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

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## **Keywords:**

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

## **Repositories:**

The GenBank accession numbers of the 16S rRNA gene and complete-genome sequences for strain J.5.4.2-T.3.5.2<sup>T</sup> are OM215197 and CP072943, respectively.

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## Abstract

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

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## INTRODUCTION

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

In the present study a novel *Synergistota* bacterium isolated from a pyrite-forming enrichment culture is described. The enrichment culture was derived from sewage sludge and has been maintained for more than 20 years in a freshwater mineral medium without organic substrates. The main products were pyrite (FeS<sub>2</sub>) and CH<sub>4</sub> which were formed from FeS, H<sub>2</sub>S, and CO<sub>2</sub> as sole substrates [2]. The community composition of this enrichment culture was analysed by 16S rRNA gene clone libraries without any evidence for the

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

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

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## ISOLATION AND ECOLOGY

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

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

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

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## PHYLOGENY

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

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

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

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## MORPHOLOGY, PHYSIOLOGY AND CHEMOTAXONOMY

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

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

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



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

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

## GENOME FEATURES

Sequencing of the complete genome of J.5.4.2-T.3.5.2<sup>T</sup> was performed using a hybrid sequencing strategy that included long-read PacBio and short-read Illumina sequencing technologies as previously reported [21]. The genome of strain J.5.4.2-T.3.5.2<sup>T</sup> consists of a single chromosome with a total sequence length of 3,108,337 bp and a G + C content of 64.0 mol%. It encodes a total of 2,787 genes, including 2,678 protein coding sequences and 67 RNA genes. Four sets of rRNA genes and two CRISPR arrays were detected. An analysis of the gene content of the complete genome of the novel strain revealed the presence of several key genes involved in the biosynthesis of a typical Gram-negative cell wall with an outer membrane and periplasmic space. For example a putative lipid-A-biosynthesis acyltransferase (KAR29\_02520) and a lipid-A-disaccharide synthase (KAR29\_09655) participating in lipopolysaccharide synthesis were detected. The presence of a proteinaceous surface layer in addition to the outer membrane is indicated by the detection of a gene with a typical S-layer domain at the N-terminal end of the protein (KAR29\_06040). This gene has a significant similarity to the outer membrane protein alpha (OmpA1) of *Thermotoga maritima* that is a major structural component of the toga characteristic for the cells of this species [22]. Cluster of genes involved in the production of flagella (e.g., KAR29\_09810 - 09945) and type IV pili (KAR29\_07540 - 07605) conferring motility were also found. The genome encodes FtsZ (KAR29\_06730) and two copies of MreB (KAR29\_08955 and KAR29\_09280), which are usually involved in the binary fission of rod-shaped cells [23]. A special feature of the novel strain is the formation of cell branches. In *Escherichia coli* it



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

Strain J.5.4.2-T.3.5.2<sup>T</sup> is specialized in the utilization of oligopeptides and amino acids as substrates which are mainly transported into the cell by ABC-type transporters specific for oligopeptides (TC 3.A.1.5.1), branched-chain amino acids (TC 3.A.1.4.1), glycine betaine and methionine. A typical example of an operon encoding subunits of an oligopeptide transporter is located at KAR29\_01550 – 01570. In addition, genes for an arginine/ornithine antiporter (KAR29\_05695), serine/threonine exchanger (KAR29\_11015) and a putative sodium/proton-glutamate symporter (KAR29\_01375) were detected.

Genes of at least four different putative aminopeptidases have been identified in the genome (e.g., KAR29\_04090) that may be involved in peptide degradation as a first step in amino acid utilization. In the new strain, amino acid catabolism likely starts with an oxidative deamination step catalysed by various aminotransferases coupled to a glutamate dehydrogenase (KAR29\_06585). Genes encoding a putative 2-oxoglutarate:ferredoxin oxidoreductase were detected upstream of the glutamate dehydrogenase at KAR29\_06565 - KAR29\_06580. The resulting succinyl-coenzyme A thioester is subsequently decarboxylated to propanoyl-CoA, which can then be used to generate ATP via substrate-level phosphorylation. Alternatively, 2-oxoglutarate may be reduced in this strain by reverse-acting enzymes of the citric acid cycle yielding citrate, which could be cleaved into acetate and oxaloacetate by a citrate lyase encoded at KAR29\_04060 - KAR29\_04070. Subsequently, the produced oxaloacetate is probably decarboxylated to acetate via pyruvate enabling the production of ATP via substrate-level phosphorylation. Besides substrate-level phosphorylation, energy can probably be generated in this strain by membrane-bound enzyme complexes. A RNF-type electron transport complex located at KAR29\_13270 – 13295 transfers electrons from reduced ferredoxin to NAD<sup>+</sup> and is probably involved in the generation of a chemiosmotic potential by the translocation of proton or sodium ions across the membrane. The dependence of the growth on NaCl indicates that essentially sodium ions are used to build up a electrochemical potential. The membrane potential could then be used by a V-type ATP synthase (KAR29\_06605 - 06640) for the production of ATP. The oxidation of ferredoxins might be also coupled to H<sub>2</sub> evolution by a NiFe hydrogenase 4 of the Ech type (KAR29\_13475 -13500) with concomitant H<sup>+</sup>/Na<sup>+</sup> translocation [25].

