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Cultivable butyrate-producing bacteria of elderly Japanese diagnosed with Alzheimer's disease[§]

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The group of butyrate-producing bacteria within the human gut microbiome may be associated with positive effects on memory improvement, according to previous studies on dementia-associated diseases. Here, fecal samples of four elderly Japanese diagnosed with Alzheimer's disease (AD) were used to isolate butyrate-producing bacteria. 226 isolates were randomly picked, their 16S rRNA genes were sequenced, and assigned into sixty OTUs (operational taxonomic units) based on BLASTn results. Four isolates with less than 97% homology to known sequences were considered as unique OTUs of potentially butyrate-producing bacteria. In addition, 12 potential butyrate-producing isolates were selected from the remaining 56 OTUs based on scan-searching against the PubMed and the ScienceDirect databases. Those belonged to the phylum *Bacteroidetes* and to the clostridial clusters I, IV, XI, XV, XIVa within the phylum *Firmicutes*. 15 out of the 16 isolates were indeed able to produce butyrate in culture as determined by high-performance liquid chromatography with UV detection. Furthermore, encoding genes for butyrate formation in these bacteria were identified by sequencing of degenerately primed PCR products and included the genes for butyrate kinase (*buk*), butyryl-CoA: acetate CoA-transferase (*but*), CoA-transferase-related, and propionate CoA-transferase. The results showed that eight isolates possessed *buk*, while five isolates possessed *but*. The CoA-transfer-related gene was identified as butyryl-CoA:4-hydroxybutyrate CoA transferase (*4-hbt*) in four strains. No strains contained the propionate CoA-transferase gene. The biochemical and butyrate-producing pathways analyses of butyrate producers presented in this study may help to characterize the butyrate-producing bacterial community in the gut of AD patients.

***Keywords*:** 16S rRNA gene sequencing, Alzheimer's disease, butyrate-producing bacteria, gut microbiota, short-chain fatty acids

Introduction

The trillions of microorganisms in the human gut play a key role in the host's life with many beneficial health effects. They may promote digestion of foods and absorption of nutrients, produce vitamins, protect the host from opportunistic pathogens, improve the immune system, and maintain the host's homeostasis of its immune system (Wallace *et al.*, 2011; Sommer and Backhed, 2013). Within the community of human gut microbiota, the group of butyrate-producing bacteria attracts particular attention because of the specific health-promoting effects they provide to their hosts (Vital *et al.*, 2014). Their major metabolic end-product, butyrate, is not only a preferred energy source for colonocytes but also a major contributor to the preservation of intestinal epithelial permeability and the protection of the host from carcinogenic, inflammatory, and oxidative factors (Hamer *et al.*, 2008). Furthermore, butyrate was shown to improve memory function in an Alzheimer's disease (AD) mouse model (Govindarajan *et al.*, 2011).

The characteristics and phylogenetic diversity of butyrate-producing bacteria in the healthy human gut have been widely investigated (Barcenilla *et al.*, 2000; Pryde *et al.*, 2002; Hamer *et al.*, 2008; Louis and Flint, 2009; Vital *et al.*, 2014). Butyrate-producing bacteria are extremely difficult to cultivate due to their obligate anaerobic lifestyle and their requirement for specific nutrients. They are mostly Gram-positive bacteria, belonging to the phylum of *Firmicutes* within clostridial clusters IV and XIVa (Barcenilla *et al.*, 2000; Pryde *et al.*, 2002; Louis and Flint, 2009; Rivière *et al.*, 2016). However, a metagenomic analysis indicated that a minor portion also included the phyla *Actinobacteria*, *Bacteroidetes*, *Fusobacteria*, *Proteobacteria*, *Spirochaetes*, and *Thermotogae* (Vital *et al.*, 2014).

