Terricaulis silvestris gen. nov., sp. nov. a new prosthecate, budding member of the family *Caulobacteraceae* isolated from forest soil

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24 ## Abstract

25 The family *Caulobacteraceae* comprises prosthecate bacteria with dimorphic cell cycle and
26 non-prosthecate members. Cells of all described species divide by binary fission. Strain
27 0127_4^T was isolated from forest soil in Baden Württemberg (Germany) and determined to
28 be the first representative of the family *Caulobacteraceae* which divided by budding. Cells of
29 0127_4^T were Gram-negative rods, prosthecate, motile by means of a polar flagellum, non-
30 spore-forming and non-capsulated. The strain formed small white colonies and grew
31 aerobically and chemoorganotrophically utilizing organic acids, amino acids and
32 proteinaceous substrates. 16S rRNA gene sequence analysis indicated that this bacterium
33 was related to *Aquidulcibacter paucihalophilus* TH1-2^T and *Asprobacter aquaticus* DRW22-8^T
34 with 91.3% and 89.7% sequence similarity, respectively. Four unidentified glycolipids were
35 detected as the major polar lipids and unlike all described members of the family
36 *Caulobacteraceae*, phosphatidylglycerol was absent. The major fatty acids were summed
37 feature 8 ($C_{18:1}$ *ω*7*c*/$C_{18:1}$ *ω*6*c*) (21.6%), summed feature 9 (iso-$C_{17:1}$ *ω*9*c*/$C_{16:0}$ 10-methyl)
38 (17.7%), $C_{16:0}$ (15.9%) and summed feature 3 ($C_{16:1}$ *ω*6*c*/ $C_{16:1}$ *ω*7*c*) (12.1%). The major
39 respiratory quinone was Q-10. The G+C content of genomic DNA was 63.5%. Based on the
40 taxonomic characterization, the novel genus and species *Terricaulis silvestris* gen. nov., sp.
41 nov. (type strain 0127_4^T = DSM 104635^T = CECT 9243^T) are proposed.

42

43 The family *Caulobacteraceae* comprises prosthecate bacteria with a dimorphic life cycle
44 which divide by binary fission. In the dimorphic life cycle two cell types occur which are
45 morphologically distinct from each other: the prosthecate cell is sessile, while the daughter
46 cell is motile and does not possess a prosthecum. This unusual cell-cycle has been studied in
47 depth for *Caulobacter crescentus* [1, 2], which is a heterotypic synonym of the first described
48 species of the family, *Caulobacter vibrioides* [3]. To date, the family *Caulobacteraceae*
49 encompasses the five genera *Aquidulcibacter* [4], *Asticcacaulis* [5–10], *Brevundimonas* [11,
50 12, 21–30, 13, 31–33, 14–20], *Caulobacter* [3, 12, 19, 34–40] and *Phenylobacterium* [19, 41,
51 50–54, 42–49]. Strains have been isolated from a wide range of habitats including soil [9, 16,
52 34, 41, 51, 53], the rhizosphere [37–39, 49], plant roots [6, 30, 40], freshwater [3, 10, 42, 48,
53 55], brackish water [23], seawater [14], sediments [7, 26, 27], activated sludge [17, 23, 47],
54 sewage [24], compost [45], blood [19, 22], a space laboratory [13], the bladder of a leech
55 [11], a *Chlorella* culture [12] and cyanobacterial aggregates [4].

56 In the present study, a new, phylogenetically deeply branching representative of the
57 family *Caulobacteraceae* with an unusual type of cell division was isolated and characterized
58 by a polyphasic approach.

59 Strain 0127_4^T was isolated from soil of an unmanaged beech forest in Baden-
60 Württemberg, located close to Gomadingen, Germany (N 48°22'57.4", E 9°22'56.6") during a
61 study conducted in the framework of the Biodiversity Exploratories [56]. The soil sample
62 consisted of 14 subsamples taken from the upper 10 cm of soil, which were then
63 homogenized. The soil had a slightly acidic pH of 6.15 (determined in 10 mM of $CaCl_2$). Strain
64 0127_4^T was isolated after six weeks of incubation of liquid media inoculated with diluted

65 soil suspension employing a high throughput cultivation approach [57]. Liquid cultures were
66 set up in 96 well microtiter plates and each of the 60 inner wells of the plates was filled with
67 180 µl SSE/C-mix medium. The SSE/C-mix medium was based on soil solution equivalent
68 (SSE) [58] amended with 50 mM aminoacids (alanine, arginine, cysteine, glutamine,
69 glutamate, glycine, histidine, lysine, methionine, phenylalanine, proline, serine, threonine,
70 tryptophan, tyrosine, asparagine, aspartate, leucine, isoleucine and valine), 50 mM sugars
71 (glucosamine, mannitol, N-acetyl-D-galactosamine, turanose, β-gentiobiose, glucose, xylose,
72 arabinose, trehalose and rhamnose), 50 mM aromatic compounds (sodium benzoate and
73 sodium salicylate), 50 mM tricarboxylic acid cycle intermediaries (lactate, succinate, citrate,
74 malate, pyruvate, α-ketoglutarate and oxaloacetate), 50 mM fatty acids (formiate, acetate,
75 propionate, butyrate and valeriate), and 0.0005% Tween 80. The medium was buffered with
76 HEPES to pH 7 and supplemented with a solution of 10 vitamins (1 ml L^{-1}) [59] and with SL10
77 trace element solution (1 ml L^{-1}) [60]. The wells were subsequently inoculated with 20 µl of
78 the soil suspension (diluted in 10mM HEPES pH 7) containing five cells, as determined by
79 total bacterial cell counting [61].

