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TAXONOMIC DESCRIPTION

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Reclassification of *Eubacterium hallii* as *Anaerobutyricum hallii* gen. nov., comb. nov., and description of *Anaerobutyricum soehngenii* sp. nov., a butyrate and propionate-producing bacterium from infant faeces

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Abstract

A bacterial strain designated L2-7^T, phylogenetically related to *Eubacterium hallii* DSM 3353^T, was previously isolated from infant faeces. The complete genome of strain L2-7^T contains eight copies of the 16S rRNA gene with only 98.0–98.5 % similarity to the 16S rRNA gene of the previously described type strain *E. hallii*. The next closest validly described species is *Anaerostipes hadrus* DSM 3319^T (90.7 % 16S rRNA gene similarity). A polyphasic taxonomic approach showed strain L2-7^T to be a novel species, related to type strain *E. hallii* DSM 3353^T. The experimentally observed DNA–DNA hybridization value between strain L2-7^T and *E. hallii* DSM 3353^T was 26.25 %, close to that calculated from the genomes (34.3 %). The G+C content of the chromosomal DNA of strain L2-7^T was 38.6 mol%. The major fatty acids were $C_{16:0}$, $C_{16:1}$ *cis*9 and a component with summed feature 10 ($C_{18:1}$c11/t9/t6*c*). Strain L2-7^T had higher amounts of $C_{16:0}$ (30.6 %) compared to *E. hallii* DSM 3353^T (19.5 %) and its membrane contained phosphatidylglycerol and phosphatidylethanolamine, which were not detected in *E. hallii* DSM 3353^T. Furthermore, 16S rRNA gene phylogenetic analysis advocates that *E. hallii* DSM 3353^T is misclassified, and its reclassification as a member of the family *Lachnospiraceae* is necessary. Using a polyphasic approach, we propose that *E. hallii* (=DSM 3353^T=ATCC 27751^T) be reclassified as the type strain of a novel genus *Anaerobutyricum* sp. nov., comb. nov. and we propose that strain L2-7^T should be classified as a novel species, *Anaerobutyricum soehngenii* sp. nov. The type strain is L2-7^T (=DSM 17630^T=KCTC 15707^T).

In a previous study investigating the phylogenetic diversity of butyrate-producing bacteria in the human intestinal tract, strain L2-7^T with highest 16S rRNA similarity to *Eubacterium hallii* DSM 3353^T was isolated from infant faeces [1]. This isolate, deposited as DSM 17630^T, was capable of producing butyrate from glucose and later it was shown to convert both D- and L-lactate to butyrate in the presence of acetate [2]. Subsequent culture-independent analysis based on 16S rRNA gene profiling has revealed bacteria related to *Eubacterium hallii* to be part of the core microbiota of the human intestinal tract, and oral administration of cells of strain L2-7^T was observed to improve insulin sensitivity in db/db mice [3–5]. Importantly, the revised roadmap of 2009 for taxonomic classification of the phylum *Firmicutes* proposed that the family *Eubacteriaceae* comprises *Eubacterium stricto sensu* which includes only *Eubacterium limosum*, *Eubacterium aggregans*, *Eubacterium barkeri and Eubacterium callanderi* as well as the genera *Acetobacterium*, *Alkalibacter*, *Anaerofustis*, *Garciella* and *Pseudoramibacter* [6]. In contrast, the species *Eubacterium hallii* has been placed in the *Lachnospiraceae* family. In order to have a better understanding of the taxonomic affiliation of strains L2-7^T and DSM 3353^T, we carried out a polyphasic taxonomic approach.

Strain L2-7^T was kindly provided by Dr Harry Flint, while strain DSM 3353^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Both strains were grown routinely in a medium with the following composition: yeast extract (4.0 g l^{-1}), casitone (2.0 g l^{-1}), soy peptone (2.0 g l^{-1}), $NaHCO_3$ (4.0 g l^{-1}), KH_2PO_4 (0.41 g l^{-1}), $MgCl_2{\cdot}6H_2O$ (0.1 g l^{-1}), $CaCl_2{\cdot}2H_2O$ (0.11 g l^{-1}), cysteine-HCL (0.5 g l^{-1}), vitamin K1 (0.2 ml),

