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1 **Genomic and morphologic characterization of a planktonic *Thiovulum***
2 **(*Campylobacterota*) dominating the surface waters of the sulfidic Movile Cave, Romania**

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32
33 **ORIGINALITY SIGNIFICANCE STATEMENT**

34 We identify *Thiovulum* sp. as a dominant bacterium in subsurface floating microbial agglom-
35 erations in a hypoxic cave system. We show for the first time that *Thiovulum* can be found in
36 high abundance in planktonic environments dissociated from solid surfaces. We provide the
37 complete genome of this organism and suggest that it is capable of dissimilatory nitrate reduc-
38 tion to ammonia using sulfur as the electron donor, thus contributing to both the cave’s nitrogen
39 and sulfur cycles. Based on sequence similarity, this clade of *Thiovulum* spp. may be common
40 and important in other sulfidic caves. Last, we propose that the short peritrichous flagella-like
41 structures of *Thiovulum* are type IV pili rather than actual flagella.

42

43 ABSTRACT

44 Life in Movile Cave (Romania) relies entirely on primary carbon fixation by bacteria oxidizing
45 sulfide, methane and ammonia with oxygen, nitrate, sulfate, and ferric iron. There, large
46 spherical-ovoid bacteria (12-16 μm diameter), rich in intracellular sulfur globules, dominate the
47 stable microbial community in the surface water of a hypoxic Air Bell. These were identified
48 as *Thiovulum* sp. (*Campylobacterota*). We obtained a closed genome of this *Thiovulum* and
49 compared it to that of *Thiovulum* ES. The genes for oxidizing sulfide to sulfate are absent,
50 therefore, *Thiovulum* likely avoids constant accumulation of elemental sulfur either by
51 oxidizing sulfide to sulfite which is then excreted, or via dissimilatory nitrate reduction to
52 ammonia using the formate-dependent nitrite reductase or hydroxylamine oxidoreductase. Thus,
53 *Thiovulum*, found also in other caves, is likely important to both S and N cycles in subterranean
54 aquatic ecosystems. Additionally, using electron microscopy, we suggest that in absence of
55 motor-like structures along the membrane, the peritrichous flagella-like structures are type IV
56 pili, for which genes were found in both *Thiovulum* genomes. These pili may play a role in veil
57 formation, connecting adjacent cells. The force exerted by coordinated movement of such pili
58 may partly explain the exceptionally fast swimming of these bacteria.

59 Key words: *Thiovulum*, sulfur, DNRA, Movile Cave, sulfide-oxidation, stable isotope analysis

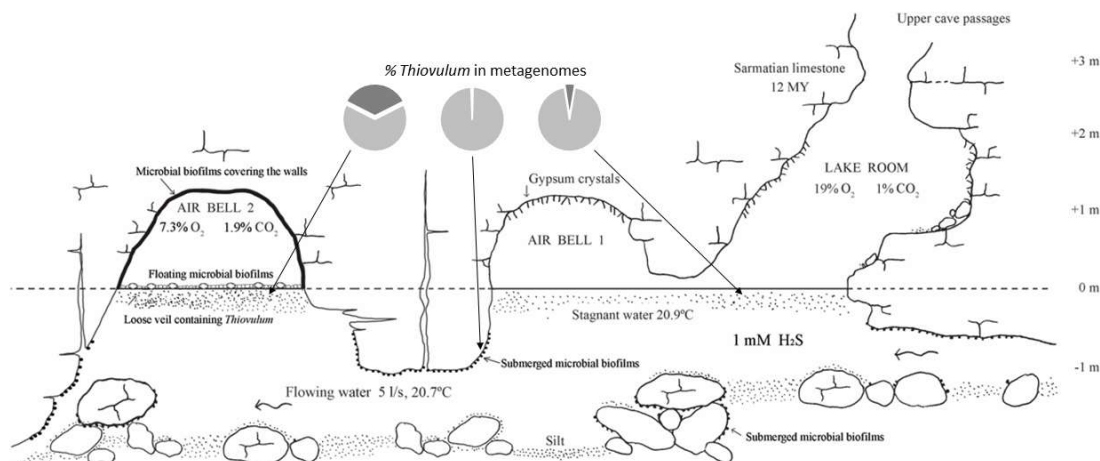
60

61 INTRODUCTION

62 Cave ecosystems, typically characterized by stable conditions, provide a window into
63 subsurface microbiology (Engel, 2015). In the absence of natural light, these ecosystems are
64 typically fueled by chemolithoautotrophy via the oxidation of reduced compounds such as H_2S ,
65 Fe^{2+} , Mn^{2+} , NH_3 , CH_4 , and H^+ . In sulfidic ecosystems, H_2S oxidation may be performed by
66 animal-microbial symbioses such as the *Niphargus-Thiotrix* association (Bauermeister *et al.*,
67 2012; Flot *et al.*, 2014).

68 Discovered at the bottom of an 18-m deep geological survey shaft in 1986, Movile Cave is
69 located near the town of Mangalia, SE Romania (43°49'32"N, 28°33'38"E), 2.2 km from the
70 Black Sea shore. It consists of a 200 m long upper dry passage that ends in a small lake allowing
71 access to a 40 m long, partially submerged lower cave level (Fig. 1). Thick and impermeable
72 layers of clays and loess cover the limestone in which the cave is developed, preventing input
73 of water and nutrients from the surface (Lascu *et al.*, 1994).

74 Sulfidic groundwater flows constantly at the bottom of Movile Cave's lower passages. This
75 flow occurs down to over 1 m below the water surface at flow rates of about 5 l s^{-1} (Sarbu and
76 Lascu, 1997), which equates to a flow velocity of approximately 5 cm s^{-1} in the Lake Room
77 area. Yet, because of the morphology of the lower cave passages (Fig. 1) and slight difference
78 in water temperatures, the water near the surface is practically stagnant. Earlier work by Riess
79 *et al.* (1999) analyzed the change in the concentration of O_2 in the water column and found
80 traces of oxygen up to a maximum depth of 0.8 mm, below which the water was anoxic.



81

82 **Figure 1.** Longitudinal profile of the sampling area in Movile Cave (modified after Sarbu and
83 Popa, 1992). The microbial community containing *Thiovulum* cells (depicted here as dots
84 present beneath the water surface) was sampled in the Lake Room and in air bells 1 and 2.
85 *Thiovulum* 16S rRNA made up 5 %, 0.9 % and 35 % of the 16S rRNA genes retrieved from
86 metagenomic samples (dark gray in pies) from these cave sections, respectively.

87

88 Chemoautotrophic microorganisms living at the water surface oxidize reduced chemical
89 compounds such as H_2S , CH_4 and NH_4^+ from the thermo-mineral groundwater (Sarbu and Kane,
90 1995; Sarbu, 2000). Most of the microbiological studies performed in Movile Cave
91 (summarized in Kumaresan *et al.*, 2014) are based on samples of microbial biofilms floating on
92 the water surface or covering rock surfaces in the cave's air bells (Fig. 1), where the atmosphere
93 is poor in O_2 (7-10 %) and enriched in CO_2 (2.5 %) and methane (1-2 %) (Sarbu, 2000).
94 *Thiobacillus*, *Thiothrix*, *Thioploca*, *Thiomonas* and *Sulfurospirillum* oxidize H_2S using O_2 or
95 NO_3^- as electron acceptors (Rohwerder *et al.*, 2003; Chen *et al.*, 2009; Flot *et al.*, 2014). The
96 methanotrophs *Methylomonas*, *Methylococcus* and *Methylocystis* (Hutchens *et al.*, 2004),
97 *Methanobacterium* (Schirmack *et al.*, 2014) and *Methanosarcina* (Ganzert *et al.*, 2014) are also
98 found in the cave, alongside other methylothrophs such as *Methylotenera*, *Methylophilus* and
99 *Methylovorus* (Rohwerder *et al.*, 2003; Chen *et al.*, 2009). Chen *et al.*, (2009) further identified
100 in the cave ammonia and nitrite oxidizers from the genera *Nitrospira* and *Nitrotoga*.