80 After six weeks of incubation at room temperature, wells containing grown bacterial
81 cultures were identified by turbidity and the phylogenetic composition of each culture was
82 determined by sequencing, employing a barcoded Illumina paired-end method targeting the
83 16S ribosomal RNA V1-2 hypervariable region [62]. Briefly, 40 µl of each culture were
84 centrifuged and pellets resuspended in 20 µl of Tris-buffer (10 mM, pH 8). Three freeze/thaw
85 cycles (5 min in 70% ethanol and ice bath followed by 5 min incubation at 99°C) were
86 employed to disrupt the cells. The V1-2 region of the 16S rRNA gene was amplified using the

87 primer pair 27F and 338R [63, 64]. Amplification was performed in a total volume of 50 µl
88 with 4 µl 5x HF Phusion buffer, 1 µl dNTP mix (10 mM each), 0.4 µl of each primer (10 mM), 1
89 µl of template DNA and 0.2 µl Phusion High-Fidelity DNA Polymerase (2 U/µl; Thermo
90 Scientific, Waltham, USA). An initial denaturation step of 98°C for 30 s was followed by 20
91 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 15 s and extension at 72°C for
92 45 s and final extension step at 72°C for 7 minutes. For barcoding, 1 µl of the reaction
93 mixture served as template in a second polymerase chain reaction (PCR) (performed under
94 the same conditions as previously). The forward primer contained a 6-nucleotide barcode
95 [65] and a 2-nucleotide CA linker [66]. Again, 1 µl of this reaction mixture served as template
96 in a third PCR designed to insert the sequence of the specific Illumina multiplexing
97 sequencing primers and index primers. This was performed as previously mentioned, but
98 only with 10 cycles. PCR products were cleaned with NucleoSpin® 96 PCR Clean-up kit
99 (Macherey-Nagel, Düren, Germany) and quantified with the Quant-iT™ PicoGreen® dsDNA
100 Assay Kit (Invitrogen, Darmstadt, Germany) following the instructions of the manufacturer.

101 Sequencing was performed using Illumina MiSeq (Illumina, San Diego, CA, USA).
102 Sequence reads were joined and assigned to their respective culture based on their
103 barcodes. Primers and barcodes were then trimmed from each read. Taxonomic assignment
104 was done using the RDP classifier [67]. Wells containing bacteria with < 97% 16S rRNA
105 sequence similarity to the next cultured representative at an abundance of > 1% of the total
106 sequence number in the well were plated on SSE/C-mix medium solidified with 0.8% gellan
107 gum (w/v). After incubation for 12 weeks at room temperature in darkness, representative
108 colonies were picked from each plate and purified. Partial 16S rRNA gene sequencing was

109 used to assess the taxonomic affiliation of all strains in culture and strain 0127_4^T was
110 classified as a member of the family *Caulobacteraceae*. Comparison of the almost full-length
111 16S rRNA gene sequence on EzBioCloud database [68] revealed *Aquidulcibacter*
112 *paucihalophilus* TH1-2^T (91.28% gene sequence similarity) and *Asprobacter aquaticus*
113 DRW22-8^T (89.66%) as the closest cultured relatives. For maintenance of cultures, and for
114 metabolic and physiological testing, medium SSE/HD1:10 [69] was employed at pH 7.0 and
115 strains were grown at a temperature of 28°C.

116 Phenotypic characterization was carried out as described previously [69–71]. Strain
117 0127_4^T formed round, convex, white colonies with entire margins that strongly attached to
118 solid media and reached 0.5-1.0 mm in diameter within eight days of growth at 28°C.
119 Reproducing cells were observed with time-lapse microscopy using a Nikon Eclipse Ti
120 inverted microscope with a Nikon DS-Ri2 camera (blue LED) and the CellASIC ONIX
121 Microfluidic Plate B04 (Merck Millipore, Burlington, MA, USA). Cells were introduced and
122 elastically trapped to the flow chamber by using a loading protocol (provided by CellASIC).
123 Dividing cells were monitored at a constant flow of medium (SSE/HD1:10; 2x 1 psi) for 32 h at
124 28°C. Images were aligned and analyzed using the NIS-Elements software V4.3 (Nikon
125 Instruments, Tokyo, Japan). To determine the cell size, 393 cells were counted by using the
126 semi-automated object count tool (smooth: 4x, clean: 4x, fill holes: on, separate: 4x). For
127 inspection of the cellular structures, cells of 0127_4^T were observed by electron microscopy.
128 Cells were fixed with 5% formaldehyde and 2% glutaraldehyde in growth media, washed with
129 TE buffer (10 mM Tris, 2 mM EDTA, pH 6.9), dehydrated in a graded series of acetone (10, 30,
130 50, 70, 90, 100%) on ice for 10 min for each step. Samples were then subjected to critical-

131 point drying with liquid CO_2 (CPD 030, Bal-Tec, Liechtenstein) and coated with a
132 gold/palladium (80/20) film by sputter coating (SCD 500, Bal-Tec, Liechtenstein). Examination
133 was performed with the field emission scanning electron microscope Zeiss Merlin (Carl Zeiss,
134 Oberkochen, Germany) using the Everhart Thornley HESE2-detector and the inlens SE-
135 detector in a 25:75 ratio at an acceleration voltage of 5 kV. Images were recorded with Zeiss
136 SEMSmart V 5.05 and contrast and brightness were adjusted with Adobe Photoshop CS5.

