INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION
Feng *et al.*, *Int J Syst Evol Microbiol* 2018;68:3546–3550
DOI 10.1099/ijsem.0.003028

MICROBIOLOGY SOCIETY

Eubacterium maltosivorans sp. nov., a novel human intestinal acetogenic and butyrogenic bacterium with a versatile metabolism

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Abstract

A novel anaerobic, non-spore-forming bacterium was isolated from a faecal sample of a healthy adult. The isolate, designated strain YI^T, was cultured in a basal liquid medium under a gas phase of H_2/CO_2 supplemented with yeast extract (0.1 g l^{-1}). Cells of strain YI^T were short rods (0.4–0.7×2.0–2.5 µm), appearing singly or in pairs, and stained Gram-positive. Catalase activity and gelatin hydrolysis were positive while oxidase activity, indole formation, urease activity and aesculin hydrolysis were negative. Growth was observed within a temperature range of 20–45 °C (optimum, 35–37 °C), and a pH range of 5.0–8.0 (optimum pH 7.0–7.5). Doubling time was 2.3 h when grown with glucose at pH 7.2 and 37 °C. Besides acetogenic growth, the isolate was able to ferment a large range of monomeric sugars with acetate and butyrate as the main end products. Strain YI^T did not show respiratory growth with sulfate, sulfite, thiosulfate or nitrate as electron acceptors. The major cellular fatty acids of the isolate were $C_{16:0}$ and $C_{18:0}$. The genomic DNA G+C content was 47.8 mol%. Strain YI^T is affiliated to the genus *Eubacterium*, sharing highest levels of 16S rRNA gene similarity with *Eubacterium limosum* ATCC 8486^T (97.3 %), *Eubacterium callanderi* DSM 3662^T (97.5 %), *Eubacterium aggregans* DSM 12183^T (94.4 %) and *Eubacterium barkeri* DSM 1223^T (94.8 %). Considering its physiological and phylogenetic characteristics, strain YI^T represents a novel species within the genus *Eubacterium*, for which the name *Eubacterium maltosivorans* sp. nov. is proposed. The type strain is YI^T (=DSM 105863^T=JCM 32297^T).

The human intestinal tract is colonized by billions of commensal micro-organisms that represent over a thousand species contributing to either health or disease. Among others, intestinal microbes convert undigested carbohydrates mainly into short-chain fatty acids, such as butyrate, propionate and acetate [1–3]. As colonic fermentations are generally anaerobic, redox balancing often involves the production of hydrogen, which can also be consumed by methanogens, sulfur compounds respirers or homoacetogenic bacteria [4].

In the course of a study to enrich sulfidogenic bacteria from the human gut (approved by CCMO Netherlands, project ID: NL2907008109), we isolated a novel acetogenic bacterial strain (YI^T) sharing 97.3 % 16S rRNA gene similarity with *Eubacterium limosum* ATCC 8486^T [5]. Strain YI^T was enriched under anaerobic conditions in a basal liquid medium prepared according to Stams *et al.* [6] supplemented with 5 mM Na_2SO_3, yeast extract (0.1 g l^{-1}; BD BBL) and H_2/CO_2 (80 : 20, v/v, 1.7 atm) in the gas phase. Throughout the enrichment, sulfite was not reduced but H_2 and CO_2 were consumed, and acetate was produced. Subsequently, a pure culture was obtained by a combination of serial dilution and plating on solidified media, with 1 % noble agar (Sigma-Aldrich), under 1.7 atm of H_2/CO_2 (80 : 20, v/v).

Cell morphology, motility, Gram-staining and spore formation were studied by phase-contrast microscopy using a Zeiss AXIO Scope A1. Gram-staining was performed according to standard procedures [7]. Survival due to spore formation was checked by placing the cultivation bottle in an 80 °C water bath for 20 min. Oxidase and catalase activities were tested as described by Florentino *et al.* [8]. Indole and urease formation, as well as gelatin and aesculin hydrolysis were examined in duplicate by the API

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Keywords: acetogens; butyrate; maltose; hydrogen; human gut microbiota.
Abbreviations: ANI, average nucleotide identity; DDH, DNA–DNA hybridization.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Eubacterium maltosivorans* strain YI^T is MH400075. The GenBank accession number of the assembled draft genome is GCA_002441855.1
One supplementary figure and one supplementary table are available with the online version of this article.

