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**Marinitoga aeolica sp. nov., a novel thermophilic anaerobic heterotroph isolated from a shallow hydrothermal field of Panarea Island in the Aeolian archipelago, Italy**

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1 ***Marinitoga aeolica* sp. nov., a novel thermophilic anaerobic heterotroph isolated from a**  
2 **shallow hydrothermal field of Panarea Island in the Aeolian archipelago, Italy.**

3

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15

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19

20 The GenBank accession numbers for the whole genome sequence and the 16S rRNA gene  
21 sequence of strain BP5-C20A<sup>T</sup> are respectively CP069362 and MZ571208.

22

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24 Panarea Italy

25

26

## 27 Abstract

28 A novel thermophilic strain, designated BP5-C20A<sup>T</sup>, was isolated from the shallow  
29 hydrothermal field of the Panarea island, in the Aeolian archipelago close to Sicily, Italy. Cells  
30 were motile rods surrounded with a ‘toga’, Gram-stain-negative, and displaying a straight to  
31 curved morphology during the exponential phase. Strain BP5-C20A<sup>T</sup> is thermophilic (optimum  
32 55°C), moderately acidophilic (optimum pH 5.6), and halotolerant (optimum 25 g L<sup>-1</sup> NaCl). It  
33 can use yeast extract, peptone, and tryptone, and uses carbohydrates: cellobiose, fructose,  
34 glucose, maltose, starch, sucrose, and xylan. Elemental sulfur is used as an electron acceptor  
35 and reduced to hydrogen sulfide. The predominant cellular fatty acid is C<sub>16:0</sub>. Phylogenetic  
36 analysis showed that strain BP5-C20A<sup>T</sup> shared 97.3% 16S rRNA gene sequence identity with  
37 the closest related species *Marinitoga lauensis* LG1<sup>T</sup>. The complete genome of strain BP5-  
38 C20A<sup>T</sup> is 2.44 Mb in size with a G+C content of 27.3%. The dDDH and ANI values between  
39 the genomes of strains BP5-C20A<sup>T</sup> and *M. lauensis* LG1<sup>T</sup> are 31.0% and 85.70% respectively.  
40 Finally, from its physiological, metabolic, and genomic characteristics, strain BP5-C20A<sup>T</sup>  
41 (=DSM 112332<sup>T</sup> = JCM 39183<sup>T</sup>) is proposed as representative of a novel species of the genus  
42 *Marinitoga* named *Marinitoga aeolica* sp. nov. and belonging to the order *Petrotogales*, in the  
43 phylum *Thermotogota*.

44

## 45 Introduction

46 Members of the phylum *Thermotogota* are anaerobic rods, with a characteristic outer sheath-  
47 like structure called ‘toga’ [1-3]. They mainly grow by fermentation of various organic  
48 compounds. Most of them are also able to grow by respiration notably by reduction of sulfur  
49 compounds. Within the phylum *Thermotogota*, the order *Petrotogales* is composed to date of a  
50 single family, the *Petrotogaceae*, including six genera: *Defluviitoga*, *Geotoga*, *Marinitoga*,  
51 *Oceanotoga*, *Petrotoga*, and *Tepiditoga* [4, 5]. The genus *Marinitoga* contains to date seven  
52 described species, here with their type strain: *Marinitoga artica* 2PyrY55-1<sup>T</sup>, *M. camini*

53 MV1075<sup>T</sup>, *M. hydrogenitolerans* AT1271<sup>T</sup>, *M. lausensis* LG1<sup>T</sup>, *M. litoralis* MC3<sup>T</sup>, *M.*  
54 *okinawensis* TFS10-5<sup>T</sup> and *M. piezophila* KA3<sup>T</sup> [6-12]. Of the seven *Marinitoga* species  
55 isolated to date, most originated from deep-sea hydrothermal ecosystems except *M. litoralis*  
56 which was isolated from a coastal thermal spring. *Thermotogota* species have already been first  
57 isolated from the shallow hydrothermal system of the Aeolian archipelago, in particular,  
58 *Thermotoga maritima* isolated from Vulcano and known as the most thermophilic bacteria  
59 described to date [13]. Here we report the description of a novel thermophilic anaerobic  
60 *Marinitoga* strain isolated from a shallow hydrothermal vent of the Panarea island in the  
61 Aeolian archipelago in the Mediterranean Sea. It is proposed as a representative of a novel  
62 *Marinitoga* species.

63

#### 64 Sampling site and chemical analyses

65 The Aeolian Islands archipelago is a volcanic arc generated by the subduction of the Ionian  
66 oceanic lithosphere under the Calabrian arc (Fig. S1). At Panarea island, located off the Sicilian  
67 coast, Italy, an underwater hydrothermal field in the caldera at approximately 20 mbsl (meters  
68 below sea level) was sampled in May 2017 [14, 15]. Hydrothermal sediments (coarse sand)  
69 bathed with hot acidic fluid were collected with a core sampler close to an iron-copper sulfide  
70 hydrothermal chimney at the Black Point site noted BP5 (38°38'23"N 15°06'28"E). The  
71 environmental parameters of the sampling station are described below. Less than 2 hours after  
72 sampling, back to the lab, these sediments and their pore fluid samples were transferred into  
73 100 mL Schott bottles to fill them to avoid air and preserve anaerobic conditions. They were  
74 stored at 4°C until use for cultivation assays (Fig. S1e).