101 Directly below the water surface in the lower level of Movile Cave, we observed a loose floating
102 veil resembling a slow-moving white cloud (Fig. 2 and Supplementary video 1). When the water
103 was not disturbed, the veil formation was evenly dispersed, yet not deeper than 2-3 mm below
104 the water surface. Occasionally, this veil was disturbed by feeding invertebrates including
105 dendrocoelid flatworms (*Dendrocoelum obstinatum*), cyclopoid copepods (*Eucyclops*
106 *subterraneus scythicus*), and niphargid amphipods (*Niphargus dancaui* and *Niphargus*
107 *racovitzae*) (Brad *et al.*, 2015; Stocchino *et al.*, 2017; Sarbu *et al.*, 2019).

108 Using genetic and microscopic analysis, we concluded that this underwater agglomeration of
109 bacteria is dominated by a species of the genus *Thiovulum*. To the best of our knowledge, this
110 is the first description of planktonic *Thiovulum* swarms/veils at considerable distance from any
111 solid surface. Here we provide further morphological and genomic information on this
112 bacterium, offering new insights into its metabolic properties and raising novel questions.

113

114 MATERIALS AND METHODS

115 Replicate samples of water were collected into sterile containers from the surface of the small
116 sulfidic lake and from the air-bells in the lower section of Movile Cave (Fig. 1). Unpreserved
117 50 ml water samples were immediately brought to the laboratory and inspected by optical
118 microscopy, while other 50 ml water samples preserved with ethanol to a final concentration of
119 50 % and later used for DNA extraction. Additionally, 15-ml water samples were preserved
120 with formaldehyde to a final concentration of 4 % and were later used to estimate the density
121 of bacterial cells under an optical microscope by direct counts on a squared slide, using a
122 defined sample volume (i.e. 5 ml).

123 *Electron microscopy and elemental analysis*

124 Two 15-ml samples collected from the water surface in Movile cave were centrifuged, and the
125 microorganisms were resuspended and fixed for 2 h with 2.7 % glutaraldehyde in phosphate
126 buffered saline (1× PBS). The cells were then rinsed three times in 1× PBS, and finally fixed
127 for 1 h with 2 % osmic acid in 1× PBS. The cells were harvested again by centrifugation,
128 dehydrated in graded acetone-distilled water dilutions, and embedded in epoxy resin. Sections
129 of about 100 nm thickness were produced with a diamond knife (Diatome, Hatfield) using a
130 Leica UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany) and were stained with lead
131 citrate and uranyl acetate (Hayat, 2001). The grids were examined with a Jeol JEM transmission
132 electron microscope. Samples for scanning electron microscopy (SEM) were fixed with 2.7 %
133 glutaraldehyde in 1× PBS, air dried on 0.22 µm mesh-sized Millipore filters, sputter coated with
134 10 nm gold and examined on a JEOL JSM 5510 LV microscope (Jeol, Japan). Energy-dispersive
135 X-ray spectroscopy (EDX) analysis was performed with an EDX analyzer (Oxford Instruments,
136 Abingdon, UK) and with the INCA 300 software.

137 *Stable isotope analysis*

138 Surface water from the Lake Room (Fig. 1) was collected in a sterile 1 l Nalgene bottle and
139 passed through a plankton net to remove any aquatic fauna and particles of floating microbial
140 mats. The water was then filtered through ashed fiberglass filters (that had previously been
141 heated at 1000 °C for an hour to remove carbonates and organic molecules). The filters were
142 then dried at 60 °C for 24 h and sent to the Water Research Center of the University of Alaska
143 in Fairbanks for carbon and nitrogen stable isotope analysis. Additional samples of water from
144 the lake were collected in sterile 1 l Nalgene bottles and the H₂S was precipitated with Cd-
145 acetate and then filtered through ashed fiberglass filters that retained the precipitate. Gypsum
146 crystals were also collected from the cave walls above the water in the Lake Room to determine
147 the stable isotope ratios of sulfur. After being dried, the filters were sent along with samples of
148 gypsum to the Stable Isotope Laboratory of the University of Indiana for sulfur stable isotope
149 analysis.

150 *gDNA Extraction*

151 Bacteria from water samples were concentrated using a vacuum pump and Nalgene's single-
152 use analytical filter funnels (Thermo Fisher Scientific, MA, USA), with the included filter
153 replaced with a 0.2 µm isopore membrane filter (Millipore Sigma, MA, USA). Prior to filtration,
154 the glass assembly components were autoclaved at 121.5 °C for 30 min wet and 20 min dry at
155 1.4 bar (20 psi). Filters were placed into 50 µl conical tubes with 300 µl 1× PBS, 1.5 µl 2 %
156 azide, and a sterile scalpel blade. Samples were minced using an OMNI bead raptor elite (OMNI
157 International, GA, USA) on a 4 m s⁻¹ 30 sec program. The resulting sample was centrifuged to
158 collect bacteria separated from the filters and extracted for gDNA using a modified version of
159 the Omega BioTek Universal Metagenomics kit protocol (OMEGA Bio-tek, GA, USA). Fifteen
160 µl of MetaPolyzyme (Millipore Sigma, MA, USA) was added to each sample and incubated at

161 35 °C for 13 h followed by three cycles of freeze and thaw alternating between 80 °C for 2 min
162 and a -80 °C freezer for 10 min. Further digestion was subsequently performed by adding 35 µl
163 Proteinase K (Omega Bio-tek, GA, USA) and incubated at 55 °C for 1 hour. Following complete
164 enzymatic digestion, the sample was extracted using the manufacturer's protocol (Omega
165 BioTek Universal metagenomics kit). Briefly, 500 µl ML1 buffer (CTAB) was added the
166 digested sample and incubated at 55 °C for 15 min. One volume of Tris-stabilized (pH >7.5)
167 phenol-chloroform-isoamyl alcohol mix (25:24:1) was used for purification and the resulting
168 upper aqueous phase was removed and combined with RBB (Guanidinium) and 100 % EtOH
169 and applied to a DNA silica column supplied with the kit. Final DNA was eluted in 35 µl of
170 elution buffer. DNA was quantified using a Qubit spectrofluorometer and Nanodrop ND-1000
171 (ThermoFisher Waltham MA USA).

172 *16S rRNA gene amplicon sequencing and processing*

173 PCR reactions were performed in triplicate. Each 25 µl reaction consisted of 0.5 µl of Phusion
174 Green Hot Start II high-fidelity DNA polymerase (Thermo Fisher Scientific, Sweden), 5.0 µl
175 of 5× Phusion Green HF buffer, 4 µl DNase- and RNase-free water, 5.0 µl of 10 µM primer mix
176 (1:1), 0.5 µl of 10 mM nucleotide mix and 10 µl of DNA extract (0.5 ng/µl). The PCR protocol
177 was 98 °C for 30 sec, 33 cycles of 98 °C for 10 sec, 55 °C for 30 sec, 72 °C for 30 sec and a
178 final 5-min extension at 72 °C. The amplicon target was the V3-V4 region of the 16S rRNA
179 gene, using the V3 forward primer S-D-Bact-0341-b-S-17, 5'-CCTACGGGNGGCWGCAG-3
180 (Herlemann *et al.*, 2011), and the V4 reverse primer S-D-Bact-0785-a-A-21, 5'-
181 GACTACHVGGGTATCTAATCC-3 (Muyzer *et al.*, 1993), resulting in fragments of ~430 bp.
182 The primers were dual barcoded in a way compatible with Illumina sequencing platforms (as
183 described in Caporaso *et al.*, (2011). Product size and successful amplification was tested by
184 running internal positive and negative controls from the triplicate plates on a 1.5 % (w/v)
185 agarose gel. Triplicate PCR products were combined, and each triplicate sample was purified
186 using SPRI beads (Agencourt® AMPure® XP, Beckman Coulter, CA, USA). DNA concentration
187 of purified samples was determined with a Quant-iT high-sensitivity DNA assay kit and a
188 Qubit® fluorometer (Invitrogen, Carlsbad, USA). All samples were diluted to similar
189 concentrations prior to pooling diluted PCR products together in equimolar volumes (50 µl) in
190 one composite sample (including positive and negative controls).

