MICROBIAL GENOMICS

RESEARCH ARTICLE

Allioux *et al.*, *Microbial Genomics* 2022;8:000865

DOI 10.1099/mgen.0.000865

MICROBIOLOGY SOCIETY

OPEN DATA OPEN ACCESS

Genome analysis of a new sulphur disproportionating species *Thermosulfurimonas* strain F29 and comparative genomics of sulfur-disproportionating bacteria from marine hydrothermal vents

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Abstract

This paper reports on the genome analysis of strain F29 representing a new species of the genus *Thermosulfurimonas*. This strain, isolated from the Lucky Strike hydrothermal vent field on the Mid-Atlantic Ridge, is able to grow by disproportionation of S^0 with CO_2 as a carbon source. Strain F29 possesses a genome of 2,345,565 bp, with a G+C content of 58.09%, and at least one plasmid. The genome analysis revealed complete sets of genes for CO_2 fixation via the Wood–Ljungdahl pathway, for sulphate-reduction and for hydrogen oxidation, suggesting the involvement of the strain into carbon, sulphur, and hydrogen cycles of deep-sea hydrothermal vents. Strain F29 genome encodes also several CRISPR sequences, suggesting that the strain may be subjected to viral attacks. Comparative genomics was carried out to decipher sulphur disproportionation pathways. Genomes of sulphur-disproportionating bacteria from marine hydrothermal vents were compared to the genomes of non-sulphur-disproportionating bacteria. This analysis revealed the ubiquitous presence in these genomes of a molybdopterin protein consisting of a large and a small subunit, and an associated chaperone. We hypothesize that these proteins may be involved in the process of elemental sulphur disproportionation.

DATA SUMMARY

The genome datasets generated and analysed during the current study are available in the National Centre for Biotechnology Information repository under BioProject number PRJNA753696. The assembled genome is available in the NCBI WGS database under accession numbers JAIFYA01 and ASM1968873v1. Shared CDS from the genome comparison of the three *Thermosulfurimonas* species dataset is available under doi: https://doi.org/10.6084/m9.figshare.19669143. Shared CDS from the genome comparison of six species isolated from hydrothermal vents able to grow by the disproportionation of elemental sulphur dataset is available under doi: https://doi.org/10.6084/m9.figshare.19669152.

INTRODUCTION

Disproportionation of inorganic sulphur compounds is a relatively undocumented catabolism and is not systematically studied in environmental studies of the microbial sulphur cycle. After having been the subject of several studies in the late 1980s, particularly focused on sedimentary ecosystems, microbial sulphur disproportionation (MSD) was then relatively poorly studied. In recent years, the study of this process has been revived, with the isolation of several new taxa, notably thermophilic ones, and with ecological and genomic studies in order to try to decipher it [1–5].

Received 18 November 2021; Accepted 15 June 2022; Published 22 September 2022

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Keywords: comparative genomics; hydrothermal vents; sulfur disproportionation; thermophile; *Thermosulfurimonas*.

Abbreviations: AAI, average amino-acid identity; ANI, average nucleotide identity; Cas, CRISPR associated protein; CDS, coding DNA sequence; CRISPR, clustered regularly interspaced short palindromic repeats; DDH, DNA-DNA hybridization; DNRA, dissimilatory nitrate reduction to ammonium; GI, genomic islands; MOSC, MOCO sulfurase C-terminal; MSD, microbial sulfur disproportionation; PGAP, prokaryotic genome annotation pipeline; SD, standard deviation.

Data statement: Four supplementary tables are available with the online version of this article.

Impact Statement

The disproportionation of inorganic sulphur compounds is poorly documented in the hydrothermal vent ecosystem and in natural environments in general. Its comprehensive apprehension might change our understanding of the hydrothermal vent sulphur cycle. Currently, the catabolic pathways and enzymatic mechanisms of this process are only partially resolved. In order to study the distribution and abundance of sulphur-disproportionators in natural environments, it might be worthwhile to find specific molecular markers or phylogenetic tags of this catabolism, if they exist. Here, we have performed a genome study of a new species disproportionating elemental sulphur, *Thermosulfurimonas* strain F29 sp. nov., and have performed comparative genomics within the genus *Thermosulfurimonas* and other sulphur-disproportionating genera of marine hydrothermal vent origin. This work has revealed a molybdopterin protein that may be involved in sulphur disproportionation. However, its role will have to be confirmed by functional approaches. The implementation of comparative genomics, followed by or combined with functional approaches should lead to a comprehensive understanding of microbial inorganic sulphur disproportionation in natural environments.

Since the discovery of this catabolism in 1987, its biochemical pathways have not been fully described, although it appears to proceed in part through a reverse pathway of the sulphate reduction route in some species [1, 3, 6, 7]. The molecular markers of microbial sulphur disproportionation pathways remain either putative, or are enzymes common to the dissimilatory sulphate reduction pathway. In recent years, several genes or gene clusters that may serve as functional markers for MSD have nevertheless been proposed in the literature [5, 8, 9]. For example, it has been suggested that molybdopterin oxidoreductases and rhodanese-like sulphurtransferases could be involved in MSD [3]. A study inferred that a YTD gene cluster composed of a *yedE*-related gene, the *tusA* gene and a dsrE related-gene followed by genes coding for hypothetical proteins, could be genomic markers of MSD [5]. Another study pointed out that most bacterial sulphur disproportionators and all *Desulfobulbaceae* encode a truncated C-terminal domain of APR reductase, and proposed that this may be a potential tag for MSD [8]. Finally, a last study proposed that a short protein of unknown function, automatically annotated as a EscU/YscU/HrcU family type III secretion system export apparatus switch protein, might be involved in elemental sulphur disproportionation. This protein referenced as Eyh, seems consistently encoded in all genomes of S^0-disproportionators and absent in most genomes of non-S^0-disproportionators, and might be involved in the first step of S^0-disproportionation [9]. To date, the first steps of the elemental sulphur disproportionation, in particular, remain still completely unknown. In the current state of knowledge, it seems likely that there are several MSD pathways [3].

To date, the share of sulphur disproportionation in total sulphur geochemical cycle in various habitats compared to sulphur oxidation and sulphate reduction is not known because disproportionation is confounded with these pathways in global budgets, since it leads to the production of sulphates and sulphides [1]. The geographical distribution of the niches where this process takes place and the abundance of sulphur-disproportionating taxa in natural habitats is not known either, notably because of the lack of molecular markers to study it. Yet, it would be important to address all these issues.

The genus *Thermosulfurimonas* was described quite recently and was the first thermophilic microbial genus in which the ability to disproportionate inorganic sulphur compounds has been reported [10]. The first species, *Thermosulfurimonas dismutans* S95^T was isolated from a deep-sea hydrothermal vent. It originates from a hydrothermal vent chimney from the Mariner hydrothermal field located on the Eastern Lau Spreading Centre, in the Pacific Ocean, at a depth of 1910 m (Table 1) [10]. A second species was then isolated from a shallow hydrothermal vent in the Sea of Okhotsk, located at 12 m water depth, and named *Thermosulfurimonas marina* SU872^T (Table 1) [11]. These two types of environments where the genus *Thermosulfurimonas* is present, are characterized by physicochemical conditions that have similarities and differences. Both deep and shallow hydrothermal vents exhibit strong gradients in temperature, pH and chemical species in the mixing zones between the hot, reduced hydrothermal vent fluid and the cold, oxygenated sea water. Located at low depths in the photic zone, shallow hydrothermal vents allow both chemotrophic and phototrophic primary producers to grow [12, 13]. Located at greater depths, deep-sea hydrothermal vents are characterized by darkness and high pressures, so that primary production is ensured only by chemolithoautotrophic microorganisms [14]. The physiology and genomic potential of these two *Thermosulfurimonas* species have been studied previously [10, 11, 15, 16]. Both have small genomes and are able to grow by disproportionation of inorganic sulphur compounds and by dissimilatory nitrate reduction to ammonium (DNRA) [11, 17]. *T. dismutans* draft genome was assembled into 61 contigs of 2119932 bp and *T. marina* complete genome consists of one circular chromosome of 1763258 bp (Table 1).