137 Cells of strain 0127_4^T were rod shaped, 0.8 – 3.0 µm long and 0.4 – 1.0 µm wide (Figure
138 1 and Supplementary Figure 1), and tended to form aggregates even in liquid medium (Figure
139 1A). The majority of the cells displayed three prosthecae: one polar prosthecum on one pole
140 and two subpolar prosthecae on the opposite pole Supplementary Figure S1B, C; Figure 1). In
141 addition, cells with only two polar prosthecae were observed (Supplementary Figure S1A;
142 Figure 1). The strain divided by budding, producing motile daughter cells with a single polar
143 flagellum at the terminus of each prosthecum (Figure 1B). This mode of division by budding
144 distinguishes the novel isolate from all other prosthecate members of the family
145 *Caulobacteraceae*, which divide by binary fission. The mode of division is more similar to
146 members of the family *Hyphomonadaceae* (*Caulobacterales* order), such as *Hyphomonas*
147 *polymorpha* PS728^T [72], but none of these display three prosthecae. For further
148 characterization, cells were treated by Gram [73], India ink and malachite green staining [74]
149 and studied by light microscopy (Zeiss Axio Lab.A1, Carl Zeiss, Oberkochen, Germany with
150 AxioCam Mrm camera). Strain 0127_4^T stained Gram-negative and no spores or capsules
151 were detected, similar to all described members of the family *Caulobacteraceae*.

152 Catalase activity was determined as described in Cowan *et al.*, 1993 [75] and cytochrome
153 *c*-oxidase activity was analyzed using Bactident Oxidase (Merck, Darmstadt, Germany). Strain
154 0127_4^T tested negative for catalase, in contrast to its closest relative *Aquidulcibacter*
155 *paucihalophilus* TH1-2^T, but tested positive for cytochrome *c*-oxidase (Table 1).
156 The relationship of strain 0127_4^T to oxygen was assessed in solid media in a candle jar
157 [76] or using Anaerocult P (Merck, Darmstadt, Germany). No growth was detected when
158 employing both approaches, confirming the strain to be a strict aerobe. Growth ranges and
159 the optima of temperature and pH were determined in triplicates under oxic conditions in
160 liquid SSE/HD1:10 medium, as previously described [69, 70, 77]. Growth was tested between
161 pH 1.0 and 12.0 and in the temperature range of 10 to 45°C. Since the SSE/HD1:10 medium
162 contains different salts, salt tolerance was determined in modified DSMZ medium 1124 (10
163 mM HEPES buffer, 0.1 ml·l^{-1} 10-vitamin solution [59] and 1 ml·l^{-1} trace element solution SL-10
164 [60]). NaCl was added in concentrations between 0 and 10% (w/v).
165 Strain 0127_4^T grew from pH 6.1 to 8.3 (optimum between pH 6.5 - 7.9) which is similar
166 to what has been reported for the closest relative *Aquidulcibacter paucihalophilus* TH1-2^T
167 and for most species of *Caulobacter*, *Phenylobacterium* and *Asticcacaulis* (Table 1). In
168 contrast, many strains of the genus *Brevundimonas*, such as *B. basaltis* J22^T, *B. denitrificans*
169 TAR-002^T, *B.humi* CA-15^T, *B. naejangsanensis* BIO-TAS2-2^T and *B. viscosa* F3^T can tolerate pH
170 values of up to 10 or 11. Strain 0127_4^T was capable of growth between temperatures of 10
171 and 30°C (optimum at 27°C) which is considerably lower than measured for its closest
172 relative *Aquidulcibacter paucihalophilus* TH1-2^T, which grows only between 20 and 40°C
173 (Table 1). Most members of the family *Caulobacteraceae* that were isolated from terrestrial

174 environments can grow at higher temperatures (up to 50°C for *Brevundimonas*
175 *naejangsanensis* BIO-TAS2-2^{T} and *Phenylobacterium terrae* YIM 730227^{T}), with the exception
176 of *Asticcacaulis benevestitus* Z-0023^{T}, *Caulobacter ginsengisoli* Gsoil 317^{T}, *Caulobacter*
177 *rhizosphaerae* 7F14^{T} and *Phenylobacterium panacis* DCY109^{T} which are similar to 0127_4^{T}
178 and grow only up to temperatures of 28 - 30°C. Strain 0127_4^{T} did not tolerate addition of
179 NaCl and grew only without its addition. Although many members of the family
180 *Caulobacteraceae* tolerate low concentrations of NaCl, they grow best without it, similarly to
181 strain 0127_4^{T}. Throughout the family *Caulobacteraceae*, only two strains of the genus
182 *Brevundimonas*, *B. abyssalis* TAR-001^{T} and *B. denitrificans* TAR-002^{T}, isolated from seafloor
183 sediment, need NaCl for growth.

184 Under optimum growth conditions, doubling times of strain 0127_4^{T} were 26.1 h. This
185 number is rarely reported and is only available for eight species of the family
186 *Caulobacteraceae*. Strain 0127_4^{T} divides considerably slower than *Asticcacaulis*
187 *biprosthecium* C-19^{T} (2 h), *Asticcacaulis excentricus* CB 48^{T} (1.5 - 2 h), *Brevundimonas*
188 *bacteroides* CB7^{T} (3.1 h), *Caulobacter vibrioides* CB51^{T} (3 - 4 h), *Caulobacter henricii* CB4^{T} (2.5
189 h), *Phenylobacterium immobile* E^{T} (7 h) and *Phenylobacterium lituiforme* FaiI3^{T} (4 h), but
190 faster than *Asticcacaulis benevestitus* Z-0023^{T} which has doubling times of 30 - 35 h.