191 Composite samples were paired-end sequenced at the Vrije Universiteit Amsterdam Medical
192 Center (Amsterdam, The Netherlands) on an Illumina MiSeq Sequencer with a 600-cycle
193 MiSeq Reagent Kit v3 (Illumina, San Diego, Ca, USA) according to the manufacturer
194 instructions. Sequences were quality trimmed using Trimmomatic (v 0.39) and paired using
195 Bbmerge (Bushnell *et al.*, 2017). The paired sequences were dereplicated using the dedupe tool
196 of the BBTools package (sourceforge.net/projects/bbmap/) aligned and annotated using the
197 SINA aligner (Pruesse *et al.*, 2012) against the SILVA SSU database (v 138) (Quast *et al.*, 2012).

198 A maximum-likelihood tree was calculated using FastTree 2 (Price *et al.*, 2010). For the sake
199 of legibility, the 908 *Thiovulum* sequence variants obtained were clustered at 97 % similarity
200 using CD-HIT-EST (Huang *et al.*, 2010), resulting in 50 clusters. Three 16S rRNA sequences
201 obtained from the genome assembly (see below) were included in the tree as well. All
202 *Thiovulum* sequences available in the SILVA database (71 in total) were clustered separately in
203 a similar manner, resulting in 22 clusters used in the final tree. Non-*Thiovulum* sequences
204 belonging to the *Sulfurimonadaceae* family were used as an outgroup.

205 To obtain information on relative *Thiovulum* abundance, the raw short-read libraries (amplicon
206 and metagenomic) were analyzed with phyloFlash (V 3.3; (Gruber-Vodicka *et al.*, 2020)).

207 Shotgun sequencing (Illumina and Oxford Nanopore)

208 Shotgun sequencing was accomplished using both Illumina and Oxford Nanopore sequencing
209 technologies. For Illumina sequencing, 1 ng of genomic DNA from each sample was converted
210 to whole-genome sequencing libraries using the Nextera XT sequencing reagents according to
211 the manufacturer's instructions (Illumina, San Deigo CA). Final libraries were checked for
212 library insert size using the Agilent Bioanalyser 2100 (Agilent Technologies Santa Clara, CA)
213 and quantified using Qubit spectrofluorometry. The final sample was sequenced using paired
214 end 2x150 sequencing on an Illumina MiniSeq system.

215 A first pass of Oxford Nanopore sequences was obtained using the SQK LSK109 ligation
216 library synthesis reagents on a Rev 9.4 nanopore flow cell with the GridION X5 MK1
217 sequencing platform, resulting in a total of 131.8 Mbp of reads with a N50 of 1.3 kbp.

218 Additionally, sequencing was performed on several cellular aggregates that were confirmed
219 microscopically to contain *Thiovulum* cells. For this, the aggregates were picked from
220 environmental samples fixed in RNAlater (4 M (NH₄)₂SO₄; 15 mM EDTA (from 0.5 M, pH 8.0

221 stock); 18.75 mM Na-citrate (from 1M stock)), and washed several times in sterile 1× PBS
222 buffer (137 mM NaCl; 2.7 mM KCl; 10 mM Na₂HPO₄; 1.8 mM KH₂PO₄; pH 7.4). The cell
223 aggregates were lysed by freeze thawing and further, following the manufacturer's instructions,
224 as part of the DNA amplification process using the Repli-G single cell amplification kit
225 (Qiagene, Hilden, Germany). Libraries for Nanopore sequencing were prepared using the LSK-
226 108 kit following the manufacturer's protocol but skipping the size selection step. The prepared
227 libraries were loaded on MIN106 R9 flow cells, generating a total of 5.7 Gbp of reads with a
228 length N50 of about 3.7 kbp. Basecalling for all Oxford Nanopore reads were done using Guppy
229 4.0.11.

230 All sequencing data generated in this study were deposited in NCBI Sequence Read Archive
231 under accession number PRJNA673084.

232 Metagenomic data analysis

233 Nanopore reads were assembled using Flye 2.8.1-b1676 (Kolmogorov *et al.*, 2019) with default
234 parameters. The resulting GFA was examined using Bandage (Wick *et al.*, 2015), allowing to
235 delineate a set of high-coverage (>300X) contigs against a background of low-coverage
236 (<100X) contigs. To verify that these high-coverage contigs corresponded to *Thiovulum*, the
237 published proteome of *Thiovulum* ES (Marshall *et al.*, 2012). was aligned on the GFA using
238 tblastn (Gertz *et al.*, 2006) within Bandage (parameters: minimum identity 70 %, minimum
239 coverage 70 %), revealing that nearly all tblastn hits were concentrated on the high-coverage
240 contigs and vice-versa. The GFA was therefore pruned to retain only the high-coverage contigs,
241 which were all interconnected. The remaining 31 contigs were exported as FASTA then
242 scaffolded using SLR (Luo *et al.*, 2019); the nine resulting scaffolds were mapped back to the
243 GFA to resolve most repeats, and the remaining repeats were resolved manually until obtaining
244 a circular genome. A final polishing step was performed with unicycler-polish from Unicycler
245 v0.4.9b (Wick *et al.*, 2017) using the complete set of Illumina reads (for a total depth of
246 coverage of 12X of the genome) and the subset of Nanopore reads longer than 5 kb (*ca.* 50X).
247 Polishing consisted of two cycles of pilon 1.23 (Walker *et al.*, 2014), one cycle of racon 0.5.0
248 (Vaser *et al.*, 2017) followed by FreeBase (Garrison and Marth, 2012), then 30 additional cycles
249 of short-read polishing using pilon 1.23, after which the assembly reached its best ALE score
250 (Clark *et al.*, 2013).

251 The completeness of the *Thiovulum* genome obtained was assessed using CheckM (Parks *et*
252 *al.*, 2015) and its continuity using the unicycler-check module in Unicycler v0.4.9b. Annotation

253 was performed using Prokka (Seemann, 2014), KEGG (Kanehisa *et al.*, 2016), the EggNOG
254 5.0 online tool (Huerta-Cepas *et al.*, 2019), PATRIC (Brettin *et al.*, 2015; Davis *et al.*, 2020)
255 and RAST (Aziz *et al.*, 2008; Overbeek *et al.*, 2014) annotation servers. A COG (Tatusov *et al.*,
256 2000) analysis was done using the ANVIO tool (Eren *et al.*, 2015). To further elucidate the
257 function of genes annotated as hypothetical proteins, a structural annotation was done using the
258 Superfamily and SCOP databases using the Superfam online interface (Gough *et al.*, 2001).

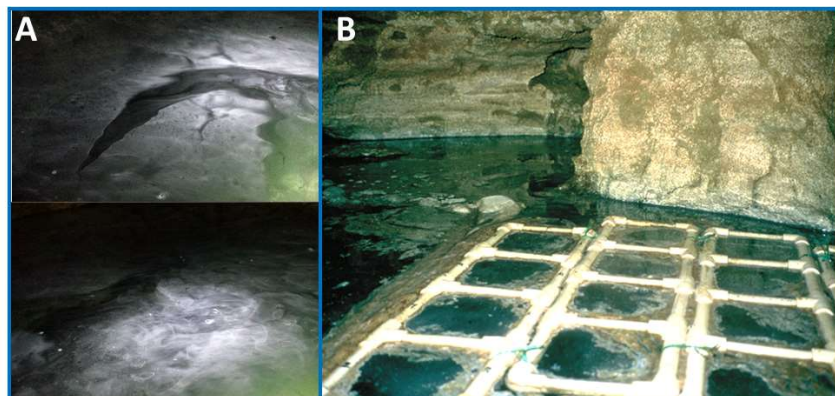
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260 RESULTS

261 Field observations

262 A pale-white loose veil, with a vertical thickness of 2 to 3 mm, was observed below and adjacent
263 to the water surface in Movile Cave (Fig. 2). This resembled microbial veils described earlier
264 as mucilaginous structures often produced by sulfur-oxidizing bacteria (Garcia-Pichel, 1989;
265 Fenchel, 1994) under specific O₂ and H₂S conditions. Nevertheless, in Movile Cave, the dense
266 agglomeration of cells does not form slime or strongly cohesive veils, but a loose cloud-like
267 white veil close to the water surface.

