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1 **A novel sulfur-reducing bacterium isolated from a**
2 **pyrite-forming enrichment culture and taxonomic**
3 **revision of the family *Synergistaceae***

4
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23
24 **Keywords:**

25 Phylogenomics, branched cells, amino acids degradation, sulfur reduction, waste water

26
27 **Repositories:**

28 The GenBank accession numbers of the 16S rRNA gene and complete-genome sequences for
29 strain J.5.4.2-T.3.5.2^T are OM215197 and CP072943, respectively.

32 Abstract

33 A novel sulfur-reducing bacterium was isolated from a pyrite-forming enrichment culture
34 inoculated with sewage sludge from a wastewater treatment plant. Based on phylogenetic
35 data the strain J.5.4.2-T.3.5.2^T could be affiliated with the phylum *Synergistota*. Among type
36 strains of species with validly published names, the highest 16S rRNA gene sequence
37 identity value was found with *Aminiphilus circumscriptus* ILE-2^T (89.2%). Cells of the new
38 strain were Gram-negative, non-spore-forming, straight to slightly curved rods with tapered
39 ends. Motility is conferred by lateral flagella. True branching of cells is frequently observed.
40 Strictly anaerobic, asaccharolytic, fermentative metabolism with peptides and amino acids
41 as preferred substrates. Sulfur is required as external electron sink during fermentative
42 growth and reduced to sulfide. Major fermentation products are acetate and propionate.
43 The cellular fatty acid composition is dominated by unsaturated and branched fatty acids,
44 especially *iso*-C_{15:0}. Major polar lipids are phosphatidylglycerol, phosphatidylethanolamine
45 and distinct unidentified polar lipids. Respiratory lipoquinones are not produced. Based on
46 the obtained data we propose the novel species and genus *Aminithiophilus ramosus*,
47 represented by the type strain J.5.4.2-T.3.5.2^T (=DSM 107166^T = NBRC 114655^T) and the
48 novel family *Aminithiophilaceae* fam. nov. to accommodate the genus *Aminithiophilus*. In
49 addition, we suggest reclassifying certain members of the *Synergistaceae* into new families
50 to comply with current standards for the classification of higher taxa. Based on
51 phylogenomic data, the novel families *Acetomicrobiaceae* fam. nov., *Aminiphilaceae* fam.
52 nov., *Aminobacteriaceae* fam. nov., *Dethiosulfovibrionaceae* fam. nov. and *Thermovirgaceae*
53 fam. nov. are proposed.

54

55 INTRODUCTION

56 The family *Synergistaceae* is essentially represented by mesophilic to moderately
57 thermophilic, fastidious, anaerobic, non-spore-forming, Gram-stain-negative bacteria that
58 are often specialized in the degradation of short chain carboxylic acids or amino acids [1].
59 Bacteria belonging to this family are generally widespread in the environment, but in most
60 cases they do not reach high abundances and are difficult to isolate. Therefore, the diversity
61 within this group is incompletely covered by isolates. The major anoxic environments
62 colonized by these bacteria include sewage, oil production waters, and the intestine or oral
63 cavity of animals.

64 In the present study a novel *Synergistota* bacterium isolated from a pyrite-forming
65 enrichment culture is described. The enrichment culture was derived from sewage sludge
66 and has been maintained for more than 20 years in a freshwater mineral medium without
67 organic substrates. The main products were pyrite (FeS₂) and CH₄ which were formed from
68 FeS, H₂S, and CO₂ as sole substrates [2]. The community composition of this enrichment
69 culture was analysed by 16S rRNA gene clone libraries without any evidence for the

70 presence of bacteria affiliated with *Synergistota* [2], so that it is unclear if the novel strain
71 plays a role in the processes leading to pyrite formation. However, the results presented in
72 this study may provide some insights into the interplay among heterotrophic and
73 lithoautotrophic microorganisms that form the pyrite-forming mixed culture.

74 In addition, a taxonomic revision of the family *Synergistaceae* is suggested, since the
75 recent proposal of the family *Thermosynergistaceae* by Yang *et al.* (2021) led to a paraphyly
76 of this taxon in reconstructed trees based on 16S rRNA sequences and conserved proteins
77 [3].

78

79 ISOLATION AND ECOLOGY

80 Strain J.5.4.2-T.3.5.2^T was isolated from a pyrite-forming enrichment culture established in
81 1995 with sludge from a sewage treatment plant in Konstanz (Germany) [2]. A sample of the
82 enrichment culture was inoculated in DSMZ medium 503 supplemented with Tryptone
83 peptone (2.5 g l⁻¹) and polysulfide (equivalent to about 80 mg l⁻¹ sulfur). After an incubation
84 period of two weeks at 35°C, growth was obtained and large conspicuous bacteria showing
85 partially Y-shaped cells were observed. Subsequently, it became apparent that the
86 successive transfer of cells of this type required yeast extract, Tryptone peptone or
87 Casamino Acids in combination with polysulfide or sulfur. Therefore, DSMZ medium 503
88 supplemented with 2.0 g l⁻¹ Casamino acids and 10.0 g l⁻¹ sulfur was used for the purification
89 of a novel strain by using the dilution to extinction approach. Purity of the obtained isolate
90 was confirmed by absence of growth in anoxic complex media without sulfur, lacking of
91 colonies on aerobic agar plates and by direct sequencing of the amplified partial 16S rRNA
92 gene.

93 The determined 16S rRNA sequence of strain J.5.4.2-T.3.5.2^T was used to investigate
94 the occurrence of closely related bacteria in the environment. The most closely related
95 cultured strain L-13 has a 16S rRNA gene sequence identity of 99.9% (CP074691) and was
96 isolated from a methanogenic hydrocarbon-degrading enrichment culture obtained from oil
97 production water in China. The most closely related uncultured bacteria were detected in
98 brackish wetland sediments [4] and had identity values above 99% with the sequence of
99 J.5.4.2-T.3.5.2^T (MG367121, MG367097). Other uncultured strains with identity values
100 above 95% were detected in wastewater (e.g., MPOV01000070, AB175356), a biogas plant
101 (GU476614) and oil production water (e.g., JQ316631). In the SILVA SSU Parc database
102 v.138.1 [5], these sequences are assigned to a clade designated EBM-39, which includes a
103 total of 247 sequences. Within this group, 71% of all sequences can be associated with
104 produced water generated during oil and gas recovery, followed by wastewater (13%) and
105 unpolluted water and sediments with elevated salinity (10%). From these data, it can be
106 concluded that the bacteria of this group are specialized in anoxic aquatic niches, mostly
107 characterized by some kind of pollution. Unlike most other clades within the phylum

108 *Synergistota*, there are no representatives from animal intestine or the oral cavity in this
109 group.

