Agathobaculum butyriciproducens gen. nov. sp. nov., a strict anaerobic, butyrate-producing gut bacterium isolated from human faeces and reclassification of Eubacterium desmolans as Agathobaculum desmolans comb. nov.

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A novel bacterial strain, SR79<sup>T</sup>, was isolated from a Korean faecal sample and characterized using a polyphasic approach. SR79<sup>T</sup> was found to be a strictly anaerobic, Gram-stain-positive, non-spore-forming, non-motile, catalase- and oxidase-negative short rod with no flagella. SR79<sup>T</sup> grew optimally at 37 °C in the presence of 0.5 % (w/v) NaCl at pH 7. The NaCl range for growth was 0–1 % (w/v). The isolate produced butyric acid (>18 mM) as a major end product. A phylogenetic analysis based on 16S rRNA gene sequences revealed that the most closely related type strains were Eubacterium desmolans ATCC 43058<sup>T</sup> and Butyricicoccus pullicaecorum 25-3<sup>T</sup> (96.4 and 96.0 % similarity, respectively). The DNA G+C content was determined to be 52.9 mol%. The major cellular fatty acids (>10 %) were C<sub>16 : 0</sub>, C<sub>18 : 1</sub> cis-9, C<sub>19 : 1</sub> cyc 9,10 and C<sub>14 : 0</sub>. Meso-diaminopimelic acid was present in the cell wall peptidoglycan and the cell wall hydrolysates contained ribose, glucose and galactose. The 16S rRNA gene sequence similarity, phylogenetic analysis, chemotaxonomic and phenotypic characteristics allowed differentiation of SR79<sup>T</sup>, which represents a novel species of a new genus within the family Ruminococcaceae, for which the name Agathobaculum butyriciproducens gen. nov. sp. nov. is proposed. The type strain is SR79<sup>T</sup> (=KCTC 15532<sup>T</sup>=DSM 100391<sup>T</sup>). Based on the results of this study, it is also proposed to transfer Eubacterium desmolans to this new genus, as Agathobaculum desmolans comb. nov. The type strain of Agathobaculum desmolans is ATCC 43058<sup>T</sup> (=CCUG 27818<sup>T</sup>).

Trillions of micro-organisms inhabit the human gastrointestinal tract; the number of human gut microbes is predicted to be ten fold greater than the number of human cells in the body (The Human Microbiome Project Consortium, 2012). Most members of the human gut microbiota belong to one of five bacterial phyla (Firmicutes, Bacteroides, Actinobacteria, Proteobacteria and Verrucomicrobia) and one phylum in the domain Archaea (Euryarchaeota) (Tremaroli & Bäckhed, 2012). These endogenous gut microorganisms and their metabolites play important roles in human health by providing nutrition, regulating epithelial development and contributing to immune system homeostasis (Lee & Hase, 2014). Therefore, understanding the diversity of the human intestinal ecosystem and functionality of each gut microorganism is important. However, performing an accurate analysis of the microbial ecosystem and each species in the human intestinal tract is difficult. Even though the microbiota of the human gut has been investigated intensively using both traditional culture methods and next-generation sequencing technologies, we need to develop and continue to innovate new methods.
methods and molecular techniques, many of the strictly anaerobic bacteria in the human gut remain uncultivated.

Members of the genus *Eubacterium*, which belongs to the phylum *Firmicutes* (family *Eubacteriaceae*, order *Clostridiales*, class *Clostridia*) are highly heterogeneous and constitute 53 species and three subspecies with validly published names according to the List of Prokaryotic Names (http://www.bacterio.net/eubacterium.html) at the time of writing (March, 2016). The type species of this genus is *Eubacterium limosum* (Judicial Commission of the International Committee on Systematic Bacteriology, 1983; Prévot, 1938). However, some species with validly published names have been misclassified within this genus (Wade, 2009) and they should be positioned within the other families such as *Peptostreptococcaceae*, *Lachnospiraceae*, *Clostridiaceae*, and *Ruminococcaceae*; thus, comprehensive taxonomic studies are needed to determine their correct position. Currently, members of the genus *Eubacterium* can be categorized into three subgroups based on their fermentation end products (Wade, 2009): those that produce butyric acid in combination with other volatile fatty acids; those that produce various combinations of lactate, acetate and formate with H₂ gas; and those that produce little acid by fermentation. Among them, butyrate producers have a critical role in human health because butyrate is a key energy substrate for cellular metabolism in the colonic epithelium (Tremaroli & Bäckhed, 2012). In addition, butyrate is an important regulator of host gene expression, cellular differentiation and apoptosis (Louis & Flint, 2009); moreover, it has anti-inflammatory effects and a potential protective role in metabolic diseases (Brahe et al., 2013).