268



269

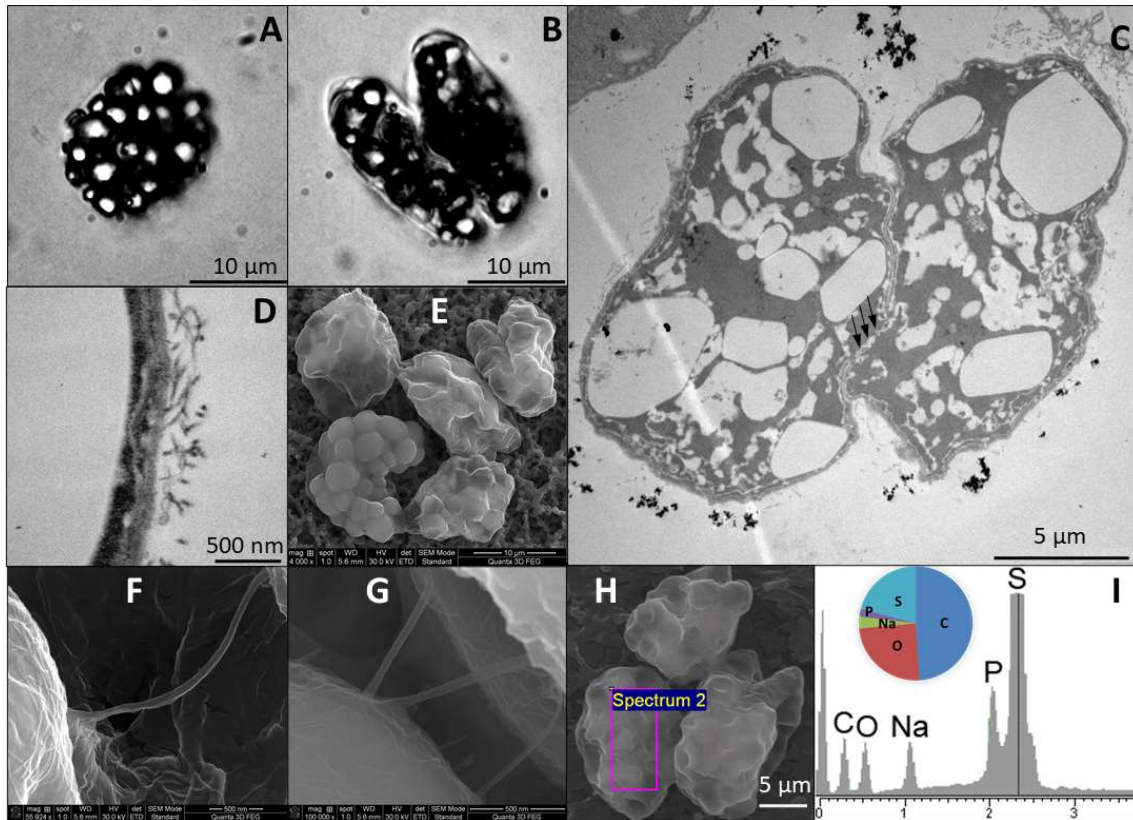
270 **Figure 2.** Images of subsurface cloud (veil) from the Lake Room (A) and Air Bell 2 (B) in
271 Movile Cave. See also Supplementary Video 1.

272 Microscopy

273 Veils similar to those seen in the Lake Room (Fig. 2A) were also observed in air bell 1 and even
274 more so in air bell 2 (Fig 2B) where they reached the highest densities. In air bell 2 these veils
275 consist of large, spherical to ovoid, bacterial cells (Fig. 3A-B) identified as belonging to the
276 genus *Thiovulum*. These cells have a diameter of 12-16 μm and occur in densities of
277 approximately 5.5×10^3 cells/ml, resembling a dense bacterial culture (Fig. 2, Supplementary
278 Video 1). Each cell contains up to 20-30 sulfur globules of various shapes and sizes that are
279 responsible for the white appearance of the veil (Fig. 3). Transmission Electron Microscope
280 (TEM) observations showed that these large cells are Gram-negative (Fig. 3C-D), like all
281 *Campylobacterota* (formerly *Epsilonproteobacteria*; *Campylobacterales*), and confirmed the
282 existence of irregularly shaped sulfur inclusions within the cells. Light microscopy suggested
283 *Thiovulum* cells divide along the long cell axis (Fig. 3B). This was confirmed by the TEM
284 images showing two dividing cells sharing the same cytoplasmic spaces prior to the complete
285 closing of the cell membrane (Fig. 3C). Short peritrichous flagella fragments (Fig. 3D) observed
286 on the surface of the cells resemble those noticed earlier in *Thiovulum* species (Wirsen and
287 Jannasch, 1978). Scanning electron microscopy (SEM) depicts the ball-like structure of the

288 sulfur inclusions in a series of connected *Thiovulum* cells (Fig. 3E). These cells are connected
289 one to the other via multiples threads (Fig. 3F-G). Energy-dispersive X-ray (EDX) analysis (Fig.
290 3H-I) revealed that the intracellular globules are dominated by sulfur (20.9 - 26.1 %), along
291 with elements common in organic matter such as carbon (49 - 49.2 %) and oxygen (21.1 -
292 24.6 %), and a few other elements in low abundance such as sodium (2.4 - 3.4 %) and
293 phosphorus (1.2 - 2.2 %).

294



295

296 **Figure 3.** Optical images of giant globular cells colonizing the subsurface cloud/veil from
297 Movile Cave (A-B) including a cell undergoing division (B). Each cell carries up to 30 sulfur
298 inclusions (large bright spots in panels A, B). TEM images of *Thiovulum* show the cellular
299 localization of sulfur inclusions of various shapes and sizes (panel C, white spots). Ovoid cells
300 divide along their long axis (B, C). The region where the cell membrane is not fully closed
301 between dividing cells is marked by with three black arrows (C). The cell wall (D) is covered
302 in pili or short flagella. *Thiovulum* cells (E) are often connected one to another through thread-
303 like structures (F-G). EDX analysis on *Thiovulum* cells (H) inspected under SEM show the
304 typical elemental composition of the cells (I) and confirm the high sulfur content of the internal
305 globules. Note that the height of the peaks in the EDX spectra do not correlate with the
306 element's ratio, but with the X-ray signal intensity.

307

308 Laboratory observations

309 Water samples were collected from the cave and brought to the lab within one hour of sampling
310 for optical microscopy observations. Upon exposure to the microscope light, the cells became
311 very active and moved fast in all directions, a behavior similar to that observed in *Thiovulum*
312 *majus* (Fenchel, 1994). After a few hours of swimming (Supplementary Video 2), the cells

313 slowed down and gathered in certain areas on the glass slide, while some cells turned around
314 and swam in the reverse direction.

315 Within 24 h after being removed from the cave, the cells became inactive and most of them
316 lysed, releasing their sulfur globules. Attempts to obtain the *Thiovulum* cells in culture were
317 unsuccessful; however, we were able to devise a laboratory setup (Fig. S1) that resulted in a
318 *Thiovulum*-enriched culture by mimicking as much as possible the cave conditions (i.e. constant
319 temperature, darkness, high relative humidity, and sulfidic water from the cave).

320

321 Stable isotope analysis

322 Both the carbon and the nitrogen stable isotope ratios of the *Thiovulum* cells collected from the
323 water surface in Movile Cave (Table 1) ($\delta^{13}\text{C} = -44.53 \text{ ‰}$ and $\delta^{15}\text{N} = -10.29 \text{ ‰}$) were among
324 the lightest values measured in Movile Cave (Sarbu *et al.*, 1996).