110

111 PHYLOGENY

112 Phylogenetic trees based on 16S rRNA genes were reconstructed as outlined in [6] using an
113 alignment of 16S rRNA gene sequences included in the SILVA database SSU Ref NR 99
114 release 138.1 [5]. Calculations were limited to a selection of sequences comprising type
115 strains affiliated with the class *Synergistia* and several environmental sequences of
116 uncultured bacteria with a significant relationship to the novel strain J.5.4.2-T.3.5.2^T. As
117 shown in Fig. 1, the novel strain forms a separate branch within the order *Synergistales*
118 along with a group of uncultured environmental bacteria. The minimum 16S rRNA gene
119 identity value within this cluster is approximately 87%, which is consistent with the
120 proposed family threshold [7]. Due to the recent proposal of the family
121 *Thermosynergistaceae* [3] the family *Synergistaceae* has become paraphyletic, which
122 however could be solved by splitting this taxon in separate families corresponding to several
123 well-defined clades (color-coded in Fig. 1). The 16S rRNA gene sequence identity values of
124 the novel strain J.5.4.2-T.3.5.2^T with the most closely related type strains of the family
125 *Synergistaceae* were 89.2% for *Aminiphilus circumscriptus*, 88.3% for *Thermovirga lienii* and
126 87.5% for *Thermanaerovibrio acidaminovorans*. However, these type strains are located in
127 different phylogenetic groups and the association of *A. circumscriptus* with the EBM-39
128 clade is not supported by bootstrap values above 80%.

129 In addition, phylogenomic trees based on 120 concatenated conserved bacterial
130 proteins were reconstructed using the dataset provided by the GTDB-Tk v. 1.5.0 [8].
131 Maximum-likelihood and neighbor-joining trees were reconstructed using the IQ-tree web
132 server v. 1.6.12 [9] and the ARB software package v. 6.0.6 [10] with default settings. Average
133 nucleotide identity (ANI) and average amino acid identity (AAI) values between genomes
134 were calculated with online tools provided by the Environmental Microbial Genomics
135 Laboratory [11]. The genome of the closest related strain L-13 shares with strain J.5.4.2-
136 T.3.5.2^T an ANI value of 99.3% and an AAI value of 99.2%, which clearly indicate an
137 assignment of both strains to the same species according to current standards [12]. This is
138 also confirmed by a digital DNA-DNA hybridization value of 92.5% between both strains
139 which is well above the established limit of 70% for strains belonging to the same species
140 [13]. In contrast, the AAI value with the genome of the closest related type strain *A.*
141 *circumscriptus* DSM 16581^T is only 56.70%. The division of the family *Synergistaceae* into
142 separate clades is supported by the phylogenomic tree reconstruction with high bootstrap
143 confidence values (Fig. 2). It is therefore proposed to define the separate phylogenetic
144 groups as families, as suggested by the GTDB database release 06-RS202 [14]. However, the
145 formation of a monophyletic group including the new strain J.5.4.2-T.3.5.2^T and *A.*

146 *circumscriptus* has no significant bootstrap support, so that a separate family is proposed for
147 the EBM-39 clade so far represented only by the cultured strains J.5.4.2-T.3.5.2^T and L-13.

148

149 **MORPHOLOGY, PHYSIOLOGY AND CHEMOTAXONOMY**

150 Morphologic characteristics of the novel strain were observed by light and transmission
151 electron microscopy. The Gram-stain reaction was determined using the BD Difco Gram
152 stain kit and flagella were observed using the BD BBL Flagella Stain Droppers (Becton
153 Dickinson, Grenoble, France). To visualize cell structures by fluorescent staining, cell
154 suspensions were incubated with Fm1-43 (N-(3-triethylammoniumpropyl)-4-(4-
155 (dibutylamino)styryl)pyridinium dibromide) and DAPI (4',6-diamidino-2- phenylindole) at a
156 final concentration of 2 $\mu\text{g ml}^{-1}$ and 1 $\mu\text{g ml}^{-1}$, respectively. Cells were incubated for 10 min
157 at room temperature in the dark and were washed twice with 1 ml anoxic 1x PBS buffer
158 (centrifugation at 1,500 rcf for 3 min). For immobilization, cells were mounted on 1% (w/v)
159 agarose support in MatTek Glass Bottom Microwell Dishes (35 mm dish, 14 mm microwell
160 with #1.5 coverslip P35G-1.5-14-C). Widefield fluorescence micrographs were acquired
161 using DAPI (370/36-440/40) and GFP (485/20-525/30) filters. Fluorescence z stacks and
162 phase contrast images were taken using a Nikon N Plan Apochromat $\lambda \times 100/1.45$ oil
163 objective and the ORCA-Flash 4.0 Hamamatsu camera. Images were processed using the
164 NIS-elements imaging software V4.3 (Nikon) together with the 3D Landweber
165 Deconvolution algorithm (Z-step: 0.2 μm , spherical aberration: 0.2). For electron
166 microscopy, whole cells and thin-sections were prepared as described previously [15] and
167 examined with a FEI Tecnai G2 transmission electron microscope operating at 200 kV
168 (Thermo Fisher, Illkirch, France). The slightly curved, rod-shaped cells stained Gram-
169 negative, were 3-7 $\mu\text{m} \times 1 \mu\text{m}$ in size, and were motile. Endospores were not detected by
170 phase contrast microscopy. An interesting feature of the new strain was the branching of
171 cells, which was particularly noticeable in cultures within the stationary phase or under
172 suboptimal growth conditions (Fig. 3A). Motility was conferred by lateral flagella (Fig. 3B).
173 True branching of cells was confirmed by transmission electron microscopy of thin sections
174 (Fig. 3C) and fluorescence microscopy of cells stained with dyes specific for DNA and lipid
175 membranes (Supplementary Fig. S1). Cells of the newly isolated strain have a sheath-like
176 structure with distensions at both ends ("toga") as occurs in members of the phylum
177 *Thermotogota*. Thin sections viewed with a transmission electron microscope revealed a cell
178 wall structure typical of Gram-negative bacteria, characterized by a thin peptidoglycan layer,
179 an outer membrane layer, and an extended periplasmic space (Fig. 3D). The fluffy, loose
180 structure visible on the outer membrane could represent a proteinaceous surface layer. In
181 cells sampled in the stationary phase that showed an expanded periplasm, it was observed
182 that the proportion of lipids in the outer envelope-like structure decreased, indicating
183 possible degradation of the outer membrane, leaving only peptidoglycan and a
184 proteinaceous surface layer (Supplementary Fig. S2).