75 Physico-chemical characterization of the fluid was performed as detailed elsewhere by Price *et*  
76 *al.* (2015) [14]. Briefly, temperatures were measured *in situ* with a custom-built probe in an  
77 underwater housing (Max Planck Institute for Marine Microbiology, Bremen, Germany). A  
78 WTW pH meter 3210 and a Mic-D electrode with temperature compensation were used to

79 measure the pH of each sample onshore. H<sub>2</sub>S concentration was measured after the addition of  
80 1 mL of a 50 mM zinc acetate solution to 3 mL of sample, by analyzing decreased light intensity  
81 caused by the ZnS precipitate with a Merck photometer at a wavelength of 670 nm. Samples  
82 for anion (Br, Cl, SO<sub>4</sub>) or cation (Mg, Na, Ca, K, B, Sr, Si, Ba, Mn, and Fe) were filtered in the  
83 field at 0.2 μm and then frozen until measurement. Samples for cations analyses were acidified  
84 using ultrapure double distilled HNO<sub>3</sub> to 1% by volume. Measurements were performed by  
85 Dionex IC system ion chromatography (anion) and inductively coupled plasma-optical  
86 emission spectrometry (cations, Perkin-Elmer Optima 3300 ICP-OES). The temperature  
87 measured in the sediment at the BP5 site varied from 50°C at the surface to around 70°C at 20  
88 cm depth. The pH of the hydrothermal pore fluid of the BP5 sample was 5.0 to 5.5. The  
89 composition in H<sub>2</sub>S, major anions and major cations were as follows: H<sub>2</sub>S 233.1 μM; Cl<sup>-</sup> 574  
90 mM; Br<sup>-</sup> 0,86 mM; SO<sub>4</sub><sup>2-</sup> 30.3 mM; Na<sup>+</sup> 442 mM; Al<sup>3+</sup> 1.1 μM; Ca<sup>2+</sup> 11.6 mM; K<sup>+</sup> 9.8 mM;  
91 Mg<sup>2+</sup> 53.5 mM; Mn(tot) 30 μM; Si<sup>4+</sup> 0.67 mM; Sr<sup>2+</sup> 0.1 mM. Ba and Fe concentrations were  
92 below detection limits. These concentrations were close to those of seawater, except for the  
93 high H<sub>2</sub>S content.

94

#### 95 Enrichment, isolation, and cultivation

96 The Panarea basal medium (PM) was composed of (per liter): 25 g NaCl, 6 g MgCl<sub>2</sub>·6H<sub>2</sub>O, 5  
97 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0,7 g KCl, 85 mg NH<sub>4</sub>Cl, 50 mg K<sub>2</sub>HPO<sub>4</sub>, 50 mg KH<sub>2</sub>PO<sub>4</sub>, 1 g CaCl<sub>2</sub>·2H<sub>2</sub>O,  
98 28 mg FeSO<sub>4</sub>·7H<sub>2</sub>O, 2 mg NaNO<sub>3</sub>, 88 mg NaBr, 84 mg NaHCO<sub>3</sub>, 200 mg MnCl<sub>2</sub>·4H<sub>2</sub>O, 1 mL  
99 Widdel traces elements solution [16], 1 mL Fe-Ni-Wo-Se solution (1.42 g FeSO<sub>4</sub>, 1.6 g NiSO<sub>4</sub>·6  
100 H<sub>2</sub>O, 38 mg Na<sub>2</sub>WO<sub>4</sub>·2 H<sub>2</sub>O, and 3 mg Na<sub>2</sub>SeO<sub>3</sub>·5 H<sub>2</sub>O per liter of distilled water), and 0.2 mL  
101 of 4% resazurin solution. For some enrichment cultures, 1 g of yeast extract and 2 g of tryptone  
102 were added as substrates to the PM medium, then noted PMYT. The pH was adjusted to 5.5  
103 with a 10 M KOH solution. The medium was boiled under a stream of O<sub>2</sub>-free N<sub>2</sub> gas, cooled  
104 to room temperature, and dispensed in 50 mL serum bottles or Hungate tubes under a N<sub>2</sub> gas

105 stream before sterilization by autoclaving. The Hungate technique for anaerobic cultivation was  
106 used throughout this study [17]. About 1 g of sediment slurry sample BP5 was used to inoculate  
107 20 mL of PM medium in 50 mL serum bottles, then incubated at 60°C. After 2 days of  
108 incubation, significant cell growth was observed in the culture BP5-C20 on PMYT medium.  
109 Microbial diversity of the culture analysis by 16S rRNA gene metabarcoding (see [18] for  
110 detailed procedure) showed that the genus *Marinitoga* was largely dominant, encompassing  
111 95.93% of the OTUs detected in the culture, followed by the scarce genera (> 0.1%)  
112 *Thermococcus* (0.29%), *Sulfurimonas* (0.20%), *Clostridium* (0.19%), *Thiopfundum* (0.17%),  
113 *Halospirulina* (0.16%) and *Kosmotoga* (0.11%). Some of these genera are known to contain  
114 (hyper)thermophilic and anaerobic species able to use sulfur compounds in their energetic  
115 metabolism. *Corynebacterium* (0.12%) and *Pseudomonas* (0.11%) were also detected and are  
116 known to be potential contaminants.

117 The enrichment culture was subcultured several times under the same growth conditions before  
118 the isolation of colonies on roll tubes containing PMYT with 1.6% (w/v) agar.

119 Several colonies obtained after 6-day incubation at 60°C were creamy beige, round, and convex  
120 with smooth edges and a diameter of 0.5–1 mm. Some colonies were picked up and inoculated  
121 new series of roll tubes. The procedure was repeated three times before the culture was deemed  
122 pure. These colonies served as inoculum to obtain axenic culture. Among them, strain BP5-  
123 C20A<sup>T</sup> was selected for further characterization.