325

326 Table 1. Stable isotope ratios of carbon and nitrogen of microbial samples collected in Movile
327 Cave. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ are expressed as ‰.

	$\delta^{13}\text{C}$ values (‰)	$\delta^{15}\text{N}$ values (‰)
<i>Thiovulum</i> microorganisms	-44.53 ‰	-10.29 ‰
Biofilms floating in the cave's air-bells	-44.54 to -45.82 ‰	- 10.09 to -10.84 ‰
Submerged microbial biofilms	-43.23 ‰	-9.98 ‰

328

329 Table 2. Stable isotope ratios of sulfur from HS^- from the cave water, S^0 from sulfur granules
330 within *Thiovulum* microorganisms swimming in the cave lake, and SO_4^{2-} from the gypsum
331 crystals covering the cave walls above the lake. $\delta^{34}\text{S}$ are expressed as ‰.

	$\delta^{34}\text{S}$ values (‰)
HS^- from the cave water	+5.5 and +8.3 ‰
S^0 from sulfur granules within <i>Thiovulum</i> microorganisms	+4.2 ‰
SO_4^{2-} from the gypsum crystals covering the cave walls above the lake.	+1.1 to + 7.3 ‰

332

333 The sulfur stable isotope ratios of the H_2S from the groundwater in Movile Cave ranged
334 between $\delta^{34}\text{S} = +5.5$ and $+8.3 \text{ ‰}$, and were close to the values measured for the oxidized sulfur
335 in the gypsum crystals covering the cave walls above the lake: $\delta^{34}\text{S} = +1.1$ to $+ 7.3 \text{ ‰}$ (Table
336 2). The sulfur granules from within *Thiovulum* in the cave lake had a $\delta^{34}\text{S}$ value of $+5.5 \text{ ‰}$,
337 which is similar to the $\delta^{34}\text{S}$ measured for the sulfide in the water and the sulfate (gypsum) in
338 the cave walls. No significant isotopic fractionation appears therefore to take place during the
339 oxidation of HS^- to S^0 .

340

341 *Phylogenetic identification*

342 Following quality control and trimming of the 16S rRNA amplicon library, *ca.* 4700 read pairs
343 could be merged into 400 bp long sequences. Of these, 35 % were annotated as *Thiovulum*. The
344 latter, together with other *Thiovulum* sequences, formed a cluster distinct from the
345 phylogenetically close genus *Sulfurimonas* (Fig. 4A). All Movile Cave *Thiovulum* sequences
346 form one cluster separated into three main subclusters, one of which harbor also the three
347 genomic 16S rRNA sequences. Only one of the three clusters harbors previously known
348 sequences of *Thiovulum*, some of which (DQ295692, FJ165502, KM410698, EF467575) were
349 obtained from sulfidic caves. The 16S rRNA gene of the *Thiovulum* ES (accession number
350 AH003133.2) was removed from the analysis as the sequence contained long stretches of Ns.
351 However, when used, this sequence does not fall within the Movile Cave clusters.

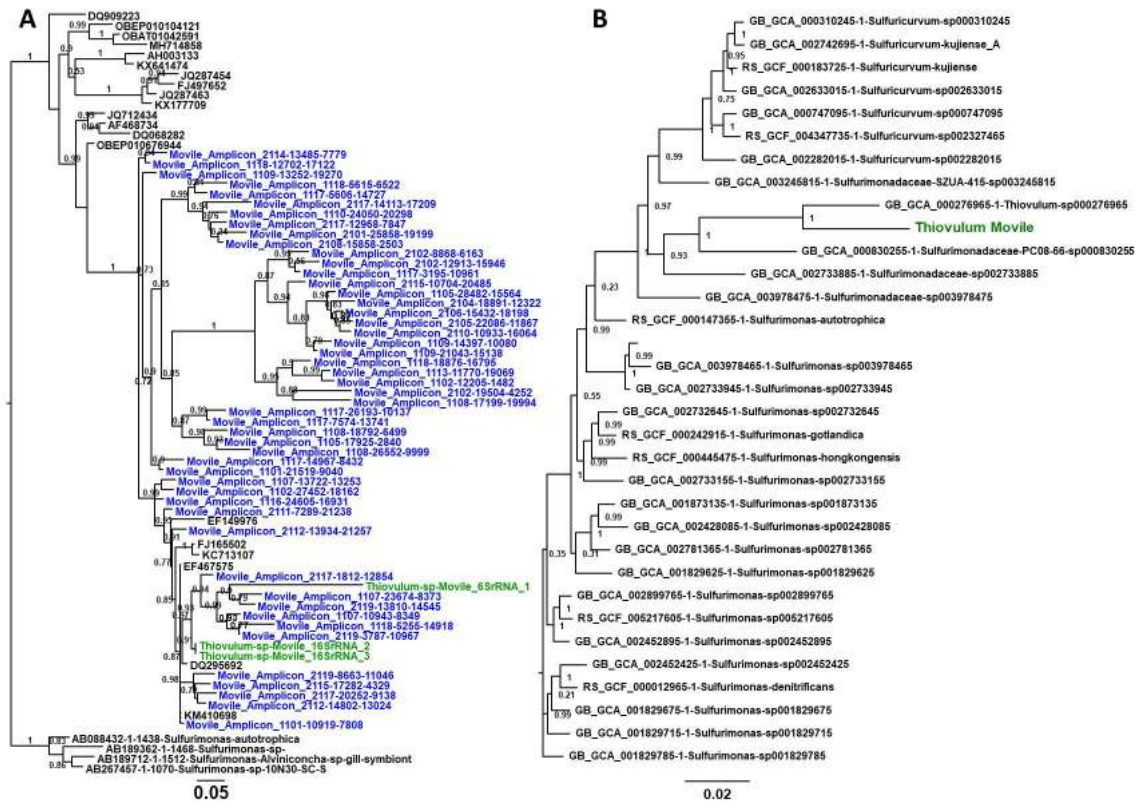
352 The circular, closed contig obtained from the metagenomic assembly of samples from Movile
353 Cave was taxonomically classified with the GTDB-Tk software against the GTDB-Tk database
354 (Chaumeil *et al.*, 2019). The genome was confirmed as *Thiovulum* sp. by having 76.34 % amino
355 acid identity to the single other available *Thiovulum* genome obtained from Elkhorn Slough
356 (ES), California (*Thiovulum* ES) (Marshall *et al.*, 2012). Accordingly, a phylogenetic tree
357 constructed from a multilocus alignment of single-copy marker genes from, *Thiovulum* ES
358 (Marshall *et al.*, 2012), the *Thiovulum* genome from Movile Cave and all available
359 *Sulfurimonas* genomes resulted as well in the two *Thiovulum* genomes clustering together (Fig.
360 4B).

361 To assess the abundance of *Thiovulum* in the different environments of the cave, phyloFlash
362 (Gruber-Vodicka *et al.*, 2020) was used to identify and annotate 16S rRNA reads from the raw
363 metagenomic data. *Thiovulum* was found in highest abundance (sequence frequency) in Air Bell
364 2 (35 %), followed by the Lake Room (5 %) and submerged microbial mats (0.9 %) (see pie
365 charts in Fig. 1).