Currently, genomic studies are powerful methods for studying sulphur-disproportionating microorganisms that are particularly difficult to grow. Here, we report the isolation, and the sequencing, assembly, taxonomic classification, and annotation of the genome of a new species of the *Thermosulfurimonas* genus, *Thermosulfurimonas* strain F29. Its genome was then compared to those of *T. dismutans*, *T. marina* and various members of the class *Thermodesulfobacteriaceae* and to those of other marine sulphur-disproportionators. These investigations were performed in order (i) to explore the genetic potential of a new species of *Thermosulfurimonas*, its phylotaxonomic position and its putative metabolism, (ii) to contribute to a better knowledge of the

Table 1. General features and genome sequencing information for *Thermosulfurimonas marina* strain SU872^T, *Thermosulfurimonas sp.* strain F29 and *Thermosulfurimonas dismutans* strain S95^T, including MIGS mandatory information, based on MaGe platform. Adapted from [15]. * Classified in the phylum *Desulfobacterota* according to [76]

Item	Description		
Investigation			
Strain	*Thermosulfurimonas marina* strain SU872^T	*Thermosulfurimonas dismutans* strain S95^T	*Thermosulfurimonas* sp. F29
Submitted to INSDC	GenBank	GenBank	GenBank
Investigation type	Bacteria	Bacteria	Bacteria
Project name	CP042909	LWLG01	JAIFYA01
Geographic location (latitude and longitude)	44° 29.469′ N, 146° 06.247′ E	22° 10.82′ S, 176° 36.09′ W	37° 17.35002′ N, 32° 15.83334′ W
Geographic location (country and/or sea, region)	Sea of Okhotsk, 250 m from the Kunashir Island shore (Sakhalin oblast, Russia)	Eastern Lau Spreading Centre (SW Pacific Ocean)	Sulphide chimney, Capelinhos vent site (Atlantic Ocean)
Collection date	June 2013	June 2009	June 2019
Environment (biome)	marine hydrothermal vent biome ENVO:01000030	marine hydrothermal vent biome ENVO:01000030	marine hydrothermal vent biome ENVO:01000030
Environment (feature)	marine hydrothermal vent ENVO:01000122	marine hydrothermal vent ENVO:01000122	marine hydrothermal vent ENVO:01000122
Environment (material)	marine hydrothermal vent chimney ENVO:01000129	marine hydrothermal vent chimney ENVO:01000129	marine hydrothermal vent chimney ENVO:01000129
Depth	−12 m	−1910 m	−1672 m
General features			
Classification	Domain Bacteria	Domain Bacteria	Domain Bacteria
	Phylum *Thermodesulfobacteria**	Phylum *Thermodesulfobacteria**	Phylum *Thermodesulfobacteria**
	Class *Thermodesulfobacteria*	Class *Thermodesulfobacteria*	Class *Thermodesulfobacteria*
	Order *Thermodesulfobacteriales*	Order *Thermodesulfobacteriales*	Order *Thermodesulfobacteriales*
	Family *Thermodesulfobacteriaceae*	Family *Thermodesulfobacteriaceae*	Family *Thermodesulfobacteriaceae*
	Genus *Thermosulfurimonas*	Genus *Thermosulfurimonas*	Genus *Thermosulfurimonas*
	Species: *Thermosulfurimonas marina*	Species: *Thermosulfurimonas dismutans*	Species: *Thermosulfurimonas* sp.
Gram stain	Negative	Negative	/
Cell shape	Oval to short rods	Rods	/
Motility	Motile	Motile	/
Growth temperature	Thermophilic, optimum at 74 °C	Thermophilic, optimum at 74 °C	Thermophilic
Relationship to oxygen	Anaerobic	Anaerobic	Anaerobic
Trophic level	Chemolithoautotroph	Chemolithoautotroph	Chemolithoautotroph

Continued

Table 1. Continued

Item	Description		
Biotic relationship	free-living	free-living	free-living
Isolation and growth conditions	doi:https://doi.org/10.1134/S0026261718040082	doi:https://doi.org/10.1099/ijs.0.034397-0	/
Sequencing			
Sequencing technology	Illumina MiSeq+PacBio Sequel (hybrid)	454 sequencing	Illumina MiSeq+Oxford nanopore MinIon (hybrid)
Assembler	Unicycler v 0.4.8-beta	Newbler v. 2.9	Unicycler v 0.4.9
Contig number	1	61	9
N50	1763258	94683	737680
Genome coverage	116.0086×	35×	76.4×
Genome Accession NCBI	ASM1231758v1	ASM165258v1	ASM1968873v1
Assembly level	Complete genome	Contigs	Contigs
Genomic features			
Genome size (bp)	1763258	2119932	2345565
GC content (%)	58.90	50.12	58.09
Protein coding genes	1827	2201	2463
Number of RNAs	54	51	51
tRNAs	47	48	48
16S-23S-5S rRNAs	1–1–1	1–1–1	1–1–1
Completeness	99.04%	/	99.18%
Contamination	0.41%	/	1.85%

genus *Thermosulfurimonas*, which is one of the branches of life little documented because it was discovered recently, and (iii) to improve understanding of the genes and enzymes involved in MSD pathways and potentially identify genomic markers that can be deployed in further ecological studies.

METHODS

Sampling

A hydrothermal vent sulphide chimney sample, from which strain F29 is derived, was collected, during the MoMARSAT 2019 oceanographic cruise (June-July 2019) [18] from the Capelinhos site (37.289167 N 32.263889 W), located 1.5 km east of the Lucky Strike lava lake at a depth of 1672 m [19]. It was collected with the clamp of the *Nautile* submersible, then the sample was brought to the surface into a decontaminated insulated box as described elsewhere [20], and referenced as M19Cap2 (PL1945-7). Once onboard, the sample was ground in a sterile mortar inside an anaerobic chamber under a N_2/H_2 (90/10%) gas atmosphere to homogenize it, and then stored into Schott flasks under anaerobic conditions. Samples were stored at 4 °C until subsequent use for enrichment culture.

Culture conditions, strain isolation and physiological experiments

Cultures were carried out in a sulphur disproportionation medium and according to the procedure described elsewhere [21]. Incubations were carried out in the mineral medium containing 62 mM $Fe(OH)_3$, at pH 6.5–7.0, at 60 °C, with 5 g l^{-1} of elemental sulphur and under a gas phase 100% CO_2 (1 bar relative pressure). The initial enrichment culture was performed by inoculating the medium with 10% (v/v) of chimney fragments suspended in artificial seawater. Then, three sub-cultures were performed, followed by serial dilutions to 1/10. The isolate was then cultivated at 70 °C as growth was observed to be better than at 60 °C. In addition, to confirm that the isolate was able to grow by S^0 disproportionation, sulphate production was investigated and measured by ion chromatography using a Dionex ICS-900 Ion Chromatography System (Dionex, Camberley, UK) equipped with an IonPac CS16 column maintained at 60 °C in an UltiMate3000 Thermostated Column Compartment (Thermo Scientific, Waltham, MA, USA), as described elsewhere [22]. In addition, three successive cultures of the isolate were performed in the absence of iron hydroxide, on which the production of both sulphate and H_2S has been measured by colorimetric tests described elsewhere [23], and which were characterized by lower growth and biomass [1, 3]. For genomic DNA extraction, the strain was cultivated anaerobically at 70 °C, with elemental sulphur as an electron donor and acceptor, and CO_2 as sole carbon source in gaseous phase.

The ability of the isolate to grow by sulphate reduction was investigated at 70 °C, in triplicate, on the same medium but prepared with 20 mM of sodium sulphate, in the absence of sulphur and iron hydroxide, and reduced with 1 mM $Na_2S{\cdot}9H_2O$. These cultures were incubated with H_2 as an electron donor, and CO_2 as a carbon source (H_2/CO_2; 80/20%; 1 bar relative pressure gas phase).

