Anaerostipes rhamnosivorans sp. nov., a human intestinal, butyrate-forming bacterium

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A novel butyrate-producing bacterium, strain 1y-2T, was isolated from a stool sample of a 1-year-old, healthy Dutch infant. The isolate was obtained by using lactate and acetate as sources of carbon and energy. The strain was Gram-variable, strictly anaerobic and spore-forming and formed curly rod-shaped cells that fermented glucose into butyrate, lactate, formate and acetate as main products. The DNA G+C content of the strain was 44.5 mol% and its major cellular fatty acids were C12:0, iso-C19:1 I and C16:0. Strain 1y-2T was related to Anaerostipes caccae DSM 14662T based on 16S rRNA gene sequence analysis, with 3% divergence, but hybridization studies of their genomic DNA revealed only 33% relatedness. Moreover, strain 1y-2T showed marked physiological and biochemical differences from known species of the genus Anaerostipes. Based on phylogenetic, chemotypic and phenotypic criteria, we propose that strain 1y-2T should be classified in the genus Anaerostipes within a novel species, Anaerostipes rhamnosivorans sp. nov. The type strain is 1y-2T (=DSM 26241T =KCTC 15316T).

The large intestinal tract is the most heavily colonized portion of the human body, with bacterial cell numbers that can exceed $10^{11}$ per gram content. A major metabolic function of the intestinal microbiota is the anaerobic conversion of polymeric sugars into short-chain fatty acids (SCFAs) such as acetate, propionate and butyrate. These SCFAs fuel the enterocytes, while some have a signalling function. Hence, bacteria that produce these SCFAs are of special interest because of their vital roles in human colonic health. Butyrate, in particular, has been identified as a major energy source for colonocytes (Pryde et al., 2002). Butyrate may protect against cancer and ulcerative colitis (Hague et al., 1997), and has been associated with preventing the development of insulin resistance and obesity in mice (Flint et al., 2012b). Therefore, studies on butyrate-producing bacteria are of specific importance for further understanding of their role in intestinal health and disease. All bacteria known to date that produce butyrate belong to the phylum Firmicutes of low-G+C-content Gram-positive bacteria. There are basically two metabolic avenues to produce butyrate. One is directly from sugars, with or without the use of external acetate, utilized by intestinal bacteria such as Faecalibacterium, Roseburia and some species of the genus Clostridium. The other is via the conversion of acetic acid and lactic acid that are produced from mixed acid fermentation from sugars. The latter conversion is of specific interest as it removes the generated lactic acid, where the accumulation of this compound may lead to intestinal damage. Bacteria such as those related to Anaerostipes caccae and Eubacterium hallii are the main intestinal representatives that are capable of producing butyrate from acetic and lactic acids rather than from sugars (Flint et al., 2012b).

Any microbial study of butyrate producers starts with their isolation from the originating ecosystem. Over the last decade, efforts to isolate butyrate producers from human stool have revealed that many of the different species are present within two clusters of Firmicutes: clostridial clusters IV and XIVa (Barcenilla et al., 2000; Louis et al., 2004). Recent analysis of the butyryl-CoA : acetate CoA transferase gene, a key gene for butyrate production, has shown that there is still a fraction of not-yet-cultured butyrate-producing strains (Louis et al., 2010; Levine et al., 2013). In an effort to isolate these butyrate producers, we performed an intensive screening procedure using a medium containing lactate and acetate as carbon and energy sources especially aimed at clostridial cluster XIVa, one of the predominant and most diverse clusters in the human intestine. A decade ago, a new genus in cluster XIVa, Anaerostipes, was isolated (Schwiertz et al., 2002). This genus represents more than 2% of total colonic microbiota in the healthy colon (Flint et al., 2012a).
Furthermore, these organisms are believed to play an important functional role in the gut ecosystem (Allen-Vercoe et al., 2012) due to their ability to produce butyrate from lactate (Muñoz-Tamayo et al., 2011). At the time of writing, only three species have been discovered: Anaerostipes caceae and Anaerostipes hadrus (formerly classified as Eubacterium hadrum), from the human intestine (Schwiertz et al., 2002; Allen-Vercoe et al., 2012), and Anaerostipes butyricus, from broiler chicken caecal content (Eckhaut et al., 2010). Here, we report the isolation, phenotypic characterization and phylogenetic analysis of a novel isolate from an infant stool. Based on our findings, we propose that this bacterium represents a novel species within the genus Anaerostipes.

