても高レベルに発現することが明らかとなった。また、硫酸還元に関わる酵素群も不均化反応の基質に依らず高発現しており、この結果は *Proteobacteria* 門の細菌での先行研究と一致していた。