Genome sequencing and assembly

Cells were harvested in the late exponential phase of growth. Genomic DNA was extracted using a standard PCI DNA extraction protocol, coupled to dithionite addition in order to dissolve iron precipitates [24]. In order to identify and check the purity of the strain, the 16S rRNA genes were amplified from genomic DNA by PCR using Bac8F and 1492Runi 16S rRNA gene universal bacterial primers (1492Runi: 5′-CGGTTACCTTGTTACGACTT-3′; Bac8F: 5′-AGAGTTTGATCATGGCTCAG-3′). Sanger sequencing was then performed on the amplified sequences, and sequences were compared to the NCBI nucleotide collection. In addition, the 16S rRNA gene sequence was also extracted from the total genome assembly using the barrnap module of Prokka (v1.14.6 - https://github.com/tseemann/prokka), for a better accuracy and to get full length 16S rRNA gene sequence [25]. Short read DNA genome sequencing was performed by Fasteris SA (Plan-les-Ouates, Switzerland) using the Illumina MiSeq technology (2×150 bp paired-reads, Nano V2 chemistry). Post-quality controls were performed by sequencing facilities and checked also with FastQC (v0.11.8 - https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Short reads were filtered with fastp (v0.20.1 - https://github.com/OpenGene/fastp) [26]. A long read additional DNA sequencing was performed with a MinIon (Oxford Nanopore) with the rapid sequencing kit (SQK-RAD004) and a R9.4.1 Flow Cell. Genome was assembled into contigs by using the Unicycler pipeline for *de novo* assembly (version: 0.4.9 - https://github.com/rrwick/Unicycler), and its dependencies (spades.py v3.13.0 ; makeblastdb v2.12.0+ ; tblastn v2.12.0+ ; bowtie2-build v2.4.4 ; bowtie2 v2.4.4 ; samtools v1.13 ; java v15.0.1 ; pilon v1.23) with 'bold' parameters [27]. Genome assembly statistics were obtained with Quast (v5.0.2; https://github.com/ablab/quast) and used to compare different assemblies. Genome assembly visualization was plotted with Bandage (v0.8.1 - http://rrwick.github.io/Bandage/) in order to detect potential plasmids from obtained contigs and then checked with viralVerify python script which predicts plasmid and virus sequences (https://github.com/ablab/viralVerify) based on HMMs [28, 29]. Genome completeness and potential contamination were controlled with CheckM (v1.1.2 - https://ecogenomics.github.io/CheckM/), and whole genome average coverage was calculated using BBMap (v38.87 - BBMap – Bushnell B. – sourceforge.net/projects/bbmap/) against total short read sequences [30, 31].

Genomic and taxonomic features

Identification and classification of the CRISPR-Cas systems were performed by using the CRISPRCas Finder webserver, with default parameters (https://crisprcas.i2bc.paris-saclay.fr/) [32]. The prediction of laterally transferred gene clusters (genomic islands) was performed with the IslandViewer4 webserver (http://www.pathogenomics.sfu.ca/islandviewer/) based on a GenBank file generated by Prokka against the reference genome of *Thermosulfurimonas marina*, and IslandPath-DIMOB and SIGI-HMM predictions were considered [33].

To study the taxonomic position of the strain, 16S rRNA gene sequence was first compared to the NCBI nucleotide collection. Pairwise 16S rRNA gene sequence similarity was then determined using the EzTaxon-e server (https://www.ezbiocloud.net/) [34]. The 16S rRNA gene sequence of strain F29 was then aligned to its nearest neighbours using MUSCLE v.5 [35], and the alignment was trimmed manually on Geneious v11.0.5. Then, PhyML (v3.0) was used to build the tree, based on 1459 homologous nucleotides, thanks to the ATGC bioinformatics platform (http://www.atgc-montpellier.fr/phyml/) [36]. The evolutionary model was selected with the SMS algorithm [37] and the branch support was computed with the aLRT SH-like method. The tree was visualized with iToL [38] and rooted with *Thermosulfuriphilus ammonigenes*.

Estimated *in silico* DNA–DNA hybridization (DDH) values with the genomes of *T. dismutans* and *T. marina* were determined using the Genome-to-Genome Distance Calculator (GGDC 2.1) using formula 2 (http://ggdc.dsmz.de/home.php) [39]. For a more accurate classification, average nucleotide identity (ANI) and average amino acid identity (AAI) scores were also calculated, using software default parameters. OrthoANIu scores were calculated against the genomes of *Thermosulfurimonas dismutans* (ASM165258v1) and *Thermosulfurimonas marina* (ASM1231758v1), using the ANI calculator tool provided by the EzBioCloud web server (https://www.ezbiocloud.net/tools/ani) [40, 41]. Average amino acid identity was calculated using the AAI calculator of the Enveomics collection (http://enve-omics.ce.gatech.edu/) with Prokka output [42]. We considered the following thresholds for classification: for 16S rRNA sequences, identity <98.7% for a new species and <94.5% for a new genus [43, 44]. For digital DDH, score <70% for a new species [45]. For ANI, score <94–96% for a new species [46], and <70.85–76.56% for a new genus with alignment fraction [47]. For AAI, scores comprised 95 and 100% for a same species, and between 65 and 95% for a same genus [48].

A standard protein BLAST (blastp) search using catalytic subunit of nitrate reductase (NapA) amino acid sequence of strain *Thermosulfurimonas* sp. F29 (WP_221171702) as query against the non-redundant protein sequences (nr) database of the NCBI was performed to obtain the closest bacterial sequences annotated as NapA, the periplasmic nitrate reductases, or molybdopterin-containing oxidoreductases genes. Twenty of these close sequences belonging to well-described species were selected for phylogenetic analysis. The alignment was performed using the CLUSTALW programme implemented in MEGA (v7.0) [49], with default alignment parameters. Phylogenetic tree reconstructions were performed with MEGA by using the Maximum-Likelihood algorithm based on the Tamura–Nei model, and the Neighbour-Joining algorithm [50, 51].

Genome annotation

Genome was analysed and annotated with the fast annotation software Prokka, the MicroScope-Microbial Genome Annotation Analysis Platform- (MaGe) (https://mage.genoscope.cns.fr/microscope/home/index.php), using KEGG and BioCyc databases, the NCBI integrated PGAP pipeline, and RAST server (v2.0 - https://rast.nmpdr.org/), with default parameters and databases for all of the four software/pipelines [25, 52–54]. Functional annotation of predicted CDSs was further blasted with UniProtKB +Swiss Prot database (release 2021_09). Hydrogenase classification was checked using the HydDB webtool (https://services.birc.au.dk/hyddb/) [55]. All *dsr* genes were extracted and analysed with the DiSCo perl script (v.1.0.0, https://github.com/Genome-Evolution-and-Ecology-Group-GEEG/DiSCo) from Prokka protein output sequences [56]. Gene locus tags associated with the genome assembly annotation given in GenBank and RefSeq (ASM1968873v1) are reported in supplementary material Table S1 (available in the online version of this article).

Comparative genomics

The genome of the novel isolate was compared by subtractive comparative genomics, first with the genomes of *Thermosulfurimonas marina* SU872^T (ASM1231758v1) and *Thermosulfurimonas dismutans* S95^T (ASM165258v1), and second, with various genomes of sulphur-disproportionating taxa, and representatives of the *Thermodesulfobacteriaceae* family, as described a little below. The MaGe platform Pan-genome Analysis tool (https://mage.genoscope.cns.fr/microscope/home/index.php) was used for comparative genomics, based on the clustering algorithm SiLiX (http://lbbe.univ-lyon1.fr/-SiLiX-.html). Genomic CDSs were clustered by 80% amino-acid identity and 80% amino-acid alignment coverage, with permissive parameters, for comparative genomic analyses performed between *Thermosulfurimonas* species and *Thermodesulfobacteriaceae* taxa. Genomic CDSs were clustered by 50% amino-acid identity and 80% amino-acid alignment coverage for analyses aimed at deciphering sulphur disproportionation pathways, as the compared genomes belong to more phylogenetically distant taxa. In order to identify genes unique to *Thermosulfurimonas* species within the *Thermodesulfobacteriaceae* family, genomes of strain F29, *T. dismutans* and *T. marina* were compared with the exclusion of the available genomes of ten other *Thermodesulfobacteriaceae: Caldimicrobium*

thiodismutans TF1^T (ASM154827v1), *Thermodesulfatator atlanticus* DSM 21156^T (ASM42158v1), *Thermodesulfatator autotrophicus* S606^T (ASM164232v1), *Thermodesulfatator indicus* DSM 15286^T (ASM21779v1), *Thermodesulfobacterium geofontis* OPF15^T (ASM21597v1), *Thermodesulfobacterium thermophilum* DSM 1276^T (ASM42160v1), *Thermodesulfobacterium commune* DSM 2178^T (ASM73401v1), *Thermodesulfobacterium hydrogeniphilum* DSM 14290^T (ASM74625v1), *Thermodesulfobacterium hveragerdense* DSM 12571^T (ASM42384v1), and *Thermosulfuriphilus ammonigenes* ST65^T (ASM1120745v1). Afterwards, to identify putative genes associated to the sulphur disproportionation ability, the following genomes of marine hydrothermal vent sulphur-disproportionators were compared: strain F29, *T. dismutans*, *T. marina, T. ammonigenes*, *Dissulfuribacter thermophilus* S69^T (ASM168733v1), and *Dissulfurirhabdus thermomarina* SH388^T (ASM1297923v1). *Dissulfurirhabdus thermomarina* SH388^T is described as being able to disproportionate S^0 and sulphite, but not thiosulphate [57]. This analysis was performed by excluding the genomes of the following hydrothermal vent bacteria that are unable to disproportionate inorganic sulphur compounds: *Caminicella sporogenes* DSM 14501^T (GCA_900142285.1), an anaerobic, strictly chemoorganoheterotrophic bacterium, and *Thermodesulfatator indicus* DSM 15286^T, able to grow by sulphate reduction but unable to grow by sulphur disproportionation nor by sulphur, thiosulphate and sulphite reduction, confirmed by culture. Two of the CDSs were subjected to subcellular localization prediction with the BUSCA server (http://busca.biocomp.unibo.it/) [58]. CDSs were then compared to the UniProtKB database, and all CDSs coding for hypothetical proteins were analysed with the InterProScan webserver (https://www.ebi.ac.uk/interpro/) to perform functional predictions and domain identification (e.g. MOSC). Molybdopterin subunits and chaperone sequences microsynteny was then analysed and plotted with SimpleSynteny (https://www.dveltri.com/simplesynteny/) [59]. The genomes of strain F29, *T. dismutans*, *T. marina, T. ammonigenes*, *D. thermophilus*, and *D. thermomarina* were compared against the three predicted protein sequences of strain F29. Protein sequences homologies between strains were calculated with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) [60].