191 The capacity of strain 0127_4^{T} for metabolizing different substrates was assessed in
192 liquid SSE supplemented with 0.1 ml·l^{-1} 10-vitamin solution [59] and 1 ml·l^{-1} trace element
193 solution SL-10 [60], in triplicates. Sugars, organic acids, keto acids, alcohols, amino acids,
194 casamino acids, casein hydrolysate, laminarin, peptone, yeast extract and Tween 80 were
195 added as sole carbon sources. The final concentrations of these have been described

196 previously [70, 78]. A result was recorded as positive when the mean OD_{660} of the three
197 parallels exceeded the mean of the control (culture without substrate) 1.5 times. Weak
198 growth was defined by OD values exceeding controls 1.2 - 1.5 times. To investigate the
199 degradation of complex substrates, solidified SSE medium supplemented with 0.005% (w/v)
200 yeast extract was used as basal a medium to which cellulose, chitin, starch, xylan, pectin and
201 lignin (0.5 $ml \cdot l^{-1}$ final concentration of each polymer) were added. Specific staining solutions
202 were employed to determine the ability of strain 0127_4^T to degrade the polymeric
203 substrates [57]. Out of 109 single carbon substrates tested, strain 0127_4^T grew strongly on
204 13 different substrates and weakly on 19. It showed preference for amino acids (histidine,
205 ornithine, serine, lysine, alanine, glutamine, isoleucine, proline and valine), organic acids
206 (lactate, pyruvate, succinate, glutamate, citrate, fumarate, aspartate and gluconate) and
207 complex protein substrates (peptone, casamino acids, casein hydrolysate, yeast extract and
208 fermented rumen extract). Furthermore, it was capable of metabolizing a few sugars
209 (galactose, arabinose and *N*-acetylglucosamine), sugar alcohols (glycerol and dulcitol),
210 ketones (β- and γ- hydroxybutyrate), fatty acids (isovaleric acid) and protocatechuate. No
211 degradation of polymeric substrates was detected for strain 0127_4^T.

212 Nitrate reduction, indole production, fermentation of glucose, β-galactosidase, arginine
213 dihydrolase, and urease activities, and gelatin and aesculin hydrolysis were determined using
214 the API 20NE test system (BioMérieux, Marcy-l'Étoile, France) following the instructions of
215 the manufacturer. The assimilation of carbon substrates using API 20NE could not be
216 determined since the media employed does not allow growth of 0127_4^T. API ZYM galleries
217 (BioMérieux, Marcy-l'Étoile, France) were employed for the determination of exoenzyme

218 activities, for both strain 0127_4^T and all type species of the genera which comprise the
219 family *Caulobacteraceae*, grown under the same conditions (SSE/HD1:10 medium, pH 7,
220 28°C). Strain 0127_4^T tested positive for gelatinase and β-galactosidase. Regarding
221 exoenzymes, 0127_4^T showed only alkaline phosphatase and leucine arylamidase activites.
222 These two exoenzymes seem to be specific characteristics of the members of the family
223 *Caulobacteraceae*, since all type species tested positive (Table 2).

224 Quinones, fatty acids and polar lipids were analyzed for strain 0127_4^T and all type
225 strains of the type species of all genera in the family *Caulobacteraceae*. All strains were
226 grown under the same conditions (SSE/HD1:10 medium, pH 7, 28°C) and harvested in late-
227 exponential phase. Isoprenoid quinones were extracted from dried biomass with
228 chloroform/methanol (2:1, v/v; [79] and subsequently analyzed via HPLC [80]. For fatty acid
229 analysis, cells were grown for 10 days at 28°C in SSE/HD1:10 medium (pH 7.0). Fatty acids
230 were extracted, saponified and methylated according to standard protocols of the Microbial
231 Identification System (MIDI Inc.; version 6.1;[81]). Compounds were identified against the
232 TSBA60 peak naming table database. The polar lipid composition was analyzed by two-
233 dimensional thin layer chromatography (modified after [82]; [83]). Like all described
234 members of the family *Caulobacteraceae*, strain 0127_4^T contained Q-10 as predominant
235 respiratory ubiquinone. The major polar lipids detected were 1,2-di-*O*-acyl-3-*O*-αD-
236 glucopyranuronosylglycerol and four unidentified glycolipids (Table 3, Supplementary Figure
237 2). No phospholipid was identified for strain 0127_4^T, a feature that distinguishes it from the
238 remaining members of the family, since all type strains of the family *Caulobacteraceae*
239 harbor phosphatidylglycerol and *Brevundimonas diminuta* AJ 2067^T [11], *Caulobacter*

240 *vibrioides* CB51^{T} [10], *Phenylobacterium immobile* E^{T} [41] in addition contain 1,2-diacyl-3-*O*-
241 [6'-phosphatidyl-αD-glucopyranosyl]glycerol (Table 3, Supplementary Figure 2). The major
242 fatty acids were summed feature 8 ($C_{18:1}$ *ω*6*c*/ $C_{18:1}$ *ω*7*c*) (21.6%), summed feature 9 (*iso*-$C_{17:1}$
243 *ω*9*c*/ $C_{16:0}$ 10-methyl) (17.7%), $C_{16:0}$ (15.9%) and summed feature 3 ($C_{16:1}$ *ω*6*c*/ $C_{16:1}$ *ω*7*c*)
244 (12.1%). In addition, *iso*-$C_{15:0}$ (9.6%), *iso*-$C_{17:0}$ (5.8%) were found in considerable amounts
245 (Table 3). Consistent with all type strains of the family *Caulobacteraceae*, strain 0127_4^{T}
246 possesses high amounts of summed feature 8 ($C_{18:1}$ *ω*6*c*/ $C_{18:1}$ *ω*7*c*), $C_{16:0}$ and summed
247 feature 3 ($C_{16:1}$ *ω*6*c*/ $C_{16:1}$ *ω*7*c*). Nevertheless, the dominant summed feature 9 (*iso*-$C_{17:1}$ *ω*9*c*/
248 $C_{16:0}$ 10-methyl) and the branched-chain fatty acids *iso*-$C_{15:0}$ and *iso*-$C_{17:0}$ are not found in any
249 other type strain of the family (except for trace amounts and therefore are a distinctive
250 feature of 0127_4^{T}; Table 3).

