INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION

Flaiz *et al.*, *Int. J. Syst. Evol. Microbiol.* 2020;70:4269–4279

DOI 10.1099/ijsem.0.004283

MICROBIOLOGY SOCIETY

Caproicibacter fermentans gen. nov., sp. nov., a new caproate-producing bacterium and emended description of the genus *Caproiciproducens*

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Abstract

A strictly anaerobic bacterial strain designated EA1^T was isolated from an enrichment culture inoculated with biogas reactor content. Cells of strain EA1^T are spore-forming rods (1–3×0.4–0.8 µm) and stain Gram-negative, albeit they possess a Gram-positive type of cell-wall ultrastructure. Growth of strain EA1^T was observed at 30 and 37 °C and within a pH range of pH 5–9. The major components recovered in the fatty acid fraction were $C_{14:0}$, $C_{16:0}$, $C_{16:0}$ DMA (dimethyl acetal) and $C_{16:1}$ $\omega7c$. Strain EA1^T fermented several mono- and disaccharides. Metabolic end products from fructose were acetate, butyrate, caproate and lactate. Furthermore, ethanol, CO_2 and H_2 were identified as products. The genome consists of a chromosome (3.9 Mbp) with 3797 predicted protein-encoding genes and a G+C content of 51.25 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain EA1^T represents a novel taxon within the family *Oscillospiraceae*. The most closely related type strains of EA1^T, based on 16S rRNA gene sequence identity, are *Caproiciproducens galactitolivorans* BS-1^T (94.9 %), [*Clostridium*] *leptum* DSM 753^T (93.8 %), [*Clostridium*] *sporosphaeroides* DSM 1294^T (91.7 %) and *Ruminococcus bromii* ATCC 27255^T (91.0 %). Further phenotypic characteristics of strain EA1^T differentiate it from related, validly described bacterial species. Strain EA1^T represents a novel genus and novel species within the family *Oscillospiraceae*. The proposed name is *Caproicibacter fermentans* gen. nov., sp. nov. The type strain is EA1^T (DSM 107079^T=JCM 33110^T).

At present, only a limited number of bacterial species have been reported to be capable of caproate formation through chain elongation via reverse β-oxidation (for a review see [1]). For example, caproate is produced by *Clostridium kluyveri* [2] from acetate and ethanol, by *Caproiciproducens galactitolivorans* [3], by *Megasphaera elsdenii* [4, 5] as well as *Megasphaera cerevisiae* [6] from sugars. A strain referred to as '*Ruminococcaceae* bacterium CPB6' uses lactate [7] whereas *Eubacterium limosum* [8, 9] and *Clostridium carboxidivorans* [10, 11] can use C1-compounds such as methanol or carbon monoxide to produce caproate. Caproate fermentation has been known ever since the discovery of *C. kluyveri* enriched by H. A. Barker in 1937 in Delft, the Netherlands [12]. The initial substrate for growth of caproate-producing bacteria can vary, but it is accepted that chain elongation starts with the central intermediate acetyl-coenzyme A (CoA) as demonstrated for butyrate formation in *C. kluyveri* [13]. In a first cycle, two molecules of acetyl-CoA are condensed via a thiolase (Thl) forming acetoacetyl-CoA. Subsequently, acetoacetyl-CoA is converted to 3-hydroxybutyryl-CoA by a respective dehydrogenase (Hbd) and 3-hydroxybutyryl-CoA is then dehydrated to crotonyl-CoA via a crotonase (Crt). Afterwards, the enzyme complex formed by a butyryl-CoA dehydrogenase and electron-transferring flavoproteins A and B (Bcd-EtfAB) catalyses the crotonyl-CoA-dependent reduction of ferredoxin (E0′=−420 mV) with NADH forming butyryl-CoA (electron bifurcation [13]). In a second cycle of reverse β-oxidation, caproate formation is assumed to be based on further CoA-thioester intermediates and direct use of acetyl-CoA for acyl-chain elongation [14]. Butyryl-CoA is elongated with acetyl-CoA by the action of a thiolase and results in 3-ketohexanoyl-CoA, which is subsequently converted to hexanoyl-CoA in two further steps. Presumably, a second electron-bifurcating reaction is involved that requires

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Keywords: strictly anaerobic; Gram-stain-negative; spore-forming; *Caproiciproducens*.
Abbreviations: AAI, average amino acid identity; ANI, average nucleotide identity; YTF, yeast extract–tryptone–fructose.
The whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number VWXL00000000. The version described in this paper is version VWXL01000000. Raw data have been deposited at the NCBI SRA database under the accession number SRS5758927. Accession number for the 16S rRNA gene sequence of strain EA1^T is MN851263.

trans-2-hexanoyl-CoA and NADH to form hexanoyl-CoA whereby ferredoxin is reduced. The enzymes for the second cycle have not been described but are assumed to act in a way similar to the enzymes necessary for butyryl-CoA formation. The Rnf (*Rhodobacter n*itrogen *f*ixation) complex, present in caproate-producing bacteria [13, 15–20], generates an electrochemical potential ($\Delta\mu H^+$ or $\Delta\mu Na^+$) from oxidation of reduced ferredoxin and reduction of oxidized NAD [21]. Subsequently, the electrochemical potential drives an F_0F_1-ATP synthase (ATPase) to form ATP by electron transport phosphorylation [22]. Furthermore, it is assumed that butyrate- and caproate-producing bacteria convert acetate or butyrate from their environment to acetyl-CoA or butyryl-CoA to avoid acidosis. Thus, bacteria maintain a pH high enough to prevent immediate toxic concentration of undissociated acids.

Caproate is an industrially relevant C6-compound with a wide range of applications [14]. It is used as basic commodity for the chemical industry, as an additive in animal feed for poultry and pigs, as a flavour additive in the food industry, as an antimicrobial agent in the pharmaceutical industry and as a possible precursor in the production of biofuels. Currently, caproate is mainly produced from fossil-based n-hexanol [23].