The effect of butyrate-producing bacteria on the host-gut microbiota relationship has been investigated in previous studies (Vital *et al.*, 2013, 2017; Liu *et al.*, 2015; Bourassa *et al.*, 2016; Geirnaert *et al.*, 2017; Li *et al.*, 2018). Some of these studies examined butyrate-producing bacteria as probiotic in regards to their effects on various diseases (Liu *et al.*, 2015; Geirnaert *et al.*, 2017). For examples, in patients with inflammatory bowel disease, a reduction in the population of butyrate-producing bacteria was reported (Ott *et al.*, 2004; Geir-

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naert *et al.*, 2017). In an *in vitro* system simulating the mucus- and lumen-associated microbiota, supplementation of either *Faecalibacterium prausnitzii* or *Butyricicoccus pullicaecorum* 25-3^T and a mix of other six butyrate-producers, to the fecal microbiota of Crohn's disease patients, helped to increase butyrate production and improved epithelial barrier integrity (Geirnaert *et al.*, 2017). Furthermore, in mice suffering from vascular disease, which is the second most common dementia-associated disease after AD, the administration of live *Clostridium butyricum* to their diet helped to regulate gut microbiota by increasing their diversity. This was reported to increase butyrate in murine brains, which improved memory. Therefore, *C. butyricum* was considered as a probiotic and it may become an economical therapeutic option to protect against vascular disease (Liu *et al.*, 2015).

AD is a progressive neurodegenerative disorder of the central nervous system, characterized by an onset of dementia in the elderly population (aged above 65 years) (Mandell and Green, 2011). It was hypothesized that butyrate or butyrate-producing bacteria may have positive effects on memory improvement in mouse models of dementia-related diseases (Govindarajan *et al.*, 2011; Liu *et al.*, 2015). Thus, here we wanted to determine if butyrate-producing bacteria are at all present or completely absent in the gut microbiota of AD patients. The application of single or mixtures of butyrate producers in studies associated with dementia-related disease (mentioned above) emphasizes the importance of identifying butyrate-producing bacteria and to assess their rate of butyrate production.

In the work presented here, we isolated bacteria from the feces of four Japanese elders diagnosed with Alzheimer's disease. The 16S ribosomal RNA (16S rRNA) gene of each isolate was sequenced. Subsequently, we selected bacteria that might possess butyrate producing ability based on species-related information available in the literature. We then analyzed them in culture for the production of short-chain fatty acids (SCFAs), including butyrate, using high-performance liquid chromatography (HPLC). Furthermore, the presence of encoding genes for butyrate production in each strain was identified. These included butyrate kinase (*buk*), butyryl-CoA:acetate CoA-transferase (*but*), CoA-transferase-related, and propionate CoA-transferase. Our findings are the first to provide valuable insight into the existence of the butyrate-producing microbial community in the guts of AD patients.

Materials and Methods

Fecal sample collection

Fresh fecal samples from four Japanese AD patients (87 ± 3.29 years old) were used to isolate butyrate-producing bacteria. Samples were immediately sealed in a plastic bag containing an AnaeroPack-Anaero (Mitsubishi) and then transported to the laboratory at 4°C within 2 days. In the laboratory, 1 g of feces was treated with 1 ml of phosphate buffer saline (PBS, Life Technologies) and 2 ml of 40% glycerol (Nacalai Tesque) in an anaerobic chamber (Bactron, Shel Lab) to generate a fecal stock sample. Subsequently, the fecal stock samples were quickly frozen in liquid nitrogen and stored at -80°C until use (Nishijima *et al.*, 2016). The study was approved by the Ethics Committee of Okayama University, Japan (Approval number 1610-025). Written informed consents were obtained from all participants or their relatives.