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

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

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## PROPOSAL OF NOVEL TAXA

### DESCRIPTION OF *AMINITHIOPHILUS* GEN. NOV.

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

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

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

The type species is *Aminithiophilus ramosus*.

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

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

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

external electron sink during fermentative growth and reduced to sulfide. No growth occurred with sulfite, thiosulfate, sulfate, nitrite, or nitrate as an alternative electron acceptor. No Stickland reaction was observed when multiple amino acids were used. In addition to the fermentation products acetate and propionate, small amounts of H<sub>2</sub> were detected. With histidine as substrate, small amounts of formate and isovalerate were also produced. In addition to *iso*-C<sub>15:0</sub>, the cellular fatty acids C<sub>19:1</sub>, C<sub>13:0</sub>, and C<sub>15:0</sub> were produced in significant amounts (>10 % of total amount). In addition to the major polar lipids listed in the genus description, diphosphatidylglycerol, phosphatidylinositol, and several unidentified glycolipids were present in small amounts.

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

#### **DESCRIPTION OF AMINITHIOPHILACEAE FAM. NOV.**

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

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

DNA G+C content (mol%): 64.0

The type genus is *Aminithiophilus*.

#### **DESCRIPTION OF ACETOMICROBIACEAE FAM. NOV.**

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

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

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

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

## **DESCRIPTION OF AMINIPHILACEAE FAM. NOV.**

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

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

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

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

## **DESCRIPTION OF AMINOBACTERIACEAE FAM. NOV.**

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

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

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

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

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

## **DESCRIPTION OF *DETHIOSULFOVIBRIONACEAE* FAM NOV.**

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

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

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

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

## **DESCRIPTION OF *THERMOVIRGACEAE* FAM. NOV.**

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

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

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

DNA G+C content (mol%): 47

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

## EMENDED DESCRIPTION OF THE FAMILY SYNERGISTACEAE

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

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## AUTHOR STATEMENTS

### Conflicts of interest

The authors declare that there are no conflicts of interest.