366 *Genome analysis*

367 The assembly of metagenomic data from Movile Cave resulted in a closed circular genomic
368 sequence classified as *Thiovulum* sp. The genome length is 1.72 Mbp with a GC content of
369 28.4 %. Genome completeness was estimated using CheckM (Parks *et al.*, 2015) at 93 %. In
370 absence of sufficient reference genomes for this genus, this value likely represents the full set
371 of marker genes for *Thiovulum*. CheckM estimated a contamination of 0 % and a strain
372 heterogeneity of 0: therefore, the *Thiovulum* genome assembly does not seem to contain any
373 sequences from additional distant or closely related organisms. According to the annotation on
374 the PATRIC server (Brettin *et al.*, 2015; Davis *et al.*, 2020), this genome contains 1849 coding
375 sequences, of which 974 encode proteins of known function, 875 are hypothetical proteins, 35
376 are tRNAs and 4 are CRISPR array comprising a total of 87 repeats. Subsystem hierarchical
377 annotation shows that among the genes attributed to a Subsystem the largest subsystems were
378 cofactors and vitamins, protein metabolism, and amino acids and derivatives (Fig. 5A). The
379 same annotation conducted de-novo on the *Thiovulum* ES genomes suggests that the Movile
380 and ES strains share a core of 493 Subsystem functions and have 36 and 48 additional unique
381 Subsystem functions, respectively (Fig. 5B and Supplementary Data 1). Despite this large
382 functional similarity between the two genomes, the two genomes turned out to be highly
383 divergent according to their level of sequence similarity with clusters of orthologous genes
384 (COGs; Fig. 5B and Supplementary Data 1).

385



386

387 **Figure 4.** Phylogenetic placement of the 16S rRNA gene of *Thiovulum* from Movile Cave (A)
388 and its genome (B). The Shimodaira-Hasegawa local support values (ranging from 0 to 1)
389 are shown next to each node.

390

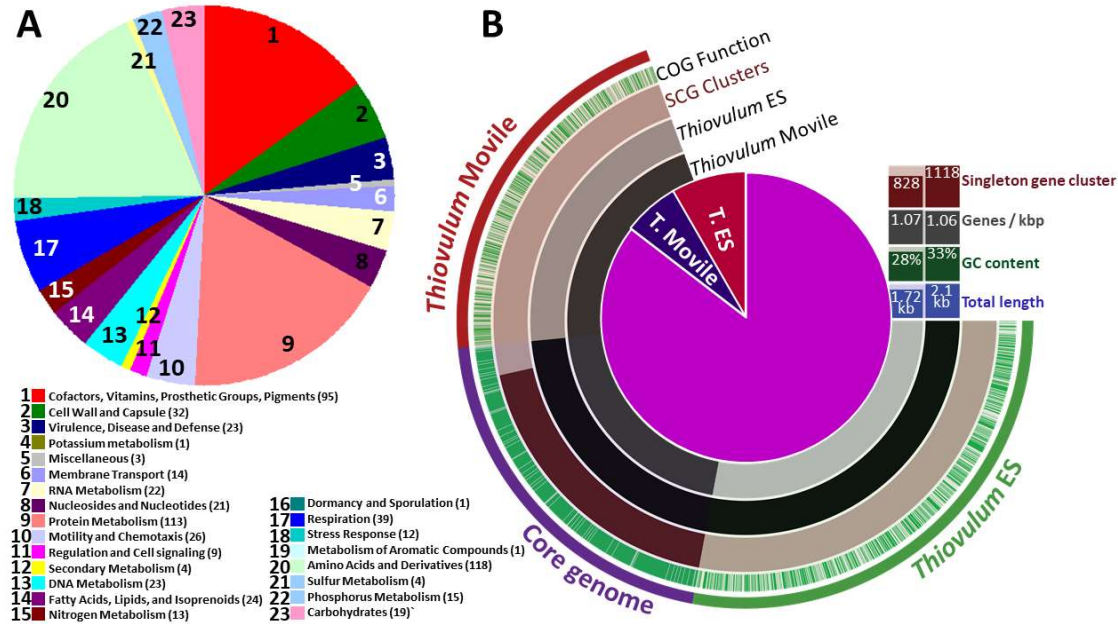
391 Carbon fixation likely takes place via the reductive TCA cycle, similarly to *Thiovulum* ES. For
392 both strains not all necessary genes for the rTCA cycle could be recognized by any single
393 annotation method including EggNOG 5.0 (Huerta-Cepas *et al.*, 2019), KEGG (Kanehisa *et al.*,
394 2016), COG (Tatusov *et al.*, 2000) or Subsystems.

395 Both the KEGG and Subsystem annotations revealed only few genes involved in sulfur
396 metabolism, with sulfide:quinone oxidoreductase being responsible for the formation of sulfur
397 globules. Additionally, we identified in both *Thiovulum* strains the tetrathionate reductase
398 subunit A and thiosulfate/3-mercaptopyruvate sulfurtransferase genes potentially involved in
399 the formation of sulfite from tetrathionate via thiosulfate. COG annotation identified in both
400 Movile and ES strains the presence of the bacterial heterodisulfide reductase (*dsrC*) (Venceslau
401 *et al.*, 2014) alongside rhodanase sulfurtransferases, involved in the conversion of sulfide to
402 sulfite. Subsequently, the presence of the *tauE* sulfite exporter gene was identified as well in
403 both strains.

404 In addition to the membrane-bound nitrate reductases (Nar) found in *Thiovulum* ES, the Movile-
405 Cave *Thiovulum* possesses also periplasmatic nitrate reductases encoded by the *nap* genes.
406 Some of the genes involved in the synthesis of the formate-dependent nitrite reductase were
407 identified in *Thiovulum* ES. These are confirmed in the current genome and are supplemented
408 by precursor genes to the *nrfA* encoded cytochrome c-552 which reduced nitrite to ammonia.
409 Additionally, the gene for hydroxylamine dehydrogenase, which is often encountered in
410 genomes from *Campylobacterota* (Haase *et al.*, 2017), formerly referred to as
411 *Epsilonproteobacteria* (Waite *et al.*, 2019), was also identified.

412 As *Thiovulum* sp. is a highly motile bacterium, we inspected motility and chemotaxis genes.
 413 All genes necessary for flagellar assembly were found, similarly to *Thiovulum* ES. The
 414 chemotaxis genes *cheA cheY, cheW, cheV* and *cheD* were identified as well alongside the *cetA*
 415 and *cetB* energy taxis genes, the parallel to the *Escherichia coli* aerotaxis (*aer*) gene. The *cheX*
 416 gene, which was not found in the genome of *Thiovulum* ES, was identified in the Movile Cave
 417 *Thiovulum*. Gene *cheB* was reported missing in *Thiovulum* ES, was identified in the Movile
 418 strain but also in *Thiovulum* ES upon COG reannotation. Additionally, 11 methyl-accepting
 419 chemotaxis proteins were identified.

420 In addition to flagella genes, the *pilA, pilE, pilT, pilN, pulO, fimV* genes responsible for the
 421 formation and retraction of type IV pili.



422

423 **Figure 5.** Distribution of genes of the Movile Cave *Thiovulum* associated with different
 424 Subsystems (A) and functional comparison to the *Thiovulum* ES genome using Subsystems and
 425 COG functional assignments (B) marking in both cases the core genome and the unique COGS
 426 and Subsystems.

427

428 DISCUSSION

429 In Movile Cave, the oxidation of reduced compounds such as H_2S , CH_4 , and NH_4^+ is the only
 430 primary energy source. There, *Thiovulum* sp., a large sulfur oxidizer, often found in close
 431 proximity to sediments, microbial mats or surfaces, is part of the chemoautotrophic microbial
 432 community involved in *in situ* carbon fixation that represents the food base for the cave's
 433 abundant and diverse invertebrate community. These *Thiovulum* cells, exceeding 15 μm in
 434 diameter, are larger than most known sulfur-oxidizers: 5-10 μm (Fenchel, 1994) and 5 μm (Thar
 435 and Fenchel, 2005) and, together with the larger *Thiomargarita namibiensis* (Schulz *et al.*,
 436 1999) and *Achromatium oxaliferum* (Babenzien, 1991), belong to the group of giant sulfur
 437 bacteria (Ionescu and Bizic, 2019). Here we investigated the morphological and genomic
 438 aspects of what appears to be a fully planktonic *Thiovulum* sp. The new genome was compared
 439 to the sole other existing genome of *Thiovulum*, strain ES. The latter, originating from a
 440 phototrophic marine mat, was reannotated for the purpose of this comparison to account for
 441 new available information, 8 years after its original publication.