185 The characterization of metabolic traits was done according to the procedures
186 described previously by Ben Hania *et al.* [16]. For routine cultivation, the DSMZ medium 503
187 was supplemented with 2.0 g l⁻¹ Casamino acids, 0.2 g l⁻¹ yeast extract and 10.0 g l⁻¹
188 elemental sulfur. In addition, the amount of NaCl was increased to 10.0 g l⁻¹. The resulting
189 medium is referred to as 503f. The optimal growth conditions of J.5.4.2-T.3.5.2^T were 35°C,
190 pH 7.0 and 5-10 g l⁻¹ NaCl. The novel isolate had a strictly anaerobic metabolism based on
191 the fermentation of amino acids. Growth with Casamino acids as substrate required either
192 vitamins or yeast extract as supplements. However, with single amino acids as a substrate,
193 yeast extract was indispensable as a growth supplement. Although the strain was isolated
194 from freshwater, sodium ions were essential for growth and KCl could not be substituted for
195 NaCl. In addition to Casamino acids, the complex substrates Tryptone peptone and yeast
196 extract as well as the amino acids glutamine and glutamate were also fermented. The amino
197 acids arginine, histidine, and lysine have also been used, but to a lesser extent. The use of
198 multiple amino acids occurred without the involvement of a Stickland reaction. No growth
199 was detected with carboxylic acids, alcohols or carbohydrates as substrates. The main
200 fermentation products formed from complex substrates and amino acids were acetate and
201 propionate (up to 6 mM each product). The acetate/propionate ratio was 1.1 (± 0.3). In
202 addition, formate and isovalerate were found in small amounts (each ca. 0.5 mM) with
203 histidine as substrate. Fermentative growth was accompanied by the production of small
204 amounts of H₂. During growth, sulfur was reduced to H₂S. Irrespective of the substrate,
205 approx. 3-5 mM sulfide was detected at the end of growth. No growth was observed with
206 sulfite (2mM), thiosulfate (10 mM), sulfate (10 mM), nitrate (10 mM) or nitrite (2 mM) as
207 alternative electron sinks for fermentative growth. However, in the presence of a
208 *Methanospirillum* sp. isolated from the same pyrite-forming enrichment culture, growth was
209 also possible without sulfur as external electron sink. After the growth of the co-culture with
210 Casamino acids, the ratio of acetate to propionate increased to over 10 and methane was
211 formed in addition to hydrogen. Syntrophic growth was also possible with the individual
212 amino acids glutamate, glutamine and, to a lesser extent, histidine.

213 We performed a detailed chemotaxonomic characterization of the new isolate and
214 the most closely related reference strain *Aminiphilus circumscriptus* DSM 16581^T to provide
215 clues for the classification of the two strains. The preparation and identification of fatty acid
216 methyl esters (FAME) was performed according to standard protocols of the Microbial
217 Identification System, version 6.1 (MIDI Inc., Newark DE, USA; [17]). Compounds were
218 identified against the TSBA40 peak naming table database. The results for fatty acids were
219 confirmed by GC-MS, which also enabled to resolve summed features as previously
220 described [18]. Polar lipids were extracted from 130 mg freeze-dried material using a two-
221 stage method and identified by two-dimensional thin layer chromatography [19]. Analysis of
222 respiratory lipoquinones was done by HPLC coupled to a diode array detector and a mass
223 spectrometer as previously described [20].

224 The cellular fatty acid patterns of *A. circumscriptus* DSM 16581^T and J.5.4.2-T.3.5.2^T
225 were similar overall and are characterized by high proportions of branched and unsaturated
226 fatty acids (Table 1). However, the proportion of unsaturated fatty acids to total cellular
227 fatty acids is significantly higher in the type strain of *A. circumscriptus* than in the new
228 isolate. Furthermore, the absence of C_{15:0} in the cell membranes of DSM 16581^T represents a
229 marked difference in the fatty acid patterns of the two strains. On the other hand, the polar
230 lipid patterns of both strains were almost identical and characterized by large amounts of
231 phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) (Supplementary Fig. S3).
232 Interestingly, different derivatives of menaquinone-9 were detected in *A. circumscriptus*
233 DSM 16581^T (80.5% MK9H4, 10.8% MK9H2, 8.7% MK9), whereas the new isolate did not
234 contain respiratory lipoquinones, as expected. This may indicate that the metabolism in *A.*
235 *circumscriptus*, unlike J.5.4.2-T.3.5.2^T, is not strictly fermentative and involves membrane-
236 bound electron transport.

237 A summary of the phenotypic characterization of strain J.5.4.2-T.3.5.2^T and a
238 comparison with type strains representing different clades of *Synergistales* at the family
239 level is presented in Table 2.

240

241 GENOME FEATURES

242 Sequencing of the complete genome of J.5.4.2-T.3.5.2^T was performed using a hybrid
243 sequencing strategy that included long-read PacBio and short-read Illumina sequencing
244 technologies as previously reported [21]. The genome of strain J.5.4.2-T.3.5.2^T consists of a
245 single chromosome with a total sequence length of 3,108,337 bp and a G + C content of 64.0
246 mol%. It encodes a total of 2,787 genes, including 2,678 protein coding sequences and 67
247 RNA genes. Four sets of rRNA genes and two CRISPR arrays were detected. An analysis of
248 the gene content of the complete genome of the novel strain revealed the presence of
249 several key genes involved in the biosynthesis of a typical Gram-negative cell wall with an
250 outer membrane and periplasmic space. For example a putative lipid-A-biosynthesis
251 acyltransferase (KAR29_02520) and a lipid-A-disaccharide synthase (KAR29_09655)
252 participating in lipopolysaccharide synthesis were detected. The presence of a
253 proteinaceous surface layer in addition to the outer membrane is indicated by the detection
254 of a gene with a typical S-layer domain at the N-terminal end of the protein (KAR29_06040).
255 This gene has a significant similarity to the outer membrane protein alpha (OmpA1) of
256 *Thermotoga maritima* that is a major structural component of the toga characteristic for the
257 cells of this species [22]. Cluster of genes involved in the production of flagella (e.g.,
258 KAR29_09810 - 09945) and type IV pili (KAR29_07540 - 07605) conferring motility were also
259 found. The genome encodes FtsZ (KAR29_06730) and two copies of MreB (KAR29_08955
260 and KAR29_09280), which are usually involved in the binary fission of rod-shaped cells [23].
261 A special feature of the novel strain is the formation of cell branches. In *Escherichia coli* it

262 was shown that branching of cells correlates with an extended Z ring zone that allows
263 variable positions of the FtsZ ring at the centre of the cell. Multiple or mislocalized FtsZ rings
264 may then induce additional patches of inert peptidoglycan that cause a branching of cells
265 during subsequent cell growth [24]. It is likely that a similar mechanism also applies to strain
266 J.5.4.2-T.3.5.2^T. Branching of cells was induced experimentally in *E. coli* by the depletion of
267 low-molecular-weight penicillin-binding proteins like PBP5 [24]. Interestingly, homologous
268 genes encoding similar D,D-carboxypeptidases were not detected in the genome sequence
269 of the novel strain.

270 Strain J.5.4.2-T.3.5.2^T is specialized in the utilization of oligopeptides and amino acids
271 as substrates which are mainly transported into the cell by ABC-type transporters specific
272 for oligopeptides (TC 3.A.1.5.1), branched-chain amino acids (TC 3.A.1.4.1), glycine betaine
273 and methionine. A typical example of an operon encoding subunits of an oligopeptide
274 transporter is located at KAR29_01550 – 01570. In addition, genes for an arginine/ornithine
275 antiporter (KAR29_05695), serine/threonine exchanger (KAR29_11015) and a putative
276 sodium/proton-glutamate symporter (KAR29_01375) were detected.