RESULTS AND DISCUSSION

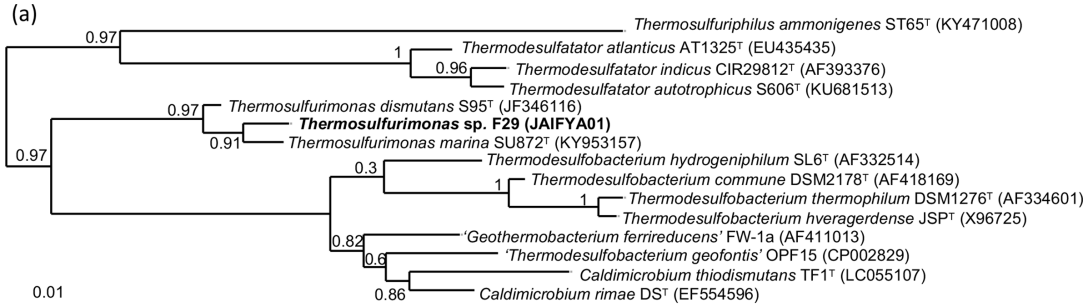
General genomic properties, genomic plasticity and taxonomy

Although hybrid sequencing was performed, only a draft genome could be reconstructed. Main genomic features are reported in Table 1. The assembled genome of strain F29 consisted of nine contigs, including four contigs of less than 5000 bp, with an overall size of 2345565 bp and a G+C content of 58.09%. The longest contig was 889860 bp, the N50 was 737680 bp and the L50 was 2. CheckM estimated the genome to be 99.18% complete based on the presence of default single-copy marker genes (two markers were missing), with 1.85% contamination (five markers were duplicated) and a coding density of about 93.3%.

Hybrid sequencing allowed the detection of two or more putative plasmid contigs, including one circular, observed with Bandage and confirmed by applying the viralVerify script to all contigs. The first putative plasmid (p1) was 145269 bp (contig 4) in size; the second one was a non-putative circularized plasmid (p2) of 69636 bp in size (contig 5). In addition, contigs 6 (1354 bp), 7 (1341 bp), 8 (1177 bp) and 9 (788 bp) were smaller and then uncertain to be chromosomal or plasmidic. Contig 4, named p1, was relatively long and could not be circularized and it is therefore not possible to state with certainty that it is a plasmid. The median size of known prokaryotic plasmids is 53212 bp according to the PLSDB database (https://ccb-microbe.cs.uni-saarland.de/plsdb/) but unknown within the class *Thermodesulfobacteria*, because no plasmids have been reported so far in bacteria of this class. p2, and possibly p1, are thus the first reported plasmids in the genus *Thermosulfurimonas* and possibly in the *Thermodesulfobacteria* class. Contigs 6, 7, 8, and 9 were treated as chromosomal for analysis although their small size did not indicate whether they correspond to chromosomal or plasmid sequences. No viral sequences were detected in the genome. As not reported in previous studies, we also searched for plasmids in *T. dismutans* genome and found two putative plasmid contigs (NZ_LWLG01000026.1; NZ_LWLG01000030.1) and other uncertain contigs. *Thermosulfurimonas* species from deep-sea hydrothermal vents thus appear to possess a plasmid diversity not revealed so far.

Annotation of the genome with Prokka resulted in the prediction of 2348 CDSs in total (Fig. S1), comprising 148 CDSs on p1 and 57 CDSs on p2. From MaGe, a total of 2557 genomic objects including 2463 CDS were found, and with PGAP, 2400 genes including 2345 CDSs were found. p1 carried few genes associated with DNA replication and a majority of sequences coding for hypothetical proteins (127/148 CDS), while p2 carried genes associated with type II secretion system, some carbohydrate-associated enzyme sequences, and a large majority of hypothetical protein sequences. The genome also contained one operon of 5S-16S-23S rRNA genes. We detected 48 tRNA genes with MaGe, and 49 with PGAP which use the tRNA scan-SE RNA finder (http://lowelab.ucsc.edu/tRNAscan-SE/index.html), while 51 tRNA were detected with Prokka based on ARAGORN RNA finder (http://130.235.244.92/ARAGORN/. The 20 tRNAs encoding essential amino acids were each encoded by at least one sequence. Furthermore, eight potential CRISPR and two potential Cas cluster loci were found using the CRISPRCasFinder server. The CRISPR sequences consisted of four low evidence CRISPR loci containing one or two spacers and four high evidence CRISPR loci, with eight spacers for 5′-GTTTGTAGTTCCCCTATAAGGGTTGAGAAG-3′ sequence, 12 spacers for 5′-GTCG CAATCCCTTATTCGTGAGGGAAAGTTTTCTCAC-3′ sequence, 17 spacers for 5′-GTTTGTAGTTCCCCTATAAGGGTT GAGAAG-3′ sequence, and 38 spacers for 5′-GTTTCCATTCCTCATAGGTAGGCTCGAAAC-3′ sequence. Moreover, two Cas cluster loci were found, one Cas Type IIIB cluster of nine genes and one Cas Type IB cluster of eight genes. The presence of this large number of CRISPRs could indicate that the new isolate is undergoing various genomic transfers and is setting up defence

(a)



(b)

	16S rRNA gene seq. similarity (%)	OrthoANIu (%)	dDDH (%)	AAI (%)
Thermosulfurimonas F29 (this study)	100	100	100	100
T. dismutans S95^T	97.85	75.18	19.30	75.16
T. marina SU872^T	97.97	73.20	18.90	73.62

Fig. 1. (a) PhyML phylogenetic tree based showing the phylogenetic positions of strain F29 and representatives of some other related taxa, based on 16S rRNA gene sequences (1459 nucleotide positions). Branch supports, computed with the aLTR SH-like method, are shown at branch nodes. Bar, 0.01 nucleotide substitution rate (Knuc) units. (b) Overall genome relatedness indexes between strain F29 and the two other species of the genus *Thermosulfurimonas*.

mechanisms against invasions by bacteriophages and plasmids from its environment [61]. Several regions of genomic plasticity were identified. It was observed 16 putative genomic islands (GI), two of which were present on plasmids, exhibited different lengths. There were two big GI of about 85 and 120 kbp, and 14 GI of less than 22 kbp. GI were mainly composed of hypothetical proteins, transposases and several DNA replication related genes.

Regarding taxonomy, strain F29 has the highest 16S rRNA gene sequence identity of 97.97% to *T. marina,* followed by *T. dismutans* with 97.85%. Phylogenetic analyses based on 16S rRNA gene sequences showed that the novel isolate forms a monophyletic group with *T. marina* and *T. dismutans*, exhibiting a high branch support value (Fig. 1a). The genome of strain F29 shows an OrthoANIu score of 75.18% with the genome of *T. marina* and of 73.20% with the genome of *T. dismutans*, and are far below the standard threshold level of 94–96% for the delineation of a new genomic species [46]. The digital DDH (dDDH) scores between the genome of strain F29 and those of *T. marina* and *T. dismutans* are respectively, 18.90 and 19.30%, well below the threshold level (70%) for a new species delineation (Fig. 1b) [45]. Finally, the AAI scores between the genome of the novel isolate and the ones of *T. marina* and *T. dismutans* are respectively 75.16% (from 1626 proteins; SD: 13.74%) and 73.62% (from 1675 proteins; SD: 14.70%). Overall, all these genomic relatedness indices indicate that strain F29 represents a new genomic species of the genus *Thermosulfurimonas* (Fig. 1b).