442 Sulfide oxidation

443 *Thiovulum* (*Campylobacterota*; *Campylobacteria*; *Campylobacterales*; *Sulfurimonadaceae*)
444 has been recognized to have several interesting features, including being among the fastest
445 bacterial swimmers and its ability to form large veils consisting of interconnected cells. Many
446 sulfur-oxidizing microorganisms including species of *Thiovulum* form veils by means of what
447 appear to be mucous threads. These threads are used (Thar and Fenchel, 2001) by the cells to
448 attach to solid surfaces (Fauré-Fremiet and Rouiller, 1958; De Boer *et al.*, 1961; Wirsén and
449 Jannasch, 1978; Fenchel, 1994; Robertson *et al.*, 2006). In marine settings, such veils keep cells
450 above sediments (Karavaiko *et al.*, 2006) at the oxic-anoxic interface where the optimal
451 concentration of O₂ and H₂S can be found (Pruesse *et al.*, 2012).

452 In Movile Cave, the dense agglomeration of cells does not form slime or strongly cohesive veils,
453 but a loose cloud-like white veil close to the water surface. Nevertheless, SEM analyses shows
454 that the cells are at least partially interconnected. It has been hypothesized that the coordinated
455 movement of *Thiovulum* cells generates currents directing H₂S or O₂ to the cells (Petroff and
456 Libchaber, 2014). The fully planktonic localization of the cells in air bell 2 means that
457 *Thiovulum* here cannot use surfaces to localize itself at the oxic anoxic interphase. Therefore,
458 as sulfide concentration is high (245 μM) (Flot *et al.*, 2014), the cells likely need to coordinate
459 their swimming to stay close to the surface where the O₂ necessary to their metabolism is present.

460 *Thiovulum* is a sulfide oxidizer as evidenced by its generation and accumulation of sulfur
461 inclusions. Inclusions such as amorphous sulfur, polysulfide granules or orthorhombic sulfur
462 inclusions have been described from other environments (Jørgensen and Revsbech, 1983;
463 Robertson *et al.*, 2006). The amount and type of sulfur inclusions in cells is influenced by the
464 concentrations of H₂S and O₂ in the environment. Typically, cells store elemental sulfur when
465 H₂S is abundant in the environment, and later use the intracellular reserves of sulfur when the
466 sulfide source in the environment is depleted (De Boer *et al.*, 1961). Sulfur inclusions were also
467 shown to form when the supply of O₂ is limited and as a result the sulfur cannot be entirely
468 oxidized to soluble sulfite, thiosulfate, or sulfate. Complete depletion of sulfur inclusions from
469 cells is not likely in Movile Cave where abundant H₂S is available continuously and where O₂
470 is scarce in most habitats (Sarbu *et al.*, 1996). Furthermore, while genomic analysis of the
471 Movile Cave and ES strains identified the SQR gene involved in this process, the genes required
472 for further oxidizing the elemental sulfur to sulfate are not present.

473 Marshall *et al.*, (2012) proposed the *Thiovulum* needs to undergo frequent (daily) oxic/anoxic
474 cycles to prevent continuous accumulation of elemental sulfur in the cell. We advance two
475 additional options by which the Movile Cave *Thiovulum* and likely the entire genus may evade
476 sulfur globule intoxication. First, the identification of a the C subunit of dissimilatory sulfite
477 reductase (*dsrC*) (Venceslau *et al.*, 2014) and rhodanese genes, known to be involved in S⁰
478 conversion to sulfite (Poser *et al.*, 2014), and a sulfite exporter (*tauE*), suggest *Thiovulum* may
479 be able oxidize elemental sulfur to sulfite and transport the latter out of the cell. Second, we
480 propose that *Thiovulum* is capable of dissimilatory nitrate reduction to ammonia (DNRA) using
481 elemental sulfur (Slobodkina *et al.*, 2017), a process already shown in *Campylobacterota* (e.g.
482 *Sulfurospirillum deleyianum*) (Eisenmann *et al.*, 1995). The Movile Cave *Thiovulum* contains
483 not only the *nar* (*narGH*) genes for nitrate reduction but also the periplasmic *nap* genes known
484 for their higher affinity and ability to function in low nitrate concentrations (Pandey *et al.*, 2020).
485 Additionally, we were able to identify the membrane component of the formate-dependent
486 nitrite reductase, (*nrfD*) gene in both Movile and ES strains, as well as the formate-dependent
487 periplasmic cytochrome C nitrite reductase, (subunit C-552) and the ferredoxin subunit of
488 nitrite reductase in the Movile Cave strain. Last, the gene for hydroxylamine dehydrogenase
489 (*hao*) was also identified in the Movile Cave *Thiovulum*. Hydroxylamine dehydrogenase is

490 known from other *Campylobacterota* (e.g. *Campylobacter fetus* or *Nautilia profundicola*) and
491 was shown to mediate the respiratory reduction of nitrite to ammonia. In line with the findings
492 of Marshall *et al.*, (2012), the *hao* gene was not found in the genome of *Thiovulum* ES upon re-
493 annotation, suggesting that the *hao* gene may not be part of the core *Thiovulum* genome.
494 Interestingly, normally *Campylobacterota* that utilize hydroxylamine dehydrogenase do not
495 have the formate-dependent nitrite reductase. Similarly, *Campylobacterota*, typically use the
496 periplasmic nitrate reductase (Nap) and do not have the membrane bound NarGHI system (Kern
497 and Simon, 2009; Meyer and Huber, 2014). Interestingly, *Thiovulum* ES has only the Nar
498 systems while the Movile Cave strain has both types. Nevertheless, while genomic information
499 is suggestive of the presence or absence of specific enzymes and pathways, experiments or gene
500 expression data are required to determine which of the genes are utilized and under which
501 environmental conditions.

502

503 Carbon fixation

504 Marshall *et al.*, (2012) found all the genes necessary for the reverse TCA cycle to be present in
505 *Thiovulum* ES, as is the case for the Movile Cave strain. However, while the isotopic
506 fractionation of $^{34}\text{S}/^{32}\text{S}$ between the sulfide in the water and the sulfur granules of the cells
507 matches the literature (*ca.* 2 ± 2 ‰, (Fry *et al.*, 1988), that of the ^{13}C is lighter than expected
508 from an organism utilizing the rTCA cycle (van der Meer *et al.*, 2001). Such depletion in ^{13}C is
509 rather typical of organisms utilizing the Calvin-Benson-Bassham (CBB) cycle (Pearson, 2010).
510 Recently it was discovered that some *Campylobacterota* lost the genes needed for the TCA in
511 favor of a gradual acquisition of those required for the CBB cycle (Assié *et al.*, 2020). Most of
512 the genes involved in the CBB cycle are common in heterotrophs as well being part of
513 alternative reactions. However, two enzymes are unique to the CBB cycle: phosphoribulokinase
514 and ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Hügler and Sievert, 2011).
515 Thus, given that $\delta^{13}\text{C}$ of cave dissolved inorganic carbon has been documented as low as -25 ‰
516 (Knierim *et al.*, 2015), and some bacteria using rTCA have a fractionation of -15 to -35 ‰,
517 relative to the dissolved inorganic carbon (Preuß *et al.*, 1989), it is likely that *Thiovulum* has a
518 stronger preference towards the lighter carbon isotopes than other rTCA utilizers.