277 Genes of at least four different putative aminopeptidases have been identified in the
278 genome (e.g., KAR29_04090) that may be involved in peptide degradation as a first step in
279 amino acid utilization. In the new strain, amino acid catabolism likely starts with an
280 oxidative deamination step catalysed by various aminotransferases coupled to a glutamate
281 dehydrogenase (KAR29_06585). Genes encoding a putative 2-oxoglutarate:ferredoxin
282 oxidoreductase were detected upstream of the glutamate dehydrogenase at KAR29_06565 -
283 KAR29_06580. The resulting succinyl-coenzyme A thioester is subsequently decarboxylated
284 to propanoyl-CoA, which can then be used to generate ATP via substrate-level
285 phosphorylation. Alternatively, 2-oxoglutarate may be reduced in this strain by reverse-
286 acting enzymes of the citric acid cycle yielding citrate, which could be cleaved into acetate
287 and oxaloacetate by a citrate lyase encoded at KAR29_04060 - KAR29_04070. Subsequently,
288 the produced oxaloacetate is probably decarboxylated to acetate via pyruvate enabling the
289 production of ATP via substrate-level phosphorylation. Besides substrate-level
290 phosphorylation, energy can probably be generated in this strain by membrane-bound
291 enzyme complexes. A RNF-type electron transport complex located at KAR29_13270 –
292 13295 transfers electrons from reduced ferredoxin to NAD⁺ and is probably involved in the
293 generation of a chemiosmotic potential by the translocation of proton or sodium ions across
294 the membrane. The dependence of the growth on NaCl indicates that essentially sodium
295 ions are used to build up a electrochemical potential. The membrane potential could then
296 be used by a V-type ATP synthase (KAR29_06605 - 06640) for the production of ATP. The
297 oxidation of ferredoxins might be also coupled to H₂ evolution by a NiFe hydrogenase 4 of
298 the Ech type (KAR29_13475 -13500) with concomitant H⁺/Na⁺ translocation [25].

299 Although sulfur is required as an electron sink during fermentative growth, no genes
300 for known enzymes involved in sulfur reduction could be detected in the genome sequence

301 of strain J.5.4.2-T.3.5.2^T. It could be that this role is fulfilled by an as yet undiscovered
302 protein or a bifunctional enzyme with only one annotated function.

303

304 **PROPOSAL OF NOVEL TAXA**

305 **DESCRIPTION OF AMINITHIOPHILUS GEN. NOV.**

306 *Aminithiophilus* (A.mi.ni.thi.o'phi.lus. N.L. neut. n. *aminum*, amine; Gr. neut. n. *theîon*,
307 sulfur; Gr. masc. adj. *philos*, loving; N.L. masc. n. *Aminithiophilus*, amine and sulfur loving).

308 Free-living, Gram-negative, non-spore-forming, straight to slightly curved rod-shaped cells
309 with tapered ends that are motile by flagella. Branching of cells is common, especially in
310 cultures in the stationary phase or under suboptimal growth conditions.

311 Chemoheterotrophic, mesophilic and neutrophilic. Strictly anaerobic, fermentative
312 metabolism with amino acids as preferred substrates. Sodium ions and vitamins are
313 required for growth. Tests for cytochrome *c* oxidase and catalase are negative. Sulfate,
314 nitrate or nitrite are not used as electron acceptors. Major fermentation products are
315 acetate and propionate. The cellular fatty acid composition is dominated by unsaturated
316 and branched fatty acids, especially *iso*-C_{15:0}. Major polar lipids are phosphatidylglycerol,
317 phosphatidylethanolamine and distinct unidentified polar lipids. Respiratory lipoquinones
318 are not produced.

319 The type species is *Aminithiophilus ramosus*.

320

321 **DESCRIPTION OF AMINITHIOPHILUS RAMOSUS SP. NOV.**

322 *Aminithiophilus ramosus* (ra.mo'sus. L. masc. adj. *ramosus*, ramifying, referring to the
323 morphology of the cells).

324 Shows the following characteristics in addition to those given for the genus. Most cells have
325 a diameter around 1 µm and a length ranging from 3 to 7 µm. Flagella are inserted laterally.
326 Optimal conditions for growth (range in brackets): 35°C (20-45°C), 5-10 g l⁻¹ NaCl (1-25 g l⁻¹)
327 and pH 7.0 (6.0-8.5). Utilization of single amino acids in defined media requires yeast extract
328 as growth supplement. The following carbon sources support fermentative growth: yeast
329 extract, Tryptone peptone, Casamino acids, histidine, arginine, lysine, glutamine, and
330 glutamate. The following compounds were tested, but not utilized: acetate, alanine,
331 arabinose, asparagine, aspartate, benzoate, butyrate, citrate, cysteine, ethanol, fructose,
332 fumarate, galactose, gelatine, glucose, glycerol, glycine, isoleucine, lactate, lactose, malate,
333 maltose, mannose, methanol, methionine, proline, propionate, pyruvate, ribose, serine,
334 starch, succinate, sucrose, thioglycolate, threonine, valine, and xylose. Sulfur is required as

335 external electron sink during fermentative growth and reduced to sulfide. No growth
336 occurred with sulfite, thiosulfate, sulfate, nitrite, or nitrate as an alternative electron
337 acceptor. No Stickland reaction was observed when multiple amino acids were used. In
338 addition to the fermentation products acetate and propionate, small amounts of H₂ were
339 detected. With histidine as substrate, small amounts of formate and isovalerate were also
340 produced. In addition to *iso*-C_{15:0}, the cellular fatty acids C_{19:1}, C_{13:0}, and C_{15:0} were produced
341 in significant amounts (>10 % of total amount). In addition to the major polar lipids listed in
342 the genus description, diphosphatidylglycerol, phosphatidylinositol, and several unidentified
343 glycolipids were present in small amounts.

344 The type strain is J.5.4.2-T.3.5.2^T (= DSM 107166^T = NBRC 114655^T), isolated from an
345 anaerobic enrichment culture initially inoculated with digested sewage sludge from a
346 sewage plant in Constance, Germany. The genome of the type strain is characterized by a
347 size of 3.11 Mb and a DNA G+C content of 64.0 mol%. The GenBank/EMBL/DDBJ accession
348 numbers for the 16S rRNA gene sequence and the complete genome sequence of strain
349 J.5.4.2-T.3.5.2^T are OM215197 and CP072943, respectively.

350

351 **DESCRIPTION OF AMINITHIOPHILACEAE FAM. NOV.**

352 *Aminithiophilaceae* (A.mi.ni.thi.o.phi.la.ce'ae. N.L. masc. n. *Aminithiophilus*, type genus of
353 the family; suff. -aceae, ending to denote a family; N.L. fem. pl. n. *Aminithiophilaceae*, the
354 family of the genus *Aminithiophilus*).

355 The description of the family is based on that given for the genus *Aminithiophilus*.
356 Distinguishing features from other families within the *Synergistota* include the formation of
357 branched cells and the dependence of growth on sulfur as an external electron acceptor.
358 Members of this group are typically found in anoxic aquatic niches, mostly characterized by
359 some kind of pollution. The affiliation of novel species to this family depends on the
360 phylogenetic position, which should be determined on the basis of comparative sequence
361 analyses of conserved proteins and 16S rRNA genes.