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### 1.2 Acknowledgements

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## ABBREVIATIONS

## SUPPLEMENTARY DATA

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

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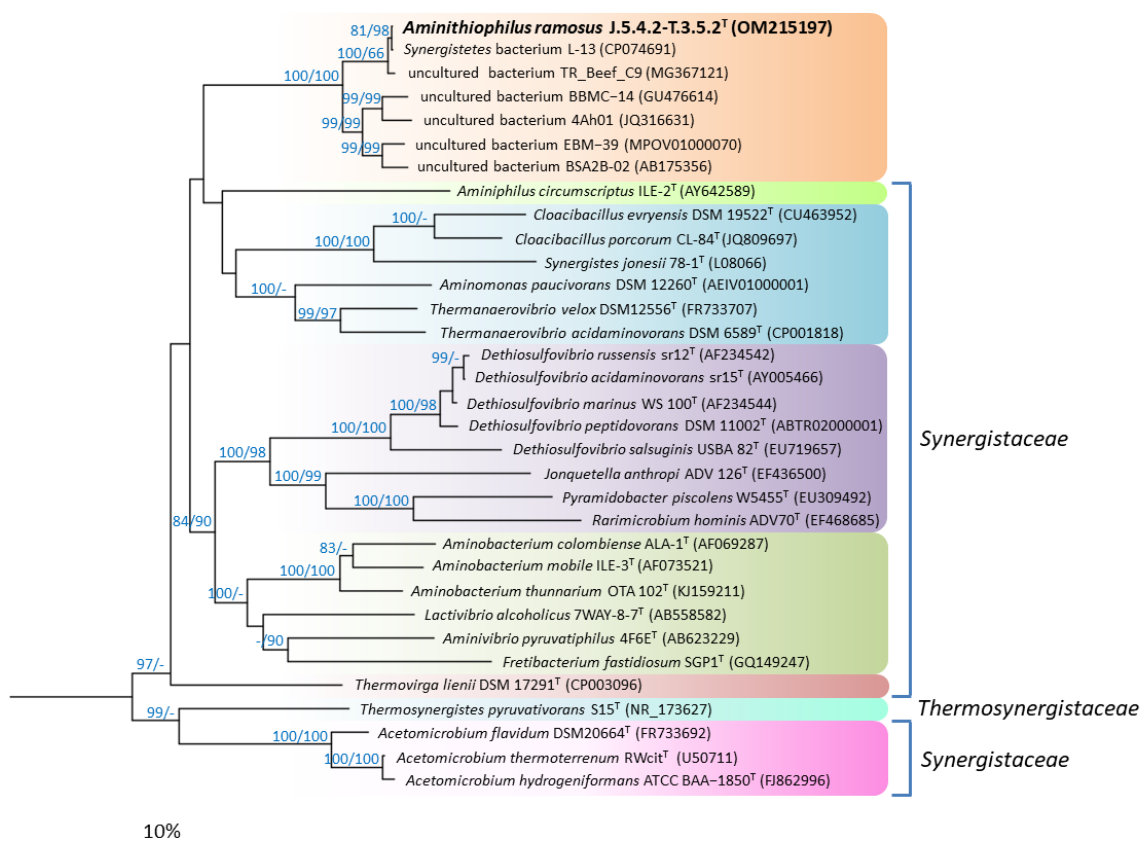
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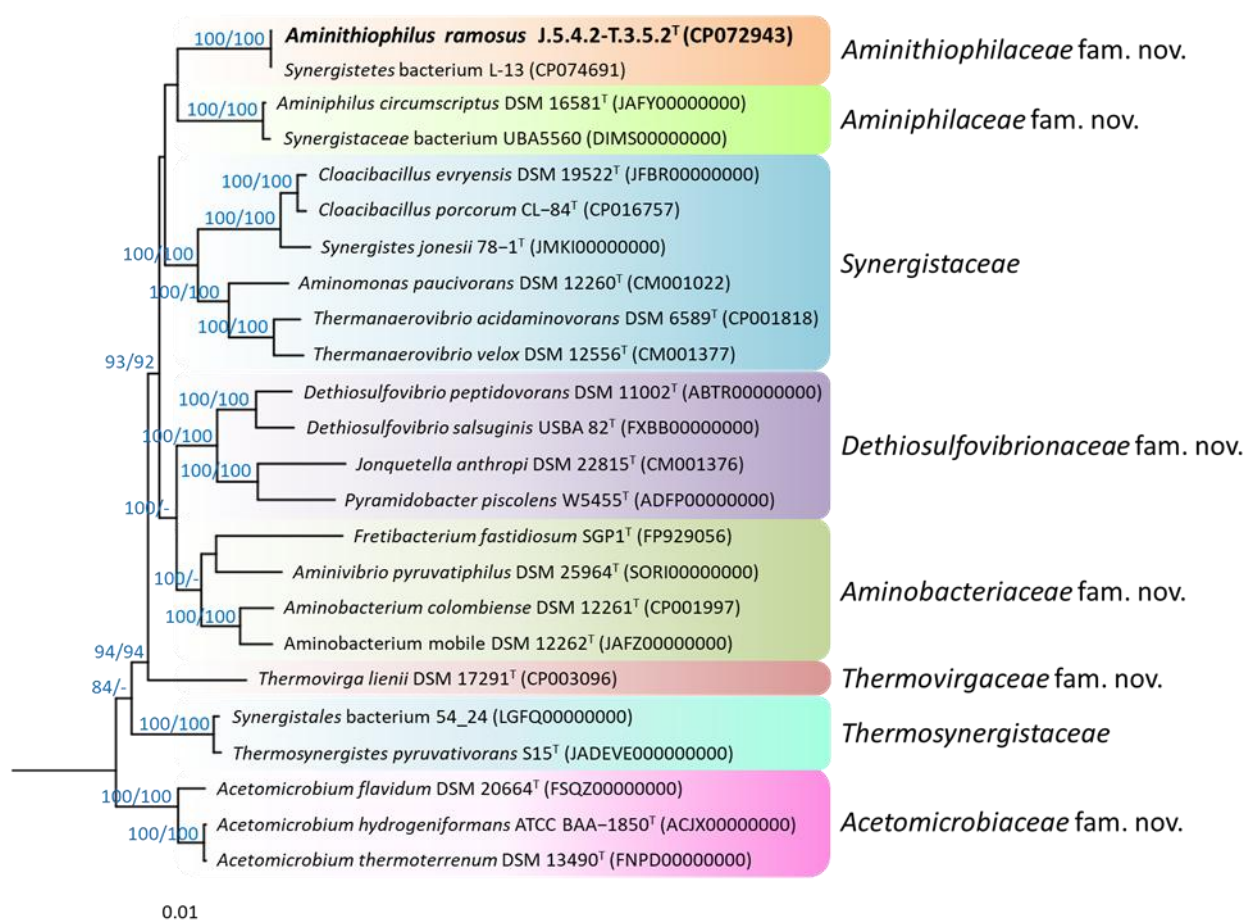
## FIGURES AND TABLES