Strain 1y-2T was obtained from a faecal sample of a 1-year-old healthy Dutch infant. Approximately 1 g fresh faecal slurry was collected by using a needle and syringe and transferred into an anaerobic 35 ml serum vial containing 10 ml medium containing 20 mM phosphate buffer, 25% (v/v) glycerol, 0.5 g resazurin l⁻¹ and 0.5 g cysteine l⁻¹. This suspension was mixed thoroughly and subsequently diluted serially up to 10¹⁰-fold into a bicarbonate-buffered anaerobic medium consisting of (l⁻¹) 0.53 g Na₂HPO₄, 2H₂O, 0.41 g KH₂PO₄, 0.3 g NH₄Cl, 0.11 g CaCl₂.2H₂O, 0.10 g MgCl₂.6H₂O, 0.3 g NaCl, 4.0 g NaHCO₃ and 0.48 g Na₂S.9H₂O as well as alkaline and acid trace elements (each 1 ml l⁻¹) and vitamins (0.2 ml l⁻¹) (Stams et al., 1993). The alkaline trace element solution contained the following (mM): 0.1 Na₂SeO₃, 0.1 Na₂WO₄, 0.1 Na₂MoO₄ and 10 NaOH. The acid trace element solution was composed of the following (mM): 7.5 FeCl₃, 1 H₂BO₄, 0.5 ZnCl₂, 0.1 CuCl₂, 0.5 MnCl₂, 0.5 CoCl₂, 0.1 NiCl₂ 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. This basal medium was supplemented with 40 mM sodium lactate and 40 mM sodium acetate added from 1 M sterile anaerobic stock solutions. Incubations were done in 35 ml serum bottles sealed with butyl-rubber stoppers at 37 °C under a gas phase of 1.7 atm (172 kPa) N₂/CO₂ (80:20, v/v). The pH of the medium was 6.8. The inoculum amount was 1% (v/v).

After 2 weeks of incubation, the highest dilution where growth was observed was transferred into the second dilution row up to 10¹⁰. For primary identification of the microbial composition, genomic DNA isolated from the highest grown dilution of the second row after 5 days was subjected to a PCR using the 16S rRNA gene primers 27F and 1492R (Weisburg et al., 1991) and used to generate a clone library of full-length 16S rRNA gene sequences using the pGEM-T Easy vector system (Promega). Twenty-four clones were selected for 16S rRNA gene sequencing using the same primers used for cloning. Based on almost-full-length 16S rRNA gene sequences, all clones were identified to have 97% similarity to A. caceae DSM 14662T. To purify the apparently new member of the genus Anaerostipes further, the culture was repeatedly transferred into new dilution series and eventually plated on 1.5% (w/v) agar-containing bicarbonate-buffered medium (agar noble; Difco) containing 40 mM lactate and 40 mM acetate as carbon and energy sources. A single colony was picked and transferred to a new plate which was incubated in an anaerobic jar at 37 °C. These plating steps were repeated until a pure culture that was designated strain 1y-2T was obtained.

Cell morphology and purity of strain 1y-2T were examined continuously with a phase-contrast microscope. Additional purity confirmation was performed using denaturing gradient gel electrophoresis analysis of 16S rRNA gene amplicons of strain 1y-2T grown in different media including reinforced clostridial medium (RCM), bicarbonate-buffered medium with glucose and bicarbonate-buffered medium with lactate and acetate as carbon and energy sources (data not shown). The PCR primers were F-968-GC (5’-CGCCGGGGCCGGCCGGGCAGCGG-GGGGACCGGGGAAACCGGAAGAACCTTAC-3’) and R-1401 (5’-CGGTGTGTACAGAACCCT-3’) (Nübel et al., 1996).