362 DNA G+C content (mol%): 64.0

363 The type genus is *Aminithiophilus*.

364

365 **DESCRIPTION OF ACETOMICROBIACEAE FAM. NOV.**

366 *Acetomicrobiaceae* (A.ce.to.mi.cro.bi.a.ce'ae. N.L. neut. n. *Acetomicrobium*, type genus of
367 the family; suff. -aceae, ending to denote a family; N.L. fem. pl. n. *Acetomicrobiaceae*, the
368 family of the genus *Acetomicrobium*).

369 The description of this family is based on the shared characteristics of members of the type
370 genus *Acetomicrobium* [3, 26–29]. Gram-negative, non-spore-forming, motile or non-motile
371 straight to slightly curved rods. Strictly anaerobic, chemoheterotrophic, moderately
372 thermophilic and neutrophilic. Preferred substrates for fermentation are carbohydrates and
373 carboxylic acids. The major fermentation product from carbohydrates is acetate. Thiosulfate
374 and sulfur are reduced to sulfide by some strains. Cellular fatty acid patterns are dominated
375 by *iso*-C_{15:0}. No respiratory lipoquinones were detected so far. Members of this group
376 typically occur in anoxic aquatic habitats often characterized by anthropogenic pollution.

377 DNA G+C content (mol%): 46.5 - 48.0 (genome analyses)

378 The type genus is *Acetomicrobium* Soutschek *et al.* 1985.

379

380 **DESCRIPTION OF AMINIPHILACEAE FAM. NOV.**

381 *Aminiphilaceae* (A.mi.ni.phi.la.ce'ae. N.L. masc. n. *Aminiphilus*, type genus of the family;
382 suff. *-aceae*, ending to denote a family; N.L. fem. pl. n. *Aminiphilaceae*, the family of the
383 genus *Aminiphilus*).

384 The description of this family is based on this study and the characterization of *Aminiphilus*
385 *circumscriptus* by Diaz *et al.* [30]. Gram-negative, non-spore-forming, motile, slightly curved
386 rods. Strictly anaerobic, chemoheterotrophic, mesophilic and neutrophilic. Preferred
387 substrates for fermentation are amino acids and certain carboxylic acids. Main fermentation
388 products are acetate and propionate. Nitrate, nitrite, sulfite, thiosulfate, sulfate and
389 elemental sulfur do not serve as electron acceptors. Cellular fatty acid patterns are
390 dominated by *iso*-C_{13:0} and C_{14:0}. Major polar lipids are phosphatidylethanolamine,
391 phosphatidylglycerol and phosphatidylinositol. The main respiratory lipoquinone is MK9H4.
392 Typically found in wastewater and sewage sludge.

393 DNA G+C content (mol%): 60.4 (genome analysis)

394 The type genus is *Aminiphilus* Díaz *et al.* 2007.

395

396 **DESCRIPTION OF AMINOBACTERIACEAE FAM. NOV.**

397 *Aminobacteriaceae* (A.mi.no.bac.te.ri.a.ce'ae. N.L. neut. n. *Aminobacterium*, type genus of
398 the family; suff. *-aceae*, ending to denote a family; N.L. fem. pl. n. *Aminobacteriaceae*, the
399 family of the genus *Aminobacterium*).

400 The description of this family is based on the shared characteristics of members of the
401 genera *Aminobacterium* [31–33], *Lactivibrio* [34], *Aminivibrio* [35] and *Fretibacterium* [36].
402 Gram-negative, non-spore-forming, motile or non-motile, straight or slightly curved rods.

403 Strictly anaerobic, chemoheterotrophic, mesophilic and neutrophilic. Preferred substrates
404 for fermentation are amino acids, carboxylic acids and in some strains carbohydrates. The
405 main fermentation product is acetate. External electron acceptors are not required for
406 growth. Cellular fatty acid patterns in most strains are dominated by *iso*-C_{13:0} and *iso*-C_{15:0}.
407 Mainly found in anoxic sludge associated with wastewater, soil and the oral cavity.

408 DNA G+C content (mol%): 43.8 - 61.7 (genome analyses)

409 The type genus is *Aminobacterium* Baena *et al.* 1999.

410

411 **DESCRIPTION OF *DETHIOSULFOVIBRIONACEAE* FAM NOV.**

412 *Dethiosulfovibrionaceae* (De.thi.o.sul.fo.vi.bri.o.na.ce'ae. N.L. masc. n. *Dethiosulfovibrio*,
413 type genus of the family; suff. *-aceae*, ending to denote a family; N.L. fem. pl. n.
414 *Dethiosulfovibrionaceae*, the family of the genus *Dethiosulfovibrio*).

415 The description of this family is based on the shared characteristics of members of the
416 genera *Dethiosulfovibrio* [37–39], *Jonquetella* [40], *Pyramidobacter* [41] and *Rarimicrobium*
417 [42]. Gram-negative, non-spore-forming, non-motile or motile by lateral flagella, straight or
418 slightly curved rods. Strictly anaerobic, chemoheterotrophic, asaccharolytic, mesophilic and
419 neutrophilic. Strains obtained from natural environments are moderately halophilic.
420 Preferred substrates for fermentation are amino acids and carboxylic acids. The main
421 fermentation product is acetate. External electron acceptors are not required for growth,
422 but sulfur and thiosulfate stimulate growth in some strains. Cellular fatty acid patterns are
423 dominated by either *iso*-C_{15:0} or saturated, straight-chain fatty acids. Inhabiting various
424 anoxic environments including oil wells, microbial mats, saline springs and human wounds.

425 DNA G+C content (mol%): 53.7 - 59.8 (genome analyses)

426 The type genus is *Dethiosulfovibrio* Magot *et al.* 1997.

427

428 **DESCRIPTION OF *THERMOVIRGACEAE* FAM. NOV.**

429 *Thermovirgaceae* (Ther.mo.vir.ga.ce'ae. N.L. fem. n. *Thermovirga*, type genus of the family;
430 suff. *-aceae*, ending to denote a family; N.L. fem. pl. n. *Thermovirgaceae*, the family of the
431 genus *Thermovirga*).

432 The description of this family is based on the characterization of *Thermovirga lienii* by Dahle
433 and Birkeland [43]. Gram-negative, non-spore-forming, motile, straight rods with the ability
434 to form large aggregates. Strictly anaerobic, chemoheterotrophic, neutrophilic, moderately
435 thermophilic and halophilic. Preferred substrates for fermentation are amino acids and
436 carboxylic acids. The main fermentation products are ethanol, acetate and propionate.

437 Sulfur and cystine are reduced to hydrogen sulfide. Originally isolated from production
438 water from an oil reservoir.

439 DNA G+C content (mol%): 47

440 The type genus is *Thermovirga* Dahle and Birkeland 2006.

441

442 **EMENDED DESCRIPTION OF THE FAMILY SYNERGISTACEAE**

443 The description is as given by Jumas-Bilak *et al.* [1] with the following emendations. The
444 range of DNA G+C content is 54.8 – 67.6 mol%. The genera *Acetomicrobium*, *Aminiphilus*,
445 *Aminivibrio*, *Aminobacterium*, *Dethiosulfovibrio*, *Fretibacterium*, *Jonquetella*, *Lactivibrio*,
446 *Pyramidobacter*, and *Thermovirga* were removed from the family.