ISOLATION AND ECOLOGY

Strain EA1^T was isolated under anaerobic conditions from an anaerobic enrichment culture (reactor microbiome) producing organic acids such as acetate, butyrate and caproate. This 1.6 l enrichment culture was set up using 320 ml inoculum from a full-scale anaerobic biogas plant (Ströbele-Beck GbR Ulm, Germany) supplied with 31 g corn silage as feedstock, 350 mM ethanol as an additional electron donor, 500 mM sodium 2-bromoethanesulfonate (BES) to inhibit methanogenic archaea and incubated anaerobically at 37 °C for 50 days. Content of this enrichment culture was diluted (10^{-3}) and 1 ml was used to inoculate 50 ml Tanner mod2 medium at pH 6 in order to obtain bacterial mixed cultures in 125 ml Müller–Krempel bottles. The Tanner mod2 medium is based on a medium published by Tanner *et al.* [24] and contained 95.6 mM MOPS (3-(*N*-morpholino) propanesulfonic acid), 2.9 mM L-cysteine-HCl·H_2O, 0.05 % (wt/vol) yeast extract, 1 % (vol/vol) trace metal solution, 1 % (vol/vol) vitamin solution, 2.5 % (vol/vol) SL-9 mineral solution and 0.1 % (vol/vol) resazurin. The trace metal solution contained: 80 g l^{-1} NaCl, 100 g l^{-1} NH_4Cl, 10 g l^{-1} KCl, 10 g l^{-1} KH_2PO_4, 20 g l^{-1} $MgSO_4·7H_2O$ and 4 g l^{-1} $CaCl_2·2H_2O$. The vitamin solution [24] contained: 10 mg l^{-1} pyridoxine-HCl, 5 mg l^{-1} thiamine–HCl·$2H_2O$, 5 mg l^{-1} riboflavin, 5 mg l^{-1} calcium D-(+)-pantothenate, 5 mg l^{-1} lipoic acid, 5 mg l^{-1} 4-aminobenzoic acid, 5 mg l^{-1} nicotinic acid, 5 mg l^{-1} vitamin B12, 2 mg l^{-1} biotin, 2 mg l^{-1} folic acid and 10 mg l^{-1} MESNA (2-mercaptoethanesulfonic acid sodium salt). The SL-9 mineral solution [24] contained: 2 g l^{-1} nitrilotriacetic acid, 1 g l^{-1} $MnSO_4·H_2O$, 0.8 g l^{-1} $Fe(SO_4)_3(NH_4)_2·6H_2O$, 0.2 g l^{-1} $CoCl_2·6H_2O$, 1 mg l^{-1} $ZnSO_4·7H_2O$, 20 mg l^{-1} $CuCl_2·2H_2O$, 20 mg l^{-1} $NiCl_2·6\ H_2O$, 20 mg l^{-1} $Na_2MoO_4·2H_2O$, 20 mg l^{-1} $Na_2SeO_4·5H_2O$ and 20 mg l^{-1} $Na_2WO_4·2H_2O$. One mixed culture was supplied with ethanol (350 mM) and acetate (102 mM) and cultivated anaerobically at 37 °C with a H_2+CO_2 (67 % H_2+33 % CO_2) gas atmosphere. This mixed culture was monitored for growth (optical density OD_{600}) and formation of metabolic end products, which were analysed by HPLC using an 1260 Infinity LC apparatus (Agilent Technologies). After confirmation of growth and product formation of the mixed culture, 1 ml cell suspension was diluted (10^{-1}–10^{-3}) and 0.2 ml of each dilution was plated on modified YTF (yeast extract–tryptone–fructose [25]) agar plates and incubated at 37 °C in an anaerobic chamber containing a N_2+H_2 (95 % N_2+5 % H_2) gas atmosphere. The modified YTF agar plates (pH 7) contained 1 % (wt/vol) yeast extract, 1.6 % (wt/vol) tryptone, 68.5 mM NaCl, 27.8 mM fructose, 5.7 mM L-cysteine–HCl·H_2O, 3.7 mM NH_4Cl, 0.2 % (vol/vol) vitamin solution, 0.2 % (vol/vol) SL-9 mineral solution and 0.1 % (vol/vol) resazurin. Colony-forming units were re-streaked on fresh anaerobic agar plates to obtain pure cultures. Strains were subsequently transferred under anaerobic conditions and maintained for further use, using the Hungate technique in either Tanner mod2 medium or modified YTF medium.

In general, similar anaerobic enrichment cultures have been studied in detail as biotechnological production platforms for medium-chain carboxylates such as n-caproate and n-caprylate [1]. Open cultures of microbial consortia ferment organic biomass or organic wastes into valuable biochemicals that can be extracted. Meanwhile, a variety of different types of reactors and feedstock combinations have been tested [26–30]. Interestingly, phylogenetic analyses of the respective reactor microbiomes often revealed the presence of bacteria related to a strain termed '*Ruminococcaceae* bacterium CPB6' and the bacterial species *Cp. galactitolivorans* [27–30]. Both bacteria belong to the family *Oscillospiraceae* [31] with very few related strains available as pure cultures.

PHYLOGENY

The 16S rRNA gene sequence of strain EA1^T was analysed using reference sequences retrieved from GenBank [32] or the Ribosomal Database Project [33]. Phylogenetic tree reconstruction was done using MAFFT (Multiple Alignment using Fast Fourier Transform [34]) and MrBayes version 3.2.1 [35]. In the reconstructed phylogenetic tree shown in Fig. 1, the closest relatives of strain EA1^T are *Clostridium* sp. W14A [36], *Clostridium* sp. MB2-A37 [37], *Cp. galactitolivorans* BS-1^T [3] and '*Ruminococcaceae* bacterium CPB6' [19]. *Cp. galactitolivorans* BS-1^T is the only relative of strain EA1^T for which its name has been validly published [3]. Moreover, the 16S rRNA gene sequence similarities for strain EA1^T were determined by pairwise alignments for more distinctly related species with valid names. This resulted in 16S rRNA gene similarity values of 94.9 % for *Cp. galactitolivorans* BS-1^T, 93.8 % for [*Clostridium*] *leptum* DSM 753^T, 91.7 % for [*Clostridium*] *sporosphaeroides* DSM 1294^T and 91.0 % for *R. bromii* ATCC 27255^T. The highest 16S rRNA gene sequence similarity of 99 % was achieved to a strain termed *Clostridium* sp. W14A.