Central metabolism and energy production pathways

From a general metabolic perspective, strain F29 has a very similar metabolic profile to other members of the genus *Thermosulfurimonas*, *T. dismutans* and *T. marina,* according to the Metabolic Profiles tool of the MaGe platform.

Carbon metabolism

Consistent with the autotrophic growth ability of strain F29, the genome encodes a complete Wood–Ljungdahl (acetyl-CoA-reductive) pathway for CO_2 fixation, including a formate-tetrahydrofolate ligase, a methylenetetrahydrofolate dehydrogenase/cyclohydrolase, a methylenetetrahydrofolate reductase, a methyltetrahydrofolate:corrinoid iron–sulphur protein methyltransferase, and a CO dehydrogenase/acetyl CoA synthase complex. As in *T. dismutans* and *T. marina*, all these genes are arranged in one gene cluster. Key enzymes from other known autotrophic carbon fixation pathways, such as the reverse tricarboxylic acid cycle and the Calvin–Benson pathways have not been identified. Conversion of acetyl-CoA formed during CO_2 fixation to pyruvate can be performed by the pyruvate:ferredoxin oxidoreductase encoded by the *porABDG* genes. The genome of strain F29 encodes the complete Embden-Meyerhof pathway of glucose catabolism, which apparently operates in reverse direction, towards gluconeogenesis. Consistently, the genome of strain F29 does not code for the pyruvate kinase, the enzyme that catalyses the irreversible reactions of glycolysis. In contrast, this gene is present in the genomes of *T. dismutans* and *T. marina*. Strain F29 also has genes encoding enzymes that specifically catalyse reverse glycolysis reactions: phosphoenolpyruvate synthase and fructose-1,6-bisphosphatase. In addition, strain F29 possesses the genes for ribulose-5-phosphate 4-epimerase, ribose-5-phosphate isomerase and ribose-phosphate pyrophosphokinase that may also be involved in glucose synthesis. Strain F29 may not be able to utilize

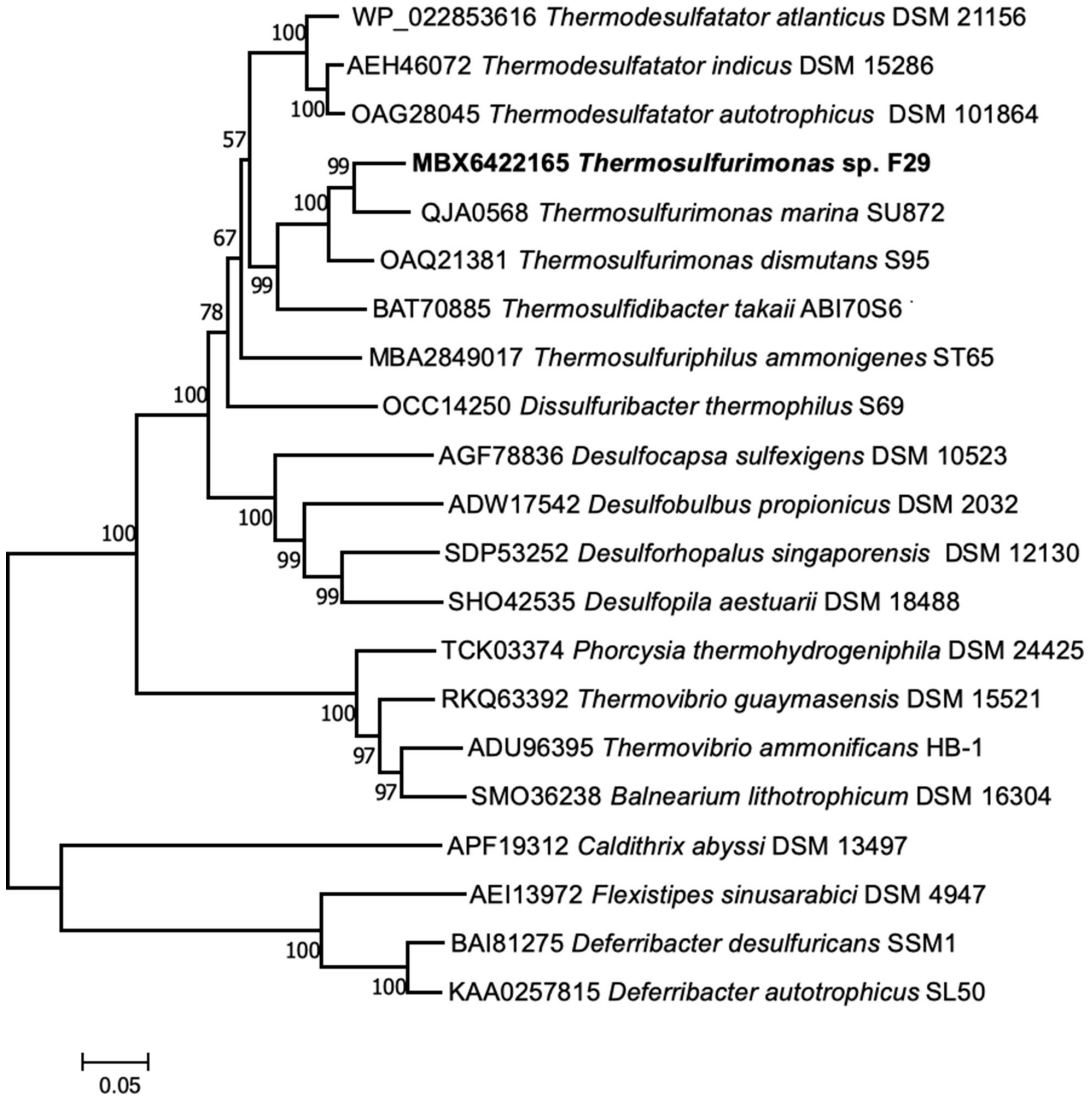


Fig. 2. Neighbour joining phylogenetic tree showing the position of the gene NapA of strain F29 within the closest bacterial sequences annotated as NapA, the periplasmic nitrate reductases, or molybdopterin-containing oxidoreductases genes. Bootstrap values based on 1000 replications are shown at branch nodes. Bar, 5% estimated substitutions. Tree reconstructed by the maximum-likelihood algorithm displayed the same topology.

organic substrates because its genome encodes a highly incomplete tricarboxylic acid cycle (TCA), as evidenced by the absence of genes coding for the key enzyme of this pathway, citrate synthase, succinyl-CoA synthetase and succinate dehydrogenase.

Nitrogen metabolism

Such as *T. dismutans* and *T. marina*, strain F29 possesses a gene cluster encoding proteins involved in nitrogen fixation. These include the nitrogenase (molybdenum-iron type; EC: 1.18.6.1), proteins related to nitrogenase cofactors, nitrogen regulatory proteins and ammonium transporter. Genome of *Thermosulfurimonas* F29 hold genes *nap* MADGH encoding a periplasmic Nap-type nitrate reductase. Phylogenetic analysis of the catalytic subunit NapA proteins of strain F29, *T. marina* and *T. dismutans* revealed that they form a clade, mainly composed of the NapA proteins of representatives of the class *Thermodesulfobacteria*, phylum *Desulfobacterota* (Fig. 2). This clade also includes nitrate reductases of the closest neighbour, *Dissulfuribacter thermophilus*, and from the distantly related species *Thermosulfidibacter takaii. D. thermophilus* belongs to class *Dissulfuribacteria* of the phylum *Desulfobacterota. T. takaii* belongs to another phylum, *Aquificae* (NCBI taxonomy) or *Thermosulfidibacterota* (GTDB taxonomy). Denitrification enzyme genes were not found in the genome of *Thermosulfurimonas* F29. The genome encodes also NrfCD and NrfH proteins of the Nrf system of nitrite reduction to ammonia. However, the catalytic subunit NrfA (pentaheme cytochrome c nitrite reductase) is absent, indicating that the nitrite reduction to ammonia potentially cannot proceed via the canonical Nrf system.