447

448

449 **AUTHOR STATEMENTS**

450

451 **Conflicts of interest**

452 The authors declare that there are no conflicts of interest.

453

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457

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465

466

467 ABBREVIATIONS

468

469

470 SUPPLEMENTARY DATA

471 Three supplementary figures are available with the online version of this article.

472

473

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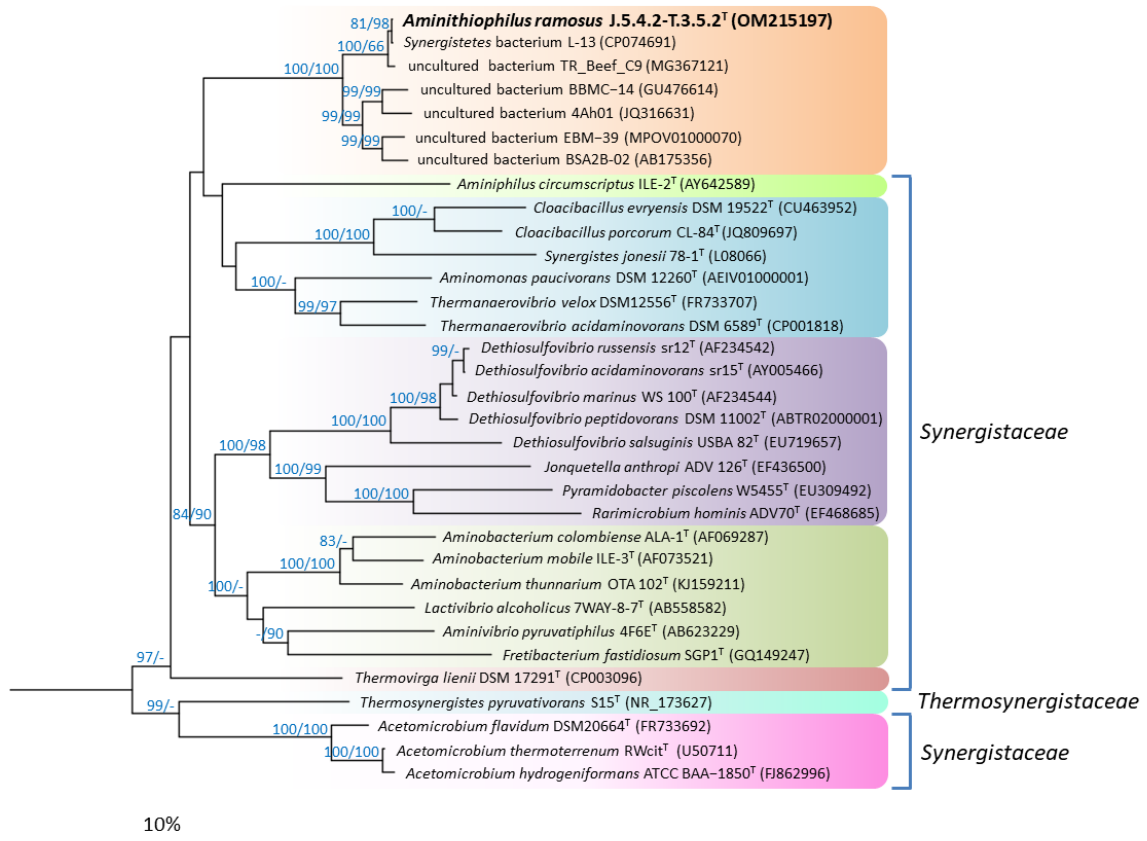
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616 **FIGURES AND TABLES**

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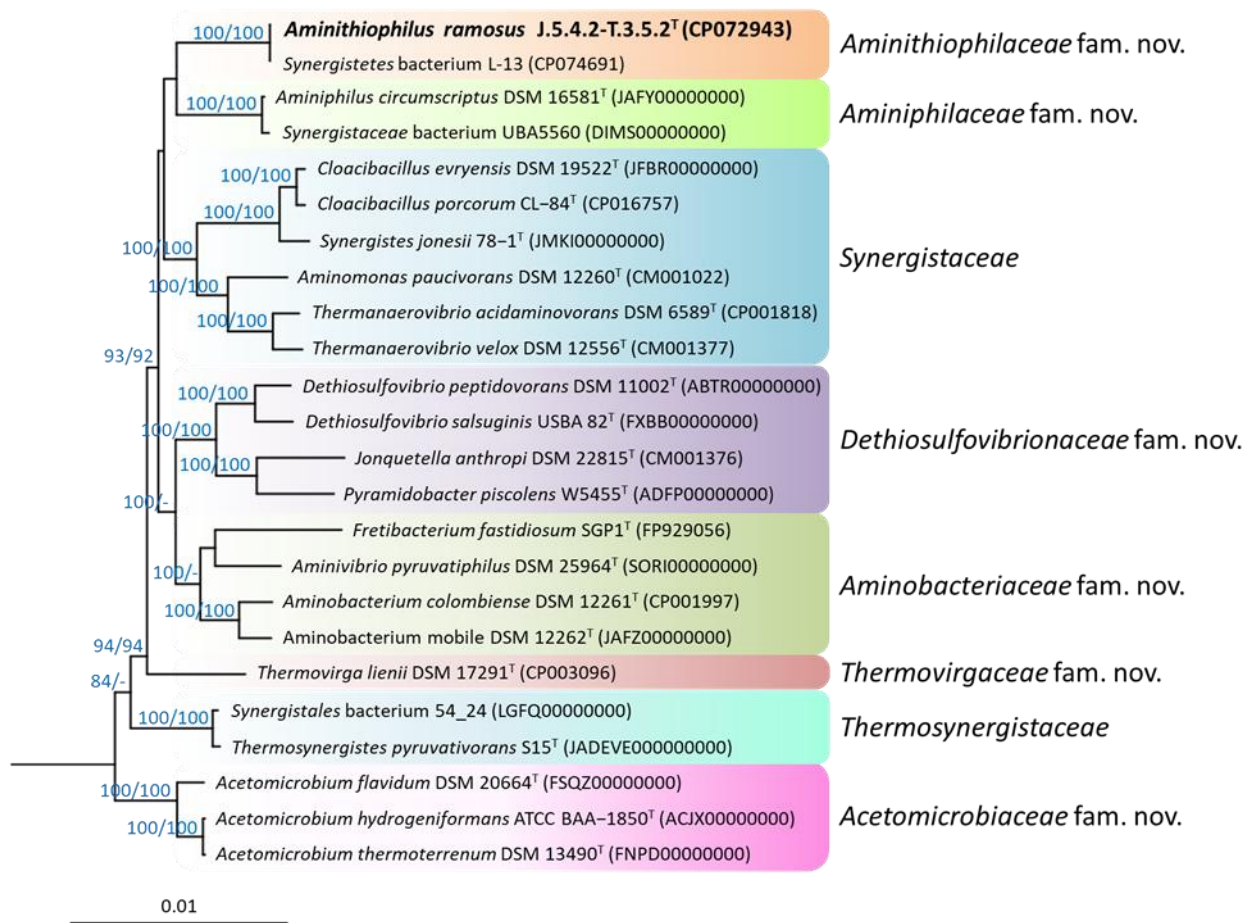
618

619 **Fig. 1.** Phylogenetic tree of the *Synergistales* based on 16S rRNA gene sequences showing the
620 placement of the novel strain J.5.4.2-T.3.5.2^T and the paraphyly of the family *Synergistaceae*.