519

520 Cell motility and veil formation

521 The loose veil formation from Movile Cave appears to move slowly in various directions, most
522 probably driven by slow movement of the individual cells toward zones of optimal chemical
523 concentrations of H_2S and O_2 . Similarly, Thar and Fenchel (2001 and 2005) showed that sulfur
524 oxidizers move in nutrient and oxygen gradients and the dynamics and aggregation of cells is
525 due to chemotactic properties toward a particular concentration of oxygen. Accordingly, genes
526 involved in chemotaxis are abundant in the *Thiovulum* genome. Unlike the ES strain the Movile
527 strain contains the CheX phosphatase rather than CheC, highlighting the high variability and
528 diversity among bacterial chemotaxis systems (Wuichet and Zhulin, 2010; Bardy *et al.*, 2017).
529 Methyl-accepting chemotaxis proteins (MCPs) are key elements in the bacterial chemotactic
530 response to environmental signal (Salah Ud-Din and Roujeinikova, 2017). Motile bacteria carry
531 between 3-30 (average 13) MCPs with *Campylobacterota* having *ca.* 10 (Lacal *et al.*, 2010).
532 We identified 11 MCPs in the Movile Cave *Thiovulum* two of which matched known sequences
533 while the other 9 were identified using secondary structure, adding to the already large known
534 variability of these proteins.

535 *Thiovulum* sp. often forms large veils of interconnected cells. The threads connecting the cells
536 are thought to be secreted by the antapical organelle, located at the posterior side of the cell (De

537 Boer *et al.*, 1961; Robertson *et al.*, 2015). Short peritrichous flagella-like fragments (Fig. 3D)
538 observed on the surface of the cells resemble those noticed earlier in *Thiovulum* species (Wirsen
539 and Jannasch, 1978). All genes necessary for type IV pili and flagella assembly were found in
540 the Movile Cave and ES *Thiovulum* sp. (Marshall *et al.*, 2012). Accordingly, together with
541 evaluating available electron microscopy we suggest that these ideas need be revisited.

542 First, our own SEM images (as well as previous ones of connected *Thiovulum* cells) show
543 connecting threads that are not exclusively polar and are much thinner than the stalk like
544 structure shown by de Boer *et al.*, (1961). We propose that these structures are rather type IV
545 pili, which are known, among other functions, to connect cells to surfaces or other cells (Craig
546 *et al.*, 2019). Alternatively, Bhattacharya *et al.*, (2019) have shown the formation of cell-
547 connecting nanotubes constructed using the same enzymatic machinery used for flagella
548 assembly. However, it is not possible to determine this in absence of TEM images of connected
549 cells showing the presence of the reduced flagellar base Bhattacharya *et al.*, (2019).

550 Second, we question the flagellar nature of the peritrichous structures around the cells.
551 Inspecting the high-resolution electron microscopy images taken by Faure-Fremie and Rouiller
552 (1958), there is no single structure visible resembling a flagellar motor. Additionally, the length
553 of these structures based on de Boer *et al.*, (1961) and a similar image in Robertson *et al.*, (2015)
554 suggest that these <3 μm structures are too short for typical flagella (> 10 μm in length (e.g.
555 (Renault *et al.*, 2017)) and are closer to the 1-2 μm lengths known type for pili. Interestingly,
556 *Caulobacter crescentus* swims at speeds of up to 100 $\mu\text{m s}^{-1}$ using a single flagellum aided by
557 multiple pili (Gao *et al.*, 2014). In light of this hypothesis, we inspected the images of the
558 fibrillar organelle at the antapical pole of the cells. The high-resolution images presented in
559 Fauré-Fremiet and Rouiller (1958) and in de Boer (1961) show an area of densely packed
560 fibrillar structures. Considering our current knowledge in flagellar motor size (*ca.* 20 nm) it is
561 highly unlikely that each of these fibers is an individual flagellum, thus, potentially representing
562 a new flagellar organization. Petroff *et al.*, (2015) investigated the physics behind the 2-
563 dimensional plane assembly of *Thiovulum* veils and suggested it to be a direct result from the
564 rotational movement which attracts cells to each other. Nevertheless, as seen, SEM images show
565 cells are physically attached one to the other suggesting several mechanisms and steps may be
566 involved. Interestingly, type IV pili retraction can generate forces up to 150 pN which are
567 known to be involved in twitching motility in bacteria (Craig *et al.*, 2019). If coordinated, these
568 may be part of the answer to the unexplained swimming velocity of *Thiovulum* (Garcia-Pichel,
569 1989), which is, at *ca.* 615 $\mu\text{m s}^{-1}$, 5 to 10 times higher than that of other flagellated bacteria.
570 Thus, genomic information and re-evaluation of electron microscopy data raise new questions
571 concerning the nature of the extracellular structures on the surface of *Thiovulum* sp. and call for
572 new targeted investigations into this topic.

573

574 CONCLUSIONS

575 Movile Cave is a system entirely depending on chemosynthesis. We showed that submerged
576 near-surface, planktonic microbial accumulations are dominated by *Thiovulum*, a giant
577 bacterium typically associated with photosynthetic microbial mats. The genome of this
578 planktonic *Thiovulum* strain suggests that it can perform dissimilatory reduction of nitrate to
579 ammonium. As this may be a main mechanism in preventing continuous accumulation of sulfur
580 globules in the cells, *Thiovulum* likely plays a major role in the nitrogen cycle of the cave,
581 providing readily available ammonia to the surrounding microorganisms. Kumaresan *et al.*,
582 (2014), based as well on genetic evidence, suggest nitrogen fixation and deamination of
583 methylamines as the source of ammonium in the cave. The coupling of DNRA to sulfide
584 oxidation provides a direct and more productive source of ammonium. Our results show that

585 the Movile Cave strain is similar to other cave *Thiovulum* sp., suggesting that in those
586 subterranean ecosystems *Thiovulum* may as well play a role in both N and S cycles.

587 This investigation of the genome, coupled with observations of current and previous
588 microscopy images, question the number of flagella the cells has, bringing forth the possibility
589 that the cell may make use of type IV pili for its rapid movement and cell-to-cell interactions.

590 The collective behavior of *Thiovulum* is still a puzzle and there may be more than one
591 mechanism keeping the cells connected in clusters or in veils. Our SEM images suggest the
592 cells are connected by thread-like structures. Petroff *et al.*, (2015) show, on another strain, there
593 is no physical connection between the cells. They suggest that the swimming behavior of
594 individual cells keeps the cells together. More research is therefore needed to understand if
595 these different mechanisms are driven by strain variability, or by different environmental
596 conditions.

597

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617

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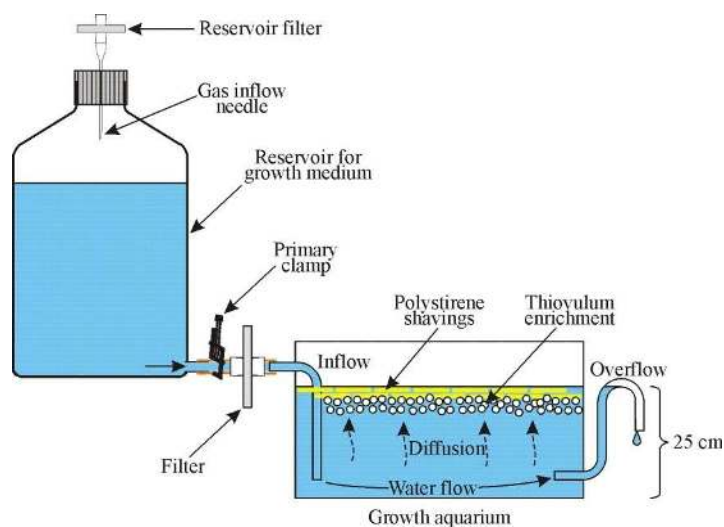
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860 Supplementary material

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864 Fig. S1. Laboratory setup that produced an enrichment culture with high abundance of
865 *Thiovulum* cells from Movile Cave. The water source contained a mineral growth solution and
866 0.25 mM H₂S at pH 7. A floating layer approximately 5-10 mm thick of polystyrene (each 1-3
867 mm thin and 2-4 cm long) shavings was added to the water surface to maintain an anchoring
868 substrate for the cells near the water surface and also to limit the diffusion of O₂ in the water.
869 The setup was installed in a ventilated hood in the lab at 20 °C and the culture was established
870 within two weeks. This setup produced abundant *Thiovulum* growth, but because of its design,
871 it could not be sterilized and therefore did not allow producing a pure *Thiovulum* culture.