Genomic DNA of strain 1y-2T was extracted and purified with a FastDNA Spin kit for soil (MP Biomedicals) following the manufacturer’s instructions. A 16S rRNA gene was amplified from chromosomal DNA of strain 1y-2T as described above. The PCR program was started at 94 °C for 5 min and continued with 35 cycles consisting of 94 °C for 90 s, 52 °C for 30 s and 72 °C for 90 s and finally 72 °C for 10 min. The PCR products were subsequently purified using the Qiaquick PCR purification kit (Qiagen) and sequenced using a universal primer set, 27F and 1492R together with 533F (5’-GTGCGACGCMGCGCGG-3’) (Lane, 1991) and 650R (5’-TCCACGCCTGAACGATGAG-3’) (unpublished), to read from the middle of the 16S rRNA gene. Sequencing of the amplified 16S rRNA gene was performed at GATC Biotech (http://www.gatc-biotech.com). Single fragments were checked for reading errors using Chromas and subsequently aligned using the program DNASTAR. The 16S rRNA gene sequence was subjected to searches using the BLASTN search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and EzTaxon 2.1 (Chun et al., 2007). The 16S rRNA gene sequences of the isolate and known related type strains were aligned with the CLUSTAL_X program (Thompson et al., 1994) and Kimura’s two-parameter model (Kimura, 1983). The phylogenetic tree was reconstructed using the neighbour-joining and maximum-parsimony algorithms (Saitou & Nei, 1987) with evolutionary distances calculated by the MEGA 5 program (Tamura et al., 2011). Bootstrap analysis was replicated 1000 times to obtain confidence levels for the branches (Felsenstein, 1985). DNA–DNA relatedness was determined reciprocally between strain 1y-2T and A. caceae DSM 14662T, obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), as described previously (De Ley et al., 1970) with some changes (Huss et al., 1983). All strains were grown under the same conditions using RCM.
values were measured by using a model Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with in situ temperature probe. Cell morphology, motility and spore formation of the isolate was observed using a Leica DM 2000 microscope, a JEOL-6480LV scanning electron microscope (SEM) and a JEOL JEM-1011 transmission electron microscope (TEM) (Fig. S1, available in IJSEM Online). For SEM observation, fresh wet samples were fixed in a solution of 2 % (w/v) glutaraldehyde in phosphate buffer. After fixation, the samples were filtered over a 0.2 μm polycarbonate membrane, rinsed with 100 % ethanol and dried overnight at room temperature. The membranes with bacteria on top were attached to 15 mm diameter mounts with a doublesided carbon adhesive tab. Before observation under high vacuum, samples were coated in a JEOL JFC-1200 fine coater with a thin (10 nm) gold layer. The SEM was operated under high-vacuum conditions at 6 kV at 10 mm working distance and spot size 20. For TEM imaging, cells grown for 2 days were fixed for 65 h in 2.5 % (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4 °C. After rising with 0.1 M sodium cacodylate buffer, post-fixation was done in 1 % (w/v) OsO4 and 1 % (w/v) potassium ferricyanide in the same buffer for 1 h at room temperature. The cells were then rinsed three times in demineralized water before embedding in 5 % (w/v) gelatin. The gelatin was solidified at 0 °C and subsequently dehydrated in a graded ethanol series (10, 30, 50, 70, 90 and 100 %) before being infiltrated with modified Spurr resin mixture (Serva). The samples were eventually sectioned to 70–80 nm on an ultramicrotome (Reichert Ultracut S). Micrographs were taken with a JEOL JEM 1011 TEM and a digital camera (Olympus; Veleta). The Gram reaction was done using standard methods (Plügg et al., 2000). Spore visualization was carried out by staining with malachite green following the manufacturer’s instructions (Sigma).

To determine the optimum growth temperature and pH, strain 1y-2T was inoculated in 10 ml RCM (Difco) and incubated at 4 and 10–60 °C at intervals of 5 °C for 8 weeks. The optimum pH was tested in duplicate in mineral medium using 20 mM Tris to buffer at ≥pH 8.0; 20 mM PIPES for pH 6.0–7.5 and 20 mM citrate for <pH 6.0 for 4 weeks. Growth was determined over the range pH 4–9 at intervals of 0.5 pH units (adjusted with 2 M HCl and 2 M NaOH). To define salt tolerance, the strain was grown in bicarbonate-buffered medium with 1–10 % (w/v) NaCl and incubated for 4 weeks. Media for pH and salt tolerance tests contained glucose as the sole carbon and energy source.