124

## 125 Phylogenetic analyses

126 For 16S rRNA gene analyses, genomic DNA isolation from a BP5-C20A<sup>T</sup> culture (50 mL) and  
127 amplification by PCR (using 27F/1492r primer set; [19]) were performed as described  
128 previously [20]. Sanger sequencing of the PCR product was performed at GATC Biotech AG  
129 (Germany). The 16S rRNA gene sequence was then compared with available sequences in the  
130 Genbank database using the BLASTN search [21]. All evolutionary analyses were performed

131 using MEGAX [22]. A multiple alignment was built using the MUSCLE program [23].  
132 Sequence positions with alignment uncertainty and gaps were omitted from the analysis.  
133 Phylogenetic trees were reconstructed using three methods: Neighbor-Joining, Minimum  
134 Evolution, and Maximum-Likelihood methods [24-26]. The evolutionary distances were  
135 computed using the Kimura 2-parameter method [27]. The analysis involved 30 nucleotide  
136 sequences having a total of 1599 positions in the final dataset. Branch robustness was estimated  
137 by the non-parametric bootstrap procedure [28] (2000 replicates). The 16S rRNA gene  
138 sequences of strain BP5-C20A<sup>T</sup> was deposited in Genbank under accession number MZ571208.  
139 Strain BP5-C20A<sup>T</sup> shared 97.5% 16S rRNA gene sequence similarity with the closest related  
140 species *M. lauensis* LG1<sup>T</sup>, 96.6% with *M. okinawensis* TFS10-5<sup>T</sup> and 96.3% with *M. piezophila*  
141 KA3<sup>T</sup> as determined by EzBioCloud [29] (Fig. 1). It belongs to Class *Thermotogae*, Order  
142 *Petrotogales*, Family *Petrotogaceae*, in the Phylum *Thermotogota* according to LPSN  
143 (<https://www.bacterio.net/>) [30].

144

#### 145 Genome features

146 For whole-genome analyses, genomic DNA extraction of strain BP5-C20A<sup>T</sup> and sequencing at  
147 the GeT-PlaGe platform, (INRAE Toulouse, France) by combining long reads (Oxford  
148 Nanopore) and short-reads (Illumina MiSeq) technologies were performed as described  
149 previously [18]. Read demultiplexing and adapter trimming were performed using qcat v1.1.0  
150 (<https://github.com/nanoporetech/qcat>) and genome assembly with Unicycler v0.4.6 [31]. A  
151 complete genome sequence was obtained, deposited at DDBJ/ENA/GenBank, and annotated  
152 by using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). For further comparative  
153 analyses, the complete genome sequence was also annotated using MaGe (Microscope Genome  
154 Annotation) [32]. A whole proteome-based taxonomic analysis of strain BP5-C20A<sup>T</sup> genome  
155 was performed using the bioinformatics platform Type Strain Genome Server (TYGS)  
156 (<https://tygs.dsmz.de>) [33]. In brief, the TYGS analysis included a determination of the closest

157 type strain genomes by (i) comparison against all type strain genomes available in the TYGS  
158 database *via* the MASH algorithm [34], and (ii) the closely related type strains determined *via*  
159 the 16S rRNA gene sequences. For the phylogenomic inference, all pairwise comparisons  
160 among the set of genomes were conducted using Genome BLAST Distance Phylogeny  
161 (GBDP), and accurate intergenomic distances were inferred under the algorithm 'trimming' and  
162 distance formula d5. The resulting intergenomic distances were used to infer a balanced  
163 minimum evolution tree with branch support *via* FASTME 2.1.6.1 [35]. Branch support was  
164 inferred from 100 replicates each. The trees were rooted at the midpoint [36]. Values of average  
165 nucleotide identity (ANI) were calculated by using OrthoANI with USEARCH  
166 (<http://www.ezbiocloud.net/tools/ani>). Digital DNA-DNA hybridizations (dDDH) were  
167 calculated using the Genome-to-Genome Distance Calculator (GGDC) 2.1  
168 (<http://ggdc.dsmz.de/>) [37]. The percentage of conserved proteins (POCP) was calculated using  
169 the Protologger software (<http://www.protologger.de/>) [38].

170 The complete genome sequence of strain BP5-C20A<sup>T</sup> is a unique circular chromosome of 2.44  
171 Mb long with a 27.3% G+C content, which is consistent with the genomes of other *Marinitoga*  
172 strains (size ranging from 2.23 Mb to 2.59 Mb and G+C content ranging from 27.2% to 29.2%)  
173 (Table S1). The dDDH values between the genomes of strain BP5-C20A<sup>T</sup> and other available  
174 *Marinitoga* genomes were found to be  $\leq 31.0\%$  (highest value with *M. lauensis* LG1<sup>T</sup>) and the  
175 ANI values were  $\leq 85.70\%$ , i.e. far below the species boundaries of 70% and 95~96%  
176 respectively, recommended by Chun et al. [39] (Table 1). Strain BP5-C20A<sup>T</sup> shared >80%  
177 POCP with other *Marinitoga* species, from 80.3% with *M. piezophila* to 83.8% with *M.*  
178 *hydrogenitolerans*.

179 Strain BP5-C20A<sup>T</sup> genome comprises a total of 2311 genes among which 2243 protein-coding  
180 genes (CDS) (Table S1). There are 51 tRNA genes with 3 rRNA operons. The genome contains  
181 4 CRISPR loci of CRISPR-associated *cas* genes. Strain BP5-C20A<sup>T</sup> genome has a complete  
182 pentose phosphate pathway and possesses a complete Embden-Meyerhoff-Parnas pathway to