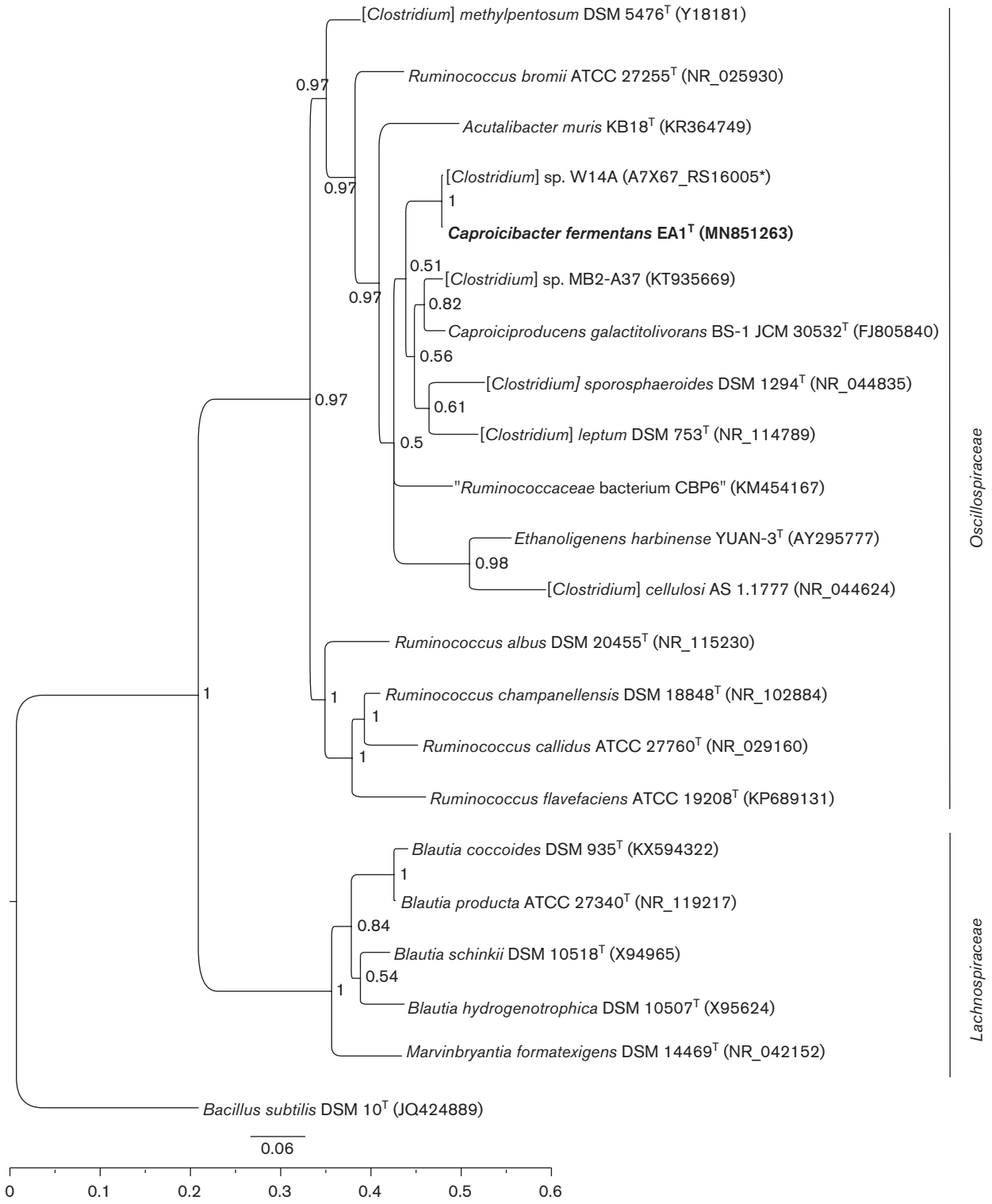

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences for classification of strain EA1^T and related bacterial strains. The phylogenetic tree was rooted to *Bacillus subtilis* DSM 10^T. GenBank accession numbers of the 16S rRNA gene sequences are given in parentheses. Estimation is based on Bayesian inference and an MAFFT alignment. The bar shows the expected changes per site resulting in different branch lengths. Numbers indicate posterior probabilities for each of the individual clades of the tree. *16S rRNA gene LocusTag number of the respective genome sequence (MBSV00000000). Square brackets ([ ]) around a genus indicate that the name awaits appropriate action by the research community to be transferred to another genus.

Strain W14A has neither been deposited in a culture collection nor has a species name been validly published for this strain. The genome sequence is available at the IMG/JGI database (https://img.jgi.doe.gov) and a short description was published by Ransom-Jones and McDonald [36].

Yarza *et al.* [38] stated that a sequence identity of 94.5 % or lower for two 16S rRNA genes is strong evidence for distinct genera. The sequence identity of 94.9 % for strain EA1^T and *Cp. galactitolivorans* BS-1^T is already close to this cut-off value. However, morphological and physiological differences between both strains provide further insights to justify the description of strain EA1^T as a new genus within the bacterial family of *Oscillospiraceae*.

GENOME FEATURES

Genome sequencing

Genomic DNA of strain EA1^T was extracted using the MasterPure Gram Positive DNA Purification kit as recommended by the manufacturer (Biozym). Quality of isolated DNA was initially checked by agarose gel electrophoresis and validated on an Agilent Bioanalyzer 2100 using an Agilent DNA 12000 kit as recommended by the manufacturer (Agilent Technologies). Concentration and purity of the isolated DNA was first checked with a NanoDrop ND-1000 (PeqLab Biotechnologie GmbH) and concentration was determined using the Qubit dsDNA HS Assay kit as recommended by the manufacturer (Life Technologies GmbH). Illumina sequencing libraries were prepared using the Nextera XT DNA Sample Preparation kit and subsequently sequenced on a MiSeq system with the reagent kit v3 with 600 cycles as recommended by the manufacturer (Illumina). Quality filtering of the 40420078 raw reads using Trimmomatic version 0.39 [39] resulted in 4078054 paired-end reads with an average read length of 301 bp. The assembly was performed with the SPAdes genome assembler software version 3.13.1 [40]. The assembly resulted in 113 contigs (>500 bp) and an average coverage of 240-fold. The assembly was validated and the read coverage was determined with QualiMap version 2.2.1 [41]. The Prokka software tool was used for automatic annotation and identification of rRNA and tRNA genes [42].