621 The tree topology was reconstructed using the neighbor-joining method with the correction of Jukes
622 and Cantor as included in the ARB software package. It was rooted using the 16S rRNA gene
623 sequence of *Coprothermobacter proteolyticus* DSM 5265^T (CP001145, not shown), which was also
624 used as the outgroup. An alternative tree was reconstructed under the maximum-likelihood criterion
625 using RaxML v. 8.2.11 with the GTRGAMMA rate distribution model and the rapid bootstrap analysis
626 algorithm. Support of a distinct branching by bootstrap analyses is indicated by confidence values
627 obtained with the neighbor-joining or maximum-likelihood reconstruction, where the first number
628 corresponds to the neighbor-joining tree. Only values of 80% or above are shown. Distinct clades are
629 marked with different colour shades. Scale bar, 10% estimated sequence divergence.

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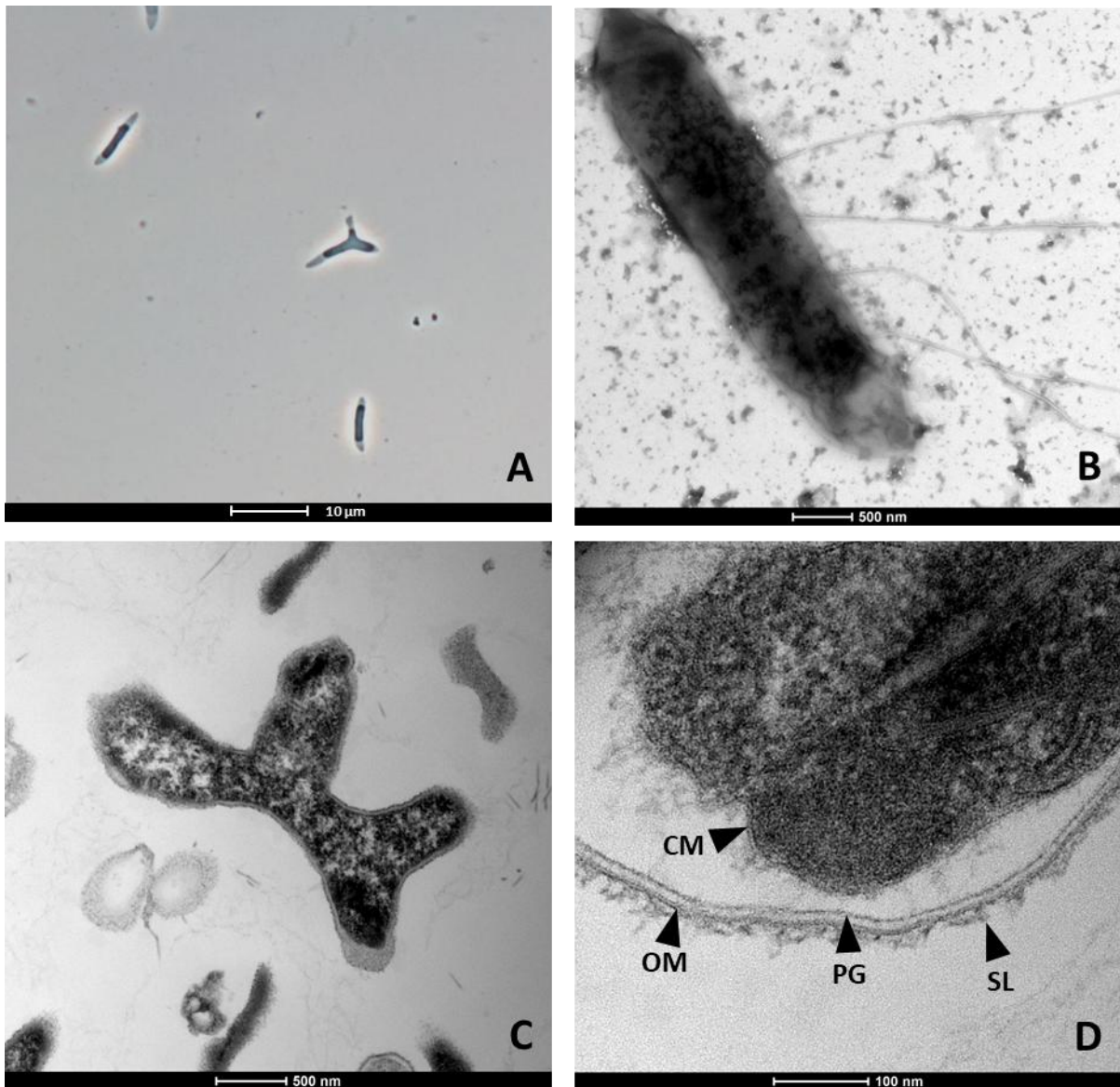
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634

635 **Fig. 2.** Phylogenomic tree of the phylum *Synergistota* based on 120 conserved bacterial proteins.

636 The shown tree was reconstructed under the maximum-likelihood criterion with the protein model
 637 LG+F+I+G4. The genome of *Coprothermobacter proteolyticus* DSM 5265^T (CP001145, not shown) was
 638 used as outgroup. Numbers at each node represent bootstrap values obtained with two different
 639 reconstruction methods. The first number corresponds to the maximum-likelihood IQ tree, while the
 640 second number represents bootstrap values (1000 samples) of an alternative tree reconstructed
 641 with the ARB software using a neighbor-joining algorithm with the PAM correction. Proposed novel
 642 families correspond with the monophyletic groups identified by the GTDB [44]. Scale bar, 0.01
 643 expected substitutions per site.

644



646

647

648 **Fig. 3.** Morphological characteristics of strain J.5.4.2-T.3.5.2^T.

649 (a) Phase contrast micrograph of a culture in the stationary phase with a Y-shaped cell; (b)
 650 transmission electron micrograph of a whole cell with lateral flagellation; (c, d) electron micrographs
 651 of thin sections showing the true branching of cells (c) and the atypical Gram-negative cell wall
 652 structure with cytoplasmic membrane (CM), extended periplasmic space, thin peptidoglycan layer
 653 (PG), outer membrane (OM) and surface layer (SL).

654

655

656

657 **Table 1.** Cellular fatty acid composition of the novel strain J.5.4.2-T.3.5.2^T compared to the related
 658 type strain *Aminiphilus circumscriptus* DSM 16581^T. Values are percentages of total fatty acids.
 659 Major fatty acids (>5% of total amount) are given in bold; fatty acids that were detected only in trace
 660 amounts (<1.0% of the total amount) in all samples are not shown. Abbreviations: -, not detected; *c*,
 661 *cis* isomer; dma, dimethyl acetal; *i*, *iso*-branched fatty acids; tr, trace amounts (<1.0% of the total
 662 amount).