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

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

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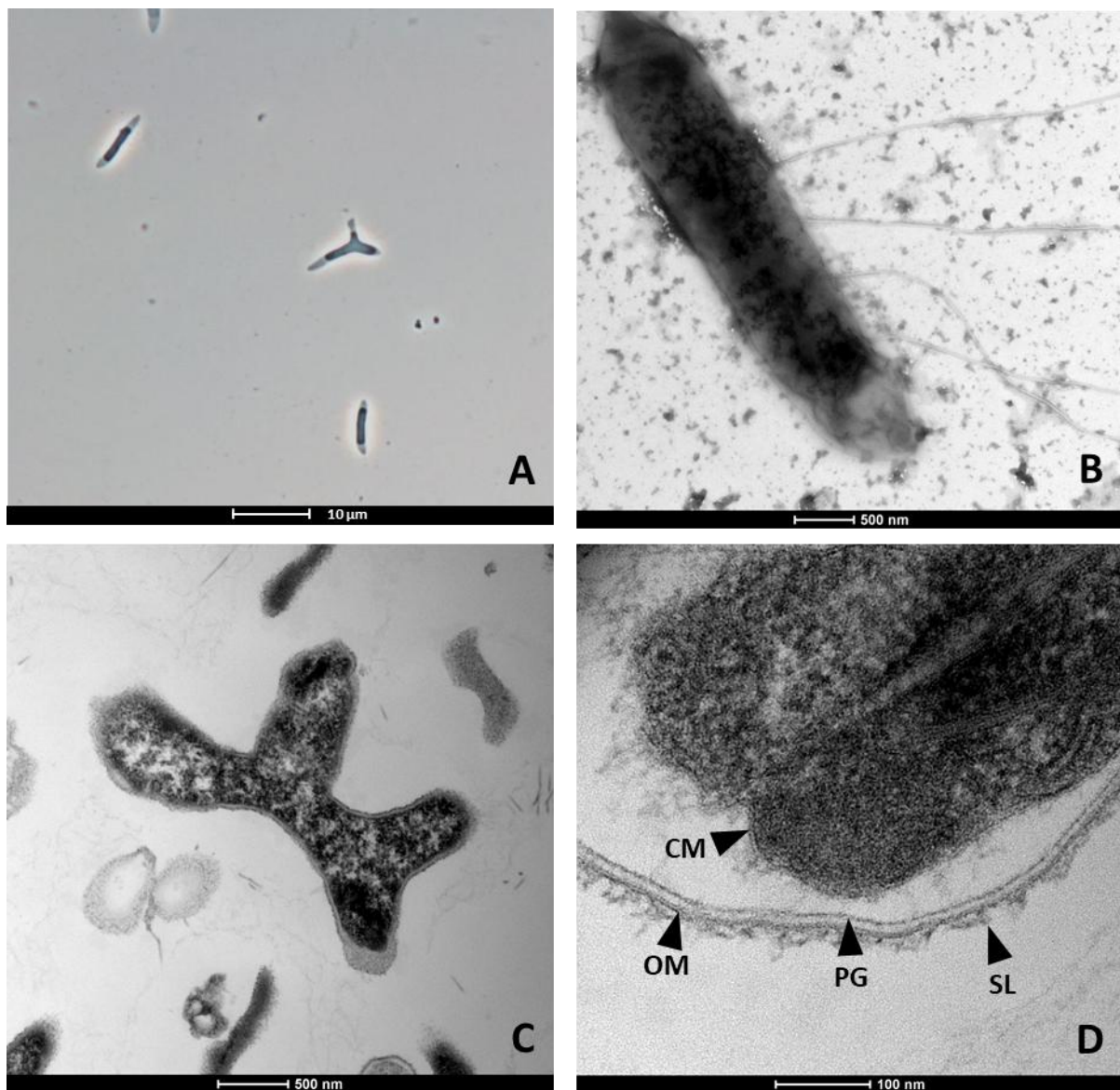
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635 **Fig. 2.** Phylogenomic tree of the phylum *Synergistota* based on 120 conserved bacterial proteins.