The genomes of *T. dismutans* and *D. thermophilus* have been shown to have an 11 gene cluster consisting of nitrate reductase subunit genes and other genes relevant to energy metabolism. It has been suggested that this oxidoreductase complex may be coupled to nitrite reduction [16, 17]. We have found a very similar gene cluster in *T. marina* and strain F29. In strain F29, the nitrate reductase genes (*nap* MADGH) are followed by the genes encoding two multiheme *c*-type cytochromes, the periplasmic iron–sulphur protein similar to subunit B of tetrathionate reductases, and the membrane anchor protein of the NrfD family. In genomes of *T. takaii, Thermosulfuriphilus ammonigenes* and species of the genus *Thermodesulfatator*, *nap* genes are not part of such a cluster. It has been demonstrated *in vitro* for *Nautilia profundicola* that the conversion of nitrite to ammonium may proceed via a non-canonical mode, potentially through the production of hydroxylamine [62]. This pathway is not completely encoded in the genome of strain F29 as well as in *T. dismutans* and *T. marina* genomes, although some genes of this pathway are present, namely hydroxylamine reductase and hydroxylamine oxidoreductase. Finally, three ammonium transporters were found in the genome of strain F29. This indicates that it is likely that strain F29 participates in the nitrogen cycle by reducing available nitrate and then performing DNRA metabolism, which is associated to a higher energy yield than sulphur disproportionation.

Sulphur metabolism

For sulphur metabolism, we found all the key enzymes associated to the sulphate reduction pathway: sulphate adenylyltransferase, adenylylsulfate reductase subunit alpha and beta, sulphite reductase dissimilatory type subunits alpha, beta, gamma (DsrC) and DsrD. The adenylylsulfate reductase beta subunit does not have a truncated tail and is 154 amino acid in length, similar to AprB in other *Thermosulfurimonas* species [63]. Moreover, a complete APS reductase-associated electron transfer complex (QmoABC) and a full DsrMKJOP complex were found. A DsrT associated sequence was also found, but no DsrL. Strain F29 was tested for sulphate reduction with dihydrogen as an electron donor but it was not able to grow. This is another case of bacterium that possesses the entire sulphate reduction pathway but is not able to grow by sulphate reduction, at least with H_2 as an energy source, but can nevertheless grow by sulphur disproportionation, as reported previously [3, 15, 16, 63, 64].

One A subunit of tetrathionate reductase was found and three B subunits, however distant from the A subunit in the genome. We also looked specifically for genes proposed in the literature as being specific marker genes or specific gene clusters for MSD. No clear rhodanese-type sulphurtransferase sequences were found in the genome of strain F29. A YTD gene cluster was found, as described with a YedE family protein, a sulphurtransferase TusA, a putative DrsE domain-containing protein and two hypothetical proteins [5]. However, we also found the YTD gene cluster in the genome of *Thermodesulfatator indicus* DSM 15286^T, a strain that we were unable to grow in the laboratory by thiosulphate, sulphite or S^0 disproportionation; Thus, this YTD gene cluster seems unlikely to be a universal genomic marker of MSD. Nevertheless, this finding does not exclude that YTD-associated proteins may be involved in the MSD process in some species. In addition, three CDSs without clear functional predictions but annotated as thiosulphate/tetrathionate/polysulphide reductase molybdopterin A and B subunits with an additional chaperone subunit TorD/DmsD-like were found. Previous studies combining genomic annotations, comparative genomics studies and transcriptomic approaches conducted on other bacterial models had led to the hypothesis that these three genes might be involved in MSD pathway or in sulphide oxidation pathway [63, 65]. Finally, the genome of strain F29 encodes the short protein Eyh, identified by Yvenou *et al.* [9], consistently retrieved in the genome of microorganisms performing S^0 disproportionation. This protein is 92 amino acids long and has 62.20% sequence homology with the protein of *Dissulfurimicrobium hydrothermale* Sh68^T (WP_237888668). This protein seems to be absent in the genome of most of microorganisms unable to perform S^0 disproportionation [9]. From this genomic work and the culture results, we can propose that strain F29 participates in the sulphur cycle of hydrothermal vents through its ability to disproportionate S^0. In this genome, we found several genes described in the literature as specific marker genes, specific gene clusters, or integral parts of MSDs (Fig. 3), whose function should be confirmed in the future by functional approaches.

Hydrogen metabolism

Two large hydrogenases subunits detected in the genome of strain F29 have had their annotation confirmed by HydDB, one Group 1 a [NiFe] hydrogenase and one Group 1 c [NiFe] hydrogenase. For Group 1 a [NiFe] hydrogenase, both large and small subunits were found. For Group 1 c [NiFe] hydrogenase, both large and small subunits were identified as well as an iron/sulphur subunit associated with the catalytic site, and four hydrogenase factors (HypA, HypB, HypC, and HypD). This structure differs from that reported for Group 1 c (HupD, HybE, HypA, HypC) which was mainly described on the basis of *Gammaproteobacteria*; no transmembrane protein was found (HybB) (https://services.birc.au.dk/hyddb/browser/class/nife-group-1c/) [55]. These results indicate that strain F29 has the genetic potential to grow by hydrogen oxidation. Yet, no growth was observed with H_2 as the electron donor and sulphate as the electron acceptor. Several hypotheses can be stated to explain this: (i) the strain may be able to oxidize hydrogen using another electron acceptor than sulphate; (ii) the optimal growth conditions in terms of temperature might be significantly different between sulphur disproportionation and hydrogen oxidation coupled with sulphate reduction, as demonstrated for the hydrothermal vent strain *Persephonella atlantica,* which preferably uses different redox couples depending on the temperature [66]; (iii) Both hydrogenases (referenced as Hyd1a and Hyd1c), predicted to be periplasmic or cytosolic and to have FeS clusters, might act as electron bifurcators, coupling exergonic and endergonic oxidation-reduction reactions to balance electron flow in metabolism and minimize free energy loss, as already described elsewhere in other metabolic pathways

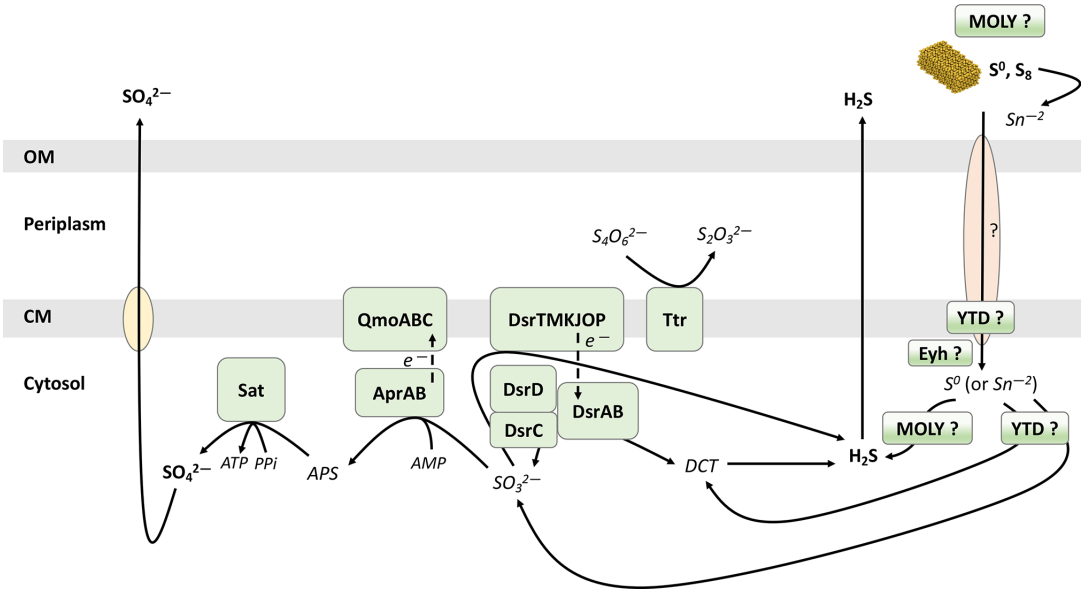


Fig. 3. Hypothetical S^0-disproportionation pathway in *Thermosulfurimonas* strain F29, based on its genomic content and functional analyses carried out on other sulphur-disproportionating taxa [1, 3, 16, 65]. In green, the enzymes present in the genome that are proposed to be involved in the process. In shaded green, speculative hypotheses about the implications of the Eyh protein and the MOLY complex in sulphur disproportionation. The Eyh protein is consistently encoded in all genomes of S^0-disproportionators and absent in most genomes of non-S^0-disproportionators [9]. The MOLY protein is encoded by a gene cluster coding for two molybdopterin oxidoreductases subunits (a and b), and a TorD/DmsD-like chaperone protein. The YTD gene cluster is composed of a *yedE*-related gene, a *dsrE*-related gene, a *tusA* sulphurtransferase gene, and two genes encoding conserved hypothetical proteins (CHPs) [5]. In black and bold, detected chemical species or proteins encoded by genes present in the genome of strain F29. In italics, speculative chemical intermediates. Abbreviations: AprAB, APS reductase; APS, 3'-phosphoadenosine-5'-phosphosulfate; DCT, DsrC-trisulfide; DsrABD, sulphite reductase, dissimilatory-type subunits alpha, beta and clustered protein DsrD; DsrC, dissimilatory sulphite reductase, small protein DsrC; CM, cytoplasmic membrane; DsrTMKJOP, sulphite reduction-associated electron transfer complex; OM, outer membrane; Sat, sulphate adenylyltransferase (=ATP sulfurylase); QmoABC, APS reductase-associated electron transfer complex; Ttr, tetrathionate reductase.