Strain 1y-2T and reference strains of other species of the genus Anaerostipes obtained from the DSMZ were pre-grown in RCM (Difco) before performing biochemical characterization using several commercial API test systems (bioMérieux). Carbohydrate assimilation was defined by using API 20A and API 50CHL kits. Enzyme profiling was performed using API Rapid ID 32A and API ZYM kits. All tests were performed in duplicate. The results were interpreted following the manufacturer’s instructions. Substrate utilization was determined by adding each substrate at a final concentration of 40 mM to anaerobic bicarbonate-buffered medium, with 10 % inoculum and incubation at 37 °C for 72 h. Tested substrates were D- and L-lactate, D-glucose, D-galactose, D-fructose, D-mannose, L-rhamnose, dulcitol, D-mannitol, D-sorbitol, D-arabinose, maltose, sucrose, trehalose, xylitol, D-tagatose, D-arabinose, inulin, laminarin and xylan. End products were determined using HPLC and GC methods as described previously (van Gelder et al., 2012).

The cellular fatty acid composition was analysed from cells grown in Wilkins–Chalgren broth (Oxoid) at 37 °C for 24 h. Cells were centrifuged at 4 °C and 16750 g and subjected to cellular fatty acid extraction. The products were analysed by using an Agilent model 6890N gas chromatograph (MIDI Sherlock) as described previously (Kämpfer & Kroppenstedt, 1996; Miller, 1982). The G+C content of the genomic DNA of strain 1y-2T was determined based on draft genome data performed at Baseclear B.V. (Leiden, Netherlands).

The isolate, strain 1y-2T, is a non-motile and strictly anaerobic, spore-forming, curved rod-shaped organism (Fig. S1). The cells were 0.70–0.83 × 3–6 μm and sometimes occurred in short chains of two to five cells. Cells differed slightly in length depending on the growth medium. Young cells stained Gram-positive, while old cells stained Gram-negative (Fig S1). After 1 day of incubation on RCB agar (RCB solidified with 1.5 % agar; Difco) at 37 °C, strain 1y-2T produced beige, circular, convex colonies with rough surfaces, 1–4 mm in diameter. Growth was observed at 15–45 °C and pH 5.5–9.0, with optima at 37 °C and pH 6.5–7.0. Strain 1y-2T tolerated up to 3 % NaCl. No growth was observed when traces of oxygen were present in the medium without the reducing agent, as indicated by the pink colour of the bicarbonate-buffered medium in which resazurin was present. No haemolytic activity was found in Columbia blood agar medium (Oxoid). Catalase and oxidase were negative. Indole was not produced. The strain could not reduce nitrate to nitrite and could not hydrolyse urea, gelatin or aesculin.

An almost full-length 16S rRNA gene sequence (1435 bp) was obtained from strain 1y-2T. Phylogenetic analysis based on 16S rRNA gene sequences showed that its closest relatives were members of cluster XIVa of the subphylum ‘Clostridium’ of the Gram-positive bacteria (Fig. 1), and it groups in the genus Anaerostipes in the lineage Firmicutes, Clostridia, Clostridiales, Lachnospiraceae. Pairwise comparison of the almost-complete 16S rRNA gene sequences (>1400 nt) of members of the most closely related species revealed approximately 3, 6 and 7 % sequence divergence, respectively, between the isolate and A. hadrus DSM 14662T, A. hadrus DSM 3319T and A. butyricus LMG
Moreover, the DNA–DNA relatedness between the isolate and *A. caccae* DSM 14662<sup>T</sup> was only 33 %, which is below the 70 % cut-off point generally accepted for species classification (Wayne *et al.*, 1987).

The biochemical properties of the isolate and the three type strains of species of the genus *Anaerostipes*, determined using the API ZYM, API Rapid ID32A and API 20A systems, showed clearly different capabilities for fermentation of carbohydrates as well as possessing various enzyme profiles (Table 1). Strain 1y-2<sup>T</sup> can be specifically distinguished from the other species of the genus *Anaerostipes* by its capacity to ferment rhamnose. The observed glucose fermentation stoichiometry was as follows (CO₂ content calculated based on redox balance, C₅H₈O₂N is used to refer to biomass): 1 glucose $\rightarrow$ 0.57 lactate +0.11 acetate +0.46 butyrate +0.45 formate +0.31 H₂ +0.58 CO₂ +0.24 C₅H₈O₂N. Biomass was determined using a method described previously (van Gelder *et al.*, 2012). Electron recovery was 94 %.