The genome of strain EA1^T consists of a circular chromosome (3.9 Mb) with an overall G+C content of 51.25 mol%. The draft genome sequence contained five rRNA genes (3×5S rRNA, 1×16S rRNA and 1×23S rRNA), 49 tRNA genes, 2615 protein-encoding genes with a predicted function and 1182 genes coding for hypothetical proteins. The analysis of the genome revealed that strain EA1^T harbours genes encoding the *bcs* operon (butyryl-CoA synthesis) [43]. The *bcs* operon, as it is present in *Clostridium acetobutylicum*, consists of the genes *crt*, *bcd*, *etfB*, *etfA* and *hbd* encoding the enzymes Crt [EC 4.2.1.17], Bcd-EtfAB [EC 1.3.8.1] and Hbd [EC 1.1.1.157], respectively. In strain EA1^T, the gene encoding the thiolase [EC 2.3.1.9], which condenses two acetyl-CoA to one acetoacetyl-CoA, is also present in the corresponding gene cluster. Genes encoding proteins required for sporulation such as spore coat proteins, various spore maturation proteins, spore membrane proteins (sporulation integral membrane proteins) and a spore protease are present in the genome of strain EA1^T. Moreover, the genome comprises a complete gene cluster encoding the Rnf complex. This protein complex functions as a ferredoxin:NAD$^+$ oxidoreductase [EC 1.18.1.3] [44], which oxidizes reduced ferredoxin (Fd^{2-}) and thereby yields NADH. An ion gradient generated by H^+ or Na^+ crossing the cytoplasmic membrane [45] drives an ATPase for ATP production. Seedorf *et al.* [13] assumed that *C. kluyveri* generates Fd^{2-} via crotonyl-CoA reduction, which is then used for the regeneration of NADH via the Rnf complex. Most likely, the Rnf complex in strain EA1^T also functions as a ferredoxin:NAD$^+$ oxidoreductase to oxidize Fd^{2-} gained from the activity of the pyruvate:ferredoxin oxidoreductase or the Bcd-EtfAB complex. Complete gene clusters required for the synthesis of cytochromes or quinones are not present in the genome.

Strain EA1^T harbours a gene cluster similar to the *lct* operon, which encodes enzymes that are responsible for lactate uptake and utilization as described for *Acetobacterium woodii* [46]. The organization of genes of that specific cluster varies compared to the *lct* operon of *A. woodii* but matches perfectly with a corresponding gene cluster present in '*Ruminococcaceae* bacterium CPB6' [19]. Strain CPB6 was shown to utilize lactate (45.1 g l^{-1}) and produce high caproate titers (16.6 g l^{-1}) with a productivity of 0.2 g l^{-1} h^{-1} [7]. However, under the conditions tested, strain EA1^T was not able to grow with lactate as a substrate.

An average nucleotide identity (ANI) analysis of genome sequences was performed for related strains using the IMG/ER system [47]. Pairwise ANI values ranged between 67.8 % and 71.6 % for the stains *Cp. galactitolivorans* BS-1^T (71.6 %), [*Clostridium*] *leptum* DSM 753^T (70.3 %), [*Clostridium*] *sporosphaeroides* DSM 1294^T (71.5 %), *R. bromii* ATCC 27255^T (67.8 %) and strain CPB6 (70.2 %). An ANI value of 99 % was recorded for *Clostridium* sp. W14A confirming the results from the 16S rRNA gene analysis. Additionally, the average amino acid identity (AAI) was calculated using the AAI-profiler software tool [48]. Pairwise AAI values compared to strain EA1^T revealed 98.5 % for strain W14A, 65.3 % for strain CPB6, 69.4 % for *Cp. galactitolivorans* BS-1^T and 64.8 % for [*Clostridium*] *leptum* DSM 753^T. [*Clostridium*] *sporosphaeroides* DSM 1294^T and *R. bromii* ATCC 27255^T were not included in the analysis since respective data were not available in the UniProt database [49].

CELL MORPHOLOGY AND PHYSIOLOGY

Morphological features

Gram-staining was carried out as previously described [50]. Cells of strain EA1^T were visualized using a Zeiss light microscope, a Hitachi S-5200 scanning electron microscope and a JEOL JEM-1400 transmission electron microscope. Gram-staining of strain EA1^T was performed in comparison to the Gram-stain-positive bacterium *Corynebacterium glutamicum*