183 convert glucose into pyruvate. It has an impaired TCA cycle because it lacks genes coding for  
184 the fumarate reductase, fumarate hydratase, malate dehydrogenase, and aconite hydratase.  
185 Strain BP5-C20A<sup>T</sup> genome possesses genes encoding flagella-related proteins, carbohydrates  
186 and amino acids transport and metabolism (92 ABC transporters), peptidases and proteases.  
187 The distribution of genes into COG functional categories is shown in Table S2. The genome of  
188 strain BP5-C20A<sup>T</sup> also contains genes encoding hydrogenases, a Rnf complex and ATP  
189 synthases that explain hydrogen and energy production. The reducing power is transferred to a  
190 ferredoxin electron carrier (MARBP5\_v1\_0066) that may fuel a hydrogenase to produce H<sub>2</sub>.  
191 Genes of a hydrogenase are found as an operon *hndA*, *hndB*, *hndC* and *hndD*  
192 (MARBP5\_v1\_2217-2220). Genes *rnfA*, *rnfB*, *rnfC*, *rnfD*, *rnfE*, *rnfG* were also found  
193 organized as an operon (MARBP5\_v1\_0453 to MARBP5\_v1\_0458). Rnf is a membrane-bound  
194 complex that couples electron transfer with translocation of ions (Na<sup>+</sup>) across the membrane.  
195 We hypothesize that this complex is associated to a F-type ATP synthase to build a simple two-  
196 component respiratory chain, as described in *Thermotoga maritima* (48). Indeed, the genome  
197 encodes both a F-type ATP synthase generating ATP via proton translocation, encoded by *atpA*,  
198 *atpB*, *atpC*, *atpD*, *atpE*, *atpF*, *atpG* and *atpH* (MARBP5\_v1\_1427 to 1434) (49), and a V-type  
199 ATP synthase (MARBP5\_v1\_1639- 1641 and MARBP5\_v1\_1643-1645-1646) known to use  
200 ATP to translocate proton across the membrane.

201 In a whole proteome-based analysis (Fig. S2), strain BP5-C20A<sup>T</sup> grouped with other  
202 *Marinitoga*, and clustered with other thermophilic members of the order *Petrotogales*.

203

204 Fatty acid analyses

205 The determination of cellular fatty acid composition was performed at the DSMZ identification  
206 service (Braunschweig, Germany). Cells were harvested in the late exponential growth phase.

207 After extraction using a modified version of the method of Miller [40, 41], fatty acids were

208 separated by gas chromatography and detected using the Microbial Identification System  
209 (MIDI, Sherlock Version 6.1) [42].

210 The cells of strain BP5-C20A<sup>T</sup> contained C<sub>16:0</sub> as major fatty acid (58.8% from total), similar  
211 to *M. okinawensis* [11]. Numerous minor fatty acids were also identified: C<sub>16:1 $\omega$ 9c</sub> (8.3%), C<sub>14:0</sub>  
212 (6.1%), C<sub>16:1 $\omega$ 7c</sub> (5.8%), C<sub>18:1 $\omega$ 9c</sub> (4.5%), C<sub>16:0 iso</sub> (4.4%), C<sub>12:0</sub> (3.2%), C<sub>18:0</sub> (2.7%), C<sub>18:1 $\omega$ 7c</sub>  
213 (1.8%), C<sub>10:0</sub> (1.7%), C<sub>17:0 anteiso</sub> (1.6%) and C<sub>15:0 anteiso</sub> (1.1%).

214

#### 215 Microscopic analyses

216 Cells were routinely observed under a Nikon ECLIPSE E600 phase-contrast microscope for  
217 culture monitoring. The cell structure of strain BP5-C20A<sup>T</sup> was analyzed using an FEI Tecnai  
218 G2 transmission electron microscope (TEM) operating at 200 kV. For observation of the cell  
219 wall, cells were first fixed 20 min with 10% (v/v) glutaraldehyde, stained overnight with 2%  
220 (w/v) osmium tetra oxide, and then embedded in EMBED-812 resin. Embedded specimens were  
221 sliced into ultrathin sections (90 nm in thickness), placed on a Cu TEM grid, and stained for 3  
222 min with 2% (w/v) uranyl acetate and 1 min with 1% (w/v) lead before observation.

223 Cells of strain BP5-C20A<sup>T</sup> are rod-shaped, surrounded with a 'toga' which is the sheath-like  
224 outer structure characteristic of the members of the phylum *Thermotogota* (Fig. 2). They are  
225 motile and Gram-stain -negative. They have a mean length of 1.5–3.4  $\mu$ m and a width of 0.5–  
226 0.7  $\mu$ m in the exponential growth phase and appear singly or in short chains inside the toga.  
227 Cells became elongated during the stationary growth phase. When cultured under unfavorable  
228 conditions, cells swell and exhibit a "balloon" shape before cell lysis occurs. No spore formation  
229 was observed, as is the case for each strain of the genus *Marinitoga*.

230

#### 231 Physiological tests

232 The pH, temperature, and NaCl concentration ranges for growth were determined in Hungate  
233 tubes using Vulcano High Salinity (VHS) medium composed of (per liter of distilled water): 18

234 g NaCl, 1 g MgCl<sub>2</sub>·6 H<sub>2</sub>O, 1 g MgSO<sub>4</sub>·7 H<sub>2</sub>O, 0.3 g KCl, 0.3 g Na<sub>2</sub>SO<sub>4</sub>, 0.3 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 mL  
235 K<sub>2</sub>HPO<sub>4</sub> 5% solution, 1 mL KH<sub>2</sub>PO<sub>4</sub> 5% solution, 1 mL CaCl<sub>2</sub>·2 H<sub>2</sub>O 2% solution, 0.1 g Fe  
236 (III)-citrate, 0.1 g L-cysteine HCl, 0.1 g Yeast extract, 0.08 g Na acetate, 1.95 g MES, 1 mL  
237 Widdel's elements, 1 mL Fe-Ni-Wo-Se solution, and 0.2 mL of resazurine 4% solution. The  
238 VHS medium was routinely supplemented with 1 g yeast extract and 2 g tryptone as substrates,  
239 except for testing autotrophic capacities. pH was adjusted to 5.5 with a 10 M KOH solution and  
240 temperature for growth was 55°C except for determining optimal pH and temperature for  
241 growth.

242 Growth was determined by directly measuring the optical density at 600 nm in Hungate tubes  
243 using a spectrophotometer equipped with a tube holder (Cary 50, Varian). To determine pH  
244 range and optimum growth conditions, the VHS medium pH was adjusted from 3.7 to 8.7 with  
245 anaerobic sterile stock solutions of 0.1M HCl or 0.1M NaOH and was verified after inoculation.