In this study, a novel strictly anaerobic and butyrate-producing strain was isolated from the faeces of a healthy 23-year-old Korean female, who had not received antibiotics in the previous year, in the process of isolating culturable anaerobic human gut microorganisms. Here, we present polyphasic taxonomic results showing that this butyrate-producing isolate represents a novel species of a novel genus within the family *Ruminococcaceae*, with *Eubacterium desmolans* being the closest neighbor.

For maintaining the collected faecal sample under strictly anaerobic conditions, a mobile anaerobic sampling device was constructed by our research group (Chang et al., 2016), which was composed of an aluminium gas cylinder containing N₂/CO₂/H₂ (86:7:7), a gas cylinder trolley and a pressurized anaerobic jar (3.5 L HP0011A, Oxoid) containing palladium catalyst. A fresh faecal sample was collected in a Falcon 50-ml conical tube (Thermo Fisher Scientific) without capping. Immediately after collection, the sample was placed in an anaerobic jar and flushed with a N₂/CO₂/H₂ (86:7:7) gas mixture for 20 min; the anaerobic jar was then pressurized to 0.6 atm to prevent oxygen contamination. The sample was transferred immediately from the hospital to the anaerobic chamber (Coy Laboratory Products) in our laboratory. The faecal sample (2 g) was suspended homogeneously in 20 ml anaerobic DSM 104 medium (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium104.pdf) in the conical tube. DSM 104 medium contained (l⁻¹ distilled water) 5 g trypticase peptone, 5 g peptone, 10 g yeast extract, 5 g beef extract, 5 g glucose, 1 ml Tween 80, 10 mg CaCl₂, 20 mg MgSO₄, 2.04 g K₂HPO₄, 40 mg KH₂PO₄, 400 mg NaHCO₃, 80 mg MgCl₂, 10 ml haemin solution (0.5 mg ml⁻¹) and 0.2 ml vitamin K1 solution (5 mg ml⁻¹). All procedures for isolation of human gut microbes and media preparation were performed under anaerobic conditions according to previously established methods (Bang et al., 2015; Lee et al., 2013b). DSM 104 agar plates (1.5 %, w/v) were prepared and the samples were incubated at 37°C in the anaerobic chamber with a N₂/CO₂/H₂ (86:7:7) gas phase for 3 days. A novel bacterial strain, designated as SR79, was isolated from the diluted (10⁻⁵-fold) faecal sample. The purity of the isolate was evaluated following more than five rounds of single-colony isolation by DNA sequencing following colony PCR amplification of the 16S rRNA gene as described previously (Chang et al., 2015).

The morphology and size of colonies were observed on DSM 104 agar plates after 3 days of incubation at 37°C. Gram staining, spore formation and cell morphology were examined as described previously (Lee et al., 2014). Colonies of SR79 were circular, convex and yellowish after growth on DSM 104 medium at 37°C for 3 days. Cells were obligately anaerobic, Gram-stain-positive, non-motile, non-spore-forming short rods (0.6–0.7 µm in width 0.9–1.1 µm in length). Transmission electron microscopy was performed as described previously (Zillig et al., 1990) and short rod cells in pairs or chains were observed (Fig. S1, available in the online Supplementary Material). Catalase and oxidase activities were evaluated as described previously (Lee et al., 2014). SR79 was observed to be catalase-negative and oxidase-negative.