251 Susceptibility to antibiotics was assessed on SSE/HD1:10 agar plates using disc assays
252 (Oxoid, Altrincham, UK) with the following antibiotics: penicillin G (10 units), oxacillin (5 µg),
253 ampicillin (10 µg), ticarcillin (75 µg) , mezlocillin (30 µg), cefalotin (30 µg), cefazolin (30 µg),
254 cefotaxime (30 µg), aztreonam (30 µg), imipenem (10 µg), tetracycline (30 µg),
255 chloramphenicol (30 µg), gentamicin (10 µg), amikacin (30 µg), vancomycin (30 µg),
256 erythromycin (15 µg), lincomycin (15 µg), ofloxacin (5 µg), norfloxacin (10 µg), colistin (10
257 µg), pipemidic acid (20 µg), nitrofurantoin (100 µg), bacitracin (10 units), polymyxin B (300
258 units), kanamycin (30 µg), neomycin (30 µg), doxycycline (30 µg), ceftriaxone (30 µg),
259 clindamycin (10 µg), fosfomycin (50 µg), moxifloxacin (5 µg), linezolid (30 µg), nystatin (100
260 units), quinupristin/dalfopristin (15 µg), teicoplanin (30 µg) and piperacillin/tazobactam (40
261 µg). Strain 0127_4^{T} was resistant to 16 of the 36 antibiotics tested. These comprise mainly

262 quinolones (ofloxacin, norfloxacin, pipemidic acid and moxifloxacin) and 1st generation
263 cefalosporins (cefalotin and cefazolin), but also oxacillin, aztreonam, erythromycin,
264 lincomycin, colistin, kanamycin, linezolid, nystatin, quinupristin/dalfopristin and
265 piperacillin/tazobactam.

266 The almost full-length 16S rRNA gene was amplified directly by colony-PCR using the
267 primer pair 8f [84] and 1492r [63]. Sequences of the purified PCR product were determined
268 by Sanger sequencing employing the AB 3730 DNA analyzer (Applied Biosystems, Foster City,
269 CA) and the AmpliTaq FS Big Dye terminator cycle sequencing kit. The 16S rRNA gene
270 sequence for strain 0127_4^T had a length of 1403 bp (GenBank/EMBL/DDBJ accession
271 number MN833493). According to the EzBioCloud database [68] the closest validly described
272 relatives of strain 0127_4^T were *Aquidulcibacter paucihalophilus* TH1-2^T (family
273 *Caulobacteraceae*; 91.3% 16S rRNA gene sequence similarity) and *Asprobacter aquaticus*
274 DRW22-8^T (family *Hyphomonadaceae*, 89.7%). Multiple sequence alignment of the 16S rRNA
275 sequence with those of the described species of the *Caulobacterales* order (families
276 *Caulobacteraceae* and *Hyphomonadaceae*) was done with SILVA Incremental Aligner (SINA)
277 [85]. Additional 16S rRNA gene sequences used were retrieved from public databases [68].
278 Phylogenetic trees were calculated using neighbor-joining (K2P evolutionary model) and
279 maximum likelihood (GTR+I+G evolutionary model) algorithms using the MEGA 7.0 software
280 [86]. Tree topology was evaluated by bootstrap analysis with 1000 replications. Both
281 neighbour joining and maximum likelihood trees placed 0127_4^T and *Aquidulcibacter*
282 *paucihalophilus* TH1-2^T as deep branching lineage of the family *Caulobacteraceae* (Figure 2;
283 Supplementary Figure 3), supported by high bootstrap values.

284 In order to better resolve the phylogenetic placement of strain 0127_4^T, a phylogenomic
285 tree was inferred for the genome of strain 0127_4^T and available genomes of the validly
286 described representatives of the families *Caulobacteraceae* and *Hyphomonadaceae*. Genome
287 sequencing was carried out on the PacBio *RSII* (Pacific Biosciences, Menlo Park, CA) using P6
288 chemistry. Genome assembly was performed with the "RS_HGAP_Assembly.3" protocol
289 included in the SMRT Portal version 2.3.0, utilizing 48,781 postfiltered reads with an average
290 read length of 10,327 bp. One complete chromosomal contig was obtained and trimmed,
291 circularized, and adjusted to *dnaA* (DSM104635_00001) as first gene. In addition, genome
292 sequencing was carried out on a NextSeq (Illumina, San Francisco, CA) in a 150-bp paired-end
293 double-indexed run. Quality improvement was performed with the Burrows-Wheeler Aligner
294 (BWA) [87] mapping the Illumina reads onto the obtained chromosome. A final genome
295 quality of QV60 was determined. The final genome sequence was annotated using Prokka
296 [88]. The genome was also uploaded to the Pathosystems Resource Integration Center
297 (PATRIC) for comparative analysis with the available genomes of type strains of the families
298 *Caulobacteraceae* and *Hyphomonadaceae* [89]. The genome of strain 0127_4^T contained
299 3,864,763 bp, 3,942 predicted protein coding genes, 3 rRNAs and 47 tRNAs. The G+C content
300 was 63.5% as inferred from the full genome sequence deposited at GenBank/EMBL/DDBJ
301 under the accession number CP047045. The RAST tool kit (RASTtk) annotation implemented
302 in PATRIC recognized 27% of the coding sequences (CDSs) as subsystem related
303 (Supplementary Figure 4) [90]. Subsystems represent collections of functionally related
304 protein families. The most populated subsystem categories were protein synthesis (168
305 genes) amino acids and derivatives (157 genes), cofactors, vitamins, prosthetic groups (146