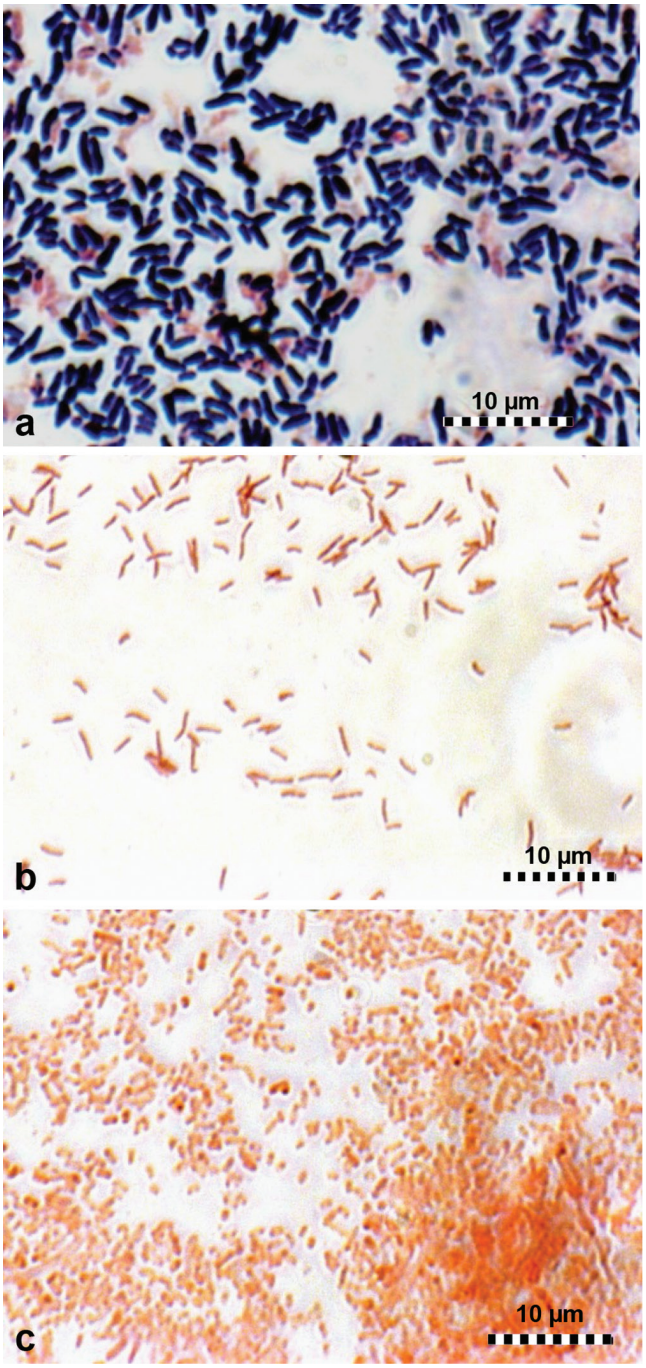

Fig. 2. Microscopy images showing Gram-stained cells of *Corynebacterium glutamicum* DSM 1933 (a) strain EA1^T (b) and *Escherichia coli* XL1-Blue (c).

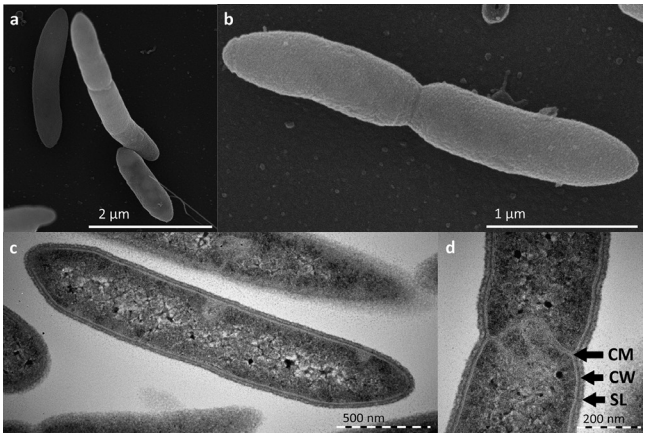

Fig. 3. Microscopy images showing cells of strain EA1^T. Scanning electron microscopy images using a 20000-fold (a) and 45000-fold (b) magnification. Transmission electron microscopy images using 50000-fold (c) and 185000-fold (d) magnification. CM, cytoplasmic membrane; CW, cell wall; SL, surface layer.

DSM 1933 and the Gram-stain-negative bacterium *Escherichia coli* strain XL1-Blue. Strain EA1^T clearly stained Gram-negative as shown in Fig. 2. Motility of EA1^T and BS-1^T cells was investigated using a Zeiss light microscope and sealed imaging dishes (µ-Dish 35 mm, high, ibidi GmbH). Dishes were filled with cell suspension in an anaerobic chamber under a N_2+H_2 (95 %+5 %) gas atmosphere. Beforehand, cells were cultivated in Hungate tubes containing Tanner mod2 medium for 18 h. Cells of BS-1^T showed clear motility, while cells of EA1^T cells did not. The rod-shaped EA1^T cells showed a length of 1–3 µm and a width of 0.4–0.8 µm. The shape and length of the cells was confirmed using scanning electron microscopy (Fig. 3a, b). Moreover, transmission electron microscopy images showed a typical Gram-positive type of cell-wall ultrastructure (Fig. 3c, d). In Fig. 3d, the cytoplasmic membrane, the densely stained cell wall and an additional surface layer are indicated by arrows. In bacteria that clearly stain Gram-positive, the thickness of cell walls ranges between 30 and 50 nm [51]. It has been speculated by Cheng and Costerton [51] that a minimum cell wall thickness is required to retain the Gram-stain-complex during decoloration. Several other bacteria have been described that stain Gram-negative and possess a Gram-positive cell-wall structure, e.g. *Butyrivibrio fibrisolvens* strain D1 [51], *Clostridium polysaccharolyticum* ATCC 33142^T [52], *Lachnoclostridium phytofermentans* ATCC 700394^T, *Cellulosilyticum lentocellum* NCIMB 11756^T and *Clostridium populeti* ATCC 35295^T [53].

Growth characteristics and analytical procedures

Growth of strain EA1^T was examined in Tanner mod2 medium at different temperatures and pH values. The tested temperatures were 25, 30, 37, 45 and 55 °C and the initial pH values of the media were set to pH 5, 6, 7, 8 and 9, respectively. Growth of strain EA1^T was observed between 30 and 37 °C and not below 25 or above 45 °C. The initial pH value of the medium that allowed growth of strain EA1^T ranged between pH 5 and 9. The growth rates (µ) of cells grown at 37 °C were determined and accounted for µ=0.1 at pH 5, µ=0.11 at pH 6, µ=0.12 at pH 7 and µ=0.05 at pH 8. Thus, the optimal pH for growth was pH 7. Subsequently, growth experiments to determine the substrate spectrum of strain EA1^T and BS-1^T were performed at 37 °C and pH 7 in Tanner mod2. Strain

EA1^T showed growth (≥ 2.5 doublings of cell mass) using the substrates cellobiose, fructose, galactose, glucosamine, glucose, mannitol, mannose, starch and sucrose. Furthermore, a slight increase of biomass (≤2 doublings of cell mass) was observed using the substrates arabinose, galactitol, sorbitol and xylose. Strain EA1^T was not able to utilize the substrates fumarate, lactate, malate, succinate, ethanol, methanol, glycerol, alanine, alanine and glycine in combination, glutamate, glycine, ribose and xylitol. Substrates were added to a final concentration of 20 mM besides alanine, ethanol, methanol, glutamate and glycine, where just 10 mM were used.