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<sup>T</sup> (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<sup>T</sup>.

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<sup>T</sup> compared to the related  
 658 type strain *Aminiphilus circumscriptus* DSM 16581<sup>T</sup>. 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 <sup>T</sup>	DSM 16581 <sup>T</sup>
<i>i</i> -C <sub>11:0</sub>	<b>9.2</b>	<b>5.4</b>
C <sub>12:0</sub>	-	3.7
<i>i</i> -C <sub>13:0</sub>	<b>7.9</b>	<b>16.4</b>
C <sub>13:0</sub>	<b>10.7</b>	tr
C <sub>14:0</sub>	2.2	<b>12.0</b>
<i>i</i> -C <sub>13:0</sub> 3OH	<b>7.0</b>	2.4
<i>i</i> -C <sub>15:0</sub>	<b>17.2</b>	<b>8.0</b>
C <sub>15:1</sub> <i>c</i> 3	1.6	-
C <sub>15:0</sub>	<b>10.4</b>	-
<i>i</i> -C <sub>15:0</sub> DMA	1.1	-
C <sub>15:0</sub> DMA	1.9	-
C <sub>16:1</sub> <i>c</i> 5	-	1.2
C <sub>16:0</sub>	1.0	4.5
<i>i</i> -C <sub>17:0</sub>	-	1.0
C <sub>17:1</sub> <i>c</i> 8	1.1	-
C <sub>17:0</sub>	1.2	-
C <sub>18:1</sub> <i>c</i> 5	-	<b>10.8</b>
C <sub>18:1</sub> <i>c</i> 7	-	<b>5.8</b>
C <sub>18:1</sub> <i>c</i> 9	2.6	-
C <sub>18:1</sub> <i>c</i> 11	-	1.0
C <sub>18:0</sub>	1.3	<b>7.9</b>
C <sub>19:1</sub> <i>c</i> 7	<b>13.2</b>	1.0
C <sub>19:0</sub>	2.5	-
C <sub>20:1</sub> <i>c</i> 5	-	<b>5.1</b>
C <sub>20:1</sub> <i>c</i> 7	-	<b>5.6</b>
C <sub>20:1</sub> <i>c</i> 9	-	3.6
C <sub>20:1</sub> <i>c</i> 11	2.2	-

663

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665

666 **Table 2.** Differential characteristics of the novel strain J.5.4.2-T.3.5.2<sup>T</sup> 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<sup>T</sup> (this study); 2, *Aminiphilus circumscriptus*  
669 ILE-2<sup>T</sup> [30]; 3, *Synergistes jonesii* 78-1<sup>T</sup> [45]; 4, *Dethiosulfovibrio peptidovorans* SEBR 4207<sup>T</sup> [37, 40];  
670 5, *Aminobacterium colombiense* ALA-1<sup>T</sup> [31, 33]; 6, *Thermovirga lienii* Cas60314<sup>T</sup> [43]; 7,  
671 *Thermosynergistes pyruvativorans* S15<sup>T</sup> [3]; 8, *Acetomicrobium flavidum* P<sup>T</sup> [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 [ $\mu\text{m}$ ]	3-7 x 1	2-4 x 1 <sup>a</sup>	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 [ $\text{g l}^{-1}$ ]								
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 <sub>15:0</sub> , C <sub>19:1</sub> <i>c</i> 7, C <sub>13:0</sub> , C <sub>15:0</sub>	<i>i</i> -C <sub>13:0</sub> , C <sub>14:0</sub> , C <sub>18:1</sub> <i>c</i> 5	C <sub>17:0</sub> , C <sub>20:0</sub> cyclo	<i>i</i> -C <sub>15:0</sub>	<i>i</i> -C <sub>15:0</sub>	NR	<i>i</i> -C <sub>15:0</sub>	<i>i</i> -C <sub>15:0</sub>
Genome size [Mb]	3.11	3.22	2.74	2.58	1.98	2.00	2.44	1.94
DNA G+C content [mol%] <sup>b</sup>	64.0	60.4	56.3	54.4	45.3	47.1	52.2	47.9

678 <sup>a</sup> Result obtained in this study

679 <sup>b</sup> Determined from the genome sequence