Butyrate was found as a product of all sugar fermentations (data not shown). All strains of the genus *Anaerostipes* have been shown to utilize lactate and acetate to form butyrate in the molar ratio of 2 : 1 : 1.5, as described previously (Duncan *et al.*, 2004), and so did strain 1y-2<sup>T</sup> (Fig. S2). Furthermore, strain 1y-2<sup>T</sup> showed a distinct glucose fermentation pattern compared with the related type strains (Table S1). Characteristics that differentiate strain 1y-2<sup>T</sup> from the three reference strains of the genus *Anaerostipes* are listed in Table 1.

Overall, the isolate has distinct biochemical properties that differentiate it from other members of the genus *Anaerostipes*.

The DNA G+C content of strain 1y-2<sup>T</sup> was 44.5 mol%. The cellular fatty acid profile was composed mainly of C<sub>12</sub>:0, iso-C<sub>19</sub>:1 I and C<sub>16</sub>:0, and differed from those of the other members of the genus *Anaerostipes* (Table S2).

Based on a polyphasic approach, our data show clearly that strain 1y-2<sup>T</sup> is significantly different from the other species of *Anaerostipes*.
the genus Anaerostipes. Therefore, it is appropriate to assign strain 1y-2T to a novel species within this genus, for which the name Anaerostipes rhamnosivorans sp. nov. is proposed.

**Description of Anaerostipes rhamnosivorans sp. nov.**

Anaerostipes rhamnosivorans (rham.no.si.vo’rans. N.L. neut. n. rhamnosum rhamnose; L. v. vorare to devour; N.L. part. adj. rhamnosivorans devouring rhamnose).

Cells are non-motile, spore-forming, anaerobic rods and are often observed to elongate and to form curly cells in old cultures. Cells are 0.70–0.83 × 6–6 μm. They occur as single cells and sometimes in short chains of two to five cells. They stain Gram-positive (young cells) or Gram-negative (old cells). On RCM agar, colonies are beige, circular and raised with rough surfaces, about 1–4 mm in diameter. Can grow at 15–45°C (optimum at 37°C), pH 5.5–8.0 (optimum pH 6.5–7.0) and up to 3% NaCl. Lactate, formate, butyrate and acetate are major metabolites from glucose fermentation and propane-1,2-diol, butyrate, acetate and formate are major products of rhamnose fermentation. Produces about 13 mM butyrate from 16 mM lactate (both D- and L-lactate can be used) plus acetate. Butyrate is detected in all sugar fermentations. Activities of esterase (C4), esterase lipase (C8), acid phosphatase and naphthol-AS-BI-phosphohydrolase and weak responses for arginine phosphatase, proline arylamidase, tyrosine arylamidase, histidine arylamidase and serine arylamidase are observed. Acid is produced from D-glucose, D-mannitol, sucrose, D-mannose, D-sorbitol, L-rhamnose, trehalose, D-galactose, D-fructose, L-sorbose, maltose, dulcitol, inositol, N-acetylglucosamine, with rough surfaces, about 1–4 mm in diameter. Can grow at 15–45°C (optimum at 37°C), pH 5.5–8.0 (optimum pH 6.5–7.0) and up to 3% NaCl. Lactate, formate, butyrate and acetate are major metabolites from glucose fermentation and propane-1,2-diol, butyrate, acetate and formate are major products of rhamnose fermentation. Produces about 13 mM butyrate from 16 mM lactate (both D- and L-lactate can be used) plus acetate. Butyrate is detected in all sugar fermentations. Activities of esterase (C4), esterase lipase (C8), acid phosphatase and naphthol-AS-BI-phosphohydrolase and weak responses for arginine phosphatase, proline arylamidase, tyrosine arylamidase, histidine arylamidase and serine arylamidase are observed. Acid is produced from D-glucose, D-mannitol, sucrose, D-mannose, D-sorbitol, L-rhamnose, trehalose, D-galactose, D-fructose, L-sorbose, maltose, dulcitol, inositol, N-acetylglucosamine,
turanose, D-lyxose and D-tagatose. Weakly fermenters D-arabinose, D-ribose, D-adenitol, methyl D-glucopyranoside, xylitol, D- and L-arabitol and xylose. The major fermentation products are lactate and butyrate. The major cellular fatty acids are C12:0 iso-C19:0 anteiso and C16:0.

The type strain is 1y-2^T (DSM 26241^T=KCTC 15316^T), isolated from a stool of a healthy infant in Wageningen, Netherlands, in 2012. The DNA G+C content of the type strain is 44.5 mol%.

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