The temperature range for growth was evaluated in DSM 104 medium at 10–50°C with 5°C increments and at 37°C. Growth occurred at between 30 and 40°C, with optimum growth at 37°C; no growth was observed at 25 or 45°C. To determine growth at different pH values, several buffers were used as described previously (Lee et al., 2013a). The isolate grew at pH 6.0–8.0, with optimal growth at pH 7.0; no growth was observed at pH 5.0 or 9.0. DSM 104 medium supplemented with NaCl was prepared and growth in 0–5 % (w/v) NaCl (at increments of 0.5 % NaCl) at 37°C was evaluated. Growth occurred under NaCl concentrations of up to 1.0 % (w/v); no growth was observed with 1.5 % (w/v) NaCl. All of the above measurements were conducted in triplicate by measuring the OD₆₀₀ using a Spec 20 spectrophotometer (Thermo Fisher Scientific) as described previously (Lee et al., 2013b). For comparison, two closely related strains, *E. desmolans* ATCC 43058 and *Butyricicoccus pullicaecorum* 25-3T (=KCTC 15070=LMG 24109), were purchased from the ATCC and KCTC, respectively and their growth ranges were determined using the methods described above. Little growth of *E. desmolans* ATCC 43058 was observed in DSM 104 medium. Thus, DSM 104 medium containing 1 % (w/v) inositol was prepared to increase the growth of the bacterium as described previously.
(Morris et al., 1986). *B. pullicaecorum* 25-3T grew as well as did the novel isolate in DSM 104 medium. The growth ranges of the novel isolate differed slightly from those of the reference strains. SR79T exhibited growth at 30°C. However, *E. desmolans ATCC 43058T* and *B. pullicaecorum* 25-3T did not grow at 30°C. The two reference strains grew optimally without NaCl, while SR79T exhibited optimal growth in the presence of 0.5 % (w/v) NaCl (Table 1).

The biochemical and physiological characteristics of the isolate and two reference strains were determined in duplicate using API 20A (bioMerièux) kit, according to the manufacturer’s instructions. After inoculation, the test strips were incubated at 37°C for 24 h under anaerobic atmosphere (100 % N2 gas). The results from API 20A for all tested strains were similar. All tested strains exhibited hydrolysis of aesculin ferric citrate and fermentation of D-glucose, D-mannitol, D-lactose, D-saccharose, D-maltose, salicin, D-xylene, L-arabinose, D-cellobiose, D-mannose, D-melezitose, L-rhamnose and D-trehalose. No urease or gelatinase activity was detected. No fermentation was observed from l-tryptophan and glycerol. Both the isolate and *E. desmolans ATCC 43058T* showed negative reactions for D-raffinose and L-sorbitol while *B. pullicaecorum 25-3T* showed weakly positive reactions for D-raffinose and L-sorbitol (Table 1). The ability to degrade starch was determined by culturing the strains on DSM 104 agar plates (1.5 %, w/v) with 0.2 % soluble starch. After 3 days incubation, the plates were flooded with 6 mM Iodine solution. All tested strains showed clear zones around cells, which indicated hydrolysis of starch.

The fermentation end products in the DSM 104 medium produced from SR79T and *E. desmolans ATCC 43058T* were compared after 2 days of incubation at 37°C as described previously (Chang et al., 2015). A gas chromatography (GC) system equipped with a flame ionization detector (GC-FID, Model 6890 N; Agilent) and a Stabilwax-DA Column (Restek) was used to measure the fermentation end products. Nitrogen gas was used as the carrier gas for the GC analysis. The major fermentation end product of glucose metabolism from SR79T was butyric acid (18.4 mM). Ethanol (3.5 mM), butanol (0.8 mM) and acetic acid (0.4 mM) were also detected. In contrast, *E. desmolans ATCC 43058T* produced acetic acid (18.7 mM) as a major fermentation end product when grown in DSM 104 medium supplemented with 1 % (w/v) inositol. Butyric acid (7.0 mM), ethanol (3.9 mM) and butanol (1.0 mM) were also present in the culture medium. Butyric acid (8.6 mM), ethanol (3.8 mM) and butanol (0.8 mM) were detected in the DSM 104 culture media of *B. pullicaecorum 25-3T* but no acetate was detected.