Table 1. Selected physiological and biochemical characteristics that differentiate strain YI^T from its closest described relatives

The major fatty acids are shown in bold. +, Positive; −, negative; w, weak (after 5 days less than 3 mM of substrates were consumed). Both strains stained Gram-positive, and were non-motile, non-spore-formers. Both strains were able to use H_2/CO_2, CO, glucose, fructose, ribose, lactate, pyruvate, mannitol, erythritol, vanillate, cysteine and betaine, and showed weak growth on soluble starch, but were not able to use lactose, arabinose, cellobiose, galactose, rhamnose, melibiose, succinate, glycine, serine, glycerol or ethanol. DMA denotes dimethylacetal.

Characteristic	*E. limosum* ATCC 8486^T*	Strain YI^T†
Growth temperature (optimum, °C)	25–45 (30–37)	20–45 (35–37)
pH range (optimum)	5.0–8.0 (7.0–7.2)	5.0–8.0 (7–7.5)
DNA G+C content (mol%)	47.2	47.8
Genome size (Mbp)	4.37	4.58
Sugars		
D-xylose	w†	−
Maltose	−†	+
Mannose	−†	+
Sucrose	−†	+
Raffinose	−	+
Acids		
Formate	w	w
Vanillate	+†	+
Alcohol		
Methanol	+	w
Cellular fatty acids (%)†		
Saturated straight-chain		
12 : 0	0.3	0.2
14 : 0	**16.8**	6.3
14 : 0 DMA	1.1	0.1
16 : 0	**38.6**	**44.5**
16 : 0 aldehyde	3.8	3.0
16 : 0 DMA	9.6	9.1
18 : 0	4.6	**10.5**
18 : 0 aldehyde	0.6	1.5
18 : 0 DMA	1.0	3.3
20 : 0	0.3	1.1
Unsaturated straight-chain		
16 : 1ω5*c*	0.2	0.7
16 : 1ω7*c*	1.7	1.2
16 : 1ω7*c* DMA	0.8	0.2
18 : 1ω7*c*	8.1	7.7
18 : 1ω7*c* DMA	1.3	0.9
Saturated branched-chain		
15 : 0	0.3	0.2
15 : 0 anteiso	0.2	−
15 : 0 iso	0.2	−
17 : 0 cyclopropane	1.4	0.8
17 : 0 cyclo DMA	0.2	0.1
19 cycloprop-11,12	6.1	7.2
19 cyclo 11,12 DMA	1.2	1.7

*Data for *E. limosum* ATCC 8486^T were from Genthner *et al.* [23] (except where otherwise indicated).

†Data from this study.

20A test (bioMérieux) according to the manufacturer's instructions.

To determine the temperature range and optimum, strain YI^T was grown in the basal medium supplemented with

20 mM glucose under 1.7 atm of N_2/CO_2 (80 : 20, v/v) gas phase without yeast extract and incubated for up to 6 weeks within the temperature range from 15 to 60 °C (at 5 °C intervals, 37 °C was tested as well) at pH 7.2. The optimum pH was tested in the same medium at 37 °C but bicarbonate and N_2/CO_2 were omitted. Different buffer systems were employed to give different pH ranges and the gas phase contained only N_2. For pH higher than 7.0, 20 mM Tris was used; for pH 6.0–7.0, 20 mM PIPES was added; and for pH 6–4, 20 mM citrate/phosphate buffer was used. Hence, growth over the pH range from 4.0 to 8.5 was tested at 0.5 unit intervals, with incubation at 37 °C for up to 6 weeks. Both temperature and pH tests were run in triplicate.