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Abbreviation: GLM, generalized linear model.
The NCBI/EMBL/DDBJ accession number for 16S rRNA gene sequence of *Anaerobutyricum soehngenii* L2-7^T is AJ270490 and the GenBank/EMBL-EBI accession number for the genome sequence of strain *Anaerobutyricum soehngenii* L2-7^T is LT907978 (assembly version EH1).
Two supplementary figures are available with the online version of this article.

haemin (1 ml) and trace elements I, trace elements II and vitamin solutions. The trace elements I (alkaline) solution contained the following (mM): 0.1 Na_2SeO_3, 0.1 Na_2WO_4, 0.1 Na_2MoO_4 and 10 NaOH. The trace elements II (acid) solution was composed of the following (mM): 7.5 $FeCl_2$, $1H_3BO_4$, 0.5 $ZnCl_2$, 0.1 $CuCl_2$, 0.5 $MnCl_2$, 0.5 $CoCl_2$, 0.1 $NiCl_2$ and 50 HCl. The vitamin solution had the following composition (g l^{-1}): 0.02 biotin, 0.2 niacin, 0.5 pyridoxine, 0.1 riboflavin, 0.2 thiamine, 0.1 cyanocobalamin, 0.1 p-aminobenzoic acid and 0.1 pantothenic acid. The medium was supplemented with 30 mM of sodium acetate. For routine use, the medium was distributed in 35 ml serum bottles sealed with butyl-rubber stoppers and incubated at 37 °C under a gas phase of 1.7 atm (172 kPa) N_2/CO_2 (80 : 20, v/v). The pH of the medium was 7.0. The vitamin solution was filter sterilized and added after autoclaving. Similarly, filter-sterilized D-glucose or other carbon sources for substrate utilization tests, was added to a final concentration of 40 mM, unless stated otherwise. All growth experiments with strains L2-7^T and DSM 3353^T were performed in duplicate. The experiments were repeated independently to confirm the observed differences.

For genomic comparison, we used the recently deposited complete genome of strain L2-7^T (accession number LT907978) and the draft genome sequence of the type strain *E. hallii* DSM 3353^T (accession number PRJNA18177) [7, 8].

A total of eight full-length 16S rRNA gene sequences were obtained from the complete genome of strain L2-7^T [8]. For more detailed 16S rRNA gene-based phylogenetic analysis, sequences were aligned using CLUSTAL_W and a phylogenetic tree was reconstructed using MEGA6 (for details, see legend to Fig. 1). This analysis indicated that the closest relative of strain L2-7^T was *E. hallii* DSM 3353^T (Fig. 1). The pairwise comparison of the almost-complete 16S rRNA gene sequences (1400 nt) of L2-7^T with that of *E. hallii* DSM 3353^T revealed approximately 1.0 to 1.4 % sequence divergence depending on the copy of L2-7^{T}16S rRNA gene. The second-closest validly published species related to strains L2-7^T and DSM 3353^T is *Anaerostipes hadrus* DSM 3319^T [9], with a mean 16S rRNA gene sequence similarity of 90.7 %.

Next, using the genome sequence of strains L2-7^T (DSM 17630^T) and DSM 3353^T, we calculated the average nucleotide identity (ANI) and *in silico* DNA–DNA hybridization using the Genome-to-Genome Distance Calculator (GGDC; DSMZ) [10–12]. The mean ANI between the two isolates was 87.81 and the *in silico* DNA–DNA hybridization estimate (GLM-based) was 34.3 % (31.9–36.8 %), which strongly suggests that isolate L2-7^T is a novel species. Moreover, supporting evidence is provided by *in vitro* DNA–DNA hybridisation between the isolate L2-7^T and *E. hallii* DSM 3353^T, which was only 26.5 %. This is well below the 70 % cut-off point generally accepted for species classification [13]. These data warranted a more detailed characterization using a polyphasic taxonomy approach.