Metabolic end products of strain EA1^T were analysed via GC (Clarus 600, PerkinElmer) and HPLC. Fructose, acetate and lactate were quantified using an HPLC equipped with a refraction index detector and a diode array detector. The prepared samples (20 µl) were analysed using the CS-Organic-Acid Resin (40×8 mm) pre-column and CS-Organic-Acid Resin (150×8 mm) column packed with polystyrene divinylbenzene copolymer. Column temperature was set to 40 °C. As the mobile phase, 5 mM H_2SO_4 at a constant flow rate of 0.7 ml min^{-1} was applied. Ethanol, butyrate and caproate were analysed using a GC equipped with a flame ionization detector operating at 300 °C. Prepared samples were injected at 225 °C into an Elite-FFAP (0.32 mm×30 m) column packed with nitroterephthalic acid modified polyethylene glycol. The carrier gas consisted of H_2 and a flow rate of 2.25 ml min^{-1} was applied. The GC temperature profile was set to 90 °C for 2 min, 90 °C - 250 °C by 40 °C min^{-1} and 250 °C for 1 min. H_2 and CO_2 were analysed using GC (Arnel Engineered Solutions Clarus 580 GC, PerkinElmer) equipped with a thermal conductivity detector operating at 200 °C. Headspace gas samples were injected using a 2.5 ml injection syringe (SGE Analytical Science) into a stainless steel packed column (320 µm×50 m; Cat. No. NR022501). Argon was used as carrier gas with a flow rate of 50 ml min^{-1}. Injected gases were analysed for 11 min at 60 °C. Fermentation, oxidation/reduction (O/R) and proton balances of supplied carbon and detected metabolic products were calculated according to Gottschalk [54].

The fermentation balance of strain EA1^T was determined using 19 mM fructose as substrate. The strain produced 3 mM acetate, 0.5 mM butyrate, 3 mM caproate, 24 mM lactate and 1 mM ethanol as well as 8 mM CO_2 and 5 mM H_2 as metabolic products. The corresponding fermentation balance (mol/100 mol substrate) accounted for 91.5 % carbon recovered in those products (Table 1). The remaining 8.5 % carbon must have been used for biomass formation. The ratio of oxidized and reduced fermentation products (oxidation/reduction balance, O/R mol/100 mol) accounted for 0.95. The ratio of available protons (H^+) compared to detected protons accounted for 1.09 (Table 1). All ratios are close to one and consequently the fermentation balance can be considered as even.

Sporulation of strains EA1^T and BS-1^T was tested according to Bahl *et al.* [55]. The 'vegetative medium' was inoculated and incubated for at least 1 week at 37 °C. Subsequently, cells and spores were concentrated by centrifugation, the supernatant was removed, the cells were aliquoted in glycerol suspensions (20 % (wt/vol)) and finally stored at −80 °C. Subsequently, the spore suspension was heated at 80 °C for 15 min and transferred to Tanner mod2 medium. Cells of strain EA1^T recovered from spores within 7 days at 37 °C, while cells of strain BS-1^T did not.

Cellular fatty acid composition

Freeze-dried cells were prepared for the analysis of fatty acids. The cell suspension (200 ml) of strain EA1^T was harvested and washed twice with 15 ml 0.8 % (wt/vol) saline. Pellets were dissolved in 2 ml saline and the respective glass vials

Table 1. Carbon recovery, O/R balance and balance of available protons of fermentation products of strain EA1^T

Carbon recovered: 549/600×100=91.5 %; O/R balance: 84/88=0.95; balance of available protons H^+ 2400/2204=1.09. The values for O/R and proton balance are different because the carbon recovery impacts the balance of available protons but not the O/R balance (O/R value of fructose=0).

			O/R balance		Balance of available protons	
Substrate/ products	**Mol/100 mol substrate**	**Mol carbon**	**O/R value**	**O/R mol/100 mol**	**Available H^+**	**Available H^+ mol/100 mol**
Fructose	100	600	0	0	24	2400
Acetate	16	32	0	0	8	128
Butyrate	3	12	−2	−6	20	60
Caproate	11	66	−4	−44	32	352
Lactate	129	387	0	0	12	1548
Ethanol	5	10	−2	−10	12	60
CO_2	42	42	+2	+84	0	0
H_2	28	0	−1	−28	2	56
Total		549		−88		2204
				+84		

were sealed with cotton wool plugs (dentist's size 2, Hartmann AG). Cells in glass vials were frozen using liquid nitrogen and placed into a pre-chilled cooling block at −80 °C. Afterwards, the vials were placed into the lyophilisator (Alpha 1-4LOC-1M, Martin Christ Gefriertrocknungsanlagen GmbH) for primary drying. A vacuum (0.01 mbar) was applied at a temperature of −54 °C for 30 h. For secondary drying, the wool plug was exchanged with a rubber stopper and placed onto a manifold (Martin Christ Gefriertrocknungsanlagen GmbH), which was placed onto the lyophilisator Alpha 1–4 LD plus (Martin Christ Gefriertrocknungsanlagen GmbH), then vacuum was applied (0.001 mbar) for about 12 h. Fatty acid analyses were carried out by the Identification Service of the DSMZ, Brunswick, Germany.