To explore the physiological properties of strain YI^T, a variety of substrates including sugars, organic acids, amino acids and sugar alcohols (see Table 1) were added to the basal medium to a final concentration of 20 mM without yeast extract, unless mentioned otherwise. Cultures were incubated under 1.7 atm of N_2/CO_2 (80 : 20, v/v) at 37 °C and pH 7.2. Each incubation was performed in triplicate. Soluble corn starch and betaine were tested at a final concentration of 5 g l^{-1} supplied with 0.1 g yeast extract l^{-1} in the basal medium. When hydrogen was used as a substrate, the headspace was at 1.7 atm with H_2/CO_2 (80 : 20, v/v) and the medium was supplemented with 0.5 g yeast extract l^{-1}. Carbon monoxide (40 %) was tested as an energy source by exchanging the N_2/CO_2 in the headspace with filter-sterilized CO. The headspace was kept at 1.7 atm and the medium was supplemented with 0.5 g yeast extract l^{-1}. Negative controls without substrate were included and showed no growth. Sulfate (20 mM), thiosulfate (20 mM), sulfite (5 mM) and nitrate (10 mM) were tested as electron acceptors. For this, six different electron donors were tested due to their relevance for the human intestinal tract: acetate, butyrate, propionate, lactate and pyruvate at a concentration of 10 mM, and H_2 at 1.7 atm of H_2/CO_2 (80 : 20, v/v). For all physiological tests, products were quantified by HPLC with a Varian Metacarb 67H 300 mm column and sulfuric acid (0.005 M) eluent at a flow rate of 0.8 ml min^{-1}. Hydrogen was measured via a gas chromatograph (Shimadzu) as described by Florentino *et al.* [9]. Sulfate, thiosulfate and sulfite were analysed using a Dionex 1000 ion chromatograph unit equipped with an IonPac AS17 anion-exchange column operating at a flow rate of 0.1 ml min^{-1} at 30 °C. Hydrogen sulfide was measured by a methylene blue method [10]. First, H_2S, HS^- and S^{2-} were fixed by using a 5 % (w/v) $ZnCl_2$ solution. Then, ZnS deposited was re-dissolved by an acid *N*,*N*-dimethyl-*p*-phenylenediamine solution, and simultaneously a ferriammonium sulfate solution was added to generate methylene blue. Reagents were prepared according to Cline [10]. The amount of sulfide was determined by using a spectrophotometer after the reaction was fully developed.

To study the differences of the cellular fatty acid composition, strain YI^T and *E. limosum* ATCC 8486^T were incubated under the same conditions (20 mM fructose with 0.5 g yeast extract l^{-1}) for 2 days. Cells were harvested and analysis was performed at the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

To obtain genomic DNA, strain YI^T was grown in the aforementioned basal medium containing 20 mM glucose under 1.7 atm of N_2/CO_2 (80 : 20, v/v) for 48 h at 37 °C. Biomass was harvested by centrifugation at 13 000 *g* for 5 min at 4 °C. Genomic DNA was isolated by using a MasterPure device (Epicentre) and purified via a Wizard Genomic DNA Purification Kit (Promega) following the manufacturers' instructions. Illumina MiSeq sequencing was performed at GATC Biotech and assembled using the Edena v3.130110 and IDBA-UD v1.1.1 assemblers and merged [11, 12]. The assembled draft genome had a size of 4.5 Mbp and the sequence has been deposited at GenBank under accession number GCA_002441855.1. The 16S rRNA gene sequence (1512 bp) was obtained from the draft genome and deposited at GenBank under accession number MG015881. To check whether there was any heterogeneity between 16S rRNA gene operons, a PCR amplicon of the 16S rRNA genes was sequenced by Sanger sequencing (performed by GATC Biotech). This 1288 bp sequence (deposited at NCBI with accession number MH400075) was found to be identical except for 1 mismatch with that obtained by the Illumina sequencing. Later, the whole 16S rRNA gene sequence of strain YI^T was aligned using the SINA online alignment tool (version 1.2.11) [13] and then merged with the Silva SSU Ref database (release 111) [14]. A phylogenetic tree was reconstructed in the ARB software package (version 6) using the neighbour-joining algorithm [15]. The G+C content of the DNA was determined based on the draft genome obtained by Illumina sequencing. The average nucleotide identity (ANI) and the *in-silico* DNA–DNA hybridization (DDH) values were calculated by the online tools developed by the Environmental Microbial Genomics Laboratory [16] and DSMZ [17], respectively. The draft or complete genomes of *E. limosum* ATCC 8486^T, *E. limosum* KIST 612, *E. limosum* SA11 and *Eubacterium callanderi* DSM 3662^T as deposited at NCBI under numbers GCA_000807675.2, NC_014624.2, GCA_001481725.1 and GCA_900142645.1 were used for the ANI and *in-silico* DDH analyses.