306 genes) and stress response, defense and virulence (90 genes). The genome of strain 0127_4^T
307 contained antimicrobial resistance genes such *aac(3)*-I which confers resistance to
308 aminoglycoside antibiotics and also encoded for a class A β-lactamase. Three cold shock
309 proteins were also detected. Regarding membrane transport, a complete type II secretion
310 system was found. Noteworthy is also the presence of all genes necessary for aerobic
311 degradation of phenylacetate and the presence of a polyphosphate kinase alongside a high
312 affinity phosphate transporter. 108 predicted proteins (48 of which were hypothetical) were
313 not present in the genomes of type strains of the families *Caulobacteraceae* and
314 *Hyphomonadaceae* (Supplementary Figure 5), including PaaA-E (EC 1.14.13.149), PaaG (EC
315 5.3.3.18), PaaK (EC 6.2.1.30) and PaaZ (EC 1.2.1.91) which are involved in the aerobic
316 degradation of phenylacetate and the aminoglycoside N(3)-acetyltransferase AAC(3)-I. The
317 UBCG v. 3.0 pipeline (Up-to-date bacterial core gene set [91]) was used to construct
318 maximum likelihood trees inferred using FastTree v2.10.1 and GTR + CAT. This was based on
319 a multiple alignment of a set of 92 universal and single copy gene sequences. The
320 phylogenomic tree confirms the results of the 16S rRNA trees and the positioning of 0127_4^T
321 as a member of the family *Caulobacteraceae* (Figure 3), with high Gene Support Index and
322 high bootstrap values. Matching the results of the 16S rRNA phylogeny, *Aquidulcibacter*
323 *paucihalophilus* TH1-2^T was the closest relative. In the 16S rRNA trees, members of the
324 *Caulobacter* genus form two paraphyletic groups, while this is not supported by the
325 phylogenomic tree.

326

327 The 16S rRNA gene sequence similarity between 0127_4^T and the closest relative
328 *Aquidulcibacter paucihalophilus* TH1-2^T was lower than 94.5%, the threshold proposed by
329 Yarza et al. [92] to differentiate bacterial genera. Based on our phylogenetic, morphological,
330 physiological, metabolic and chemotaxonomic comparison of strain 0127_4^T with its closest
331 phylogenetic neighbors this strain represents a new genus and species within the family
332 *Caulobacteraceae*, for which the name *Terricaulis silvestris* gen. nov., sp. nov. is proposed.
333

334 **Description of *Terricaulis* gen. nov.**

335 *Terricaulis* (Ter.ri.cau'lis. L. fem. n. *terra*, earth; L. masc. n. *caulis* stalk, referring to
336 prosthecae; N.L. masc. n. *Terricaulis* stalked organism isolated from soil). Gram-stain-
337 negative, motile by means of a polar flagellum, non-spore-forming, non-capsulated,
338 prosthecate short rods that divide by budding. Catalase negative and oxidase positive.
339 Aerobic, chemoorganotrophic, mesophile. The major respiratory quinone is Q-10. The major
340 fatty acids are summed feature 8 ($C_{18:1}$ $\omega 6c$/ $C_{18:1}$ $\omega 7c$), summed feature 9 (*iso*-$C_{17:1}$ $\omega 9c$/ $C_{16:0}$
341 10-methyl), $C_{16:0}$ and summed feature 3 ($C_{16:1}$ $\omega 6c$/ $C_{16:1}$ $\omega 7c$). 1,2-di-*O*-acyl-3-*O*-αD-
342 glucopyranuronosylglycerol and four unidentified glycolipids are the major polar lipids. The
343 type species is *Terricaulis silvestris*. The novel genus is classified as belonging to the family
344 *Caulobacteraceae*.
345

346 **Description of *Terricaulis silvestris* sp. nov.**

347 *Terricaulis silvestris* (sil.ves'tris. L. masc. adj. *silvestris* referring to the forest soil from which
348 the type strain was isolated).

349 Cells are 0.8 - 3.0 µm long and 0.4 - 1.0 µm wide. Colonies are white, circular, convex
350 with entire margins, reaching 1 - 2 mm in diameter. Grows at temperatures between 10 and
351 30°C (optimum 27°C) and at pH 6.1 - 8.3 (optimum 6.5 - 7.9). Under optimal growth
352 conditions doubling time is 26.1 h. The strain grows only in the absence of NaCl.