For identifying the optimum growth temperature, the isolates were incubated at different temperatures (10, 20, 25, 30, 35, 37, 40, 45, 50 and 55 °C). Strains L2-7^T and DSM 3353^T both grew at temperatures from 35 to 45 °C with optimum growth at 37 °C. Cell morphology, motility and spore formation of strain L2-7^T was observed using a Leica DM 2000 microscope and a JEOL-6480LV scanning electron microscope (SEM). The sample processing for SEM was performed as previously described [14]. Strain L2-7^T is a non-motile and strictly anaerobic, straight rod-shaped organism. While sub-terminal and terminal swellings were observed in both the isolates L2-7^T and DSM 3353^T as previously reported for DSM 3353^T [13, 15], the cultures did not survive after heat treatment (80 °C for 10 min). Young cells of both strains stained Gram-positive (Fig. S1 a, b). The cells were approximately 0.70–4 µm in size after 12 h of incubation in glucose-containing growth media (Fig. S1c, d, available in the online version of this article). Cells differed slightly in length depending on the growth medium and appeared sometimes elongated under nutrient, temperature and pH stress conditions. After 48 h of incubation on Reinforced Clostridial Medium (solidified with 1 % agar; Difco) at 37 °C, both strains L2-7^T and DSM 3353^T produced white centred colonies, surrounded by a colourless layer, circular, raised with entire margins and 4.5 mm in diameter. Under the microscope cells appear elongated. Strains L2-7^T and DSM 3353^T were grown at pH 4.5–8.5, and growth was observed in the pH range 6.0–8.0, and optimum growth was observed at pH 6.5 for strain L2-7^T and pH 7.5 for DSM 3353^T. For the pH experiment, the buffers used were as follows: 20 mM Tris to buffer at ≥pH 8.0, 20 mM PIPES for pH 6.0–7.5 and 20 mM sodium citrate for <pH 6.0 and adjusted with 2 M HCl and 2 M NaOH. To check for the ability to grow in the presence of oxygen, the strains were inoculated in media without reducing agent (cysteine-HCl) and checked for growth after 24 h. No growth was observed, confirming they are strictly anaerobic bacteria. Aesculin is not hydrolysed. Indole is not produced.

The biochemical characterization of the isolate L2-7^T and the type strain DSM 3353^T gave inconsistent results on API ZYM and hence those data were not included in the results. This issue with the use of commercial rapid identification kits for testing biochemical properties for this group of bacteria is already known [16]. In addition, we also tested fermentation end products on various carbohydrates (Table 1). After 24 h of incubation, the major fermentation products of glucose metabolism for strain L2-7^T were butyrate (23.2 mM) and formate (11.9 mM). Similar values for butyrate (22.6 mM) and formate (10.2 mM) were observed for DSM 3353^T as end products of glucose metabolism. For L2-7^T, the major end product of 1,2-propanediol (80 mM) utilisation were propionate (23.2 mM), propanal (15.4 mM) and 1-propanol (14.5 mM). For DSM 3353^T, the end products of 1,2-propanediol were propionate (26.3 mM), propanal (19.1 mM) and 1-propanol (20.5 mM). The end products observed for 1,2-propanediol are in accordance