Cells of strain YI^T were short rods, 0.4–0.7×2–2.5 µm in size, non-motile, appearing singly or in pairs, rarely in chains [Fig. S1(a), available in the online version of this article]. Spores were never detected by phase contrast microscopy in growing or stationary cultures, or in cultures that had been heated at 80 °C for 20 min. Cells stained Gram-positive [Fig. S1(b)]. Catalase activity was positive. Oxidase and urease activities were negative, indole formation and aesculin hydrolysis were absent, but gelatin hydrolysis occurred. The predominant cellular fatty acids of the isolate were $C_{16:0}$ (44.5 %) and $C_{18:0}$ (10.5 %). The main differences compared to that of the *E. limosum* type strain were the different proportional abundancies of $C_{18:0}$ (4.6 %) and $C_{14:0}$ (16.8 %) (Table 1).

Strain YI^T grew from 20 to 45 °C, with an optimum at 35–37 °C. It was able to grow at pH 5.0–8.0, but grew optimally at pH 7.0–7.5. The doubling time when grown at optimal pH and temperature with glucose was 2.3 h, and 2.5 h with fructose. Strain YI^T fermented a large range of monomeric sugars besides acetogenic growth with H_2/CO_2 (Table 1). When sugars were fermented, acetate, butyrate and H_2 were the principal end products, while when growing with H_2/CO_2 and CO, acetate was the only product. Strain YI^T was also capable of fermenting cysteine, releasing acetate and hydrogen sulfide. The strain was not able to reduce sulfate, thiosulfate, sulfite or nitrate. Physiologically, strain YI^T could be distinguished from the type strain of *E. limosum* by its ability to ferment maltose, sucrose, mannose and raffinose.

Phylogenetic analysis showed that strain YI^T is located in the genus *Eubacterium* of the family *Eubacteriaceae* (order *Clostridiales*, class *Clostridia*, phylum *Firmicutes*), sharing highest levels of 16S rRNA gene similarity with *E. limosum* KIST 612 (98.3 %) [18], *E. limosum* ATCC 8486^T (97.3 %), *E. callanderi* DSM 3662^T (97.5 %), *Eubacterium aggregans* DSM 12183^T (94.4 %) and *Eubacterium barkeri* DSM 1223^T (94.8 %) (Fig. 1). The family *Eubacteriaceae* also includes *Acetobacterium*, members of which are well known for their ability to grow on C1 compounds. However, species of the genus *Eubacterium* can utilize a larger range of substrates, including hexoses, pentoses, alcohols and some amino acids. Moreover, fermentative growth leads to acetate and butyrate as products. The type species of the genus *Eubacterium* was first designated as *Eubacterium foedans* [19], which was isolated by Klein [20] from spoiled hams. Later, Cato *et al.* [21] proposed *E. limosum* as the type species because no cultures of *E. foedans* were extant, and this request was approved in 1983. The type strain of *E. limosum* was first isolated from human faeces [22]. Later this bacterium was commonly reported from many other ecosystems [22]. *E. limosum* is known for itsability to convert C1 compounds such as CO, H_2/CO_2, formate and methanol, as well lactate, hexoses, pentoses and some more complex carbohydrates into acetate, ethanol or butyrate [23]. The closest relative of *E. limosum* is *E. callanderi*, sharing 99.5 % 16S rRNA gene sequence similarity. *E. callanderi* differs from *E. limosum* in that it cannot utilize H_2/CO_2, CO, methanol or other one-carbon compounds. Moreover, *E. callanderi* cannot grow on glucose without a supply of acetate in a defined medium, whereas this is not the case for *E. limosum* [24].

Because of the phylogenetic similarity and acetogenic growth characteristics of the new strain YI^T and *E. limosum,* we compared the physiological and biochemical properties of strain YI^T with that of *E. limosum* ATCC 8486^T (Table 1). The substrate utilization of strain YI^T included maltose, sucrose, mannose and raffinose, none of which could be used by *E. limosum*. However, *E. limosum* could use xylose in contrast to strain YI^T. The *in-silico* ANI and DDH values between the genomes of strain YI^T and *E. limosum* ATCC 8486^T were 89.2 % and 38.6 %, respectively. Both values are well below the cut-off values for novel species (<95–96 % and <70 %, respectively, [25, 26]) (Table S1). Similarly, the same also applied to the *in-silico* ANI and DDH values of the genomes of strain YI^T and *E. callanderi* DSM 3662^T (89.7 % and 39.7 %, respectively; Table S1). Hence, we conclude that strain YI^T differs genotypically and physiologically from *E. limosum* ATCC 8486^T and hence belongs to a novel species. The genome of strain YI^T shared approximately 86 % sequence similarity (Table S1) with that of the recently reported but not publicly deposited strain SA11 of *E. limosum*, isolated from the rumen of a New Zealand sheep [27]. Based on these considerations, we propose that strain YI^T represents a novel species, *Eubacterium maltosivorans* sp. nov., within the genus *Eubacterium*.