246 Temperature tests were performed using water baths from 20 °C to 80 °C with an increment of  
247 5°C. For NaCl requirement, NaCl was weighted directly in the tubes prior to adding the medium  
248 (0 to 70 g L<sup>-1</sup> with an increment of 5 g L<sup>-1</sup> from 0 to 40 g L<sup>-1</sup>, then 10 g L<sup>-1</sup> from 40 to 70 g L<sup>-1</sup>).

249 All cultures were performed in duplicate and reproduced twice. Growth rates were determined  
250 for subcultures under the same experimental conditions.

251 Substrate utilization experiments were performed for strain BP5-C20A<sup>T</sup> in the VHS medium at  
252 pH 5.5 and 55°C. Sugars, organic acids, and alcohol were tested individually at 20 mM and  
253 proteinaceous compounds at 5 g L<sup>-1</sup>. Autotrophic growth was tested by replacing N<sub>2</sub> headspace  
254 with H<sub>2</sub>/CO<sub>2</sub> (80/20, v/v) at 200 kPa with elemental sulfur as an electron acceptor. Electron  
255 acceptor utilization experiments were performed in the VHS medium added with 20 mM  
256 maltose as carbon and energy source. Each compound was tested in duplicate Hungate tubes  
257 and those for which growth was observed (OD significantly above that obtained on the  
258 inoculated duplicate controls on VHS medium only) were checked by two successive  
259 subcultures under the same conditions. Bacterial growth was monitored by measuring the

260 increase in turbidity at 600 nm as mentioned above. H<sub>2</sub>S production was determined measuring  
261 the formation of colloidal CuS photometrically at 480 nm following the method described by  
262 Cord-Ruwisch [43]. Metabolic end-products were measured by HPLC using an Aminex HPX-  
263 87H (Bio-Rad) column with 2.5 mM H<sub>2</sub>SO<sub>4</sub> as mobile phase and gas-phase chromatography  
264 (Shimadzu GC 8A) after 5 days of incubation at 55 °C. Oxygen and hydrogen tolerances were  
265 tested at 55°C with shaking at 120 rpm. The gas phase was set from 20% to 0% O<sub>2</sub> with a 2%  
266 increment, and from 100% H<sub>2</sub> to 0% H<sub>2</sub> with a 20% increment. The various gas phases were  
267 tested at atmospheric pressure. Hydrogen tolerance was tested with or without 0.5% elemental  
268 sulfur.

269 Susceptibility to antibiotics was investigated at 100 µg mL<sup>-1</sup>, 50 µg mL<sup>-1</sup>, 25 µg mL<sup>-1</sup>, and 10  
270 µg mL<sup>-1</sup>. Ampicillin, bacitracin, chloramphenicol, cycloheximide, kanamycin, penicillin,  
271 streptomycin, and vancomycin were tested separately. Concentrated anaerobic solutions of  
272 antibiotics were added to the VHS medium just before inoculation. To measure antibiotics  
273 susceptibility, strain BP5-C20A<sup>T</sup> was cultured twice in VHS medium with the antibiotic, and  
274 growth was compared with an antibiotic-free control.

275 Growth occurs from 40°C to 70°C, with an optimum at 55°C, from 10 to 50 g L<sup>-1</sup> (w/v) NaCl  
276 with an optimum at 25 g L<sup>-1</sup> and from pH 4.7 to 6.5 with an optimum at pH 5.6 (Fig. S3). The  
277  $\mu_{\max}$  determined in optimal conditions (pH 5.6) was 0.18 h<sup>-1</sup> (doubling time of 3 h 51 min).  
278 From the substrates tested as carbon and energy sources for growth of strain BP5-C20A<sup>T</sup>,  
279 cellobiose, fructose, glucose, maltose, starch, sucrose, and xylan as carbohydrates, and yeast  
280 extract, peptone, and tryptone as complex compounds were used and improved growth. The  
281 following tested compounds were not used and did not improve growth: acetate, casamino  
282 acids, casein, ethanol, formate, galactose, glycerol, methanol, succinate, and xylose (Table 2).  
283 Of the electron acceptors tested: elemental sulfur (5 g L<sup>-1</sup>), L-cystine (2 g L<sup>-1</sup>), sodium  
284 thiosulfate (20 mM), sodium sulfite (2 mM), sodium sulfate (20 mM), nitrate (20 mM), nitrite  
285 (2 mM), Fe III hydroxide (12 mM), and Fe III EDTA (12 mM), only elemental sulfur was used

286 as an electron acceptor and reduced to H<sub>2</sub>S. During the growth of strain BP5-C20A<sup>T</sup> on glucose,  
287 acetate, carbon dioxide, and hydrogen were produced. Growth on yeast extract and tryptone  
288 was accompanied by acetate, H<sub>2</sub>, and CO<sub>2</sub> production, with lactate, isovalerate, and isobutyrate  
289 detected as traces. H<sub>2</sub>S was only detected during growth on cultures containing elemental sulfur  
290 as an electron acceptor.

291 Autotrophic growth was examined in VHS medium in the absence of any organic compound.  
292 Strain BP5-C20A<sup>T</sup> was not able to grow under autotrophic conditions using H<sub>2</sub> as a sole energy  
293 source and elemental sulfur as electron acceptors, as all members of the genus *Marinitoga*.  
294 Tolerance to oxygen was examined and strain BP5-C20A<sup>T</sup> was a strict anaerobe as *M. lauensis*  
295 [8] and *M. okinawensis* [11].

296 Growth did not occur under 100% H<sub>2</sub> in the gas phase, but this growth inhibition was lifted in  
297 the presence of elemental sulfur as in *M. camini* [10]. Growth was observed without elemental  
298 sulfur in the medium under H<sub>2</sub>/CO<sub>2</sub> (80/20, v/v) atmosphere. Strain BP5-C20A<sup>T</sup> growth was  
299 inhibited at 10 µg mL<sup>-1</sup> ampicillin, chloramphenicol, penicillin, and vancomycin; 50 µg mL<sup>-1</sup>  
300 bacitracin, cycloheximide and streptomycin; and 100 µg mL<sup>-1</sup> kanamycin.