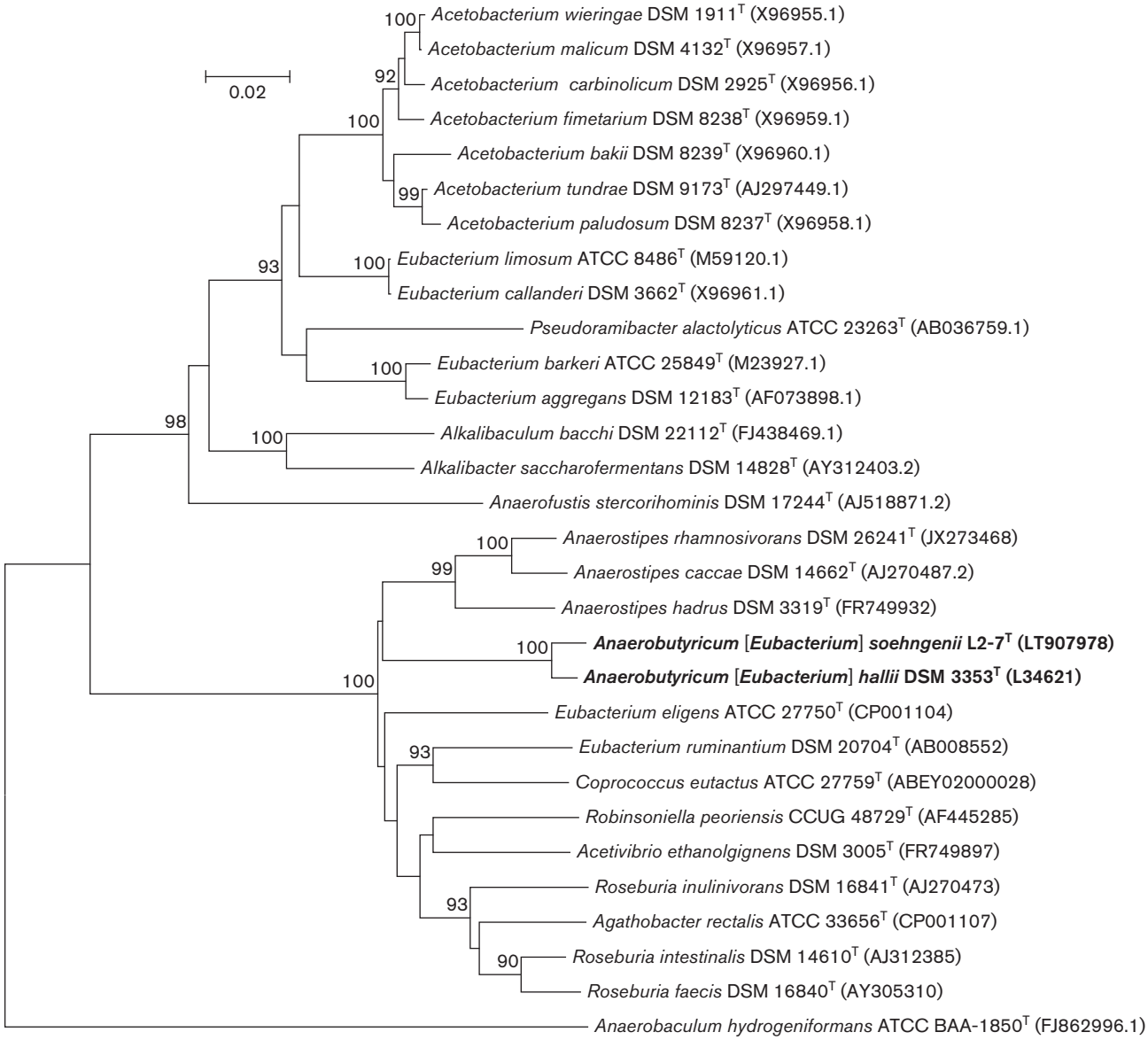

Fig. 1. Rooted phylogenetic tree showing the relationship of *Anaerobutyricum* [*Eubacterium*] *hallii* comb. nov., DSM 3353^T and strain L2-7^T with selected members of the families *Lachnospiraceae* and *Eubacteriaceae* reconstructed in MEGA6 [24]. The 16S rRNA gene of *Anaerobaculum hydrogenifromans* ATCC BAA-1850^T was used as an outgroup. The tree was reconstructed using the neighbor-joining method based on pairwise distances obtained using Kimura's two-parameter model. The bootstrap test included 1000 replicates, and only bootstrap values >90 % are shown [25, 26].

with a previous study [3]. After 24 h, strain L2-7^T utilized 37.8 mM of D,L-lactate and 16.8 mM of acetate and produced 24.3 mM of butyrate, while minor amounts of formate were detected. On the other hand, after 24 h, strain DSM 3353^T, utilized 26.8 mM of D,L-lactate and 10 mM acetate to produce 22.9 mM of butyrate. CO_2 was not measured. Resistance to porcine (Sigma-Aldrich) and Oxgall bile (Fluka Analytical) was tested in the standard medium. *E. hallii* DSM 3353^T was resistant to 0.1–1.0 % (w/v) porcine bile and 0.1 and 0.5 % (w/v) Oxgall. On the other hand, strain L2-7^T (DSM 17630^T) was not resistant to porcine bile but was resistant to 0.1 % Oxgall and susceptible to higher concentrations.

The DNA G+C content of strain L2-7^T was 38.6 mol% and for DSM 3353^T was 38.2 mol%, as determined from the genome sequence. The cellular fatty acid contents of strain L2-7^T and *E. hallii* DSM 3353^T were compared from cells grown for 12 h on the standard medium. The cellular fatty acids were then extracted by following the standard protocol of the Sherlock Microbial Identification Systems (MIDI) and identified using Sherlock version 4.0 and the BHIBLA 3.80

Table 1. Substrate utilization for strains L2-7^T (DSM 17630^T) and DSM3353^T

The major end products for all substrates (except for 1,2-propanediol), was butyrate. For 1,2-propanediol, the major end products were propionate and 1-propanol. Substrates for which differences in utilization between the two strains were observed are highlighted in bold.