[67–69]. Given the lack of knowledge about pathways, it cannot be excluded that the identified hydrogenase-like proteins may be involved in electron bifurcation, but we recognize this is a speculative hypothesis.

Based on this hypothesis, hydrogenases were searched for and found in the genome of *Thermosulfuriphilus ammonigenes* and *Thermosulfurimonas marina*, two phylogenetically related bacteria capable of disproportionating sulphur but unable to use dihydrogen as an electron donor. Hydrogenases had not been previously searched for in these genomes because of the inability of these strains to grow by hydrogen oxidation [15, 64]. Both genomes contain operons coding for hydrogenases. The genome of *T. marina* encodes a Group 1 c [NiFe] hydrogenase and a Group 3 c [NiFe] hydrogenase which are closely located on the genome (WP_168719511 to WP_168719521). The genome of *T. ammonigenes*, like that of strain F29, has a Group 1 a [NiFe] hydrogenase (WP_166031792 to WP_166031793) and a Group 1 c [NiFe] hydrogenase (WP_166031912 to WP_166031919). In addition, other thermophilic sulphur-disproportionators from hydrothermal vents, *Thermosulfurimonas dismutans* and *Dissulfurirhabdus thermomarina* have Group 1 c [NiFe] hydrogenases, but are able to use H_2 as an electron donor [16, 63]. Therefore, the role of hydrogenases in strain F29 is not elucidated and may be subject to functional analysis in the future to determine whether these proteins are involved in hydrogen oxidation, in electron transfer and bifurcation or are not expressed.

Comparative genomics

Comparison of the genomes of the three species of the genus *Thermosulfurimonas*, showed that a very high number of CDSs are shared. Thus, 368 CDSs are shared (Fig. 4) between the genomes of *T. dismutans*, *T. marina* and strain F29 (https://doi.org/10.6084/m9.figshare.19669143). These numerous shared genes are associated with several metabolisms: energy, carbon, sulphur, nitrogen, cell mobility, stress resistance, membrane and transport proteins, nucleic acids, amino acids, proteins, vitamins and cofactors associated metabolisms, and conserved proteins of unknown functions. These three strains have the genetic potential to perform reduction or oxidation of inorganic sulphur compounds, potential reduction of nitrate, hydrogen oxidation, reduction of carbon monoxide, reduction of tetrathionate and carbon fixation by the Wood-Ljungdahl pathway. Most of these properties have been experimentally confirmed for *T. marina* and *T. dismutans* by physiological experiments [10, 11]. However, comparative genomic approaches only predict functional potential. Thus, although *T. dismutans*, *T. marina* and strain F29 possess the complete

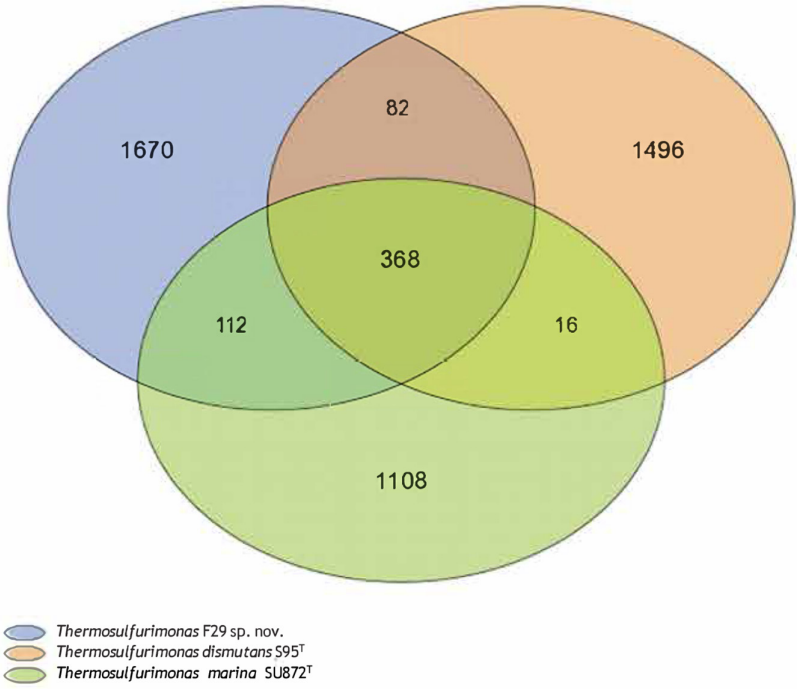


Fig. 4. Venn diagram of the CDSs shared among *Thermosulfurimonas* F29 sp. nov., *Thermosulfurimonas dismutans* S95^T, and *Thermosulfurimonas marina* SU872^T against the genome of ten other members of the *Thermodesulfobacteriaceae* family (*Caldimicrobium thiodismutans* TF1^T, *Thermodesulfatator atlanticus* DSM 21156^T, *Thermodesulfatator autotrophicus* S606^T, *Thermodesulfatator indicus* DSM 15286^T, *Thermodesulfobacterium geofontis* OPF15^T, *Thermodesulfobacterium thermophilum* DSM 1276^T, *Thermodesulfobacterium commune* DSM 2178^T, *Thermodesulfobacterium hydrogeniphilum* DSM 14290^T, *Thermodesulfobacterium hveragerdense* DSM 12571^T, and *Thermosulfuriphilus ammonigenes* ST65^T).

sulphate reduction pathway, this property has not been demonstrated experimentally by testing different electron donors [10, 13; Yvenou *et al.*, in preparation].

In conclusion, the comparative genomic approaches showed that *Thermosulfurimonas* genes are relatively conserved within the genus, associated with a variety of pathways, regardless of the natural habitat of the bacteria, whether deep or shallow hydrothermal vents.

By comparing the six genomes of sulphur-disproportionating strains of marine hydrothermal vent origin, eight shared CDSs were found (https://doi.org/10.6084/m9.figshare.19669152). These eight CDSs code for: a cation efflux system protein, a MOSC domain protein (putative molybdenum cofactor sulphurase), a metallo-beta-lactamase family protein, an uncharacterized DUF1566 domain-containing protein, a molybdopterin oxidoreductase iron-sulphur subunit B, a molybdopterin oxidoreductase catalytic subunit A, a formate dehydrogenase subunit alpha and a putative phosphate-selective porin.

The uncharacterized DUF1566 domain-containing protein, the metallo-beta-lactamase family protein and the putative phosphate-selective porin were not associated with any clear and known function by InterProScan searching. The cation efflux system transmembrane protein has an Acriflavine resistance protein domain, which may be related to cobalt, zinc, cadmium, copper, or silver resistance. The formate dehydrogenase subunit alpha is composed of two dihydroprymidine dehydrogenase, one FAD/NAD(P)-binding domain, two iron/sulphur and one molybdopterin oxidoreductase domains. Molybdopterins are present as two different subunits, a small subunit with a 4Fe-4S di-cluster domain and a large subunit with putative oxidoreductase activity. The small subunit is composed of a 191 amino acids Fe-4S ferredoxin-type, iron-sulphur binding domain. The large subunit is composed of a molybdopterin dinucleotide-binding 4Fe-4S domain, a molybdopterin oxidoreductase 4Fe-4S domain, and a large molybdopterin oxidoreductase domain of 421 amino acids. The function of the MOSC domain protein was predicted to be related to the binding to pyridoxal phosphate or molybdenum ion. This protein could also be associated with molybdopterins as putative molybdenum ion binding proteins, according to InterProScan [70].