301  
302 Optimal growth conditions of strain BP5-C20A<sup>T</sup>, in terms of temperature, NaCl concentration,  
303 and pH are very similar to other species: the novel strain is thermophilic, slightly halophilic,  
304 and acidophilic. It differs from the closest *Marinitoga* type species in the use of substrates,  
305 especially in the inability to use acetate, casein, formate, and galactose contrary to *M. lauensis*  
306 LG1<sup>T</sup> (Table 2). Strain BP5-C20A<sup>T</sup> could use maltose and sucrose but not glycerol, at the  
307 difference of *M. okinawensis* TFS-10-5<sup>T</sup>. They were all able to use elemental sulfur as an  
308 electron acceptor but differ in the use of L-cystine and thiosulfate. The phylogenetic and  
309 genome features, especially the dDDH and ANI values between the genomes of strains BP5-  
310 C20A<sup>T</sup> and its closest relatives, allow us to propose this strain as a new species of the genus  
311 *Marinitoga*.

312

313 **Description of *Marinitoga aeolica* sp. nov.**

314 *Marinitoga aeolica* (ae.o'li.ca. L. fem. adj. *aeolica*, from the Aeolian archipelago, referring to  
315 the Panarea Island near Sicily in Italy, the source of the first isolated strain). Cells are motile  
316 rods and occur singly, in pairs or in chains. They have a mean length of 1.5–3.4  $\mu\text{m}$  and width  
317 0.5–0.7  $\mu\text{m}$  in exponential growth phase. Colonies are creamy, circular, and convex. Strictly  
318 anaerobic. The fatty acids identified were: C<sub>16:0</sub>, C<sub>16:1 $\omega$ 9c</sub>, C<sub>14:0</sub>, C<sub>16:1 $\omega$ 7c</sub>, C<sub>18:1 $\omega$ 9c</sub>, C<sub>16:0 iso</sub> C<sub>12:0</sub>,  
319 C<sub>18:0</sub>, C<sub>18:1 $\omega$ 7c</sub>, C<sub>10:0</sub>, C<sub>17:0 anteiso</sub> and C<sub>15:0 anteiso</sub>. Obligate chemoorganotroph; the growth occurs  
320 preferentially on complex peptide organics such as yeast extract, peptone, tryptone. Able to use  
321 a range of carbohydrates: cellobiose, fructose, glucose, maltose, starch, sucrose, and xylan.  
322 Elemental sulfur used as electron acceptor and reduced to sulfide. Optimal growth occurs at  
323 55°C, pH 5.6 and 25 g L<sup>-1</sup> (w/v) NaCl. The DNA G + C content of this strain is 27.3 %. The  
324 Genbank 16S rRNA gene sequence accession number of strain BP5-C20A<sup>T</sup> is MZ571208. The  
325 GenBank accession number of the complete genome sequence is CP069362. The type strain  
326 BP5-C20A<sup>T</sup> (=DSM 112332<sup>T</sup> = JCM 39183<sup>T</sup>) was isolated from a shallow hydrothermal vent  
327 located at Panarea Island in the Aeolian archipelago near Sicily, Italy.