Isolation of bacteria from fecal samples

Bacteria from fecal samples were recovered following previous methods with a minor modification (Morita *et al.*, 2007). All isolation processes were performed under anaerobic conditions. Therefore, PBS solutions, culture media, and other materials were kept in an anaerobic chamber up to 24 h before use. The anaerobic condition was maintained in the workstation by using a tank of anaerobic mixed gas (5% carbon dioxide, 5% hydrogen, and 90% nitrogen) along with a tank of nitrogen gas. 0.4 ml of each fecal stock sample was recovered in 9.6 ml of PBS solution (Life Technologies) to generate a 10^2 dilution of the original fecal material. This solution was thoroughly vortexed and then subsequently diluted serially in 10-fold steps until a 10^8-fold dilution was obtained. 0.1 ml of each dilution was spread onto blood liver (BL) agar medium (Eiken) supplemented with 5% defibrinated horse blood and yeast extract, casitone, fatty acids (YCFA) agar medium. All plates were incubated at 37°C for 3–7 days in the anaerobic chamber. YCFA medium contained (per 100 ml) 1.5 g agar, 1 g casitone, 0.25 g yeast extract, 0.4 g $NaHCO_3$, 0.1 g cysteine, 0.045 g K_2HPO_4, 0.045 g KH_2PO_4, 0.09 g NaCl, 0.009 g $MgSO_4{\cdot}7H_2O$, 0.009 g $CaCl_2$, 0.1 mg resazurin, 1 mg hemin, 1 µg biotin, 1 µg cobalamin, 3 µg p-aminobenzoic acid, 5 µg folic acid, and 15 µg pyridoxamine. SCFAs were added to the medium at final concentrations of 33 mM acetate, 9 mM propionate, and 1 mM each of isobutyrate, isovalerate, and valerate. After sterilization by autoclaving, a sterile-filtered solution of vitamins and sugars was added to the medium, at a final concentration of 50 mg/L each of thiamine and riboflavin (Duncan *et al.*, 2002) and 2 g/L each of glucose, maltose and cellobiose (Browne *et al.*, 2016). Single colonies were picked randomly and re-streaked on the same media until pure colonies appeared as confirmed by morphology. Each pure isolate was used for colony PCR (Polymerase Chain Reaction) and cultivated in 5 ml of GAM broth (Gifu Anaerobic Media, Eiken) at 37°C under anaerobic conditions. The resulting cultures were used to create stocks which were stored at -80°C (Atarashi *et al.*, 2013).

Colony PCR, 16S rRNA gene amplicon sequencing, and sequence analysis

Each pure colony was picked up with a sterile toothpick and transferred into a PCR tube (0.2 ml) containing 5 µl of sterile distilled water. Next, a mixture consisting of 0.4 µl of each universal primer, 27Fmod and 1525R (Eurofins, Supplementary data Table S1), 10 µl of Emerald PCR Master Mix (TaKaRa), and 4.2 µl of sterile distilled water was added to the PCR reaction tube. The amplification program was performed according to a previous study (Schulze-Schweifing *et al.*, 2014) with a small modification: initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 20 sec, elongation at 72°C for 2 min, and a final elongation at 72°C for 10 min using a thermocycler (Biometra, T1 Thermocycler). The am-

plified fragments were then cleaned up with ExoSAP-IT (Thermal Fisher Scientific) according to the manufacturer's instructions. The purified amplicons were sequenced using a 3730 × l DNA Analyzer with a BigDye Terminator v3.1 Cycle sequencing kit (Thermo Fisher Scientific) by the Eurofins MWG lab in Tokyo, Japan. The resulting sequences of each isolate were analyzed against each other with BLASTn and the similarity of their 16S rRNA gene sequence (~850 bp) was determined by comparison against GenBank entries using Match/Mismatch scores of 1,-2 and a linear gap costs parameter (Altschu *et al.*, 1990). A 97% identity cut-off value was used to group species into operational taxonomic units (OTUs) while other isolates showing lower similarity values were each considered as individual OTUs (Li *et al.*, 2016a). Thereafter, each representative OTU was subjected to near-full-length 16S rRNA gene sequencing to obtain virtually complete 16S rRNA sequences. Isolate names mentioned hereafter indicate that they are representative isolates of their OTU. Contigs of each OTU (~1,400 bp) were created using GeneStudio software (http://genestudio.com/), which were then BLASTn compared against two databases of 16S ribosomal RNA sequences and RefSeq Genome (organism: Bacteria [taxid:2]) in the GenBank database (https://blast.ncbi.nlm.nih.gov), the SILVA database (https://www.arb-silva.de/) (Pruesse *et al.*, 2012), and the EzTaxon database (https://www.ezbiocloud.net/resources/16s_download) (Yoon *et al.*, 2017), to identify the underlying species.