Furthermore, by studying the microsynteny of molybdopterin CDSs, it appears that in these six genomes, a TorD/DmsD-like chaperone protein is also present (Fig. 5). The chaperone protein is predicted to be a DMSO/Nitrate reductase chaperone by InterProScan. This chaperone protein could be related to the assembly of the molybdopterin protein as TorD for the assembly of the trimethylamine oxide reductase [71]. The protein sequences homologies were higher for respective molybdopterin subunits

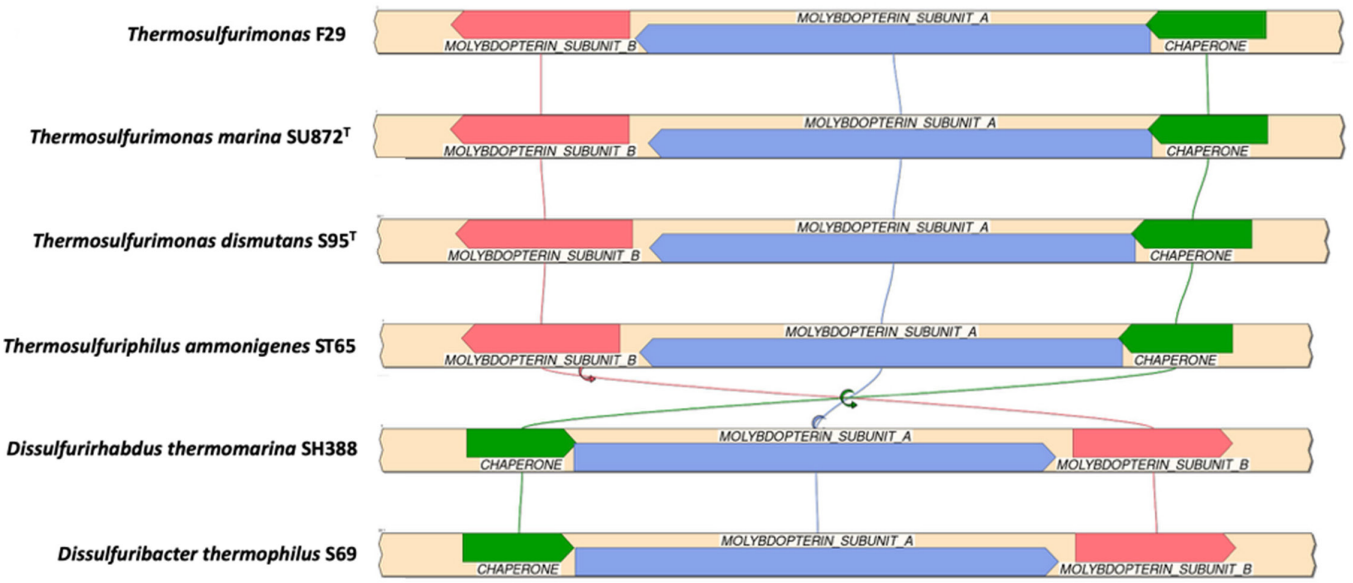


Fig. 5. Microsyntenic ordering of the three putative CDSs associated with sulphur disproportionation (referenced as MOLY cluster in the text), found by comparative genomics, showing gene locus tags, molybdopterin subunits A and B, and the associated chaperone protein, within the genomes of strain F29 (K3767_RS01060 to K3767_RS01070), *T. marina* (FVE67_RS02285 to FVE67_RS02295), *T. dismutans* (TDIS_RS03055 to TDIS_RS03065), *T. ammonigenes* (G4V39_RS07125 to G4V39_RS07135), *D. thermomarina* (HCU62_RS07530 to HCU62_RS07540), and *D. thermophilus* (DBT_RS08130 to DBT_RS08140).

than for chaperone proteins (Table S1.2.). This cluster of three CDSs, namely two molybdopterins and one chaperone protein, was previously identified in the study by [65]. In this previous study, it was observed that this gene cluster was more expressed in the S^0 disproportionation condition than in the DNRA condition. The authors hypothesized that it was related to the oxidation of sulphide to form S^0. By hypothesis, the reaction could occur in the opposite direction and then reduce the S^0 to sulphide (Fig. 3). Putative homologs of molybdopterin subunits were found with Prokka in other known bacteria capable of disproportionating S^0 from other environments, such as *Caldimicrobium thiodismutans*, *Desulfocapsa sulfexigens*, *Desulfofustis glycolicus*, *Desulfurivibrio alkaliphilus* and *Dissulfurispira thermophila,* but with much lower sequence identity (Table S1.3) [65, 72–74]. However, there is a lack of microbial models that could serve as negative controls to support this hypothesis and improve the comparison (i.e. sulphate reducers tested and confirmed to be unable to grow by sulphur disproportionation).

Both molybdopterin subunits from strain F29 were predicted by BUSCA to be non-membrane proteins. The large subunit was predicted to be extracellular (score: 0.87), with a signal peptide at the beginning of the sequence, and the small subunit was estimated to be cytosolic (score: 0.7). This localization seems consistent with the hypotheses of [16, 75] who demonstrated that *T. dismutans* and *D. amilsii*, respectively, can perform S^0 disproportionation without a direct cell-sulphur contact [16, 75]. However, these localization predictions are not confirmed in *T. dismutans*, *D. thermophilus*, and *D. thermomarina* (Table S1.4).

The three putative molybdopterin oxidoreductase subunit homologs were not clearly found in the genome of *Desulfurella amilsii,* a species which has been the subject of functional studies. Two gene clusters could be related to these clusters and were previously postulated to have a sulphur reductase or tetrathionate reductase activity (WP_086034030-WP_086034034) or thiosulphate reductase activity (WP_086032839-WP_086032840) [75]. In previous proteomic analyses carried out with *D. amilsii* [75], none of those proteins associated to these two clusters were more abundant in S^0 disproportionation conditions, but extracellular proteins were not analysed.

Therefore, it is not possible to conclude whether this novel molybdopterin protein, represented by at least one large and one small subunit and a chaperone protein, is clearly involved in the S^0 disproportionation process. It is also not possible to conclude whether this gene cluster can be used as a genomic marker for sulphur disproportionation because of the lack of microbial models carrying this gene cluster and unable to disproportionate S^0. This cluster of three genes including the two subunits of molybdopterin oxidoreductase and the associated chaperone protein is named MOLY, in order to facilitate its citation in future work. In the future, in order to determine the role of proteins encoded by the MOLY cluster and to describe the pathways of sulphur disproportionation, it will be necessary to implement functional approaches. The implementation of comparative proteomics, RNA-seq, enzymology, and eventually genetics approaches should elucidate the MSD pathways that are long-standing enigmas.

In conclusion, the genome analysis of a new species of the genus *Thermosulfurimonas*, strain F29, was performed and showed that this strain is almost equidistant from *T. dismutans* and *T. marina*. This strain has at least one plasmid, which is the first plasmid identified within the class *Thermodesulfobacteria*. This work demonstrates that genome annotation is a very interesting approach to predict the putative catabolism of difficult to grow microorganisms, particularly sulphur-disproportionating bacteria, but does not replace physiological/cultural studies. The new strain F29 grows by sulphur disproportionation and has the genetic potential to grow by sulphite disproportionation, possible nitrate reduction, sulphate reduction and hydrogen oxidation. A comparative genomics study demonstrated that the genes were highly conserved within the genus *Thermosulfurimonas* and associated with a wide range of metabolic pathways. Specific molybdopterin-like proteins, consisting of two subunits and associated with a chaperone protein, were found and may play a role in the sulphur disproportionation reaction, in the bacterial models studied here. However, it is important to emphasize that these results remain hypothetical, predictive and are based only on genomic models of marine hydrothermal vent origin. The S^0 disproportionation pathway remains to be deciphered and could very likely proceed through various pathways.

Funding information
This work was supported by the French-Russian collaborative Project (CNRS/RFBR) Neptune (PRC Russie 2017 n°281295) to M.J., by the Sino-French IRP 1211 MicrobSea to K.A., by the ISblue project, Interdisciplinary graduate school for the blue planet (ANR-17-EURE-0015) and co-funded by a grant from the French government under the program "Investissements d'Avenir" (Theme 2, project DISMUT) to K.A. The study was supported by grants from the French Ministry of Higher Education and Research, and from the Region Bretagne, to M.A and S.Y.

Acknowledgements
We thank the captains and crews of the R/V Pourquoi pas? and the Nautile submersible team for their efficiency, as well as the chief scientists and scientific parties of the MoMARSAT 2019 [19]. We acknowledge especially Françoise Lesongeur for the sampling and conditioning of samples, and Erwan Roussel and Xavier Phillippon for gas and ionic chromatography analyses. The LABGeM (CEA/Genoscope CNRS UMR8030), the France Génomique and French Bioinformatics Institute national infrastructures (funded as part of Investissement d'Avenir program managed by Agence Nationale pour la Recherche, contracts ANR-10-INBS-09 and ANR-11-INBS-0013) are acknowledged for support within the MicroScope annotation platform.

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Conflicts of interest
The author(s) declare that there are no conflicts of interest.

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