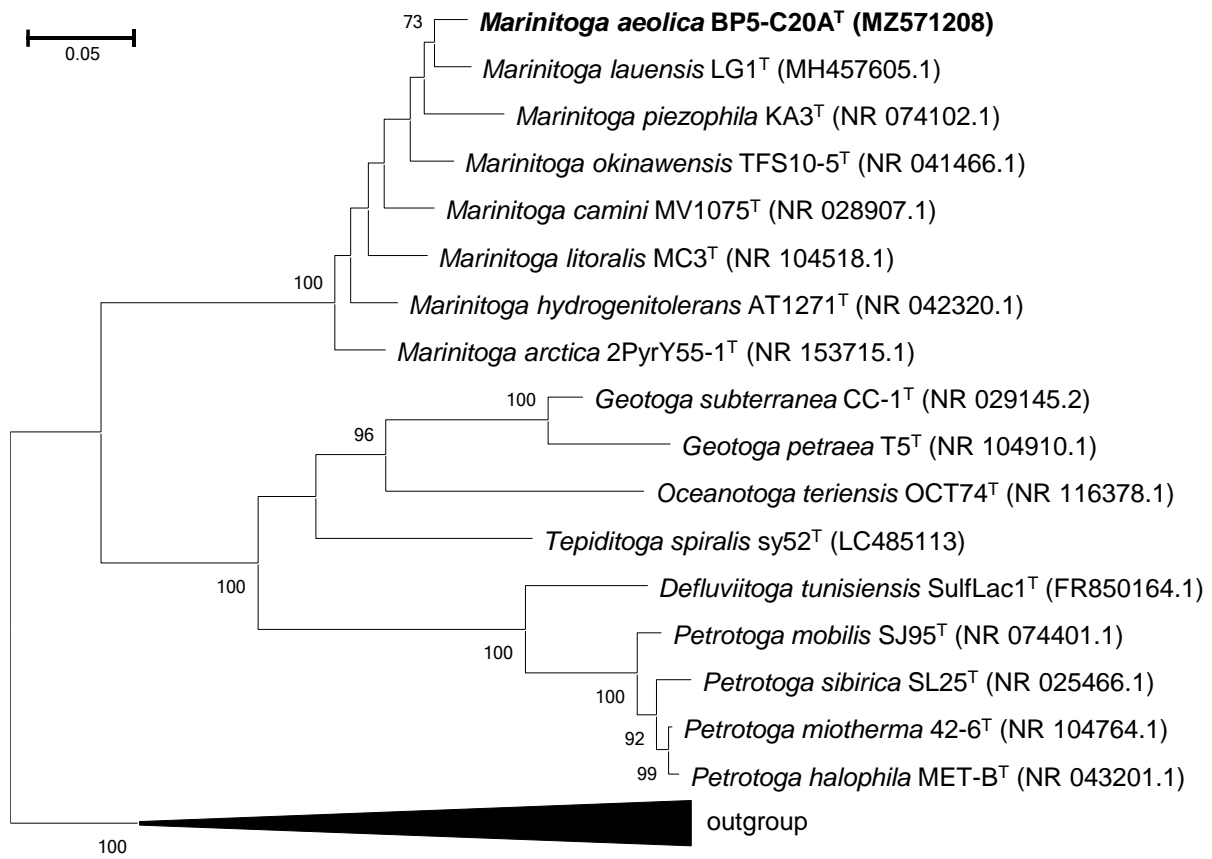
328

329

330 **Conflict of Interest Statement**

331 No Conflict of Interest to declare.

332



334

335 **Figure 1.** Phylogenetic tree based on 16S rRNA gene sequence showing the position of strain  
 336 BP5-C20A<sup>T</sup>, together with type species of the *Petrogaceae* family (*Petrotogales* order,  
 337 *Thermotogota* phylum). Accession numbers of the 16S rRNA gene sequences are given in  
 338 brackets. Evolutionary analysis was done using by Maximum Likelihood method and Kimura  
 339 2-parameter model [27]. Bootstrap values above 70 are indicated at the node (from 2000  
 340 replicates). This analysis involved 30 nucleotide sequences. There were a total of 1599 positions  
 341 in the final dataset. The outgroup is composed of: *Mesoaciditoga lauensis* cd-1655R<sup>T</sup> (NR  
 342 125611.1), *Kosmotoga olearia* TBF 19.5.1<sup>T</sup> (EU980631), *Mesotoga infera* VNs100<sup>T</sup>  
 343 (KC800693), *Fervidobacterium riparium* 1445t<sup>T</sup> (NR 108234.1), *Fervidobacterium*  
 344 *gondwanense* AB39<sup>T</sup> (NR 036997.1), *Thermosipho atlanticus* DV1140<sup>T</sup> (NR 029020.1),  
 345 *Pseudothermotoga elfii* SERB<sup>T</sup> (NR 026201.1), *Pseudothermotoga subterranea* SL1<sup>T</sup> (NR  
 346 025969.1), *Thermotoga profunda* AZM34c06<sup>T</sup> (NR 133904.1), *Thermotoga naphthophila*  
 347 RKU-10<sup>T</sup> (NR 074952.1), *Thermotoga petrophila* RKU-10<sup>T</sup> (NR 042374.2), *Thermotoga*

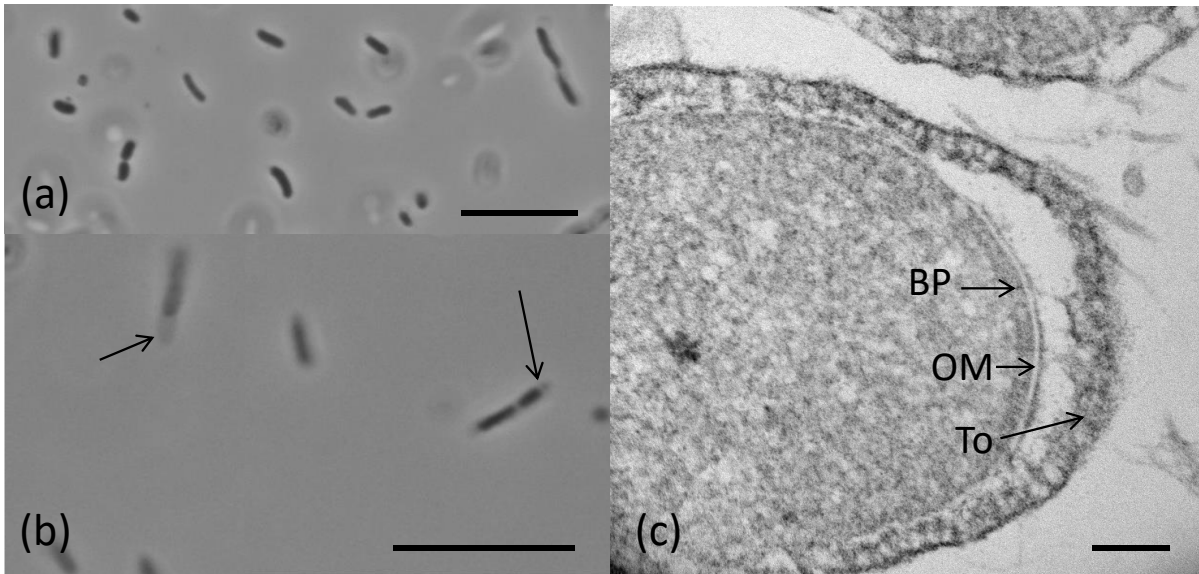
348 *maritima* MSB-8<sup>T</sup> (NR 029163.2) and *Thermotoga neapolitana* DSM 4359<sup>T</sup> (NR 074959.1).

349 The scale bar represents 0.05 substitution per nucleotide.

350



351



352

353 **Figure 2.** Microphotograph of strain BP5-C20A<sup>T</sup>. (a) Phase-contrast microphotograph  
354 (magnification X1000) showing cell dividing by constriction (scale bar: 10  $\mu$ m) and (b) 'toga'  
355 structures indicated by arrows (scale bar: 10  $\mu$ m); (c) thin section observed in MET microscopy  
356 showing the phospholipid bilayer (BP) and a thin outer membrane (OM) typical of a Gram-  
357 negative cell wall, surrounded by a sheath-like structure, 'toga' (To), scale bar: 100 nm.