353 Uses galactose, lactate, pyruvate, succinate, glutamate, citrate, fumarate, serine,
354 histidine, ornithine, glycerol, peptone and β- hydroxybutyrate as sole carbon source for
355 growth. Weak growth is observed for arabinose, *N*-acetylglucosamine, aspartate, gluconate,
356 lysine, alanine, glutamine, isoleucine, proline, valine, dulcitol, casamino acids, casein
357 hydrolysate, yeast extract, fermented rumen extract, γ- hydroxybutyrate, isovaleric acid and
358 protocatechuate acid. No growth is observed on fructose, fucose, sorbose, lyxose, rhamnose,
359 glucose, lactose, cellobiose, mannose, melezitose, raffinose, maltose, sucrose, trehalose,
360 xylose, lactate, adenitol, arabitol, mannitol, myo-inositol, sorbitol, xylitol, hydroxyproline,
361 glycolate, malonate, propionate, oxaloacetate, butanol, ethanol, methanol, propanol,
362 caproate, caprylate, ethyleneglycol, erythrose, erythrulose, α-hydroxybutyrate, isocitrate,
363 laevulinate, arabinose, glucosamine, N-acetylgalactosamine, glucoronate, lyxitol, crotonate,
364 heptanoate, 2-oxoglutarate, acetoin, ascorbate, glyoxilate, 2-oxovalerate, 2-oxogluconate,
365 maleic acid, 1,2-butandiol, 2,3-butandiol, 1,2-propandiol, leucine, arginine, asparagine,
366 cysteine, benzoate, tryptophan, acetate, butyrate, formiate, isobutyrate, tyrosine,
367 phenylalanine, glycine, leucine, methionine, threonine, nicotinic acid, tween 80, laminarin,

368 adipate, shikimate, malate, tartrate, fermented rumen extract, heptanoate and
369 trimethoxybenzoate.

370 Tests positive for alkaline phosphatase and leucine arylamidase. No activities of the
371 enzymes esterase C4, esterase lipase C8, lipase, valine arylamidase, cysteine arylamidase,
372 trypsin, α-chymotrypsin, acid phosphatase, naphtol-AS-BI-phosphohydrolase, α-
373 galactosidase, β-galactosidase, β-glucoronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-
374 glucosaminidase, α-mannosidase and α-fucosidase are observed. Gelatin hydrolysis and β-
375 galactosidase are observed. Nitrate reduction to nitrite and/or nitrogen, indole production,
376 fermentation of glucose, arginine dihydrolase, urease, and aesculin hydrolysis are absent.

377 The type strain 0127_4^T (= DSM 104635^T = CECT 9243^T) was isolated from a temperate
378 beech forest soil close to Gomadingen, Baden-Württemberg, Germany. The DNA G+C
379 content of the type strain is 63.5%. The GenBank/EMBL/DDBJ accession numbers for the 16S
380 rRNA gene sequence and for the genome sequence of strain 0127_4^T are MN833493 and
381 CP047045, respectively.

382

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Conflict of Interest

The authors declare no existing conflict of interest.

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652

653

654 **Figures**

655

656 **Figure 1.** Morphology of 0127_4^T as seen by field emission scanning electron microscopy. A)
657 Aggregates of 0127_4^T displaying two or three prostheca. B) Forming daughter cells
658 displaying a single polar flagellum (marked by white arrow heads).

659

660 **Figure 2.** Maximum likelihood (ML) phylogenetic tree based on almost full length 16S rRNA
661 gene sequences, illustrating the phylogenetic position of *Terricaulis silvestris* 0127_4^T. The
662 best evolutionary model of nucleotide substitution applied was GTR+I+G. *Rhodobacter*
663 *capsulatus* ATH 2.3.1^T (D16428) was used as outgroup. Bar indicates 5% nucleotide
664 divergence. Only bootstrap values above 50% are indicated at the branches (1000 replicates).

665

666 **Figure 3.** Phylogenomic tree of strain 0127_4^T and members of the families
667 *Caulobacteraceae* and *Hyphomonadaceae*. Unrooted maximum likelihood tree inferred using
668 FastTree v2.10.1 and GTR + CAT model, which was based on a multiple alignment of a set of
669 92 gene (aminoacidic) sequences from using the UBCG v. 3.0 pipeline. Bootstrap analysis was
670 carried out using 100 replications. Gene support indices and percentage bootstrap values are
671 given at branching points. Bar, 0.3 substitution per position.

672 **Tables**

673

674 **Table 1.** Differential characteristics of strain 0127_4^T compared with the genera of the family

675 *Caulobacteraceae*.

676 Strains: 1, 0127_4^T; 2, *Aquidulcibacter paucihalophilus* TH1-2^T [4]; 3, *Asticcacaulis* [5–10]; 4,

677 *Brevundimonas* [11, 12, 22–31, 13, 32, 33, 64, 14–18, 20, 21]; 5, *Caulobacter* [3, 12, 19, 34–

678 40]; 6, *Phenylobacterium* [19, 41, 50–54, 42–49]

679 +, positive; -, negative; V, variable;

Features	**1**	**2**	**3**	**4**	**5**	**6**
Colony pigmentation	White	Pale yellow	White to light yellow	White to dark orange	White to yellow	White to orange
Reproduction	Budding	Binary fission	Binary fission	Binary fission	Binary fission	Binary fission
Flagellar motility	+	+	+	+*	V	V
Catalase	-	+	+	V	V	+**
Oxidase	+	+	+	V	V	V
Nitrate reduction	-	+	V	V	-***	V
Temperature range (°C)	10 - 30	20 - 40	4 - 37	2 - 50	4 - 40	10 - 50
(optimum)	(27)	(30)	(15 - 30)	(20 – 40)	(25 - 35)	(25 - 45)
pH range	6.1 - 8.3	5.5 - 8.5	4.5 - 10	5.5 - 11	5 - 10	5.5 - 9.5
(optimum)	(6.5 - 7.9)	(7.0)	(5 - 8)	(6.5 - 9)	(6.5 - 8)	(6.5 - 8)
DNA G+C content	63.6	55.6	57.8 - 63.7	61.8 - 70.3	62 - 70	65.5 - 72.3
Isolation source	Forest soil	Cyanobacterial aggregates	Soil, plant roots, freshwater, sediment	Soil, plant roots, freshwater, seawater, sludge, sediment, blood, leech, *Chlorella* culture, space laboratory	Soil, plant roots, freshwater, sludge	Soil, plant roots, freshwater, sludge, blood, compost