A cellular fatty acid analysis was performed as described previously (Chang et al., 2015). A loop of cell mass collected from DSM 104 agar plates after incubation for 2 days at 37°C was used and fatty acid profiles were prepared using the Microbial Identification software package (Moore 150 library). The following major cellular fatty acids (>10%) were detected in strain SR79T: C16:0 (21.6 %), cis-C18:1 9 (14.1 %), C19:1 cyc 9,10 (14.0 %) and C14:0 (11.8 %). The fatty acid profiles of the isolate and *E. desmolans ATCC 43058T* were similar (Table S1, available in the online Supplementary Material). The only difference was that one of the major fatty acids in SR79T, cis-C18:1 9 FAME (14.1 %), was not detected in *E. desmolans ATCC 43058T*. Instead, there is a higher cis-C18:2 9,12 (15.2 %) level in *E. desmolans ATCC 43058T* than that in the novel isolate (<1%). However, marked differences were observed between the isolate and *B. pullicaecorum 25-3T* (Table S1).

### Table 1. Characteristics that distinguish strain SR79T from the closest phylogenetically related type strains of the genera *Eubacterium* and *Butyricicoccus*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flagellation</td>
<td>No flagella</td>
<td>Multiple peritrichous*</td>
<td>ND</td>
</tr>
<tr>
<td>Temperature range (optimum)</td>
<td>30–40 (37)</td>
<td>35–40 (37)</td>
<td>35–45 (37)</td>
</tr>
<tr>
<td>pH range (optimum)</td>
<td>6–8 (7)</td>
<td>6–8 (7)</td>
<td>6–8 (7)</td>
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<tr>
<td>NaCl range (optimum) (percentage)</td>
<td>0–1 (0.5)</td>
<td>0–1 (0)</td>
<td>0–1 (0)</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-raffinose</td>
<td>–</td>
<td>–</td>
<td>W</td>
</tr>
<tr>
<td>D-sorbitol</td>
<td>–</td>
<td>–</td>
<td>W</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>52.9</td>
<td>48.5</td>
<td>53.1</td>
</tr>
<tr>
<td>Cell wall sugar</td>
<td>Ribose, glucose galactose</td>
<td>Ribose, glucose</td>
<td>Ribose, glucose</td>
</tr>
<tr>
<td>Major fermentation end product</td>
<td>Butyrate</td>
<td>Acetate</td>
<td>Butyrate</td>
</tr>
<tr>
<td>Source</td>
<td>Human faeces</td>
<td>Cat faeces*</td>
<td>Caecal content of broiler chicken†</td>
</tr>
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</table>

*Data from *, Morris et al. (1986); †, Eckhaut et al. (2008).
As described previously (Nakagawa & Yamasato, 1993), respiratory quinones were extracted from 300 mg lyophilized cells from the isolate, *E. desmolans* ATCC 43058T and *B. pullicaecorum* 25-3T. The samples were purified and analyzed by HPLC, (L-5000, Hitachi) using a reverse-phase column (YMC Pack ODS-A) with methanol/isopropyl ether [3:1, (v/v)] as the mobile phase (1 ml min−1). Quinone was not detected in the three strains. The cell-wall diaminopimelic acid was determined from whole-cell hydrolysates. Patterns of diaminopimelic acid were obtained by thin layer chromatography (TLC) on cellulose plates (Rhuland et al., 1955). The diagnostic diamino acid of the peptidoglycan was meso-diaminopimelic acid. As described previously (Schleifer & Kandler, 1972), the cell-wall sugars in strain SR79T and two reference strains were analyzed by TLC (cellulose glass plate 20×20 cm; Merck). All tested strains, including the isolate, contained ribose and glucose as cell sugars. Galactose was detected only in SR79T (Fig. S2).