The major components in the cellular fatty acid fraction of EA1^T cells grown using fructose were $C_{14:0}$ (23.1 %), $C_{16:0}$ (13.2 %), $C_{16:0}$ DMA (17.0 %) and $C_{16:1}$ *ω*7c (11.7 %). The presence of dimethyl acetals in the fatty acid fraction is indicative of the presence of plasmalogens in the lipids of this organism. The fatty acid composition of strain EA1^T is clearly different from *Cp. galactitolivorans* BS-1^T and [*Clostridium*] *sporosphaeroides* DSM 1294^T (Table 2). Especially the major cellular fatty acid $C_{14:0}$ of strain EA1^T with 23.1 % clearly differs from *Cp. galactitolivorans* BS-1^T (3.2 %) and [*Clostridium*] *sporosphaeroides* DSM 1294^T (4.5 %). Depending on the reaction conditions, cleavage of the plasmalogens can lead to the formation of both the corresponding dimethyl acetals and aldehydes (i.e. $C_{16:0}$ DMA and $C_{16:0}$ aldehyde) and when evaluating their presence, these two derivatives should be considered to have originated from a single plasmalogen. $C_{16:0}$ dimethyl acetals/aldehydes are one of the major components in the fatty acid fraction in both *Cp. galactitolivorans* BS-1^T (36.2 %) and [*Clostridium*] *sporosphaeroides* DSM 1294^T (38.5 %), but in strain EA1^T, this comprises only 20.0 %. Some fatty acids, such as $C_{16:1}$ ω5*c*, $C_{16:1}$ ω7*c* or $C_{16:1}$ ω7*c* DMA are unique for strain EA1^T (Table 2). The presence of 18:1 ω7*c* derivatives of fatty acids and plasmalogens in strain EA1^T vs 18:1 ω9*c* and 18:1 ω11*c* derivatives of fatty acids and plasmalogens in *Cp. galactitolivorans* BS-1^T also indicates, that enzymes with different specificities for the positions of unsaturation are present in these two organisms.

Distinguishing characteristics

Results of 16S rRNA gene sequence comparisons and phylogenetic tree reconstruction already indicate that strain EA1^T represents a novel taxon within the family *Oscillospiraceae* [31]. Furthermore, strain EA1^T differs from *Cp. galactitolivorans* BS-1^T with respect to the following phenotypic features. Strain EA1^T stained Gram-negative whereas strain BS-1^T stained Gram-positive [3]. Strain EA1^T is able to sporulate and regrow from spores but strain BS-1^T is not. Strain EA1^T was not able to grow at 45 °C but strain BS-1^T is able to do so. Moreover, the genome of strain EA1^T accounts for 3.9 Mbp whereas the genome of strain BS-1^T comprises 2.57 Mbp [20]. Differences in the quantitative and qualitative amounts of both fatty acids (as their methyl esters) and the dimethyl acetal/aldehyde derivates of lipid plasmalogens also indicate clear phenotypic differences at the level of these compounds.

Table 3 summarizes the main phenotypic features of the closely related type strains [*Clostridium*] *leptum* DSM 753^T, [*Clostridium*] *sporosphaeroides* DSM 1294^T and *R. bromii* ATCC 27255^T. All strains show phenotypic differences compared to strain EA1^T. In summary, the phenotypic and genotypic characteristics suggest that strain EA1^T represents a novel genus within the family *Oscillospiraceae*, when compared to closely related type strains (Table 3).

DESCRIPTON OF *CAPROICIBACTER* GEN. NOV.

Caproicibacter (Ca.pro.i.ci.bac'ter. N.L. neut. n. *acidum caproicum* caproic acid; N.L. masc. n. *bacter* a rod; N.L. masc. n. *Caproicibacter* a rod-shaped organism that produces caproate).

Cells are strictly anaerobic, caproate-producing, spore-forming and possess a Gram-positive type of cell-wall structure. Carbohydrate utilization includes but is not limited to arabinose, cellobiose, fructose, galactose, glucose, mannose, starch, sucrose, xylose, glucosamine, galactitol, mannitol and sorbitol. The major metabolic end products are acetate, butyrate, caproate and lactate, as well as CO_2 and H_2. The major components of the cellular fatty acid fraction are $C_{14:0}$, $C_{16:0}$ DMA/aldehyde, $C_{16:0}$ and $C_{16:1}$ *ω7c*. Phylogenetically, the genus represents a distinct lineage in the family *Oscillospiraceae*. The type species is *Caproicibacter fermentans*.

DESCRIPTON OF *CAPROICIBACTER FERMENTANS* SP. NOV.

Caproicibacter fermentans (fer.men´tans. L. part. adj. *fermentans* fermenting).

Cells are strictly anaerobic, Gram-stain-negative, spore-forming, non-motile and caproate-producing. Colonies growing on modified YTF agar plates are punctiform, circular and white. Cells are 1.3–3.3 µm long and 0.4–0.8 µm wide. Growth of cells occurs in a temperature range of 30–37 °C with a growth optimum at 37 °C. Furthermore, cell growth occurs in a pH range of pH 5–9 with an optimum at pH 7. Organic acids are produced from sugars including arabinose, cellobiose, fructose, galactose, glucose, mannose, starch, sucrose, xylose, the sugar alcohols galactitol, mannitol and sorbitol as well as from the amino sugar glucosamine. No cell growth occurs using the substrates ribose, fumarate, succinate, malate, lactate, glycerol, ethanol, methanol, alanine, glutamate, glycine, galactitol and xylitol. Fermentative end products from growth on fructose are acetate, butyrate, caproate and lactate, as well as CO_2 and H_2. The genome harbours genes encoding the *bcs* operon and genes encoding lactate dehydrogenases. Furthermore, genes encoding spore coat proteins, spore maturation proteins, spore membrane proteins and a spore protease are present. A complete gene cluster encoding the Rnf complex and a gene cluster similar to the *lct* operon (lactate uptake and utilization) is present in the genome. The major components of the cellular fatty acid fraction, when cells grown using fructose, are $C_{14:0}$, $C_{16:0}$, $C_{16:0}$ DMA/aldeyhde and $C_{16:1}$ *ω7c*. The type strain of

Table 2. Fatty acid analysis of strain EA1^T and the two closely related strains *Cp. galactitolivorans* BS-1^T and [*Clostridium*] *sporosphaeroides* DSM 1294^T

Strains: 1, EA1^T; 2, *Cp. galactitolivorans* BS-1^T; 3, [*Clostridium*] *sporosphaeroides* DSM 1294^T. Strain EA1^T cells were cultivated for 3 days at 37 °C in Tanner mod2 medium. Values are percentages of total fatty acids. –, Not detected; DMA, dimethyl acetal. Major components are indicated with bold text. Data for *Cp. galactitolivorans* BS-1^T and [*Clostridium*] *sporosphaeroides* DSM 1294^T were obtained from Kim *et al.* [3].