358

359

360

361 **Table 1.** Digital DNA-DNA hybridization (dDDH) and Average Nucleotide Identity (ANI)  
362 values between strain BP5-C20A<sup>T</sup> genome and other related *Marinitoga* species for which  
363 genome data were available: *M. lauensis* LG1<sup>T</sup> [44], *M. camini* str. 1197 [45], *M. camini* str.  
364 1155 [45], *M. piezophila* KA3<sup>T</sup> [46], *M. hydrogenitolerans* AT1271<sup>T</sup>, *Marinitoga* sp. 1137.  
365 Accession numbers of the genomic sequences are given in brackets. dDDH were calculated  
366 using the Genome-to-Genome Distance Calculator (GGDC) 2.1 and formula d4  
367 (<http://ggdc.dsmz.de/>) [37]. ANI values were calculated using OrthoANI with usearch  
368 (<http://www.ezbiocloud.net/tools/ani>) [47].

369

	BP5-C20A <sup>T</sup> (CP069362)	
	dDDH*	OrthoANI (%)
<i>M. lauensis</i> LG1 <sup>T</sup> (QHLX00000000.1)	31.0	85.70
<i>M. litoralis</i> DSM 21709 <sup>T</sup> (GCA_016908145.1)	25.3	82.27
<i>M. hydrogenitolerans</i> DSM 16785 <sup>T</sup> (GCA_900129175.1)	23.4	80.68
<i>M. camini</i> str. 1197 (AZAY00000000.1)	22.5	79.97
<i>M. camini</i> str. 1155 (AZAX00000000.1)	22.3	79.61
<i>Marinitoga</i> sp. 1137 (NZ_CP009471.1)	21.3	77.38
<i>M. piezophila</i> KA3 <sup>T</sup> (CP003257.1)	21.0	76.82

370 \* Formula d4 (a.k.a. GGDC formula 2): sum of all identities found in HSPs divided by overall  
371 HSP length (Formula d4 is independent of genome length and is thus robust against the use of  
372 incomplete draft genomes).

373

374 **Table 2.** Differential characteristics between strain BP5-C20A<sup>T</sup> and other members of the genus  
 375 *Marinitoga*. Type strains of the *Marinitoga* species: 1, BP5-C20A<sup>T</sup> (this study); 2, *M. lauensis*  
 376 LG1<sup>T</sup> [8]; 3, *M. okinawensis* TFS-10-5<sup>T</sup> [11]; 4, *M. piezophila* KA3<sup>T</sup> [12]; 5, *M. camini*  
 377 MV1075<sup>T</sup> [10]; 6, *M. litoralis* MC3<sup>T</sup> [6] ; 7, *M. hydrogenitolerans* AT1271<sup>T</sup> [9]; 8, *M. artica*  
 378 2PyrY55-1<sup>T</sup> [7]. +, Positive; (+), weakly positive; -, negative; ND, not determined.

Species type strain	1	2	3	4	5	6	7	8
<b>Origin</b>	Mediterranean Sea	South Western Pacific Ocean	Okinawa Trough	East Pacific Rise	Mid-Atlantic Ridge	Southern Indian Ocean	Mid-Atlantic Ridge	Arctic Mid-Ocean Ridge
<b>Latitude</b>	38°38'N	20°10'S	24°51'N	12°48'N	37°51'N	38°43'S	36°12'N	71°26'N
<b>Longitude</b>	15°06'E	176°36'E	122°42'E	103°56'W	31°31'W	77°31'E	33°54'W	48°75'W
<b>Depth (meter below sea level)</b>	20	1914	1365	2630	980	0	2275	700
<b>pH for growth:</b>								
<b>Range</b>	4.7 - 6.5	5.4–7.9	5.0–7.4	5.0–8.0	5.0–9.0	5.5–7.5	4.5–8.5	5.0–7.5
<b>Optimum</b>	5.6	6.5	5.5–5.8	6.0	7.0	6.0	6.0	5.5
<b>Temperature for growth (°C):</b>								
<b>Range</b>	40-70	40–70	30–70	45–70	25–65	45–70	35–65	45–70
<b>Optimum</b>	55	50–55	55–60	65	55	60	60	65
<b>NaCl concentration for growth (g L<sup>-1</sup>):</b>								
<b>Range</b>	10–50	7.5–50	10–55	10–50	10–45	8–46	10–65	15–55
<b>Optimum</b>	25	30	30–35	30	20	26	30–40	25
<b>Carbon sources:</b>								
<b>Acetate</b>	–	+ <sup>a</sup>	–	(+) <sup>a</sup>	– <sup>a</sup>	–	–	–
<b>Casein</b>	–	+	–	+	–	+	+	–
<b>Formate</b>	–	+	–	+	–	–	ND	–
<b>Galactose</b>	–	+	–	+	–	+	–	–
<b>Glycerol</b>	–	ND	+	ND	–	–	ND	ND
<b>Maltose</b>	+	+	–	+	+	+	+	+
<b>Sucrose</b>	+	ND	–	ND	+	ND	ND	+
<b>Tolerance of O<sub>2</sub></b>	No	No	No	ND	ND	<4	<4	ND
<b>Tolerance of 100% H<sub>2</sub> without S<sup>o</sup></b>	No	No	Yes	No	No	Yes	Yes	Yes
<b>G + C content (%)</b>	27.3	27.9	28	29.2	29	26.2	27.2	27
<b>Electron acceptors</b>	Elemental sulfur	Elemental sulfur, L-cystine, sodium thiosulfate	Elemental sulfur, L-cystine	Elemental sulfur, L-cystine, thiosulfate	Elemental sulfur, L-cystine	Elemental sulfur	Elemental sulfur, L-cystine, thiosulfate	Elemental sulfur, L-cystine

379 <sup>(a)</sup> means acetate was tested in the presence of CO<sub>2</sub> in the gas phase.

380

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390

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394

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