Large cell masses were prepared from 31 cultures of SR79T, *E. desmolans* ATCC 43058T and *B. pullicaecorum* 25-3T at the late-exponential phase of growth (2 or 3 days) for genomic DNA purification as described previously (Lee et al., 2013b). 16S rRNA gene amplification and DNA G+C content analyses were performed using the purified genomic DNAs. The DNA G+C content was evaluated by the method of Mesbah et al. (1989) using a YMC-Triat C18 column (150×4.6 mm). Genomic DNA of *Escherichia coli* KCTC 2441T was used as the standard. The DNA G+C contents of SR79T, *E. desmolans* ATCC 43058T and *B. pullicaecorum* 25-3T were 52.9, 48.5 and 53.1 mol%, respectively (Table 1). The 16S rRNA gene of strain SR79T was amplified using the universal primers 27F and 1492R as described previously (Lee et al., 2014). The purified PCR products were sequenced by Solgent (Daejeon, Korea). The SeqMan software (DNAStar) was used to obtain the assembled 16S rRNA gene sequence. An almost-complete 16S rRNA gene sequence (1383 bp) of strain SR79T was obtained and used for phylogenetic analysis. The 16S rRNA gene sequences of the related type strains were downloaded from EzTaxon-e server 2.1 (http://eztaxon-e.ezbiocloud.net; Kim et al., 2012) and pairwise sequence similarities were calculated using the EzTaxon-e server software. Multiple alignments with the related sequences of type strains were performed using CLUSTALW as described previously (Thompson et al., 1997). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods with MEGA 5.2 software (Tamura et al., 2011). A bootstrap analysis was conducted by 1000 re-samplings (Felsenstein, 1985). In a phylogenetic comparison of SR79T with reference type strains, the isolate showed 16S rRNA gene sequence similarities of 96.4 and 96.0% with *E. desmolans* ATCC 43058T and *B. pullicaecorum* 25-3T, respectively. The relationship of R79T with its phylogenetic neighbours is shown in Fig. 1. The robust clustering of SR79T with *E. desmolans* ATCC 43058T was supported by high bootstrap values (Fig. 1).

Our phylogenetic analysis indicates that SR79T could be clustered with *E. desmolans* ATCC 43058T but *B. pullicaecorum* 25-3T was positioned on a different phylogenetic branch (Fig. 1); these findings are supported by high bootstrap values and the fatty acid profiles of the microbes. As mentioned above, the fatty acid profiles of the isolate and *E. desmolans* ATCC 43058T were similar whereas they were quite different from that of *B. pullicaecorum* 25-3T (Table S1). However, the isolate also exhibited distinct characteristics from those of *E. desmolans* ATCC 43058T. The
isolate possessed galactose as a cell-wall sugar and cis-C_{18:1} \; 9 \; (14.1 \%) as a major fatty acid, while \textit{E. desmolans} ATCC 43058^{T} did not. SR79^{T} produced much more butyric acid (18.4 mM) than did \textit{E. desmolans} ATCC 43058^{T} (7.0 mM); \textit{E. desmolans} ATCC 43058^{T} produced acetic acid (18.7 mM) as a major fermentation end product. SR79^{T} was observed to have no flagella but multiple peritrichous flagella were observed in \textit{E. desmolans} ATCC 43058. Little growth of \textit{E. desmolans} ATCC 43058 was observed using DSM 104 broth. However, based on the high level of 16S rRNA gene sequence similarity (96.4 %), molecular phylogenetic analysis and similar cellular fatty acid profiles, SR79 along with \textit{E. desmolans} ATCC 43058 could be categorized into a novel genus within the family \textit{Ruminococcaceae}, for which the name \textit{Agathobaculum} gen. nov., is suggested. SR79^{T} should be classified as representing a novel species of this new genus for which the name \textit{Agathobaculum butyriciproducens} gen. nov. sp. nov., is proposed.

Description of \textit{Agathobaculum} gen. nov.

\textit{Agathobaculum} (A.ga.tho.ba’cu.lum. Gr. adj. agathos good; L. neut. n. baculum small rod; N.L. masc. n. \textit{Agathobaculum} small rod beneficial bacterium).