DESCRIPTION OF *EUBACTERIUM MALTOSIVORANS* SP. NOV.

Eubacterium maltosivorans (mal.to.si.vo′rans. N.L. neut. n. *maltosum* maltose; L. pres. part. *vorans* eating; N.L. part. adj. *maltosivorans* maltose eating).

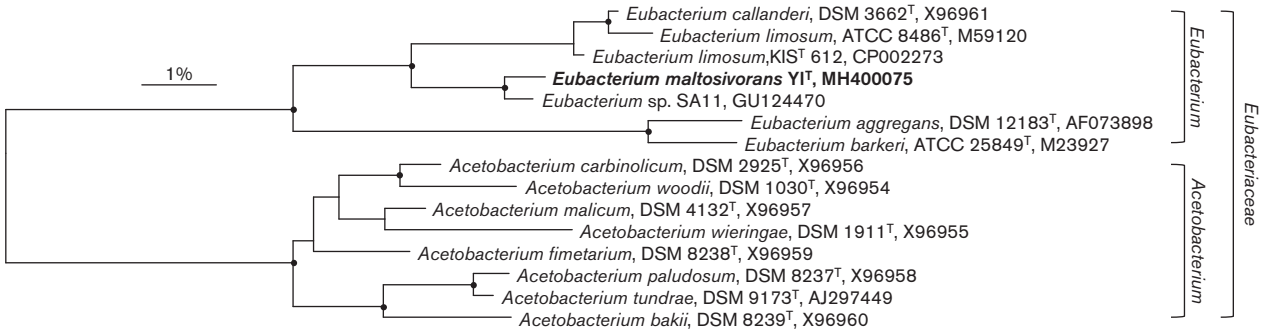

Fig. 1. Neighbour-joining tree showing the phylogenetic affiliation of strain YI^T to other representatives of the family *Eubacteriaceae* based on 16S rRNA gene sequence similarity calculated with Jukes–Cantor correction in ARB. The tree was rooted with *Escherichia coli*, which was subsequently removed. Bar, 1 % sequence divergence. Bootstrap values greater than 90 % (1000 replicates) are indicated by filled circles.

Cells are non-motile, non-spore forming and Gram-stain-positive. Short rods (0.4–0.7×2.0–2.5 µm) appear singly and in pairs, rarely in chains when grown with glucose. Catalase activity and gelatin hydrolysis are positive. Oxidase and urease activity, indole formation and aesculin hydrolysis are negative. The temperature range is 20–45 °C, with optimum of 35–37 °C. The pH range is 5.0–8.0, with optimum of 7.0–7.5. Yeast extract is only essential for growth with H_2/CO_2. Utilizes glucose, fructose, ribose, maltose, mannose, sucrose, raffinose, lactate, pyruvate, sorbitol, erythritol and betaine. Fermentative growth with sugars occurs, leading to the production of acetate, butyrate and hydrogen. Ferments cysteine, releasing sulfide and acetate. Does not use xylose, lactose, arabinose, cellobiose, galactose, rhamnose, melibiose, formate, succinate, glycine, serine, glycerol or ethanol. No respiratory metabolism is detected.

The type strain is YI^T (=DSM 105863^T=JCM 32297^T), isolated from human faeces. The G+C content of the genomic DNA of the type strain is 47.8 mol%.

Funding information
This work was supported by the SIAM Gravitation Grant 024.002.002 of the Netherlands Organization for Scientific Research.

Acknowledgements
We would like to thank Steven Aalvink for his valuable tips on genomic DNA extraction, and Bastian Hornung and Benoit Carreres for their help with genome assembly.

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

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