Fatty acid	J.5.4.2-T.3.5.2 ^T	DSM 16581 ^T
<i>i</i> -C _{11:0}	9.2	5.4
C _{12:0}	-	3.7
<i>i</i> -C _{13:0}	7.9	16.4
C _{13:0}	10.7	tr
C _{14:0}	2.2	12.0
<i>i</i> -C _{13:0} 3OH	7.0	2.4
<i>i</i> -C _{15:0}	17.2	8.0
C _{15:1} c3	1.6	-
C _{15:0}	10.4	-
<i>i</i> -C _{15:0} DMA	1.1	-
C _{15:0} DMA	1.9	-
C _{16:1} c5	-	1.2
C _{16:0}	1.0	4.5
<i>i</i> -C _{17:0}	-	1.0
C _{17:1} c8	1.1	-
C _{17:0}	1.2	-
C _{18:1} c5	-	10.8
C _{18:1} c7	-	5.8
C _{18:1} c9	2.6	-
C _{18:1} c11	-	1.0
C _{18:0}	1.3	7.9
C _{19:1} c7	13.2	1.0
C _{19:0}	2.5	-
C _{20:1} c5	-	5.1
C _{20:1} c7	-	5.6
C _{20:1} c9	-	3.6
C _{20:1} c11	2.2	-

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665

666 **Table 2.** Differential characteristics of the novel strain J.5.4.2-T.3.5.2^T and type strains of type species
667 belonging to distinct family-level clades within the *Synergistota*. Strains and sources of data: 1,
668 *Aminithiophilus ramosus* gen. nov., sp. nov. J.5.4.2-T.3.5.2^T (this study); 2, *Aminiphilus circumscriptus*
669 ILE-2^T [30]; 3, *Synergistes jonesii* 78-1^T [45]; 4, *Dethiosulfovibrio peptidovorans* SEBR 4207^T [37, 40];
670 5, *Aminobacterium colombiense* ALA-1^T [31, 33]; 6, *Thermovirga lienii* Cas60314^T [43]; 7,
671 *Thermosynergistes pyruvativorans* S15^T [3]; 8, *Acetomicrobium flavidum* P^T [26, 27]. All strains shared
672 the following characteristics: Gram-negative, straight to slightly curved rod-shaped cells, non-
673 sporulating, strictly anaerobic, fermentative, yeast extract required for growth on single carbon
674 sources, sulfate is not used as electron acceptor. +, positive; -, negative; w, weak reaction; NR, not
675 reported; PE, phosphatidylethanolamine ; PG, phosphatidylglycerol; PI, phosphatidylinositol; L,
676 unidentified polar lipids; GL, unidentified glycolipids.

677

Characteristic	1	2	3	4	5	6	7	8
Isolation source	Sewage	Sewage	Goat rumen	Oil field	Sludge	Oil field	Oil field	Sewage
Size [μm]	3-7 x 1	2-4 x 1 ^a	1.2-1.8 x 0.6-0.8	3-5 x 1	3-4 x 0.2-0.3	2-3 x 0.4-0.8	1.4-3.0 x 0.4-0.8	2-3 x 0.8
Flagella	+, lateral	+, peritrichous	-	+, lateral	-	+	+	+, lateral
True branching	+	-	-	-	-	-	-	-
Temperature [$^{\circ}\text{C}$]								
Range	20-45	25-55	NR	20-45	20-42	37-68	30-65	35-65
Optimum	35	42	37	42	37	58	55	58
NaCl concentration [g l^{-1}]								
Range	1-25	0-25	NR	<110	0-15	5-80	0-30	0-40
Optimum	5-10	5	NR	30	0.5-5.0	20-30	10	0
pH								
Range	6.0-8.5	5.5-8.5	NR	5.5-8.8	6.6-8.5	6.2-8.5	6.0-8.5	6.2-8.0
Optimum	7.0	7.1	NR	7.0	7.3	6.5	6.5	7.0
Catalase	-	-	-	NR	NR	NR	NR	-
Oxidase	-	-	NR	NR	NR	NR	NR	NR
Electron acceptors								
Nitrate	-	-	-	NR	-	NR	-	NR
Nitrite	-	-	NR	NR	NR	NR	-	NR
Sulfite	-	-	NR	NR	-	NR	-	NR
Thiosulfate	-	-	NR	+	-	-	+	NR
Sulfur	+	-	NR	+	-	+	NR	NR
Electron donors								
Tryptone peptone	+	+	+	+	+	+	-	+
Casamino acids	+	+	+	+	+	+	-	-
Gelatine	+	-	-	-	-	NR	NR	-
Glycine	-	-	-	-	+	-	-	-
Alanine	-	+	-	+	-	+	-	-
Serine	-	-	-	+	+	+	-	-
Glutamate	+	+	-	+	-	+	-	-
Glutamine	+	+	-	NR	-	NR	NR	-
Histidine	w	+	+	+	-	NR	-	-
Arginine	w	+	+	+	-	+	-	-

Lysine	W	+	-	-	-	NR	-	-
Methionine	-	+	-	+	-	NR	NR	-
Threonine	-	-	-	-	+	-	NR	-
Aspartate	-	-	-	NR	-	NR	-	-
Valine	-	+	-	+	-	+	-	-
Isoleucine	-	+	-	+	-	+	NR	-
Ethanol	-	-	NR	NR	-	-	-	-
Glycerol	-	+	NR	NR	-	-	-	+
Acetate	-	-	NR	-	-	-	-	NR
Pyruvate	-	+	NR	NR	+	+	+	-
Lactate	-	-	-	-	-	-	-	-
Propionate	-	-	NR	-	-	-	-	NR
Butyrate	-	-	NR	-	-	NR	-	NR
Malate	-	+	NR	NR	-	-	-	-
Fumarate	-	+	NR	NR	-	-	-	-
Glucose	-	-	-	-	-	-	-	+
Maltose	-	-	-	-	-	-	-	+
Mannose	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-
Major fermentation products	Acetate, propionate	Acetate, propionate	Acetate, propionate	Acetate	Acetate	Ethanol, acetate, propionate	Acetate	Acetate
Respiratory quinones	None	MK9H4, MK9H2, MK9	NR	NR	NR	NR	None	NR
Major polar lipids	PG, PE, L	PE, PG, L, PI	NR	NR	NR	NR	GL, PE	NR
Major cellular fatty acids (>10% of total)	<i>i</i> -C _{15:0} , C _{19:1} <i>c</i> 7, C _{13:0} , C _{15:0}	<i>i</i> -C _{13:0} , C _{14:0} , C _{18:1} <i>c</i> 5	C _{17:0} , C _{20:0} cyclo	<i>i</i> -C _{15:0}	<i>i</i> -C _{15:0}	NR	<i>i</i> -C _{15:0}	<i>i</i> -C _{15:0}
Genome size [Mb]	3.11	3.22	2.74	2.58	1.98	2.00	2.44	1.94
DNA G+C content [mol%] ^b	64.0	60.4	56.3	54.4	45.3	47.1	52.2	47.9

678 ^a Result obtained in this study

679 ^b Determined from the genome sequence