680 * With exception of *B. poindexterae* FWC40^T [23]

681 ** With exception of *P. aquaticum* W2-3-4^T [48] and *P. soli* LX32^T [53]

682 *** With exception of *C. profundus* DS48-5-2^T [36]

683

684 **Table 2.** Exoenzyme activities of strain 0127_4^T compared to the type species of all genera of
685 the family *Caulobacteraceae*. Data were generated in the present study andall strains were
686 obtained from the German Collection of Microorganisms and Cell Cultures.
687 Strains: 1, 0127_4^T; 2, *Aquidulcibacter paucihalophilus* TH1-2^T; 3, *Asticcacaulis excentricus* CB
688 48^T; 4, *Brevundimonas diminuta* AJ 2067^T; 5, *Caulobacter vibrioides* CB51^T; 6,
689 *Phenylobacterium immobile* E^T
690 +, positive; -, negative

Exoenzyme activity	**1**	**2**	**3**	**4**	**5**	**6**
Alkaline phosphatase	+	+	+	+	+	+
Esterase	-	+	-	+	-	+
Esterase lipase	-	+	+	+	-	-
Leucine arylamidase	+	+	+	+	+	+
Valine arylamidase	-	-	-	-	+	-
Cysteine arylamidase	-	-	-	-	+	-
Trypsin	-	+	+	+	+	-
α-chemotrypsin	-	-	-	+	-	-
Acid phosphatase	-	+	+	+	-	+
Naphtol-AS-BI-phosphohydrolase	-	+	+	+	-	+
α-galactosidase	-	-	+	-	-	-
β-galactosidase	-	+	+	-	+	-
α-glucosidase	-	-	-	-	-	+
β-glucosidase	-	+	+	-	+	-
N-acetyl-β-glucosaminidase	-	-	+	-	+	-

691

692 **Table 3.** Fatty acid and polar lipid profiles of strain 0127_4[T] compared with the type species
693 of all genera of the family *Caulobacteraceae*. Data were generated in the present study and
694 all strains were obtained from the German Collection of Microorganisms and Cell Cultures.
695 Only fatty acids that amount to >1% of all fatty acids in at least one strain are displayed.
696 Strains: 1, 0127_4[T]; 2, *Aquidulcibacter paucihalophilus* TH1-2[T]; 3, *Asticcacaulis excentricus* CB
697 48[T]; 4, *Brevundimonas diminuta* AJ 2067[T]; 5, *Caulobacter vibrioides* CB51[T]; 6,
698 *Phenylobacterium immobile* E[T]
699 +, positive; -, negative; tr, trace amount (<1%)

Features	**1**	**2**	**3**	**4**	**5**	**6**
Major fatty acids (%)						
$C_{10:0}$	-	1.2	tr	-	-	-
$C_{11:0}$	-	2.2	-	-	-	tr
$C_{12:0}$	-	-	-	-	tr	2.4
$C_{14:0}$	1.1	tr	tr	1.6	4.6	tr
$C_{16:0}$	15.9	7.1	20.4	23.4	14.1	11.6
$C_{17:0}$	2.1	11.4	1.2	tr	-	7.5
$C_{14:1}\ \omega 5c$	3.8	-	-	-	-	-
$C_{16:1}\ \omega 5c$	-	8.3	tr	-	tr	-
$C_{17:1}\ \omega 6c$	tr	13.4	1.5	tr	tr	3.0
$C_{17:1}\ \omega 8c$	1.2	7.6	tr	tr	tr	3.3
Iso-$C_{15:0}$	9.6	tr	-	-	tr	-
Iso-$C_{17:0}$	5.8	tr	-	-	-	-
$C_{16:1}$ 2-OH	-	-	-	-	5.9	-
$C_{12:0}$ 3-OH	-	-	tr	2.4	tr	tr
$C_{12:1}$ 3-OH	tr	2.9	3.9	tr	1.2	2.0
$C_{19:0}\ \omega 8c$ cyclo	-	-	-	4.3	-	2.4
$C_{18:1}\ \omega 7c$, 11-methyl	3.7	tr	2.4	-	1.0	tr
Summed feature 3 ($C_{16:1}\ \omega 6c$/ $C_{16:1}\ \omega 7c$)	12.1	14.25	11.9	4.0	27.1	2.2
Summed feature 4 (Iso-$C_{17:1}$ / anteiso B-$C_{17:1}$)	1.8	-	-	-	-	-
Summed feature 7 ($C_{19:1}\ \omega 6c$/ .846/19cy)	-	2.4	tr	-	-	-
Summed feature 8 ($C_{18:1}\ \omega 6c$/ $C_{18:1}\ \omega 7c$)	21.6	25.1	55.1	62.2	42.9	61.8
Summed feature 9 (*iso*-$C_{17:1}\ \omega 9c$/ $C_{16:0}$ 10-methyl)	17.7	-	-	-	tr	-
Major polar lipids						
Unidentified glycolipids	+	-	+	-	-	-
Phosphatidylglycerol	-	+	+	+	+	+
1,2-diacyl-3-*O*-[6'-phosphatidyl-αD-glucopyranosyl]glycerol	-	-	-	+	+	+
1,2-di-*O*-acyl-3-*O*-αD-glucopyranuronosylglycerol	+	+	+	+	+	+
1,2-di-*O*-acyl-3-*O*-[αD-glucopyranosyl]-sn-glycerol	-	+	+	+	+	+
1,2-di-*O*-acyl-3-*O*-[D-glucopyranosyl-(1→4)-α D-glucopyranuronosyl]glycerol	-	-	-	+	-	+

700