Fatty acid	1	2	3
Straight-chain:			
$C_{12:0}$	–	–	4.3
$C_{13:0}$	–	–	1
$C_{14:0}$	**23.1**	3.2	4.5
$C_{16:0}$	**13.2**	3.1	1.9
$C_{14:0}$ DMA	8.2	5.4	8.9
$C_{16:0}$ DMA	**17.0**	**22.1**	**35.9**
$C_{18:0}$ DMA	–	4.0	2.5
$C_{16:0}$ aldehyde	3.0	**14.1**	**22.6**
$C_{18:0}$ aldehyde	–	3.1	1.8
Branched-chain:			
anteiso-$C_{17:0}$	–	5.9	–
Unsaturated:			
$C_{16:1}$ $\omega 5c$	8.0	–	–
$C_{16:1}$ $\omega 7c$	**11.7**	–	–
$C_{16:1}$ $\omega 9c$	–	–	1.2
$C_{18:1}$ $\omega 9c$	–	1.1	–
$C_{16:1}$ $\omega 7c$ DMA	5.0	–	–
$C_{18:1}$ $\omega 7c$ DMA	1.6	–	–
$C_{18:1}$ $\omega 9c$ DMA	–	7.6	–
$C_{18:1}$ $\omega 11c$ DMA	–	4.6	–
$C_{18:2}$ $\omega 9c$ $\omega 12c$	–	1.1	1.4
Summed features:*			
1	1.3	2.5	4.3
5	–	2.4	4.7
7	–	5.6	–
8	–	3.5	–
10	1.1	–	–
11	–	**10.0**	1.1

*Groups of fatty acids that could not be separated by gas chromatography using the MIDI Sherlock system are summarized under the term summed features. Summed feature 1 contained $C_{13:1}$ $\omega 2c$ and/or $C_{14:0}$ aldehyde. Summed feature 5 contained $C_{15:0}$ DMA and/or $C_{14:0}$ 3OH. Summed feature 7 contained $C_{17:2}$ $\omega 2c$ and/or $C_{17:1}$ $\omega 8c$. Summed feature 8 contained $C_{17:1}$ $\omega 8c$ and/or $C_{17:2}$. Summed feature 10 contained $C_{18:1}$ $\omega 7c$/$\omega 9t$/$\omega 12t$ and/or unknown ECL 17.834. Summed feature 11 contained iso-$C_{17:0}$ 3OH and/or $C_{18:2}$ DMA.

Table 3. Comparison of genotypic and phenotypic characteristics of strain EA1^T and most closely related type strains

Strains: 1, EA1^T; 2, *Cp. galactitolivorans* BS-1^T [3]; 3, [*Clostridium*] *sporosphaeroides* DSM 1294^T [56, 57]; 4, [*Clostridium*] *leptum* DSM 753^T [58]; 5, *R. bromii* ATCC 27255^T [59]. +, Positive; –, negative; (+), products were not always detected.

Characteristics	1	2	3	4	5
Cell shape	Rod	Rod	Rod	Rod	Coccus
Cell size:					
Length (µm)	1.3–3.3	0.4–2.0	1.8–8.0	1.3–2.8	0.7–1.1
Width (µm)	0.4–0.8	0.3–0.5	0.5–0.6	0.6–0.8	0.7–1.1
Spore formation	+	–	+	+	–
Gram-staining	–	+	+	+	+
Motility	–	+	–	–	–
Metabolic end products:					
Acetate	+	+	+	+	+
Propionate	–	–	+	–	(+)
Butyrate	+	+	+	–	–
Caproate	+	+	–	–	–
Lactate	+	+	–	(+)	(+)
Ethanol	+	+	–	+	+
G+C content (mol%)	51.25	50.00	53.54	51.00	40.00
Genome size (Mbp)	3.90	2.57	3.18	3.27	2.25
Optimum temperature for growth (°C)	37	40	37–45	37	37

Caproicibacter fermentans is strain EA1^T (DSM 107079^T, JCM 33110^T).

EMENDED DESCRIPTION OF *CAPROICIPRODUCENS* KIM *ET AL.* 2015

The description is as given by Kim *et al.* [3] with the following modifications. Cells are motile and produce lactate as a major metabolic product. These insights were obtained by performing experiments using cells of *Cp. galactitolivorans* BS-1^T and the media and analytics as described above.

EMENDED DESCRIPTION OF *CAPROICIPRODUCENS GALACTITOLIVORANS* KIM *ET AL.* 2015

The description is as given by Kim *et al.* [3] with the following modifications. Cells are motile and produce lactate as a major metabolic product in addition to the known products (acetate, butyrate, caproate and H_2). The genome of strain BS-1^T contains genes encoding the *bcs* operon, genes encoding lactate dehydrogenases and a complete gene cluster encoding the Rnf complex [20]. Approximately one third of the components in the fatty acid fraction comprises dimethyl acetals/aldehydes ($C_{16:0}$ DMA/aldehyde dominate), while a smaller proportion of the fraction comprises unsaturated derivatives with the double bonds located at the ω9*c* or ω11*c* positions. This indicates that different enzymes are involved in the production of these compounds.

Funding information
This study was funded by the Institute of Microbiology and Biotechnology (Ulm University) and the Genomic and Applied Microbiology and Göttingen Genomics Laboratory (Georg-August University Göttingen).

Acknowledgements
We thank Kathleen Gollnow, Frauke-Dorothee Meyer, and Christian Kröly for the technical support and Paul Walther from the Zentrale Einrichtung Elektronenmikroskopie of Ulm University for his support. A special thanks goes to Dr. Brian J. Tindall for his excellent advice regarding the fatty acid analysis, the taxonomic descriptions, and his further valuable input to our manuscript.

Author contributions
M.F., T.B., S.B. and A.P. performed investigations, data curation, formal analysis and validation. M.F. wrote the original draft of the manuscript. M.F., T.B., A.P., R.D. and F.R.B. reviewed and edited the manuscript. R.D. contributed to funding acquisition and F.R.B. was responsible for conceptualization, supervision and funding acquisition.

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

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