Cells are Gram-stain-positive. No spores are observed. Obligately anaerobic. Motile or non-motile. Oxidase- and catalase-negative. The major cellular fatty acids are C_{16:0}, C_{19:1} \; \text{cyc} \; 9, \; 10 \; and \; C_{14:0}. \; The \; cell \; wall \; contains \; meso-diaminopimelic acid. The G+C content of DNA is 48.5–52.9 mol%. Major fermentation end product is butyrate or acetate. Phylogenetically, belongs to the family \textit{Ruminococcaceae}. The type species is \textit{Agathobaculum butyriciproducens}.

Description of \textit{Agathobaculum butyriciproducens} sp. nov.

\textit{Agathobaculum butyriciproducens} (bu.ty.ric.pro.du’cens. N. L. n. acidum butyricum butyric acid; L. press. part. producens producing; N.L. part. adj. butyriciproducens producing butyric acid).

In addition to the characteristics given in the genus description, cells are non-motile, plump rods (0.6–0.7 \; \mu \text{m} \; in \; width \; 0.9–1.1 \; \mu \text{m} \; in \; length). Growth occurs at pH 6–8 (optimum pH is 7), at 30–40 °C (optimum temperature is 37 °C), and maximum salinity tolerated is 1 % (w/v) (optimum for growth, 0 % NaCl). Butyrate is a major fermentation end product. Ethanol, butanol and acetate are also produced. Using API20A kit, the following tests are positive; aesculin ferric citrate, D-glucose, D-mannitol, D-lactose, D-saccharose, D-maltose, salicin, D-xylene, L-arabinose, D-cellobiose, D-mannose, D-melezitose, L-rhamnose, D-trehalose. Negative for L-tryptophan, urea, gelatin, glycerol, D-raffinose and D-sorbitol. The major fatty acids (>10%) are C_{16:0}, cis-C_{18:1} \; 9, \; C_{19:1} \; \text{cyc} \; 9,10 \; and \; C_{14:0} \; and \; whole-cell hydrolysates contain ribose, glucose and galactose. The diagnostic diaminoc acid of the peptidoglycan is \textit{meso-diaminopimelic acid}.

The type strain, SR79^{T} (=KCTC 15532^{T}=DSM 100391^{T}), was isolated from human faeces of a 23-years-old healthy Korean woman. The DNA G+C content of the type strain is 52.9 mol%.

Description of \textit{Agathobaculum desmolans} comb. nov.


\textit{Agathobaculum desmolans} (des’mo.lans. Gr. n. desmos a bond; mod. chem. Term desmolase, an enzyme splitting a carbon–carbon bond; N.L. part. adj. desmolans making desmolase).

The description is identical to that given by Morris \textit{et al.} (1986) with the following additions. Respiratory quinones are absent. Growth occurs at pH 6–8 (optimum pH is 7), at 35–40 °C (optimum temperature is 37 °C), and salinity tolerance is 1 % (w/v) (optimum for growth, 0 % NaCl). Acetate from inositol is a major fermentation end product. Using the API20A kit, the followings tests are positive; aesculin ferric citrate, D-glucose, D-mannitol, D-lactose, D-saccharose, D-maltose, salicin, D-xylene, L-arabinose, D-cellobiose, D-mannose, D-melezitose, L-rhamnose, D-trehalose; negative for L-tryptophan, urea, gelatin and glycerol. The major fatty acids (>10%) are C_{16:0}, cis-C_{18:2} \; 9,12, \; C_{19:1} \; \text{cyc} \; 9,10 \; and \; C_{14:0}. \; Whole-cell hydrolysates contain ribose and glucose. The diagnostic diaminoc acid of the peptidoglycan is \textit{meso-diaminopimelic acid}.

The type strain is ATCC 43058^{T} (=CCUG 27818^{T}=ICM 6556^{T}=VTT E-062954^{T}) isolated from cat faeces. The DNA G+C content of the type strain is 48.5 mol%.

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References