	L2-7^T (DSM 17630^T)	DSM3353^T
Glucose	+	+
Fructose	+	+
Galactose	+	+
Maltose	+	+
Sucrose	**+**	−
Xylose	−	−
Arabinose	−	−
Mannose	+	+
Lactose	−	**+**
Ribose	−	**+**
Maltose	+	+
Sorbitol	+	+
Mannitol	−	**+**
Cellobiose	−	−
Rhamnose	−	−
1,2-Propanediol	+	+

libraries at DSMZ, Braunschweig [17–19]. GC indicated that $C_{16:0}$ was the predominant fatty acid in both the isolates, with $C_{16:0}$ being higher in strain L2-7^T compared to *E. hallii* DSM 3353^T (Table 2). Overall similarity in the fatty acid profile indicated that both strains belong to the same genus. The cellular fatty acid profile was composed mainly of $C_{16:0}$, $C_{16:1}$*cis* 9 and summed feature 10 ($C_{18:1}$c11/t9/t6*c*). The polar lipid patterns clearly differed between strains L2-7^T and DSM 3353^T (Fig. S2) and comprised unknown phospholipids, unknown lipids and phospoaminoglycolipid. Strain DSM 3353^T had an additional aminolipid and phosphoglycolipid and two unknown lipids. Strain L2-7^T was observed to have phosphatidylglycerol, phosphatidylethanolamine and two unknown phospholipids.

The type of peptidoglycan membrane for L2-7^T and DSM 3353^T was determined at DSMZ, using the methods described previously [20]. The peptidoglycan of both the strains is of the A1γ (A31) type containing *meso*-diaminopimelic acid (*meso*-Dpm). This type of peptidoglycan has also been reported recently to be present in isolates of the genus *Faecalicatena* that were reclassified from *Eubacteriaceae* to *Lachnospiraceae* [21].

The original classification of DSM 3353^T as a species of *Eubacterium* was incorrect has been confirmed by phylogenetic analyses presented here, and thus we propose that it should be reclassified as *Anaerobutyricum hallii* comb. nov. within the family *Lachnospiraceae* [13]. In addition, based on the results of our comparative polyphasic characterization, it is appropriate to assign strain L2-7^T to a novel species within the novel genus *Anaerobutyricum*, for which the name *Anaerobutyricum soehngenii* sp. nov. is proposed.

Table 2. Cellular fatty acid profiles for type strain DSM3353^T and strain L2-7^T (DSM17630^T). Values of 10 % and over are highlighted in bold

Fatty acids	L2-7^T (DSM 17630^T)	DSM3353^T
$C_{10:0}$	0.1	0.2
$C_{12:0}$	0.4	0.4
$C_{14:0}$	5.0	5.7
$C_{14:0}$ FAME	ND	5.7
$C_{14:0}$ DMA	ND	0.5
$C_{16:0}$ ALDE	0.5	1.5
$C_{16:1}$ *cis* 7 FAME	3.2	5.2
$C_{16:1}$*cis* 9 FAME	**5.5**	**11.7**
$C_{16:1}$*cis* 11 FAME	0.9	1.4
$C_{16:0}$ FAME	**30.4**	**19.5**
$C_{16:1}$*cis* 9 DMA	2.6	5.0
$C_{16:0}$ DMA	1.9	5.5
$C_{18:0}$ ADLE	0.2	ND
$C_{16:0}$ 3OH FAME	0.4	0.5
$C_{18:1}$*cis* 9 FAME	4.3	3.2
$C_{18:1}$*cis* 13 FAME	0.6	0.3
$C_{18:0}$ FAME	3.7	1.2
$C_{18:1}$*cis* 9 DMA	4.6	3.2
$C_{18:1}$*cis* 11 DMA	**11.4**	8.8
$C_{18:0}$ DMA	0.8	0.5
$C_{20:1}$cl3/t11 FAME	0.5	ND
$C_{19:0}$ cyc 11,12 DMA	ND	0.3
Summed feature 1*	ND	0.2
Summed feature 4*	1.0	2.6
Summed feature 6*	0.5	2.4
Summed feature 7*	1.1	0.8
Summed feature 8*	3.1	2.5
Summed feature 10*	**16.9**	**16.8**

*Summed feature 1 comprises $C_{13:1}$*cis* 12 FAME, $C_{14:0}$ ALDE; summed feature 4 is unknown or $C_{15:2}$ FAME, Summed feature 6 comprises $C_{15:0}$ ANTEI 3OH FAME, $C_{16:1}$*cis* 7 DMA; summed feature 7 comprises $C_{17:2}$ FAME, $C_{17:1}$*cis* 8 FAME; summed feature 8 comprises $C_{17:1}$*cis* 9 FAME, $C_{17:2}$ FAME; summed feature 10 most likely comprises $C_{18:1}$c11/t9/t6 FAME.

DESCRIPTION OF *ANAEROBUTYRICUM* GEN. NOV.

Anaerobutyricum (An. ae. ro. bu.ty′ri.cum. Gr. pref. *an* not; Gr. n. *aer* air; L. masc. n. *butyricum,* butyrate producing, anaerobic butyrate producing).

Gram-positive, non-motile, with rod-shaped cells. Obligately anaerobic and catalase-negative and oxidase-negative. Optimal growth is observed at 37 °C. Monosaccharides galactose, fructose and glucose, disaccharides such as maltose and the sugar alcohol sorbitol are fermented. Mannitol can be fermented by *A. hallii*, but not by *A. soehngenii*. The

major end-products of sugar fermentation are butyrate, formate, H_2 and CO_2. When grown on 1,2-propanediol, propionate, propanal and 1-propanol are produced. It requires acetate for better growth on substrates such as D,L-lactate and produces butyrate as major end product and small amounts of formate. The G+C content of the DNA is 38.2 ±4 mol%. The type species of the genus is *Anaerobutyricum hallii* [22]. Known species within this genus tolerate 0.1 % Oxgall.

DESCRIPTION OF *ANAEROBUTYRICUM HALLII* COMB. NOV.

Anaerobutyricum hallii (hall'i.i. N.L. gen. n. *hallii* of Hall, named for Ivan C. Hall, an American microbiologist).

Basonym: *Eubacterium hallii* (Holdeman and Moore 1974) Approved Lists 1980

Description is as given by Holdeman and Moore 1974, in addition to the original description of *E. hallii* [23], the species requires acetate to grow on lactate. It can grow well on lactose, ribose and mannitol, but not on sucrose. In accordance with Holdeman and Moore 1974, weak growth for *A. hallii* is observed in ribose and growth is seen after a 24 h lag phase. The major cellular fatty acids are $C_{14:0}$ FAME, $C_{16:1}$*cis* 9 FAME and $C_{18:1}$*cis* 11 DMA. The optimum pH for growth is 7.5 at 37 °C. The type strain for this species is *Anaerobutyricum hallii* (=DSM 3353^T=ATCC 27751^T).

DESCRIPTION OF *ANAEROBUTYRICUM SOEHNGENII* SP. NOV.

Anaerobutyricum soehngenii (soehn.ge'ni.i. N.L. gen. n. *soehngenii* in honour of the first Wageningen Professor of Microbiology, Nicolaas L. Soehngen, for his contribution to anaerobic microbiology).

In addition to the characteristics of the genus, *A. soehngenii* exhibits the following features. Indole and catalase are not produced and it is negative for oxidase. Aesculin is not hydrolysed. Acid is produced from D-glucose, maltose, galactose, sucrose, D-mannose, D-fructose and sorbitol. There is a net utilization of acetate when grown on sorbitol. It can produce butyrate when D,L-lactate and acetate are present in the medium. No growth was observed in D-xylose, L-arabinose, cellobiose, D-mannitol, lactose or D-rhamnose. The major cellular fatty acids are $C_{16:0}$ FAME and $C_{18:1}$*cis* 9 FAME, $C_{18:0}$ FAME and $C_{18:1}$*cis* 11 DMA.

The type strain, L2-7^T (=DSM 17630^T=KCTC 15707^T), was isolated from human infant faeces. The DNA G+C content of the type strain is 38.6 mol%.

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

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