Screening of butyrate-producing bacteria

Based on the 16S rRNA gene sequencing results, all identified species were analyzed for their butyrate-producing ability by scan-searching against the PubMed and the ScienceDirect databases (Hamer *et al.*, 2008). Keywords used for the scanning were the species name of each representative OTU, butyrate, and short-chain fatty acid. To be inclusive, all isolates with less than 97% sequence homology to known species were separately considered as potential butyrate producers. Subsequently, the suspected butyrate-producing candidates were assessed for their butyrate-producing ability by HPLC

Table 1. Relationships and short-chain fatty acids quantification of cultivable putative butyrate-producing bacterial isolates from AD patients

Isolate	Species identified (16S rRNA gene % homology, accession number)	OD_{620}	pH	Short-chain fatty acids concentration (mM)					References
				Formic	Lactic	Acetic	Butyric	Valeric	
	Blank (GAM broth)		7.10 ± 0.04	4.20 ± 0.90	16.17 ± 0.49	0.72 ± 0	1.45 ± 0.14	17.43 ± 1.05	
35Y8B	*Clostridium baratii* ATCC27638^T (99.86%, X68174)	0.83 ± 0.27	6.1 ± 0.58	2.89 ± 2.97	5.22 ± 1.26	ND	0.78 ± 0.29	2.28 ± 0.12	Rainey (2009)
30Y4	*Clostridium paraputrificum* DSM 2630^T (99.57%, X73445)	0.025 ± 0.02	6.23 ± 0.02	ND	7.36 ± 4.28	ND	1.57 ± 0.46	4.35 ± 0.74	Rainey (2009)
36A18	*Clostridium perfringens* ATCC 13124 (99.78%, CP000246)	0.575 ± 0.03	6.81 ± 0.00	ND	17.95 ± 3.85	ND	0.41 ± 0.73	4.58 ± 0.42	Li *et al.* (2016a)
35Y30	*Clostridium tertium* DSM 2485^T (99.93%, Y18174)	0.29 ± 0.04	5.96 ± 0.09	0.20 ± 0.01	19.14 ± 4.08	ND	1.22 ± 0.48	1.42 ± 0.08	Rainey (2009)
35Y33	*Flavonifractor plautii* ATCC 29863^T (99.65%, JH417629)	0.02 ± 0.01	6.83 ± 0.01	ND	2.79 ± 0.95	ND	1.86 ± 0.04	14.21 ± 3.64	Li *et al.* (2016a)
35Y26	*Intestinimonas butyriciproducens* SRB-521-5-I^T (99.93%, KC311367)	0.07 ± 0.01	6.86 ± 0.05	1.05 ± 0.99	11.7 ± 0.00	ND	0.54 ± 0.55	1.58 ± 1.21	Bui *et al.* (2016)
30Y2	***Oscillibacter valericigenes* NBRC 101213^{T*} (95.79%, AP012044)**	**0.03 ± 0.01**	**7.14 ± 0.01**	**1.65 ± 0.07**	**14.3 ± 2.01**	**ND**	**0.95 ± 0.23**	**0.41 ± 0.52**	**< 97%**
35Y37	***Intestinimonas butyriciproducens* SRB-521-5-I^T (95.04%, KC311367)**	**0.06 ± 0.02**	**7.20 ± 0.02**	**0.06 ± 0.56**	**15.83 ± 2.48**	**ND**	**1.93 ± 0.10**	**1.26 ± 1.53**	**< 97%**
35A14	*Paeniclostridium sordellii* ATCC 9714^T (98.63%, AB075771)	0.09 ± 0.03	6.4 ± 0.40	ND	10.85 ± 6.70	ND	0.25 ± 0.55	ND	Rainey (2009)
6A16	*Anaerostipes caccae* DSM 14662^T (100%, ABAX03000031)	0.13 ± 0.05	6.93 ± 0.02	6.31 ± 1.47	ND	3.91 ± 3.75	2.51 ± 0.39	0.95 ± 1.38	Schwiertz *et al.* (2002)
30A19	*Eubacterium limosum* ATCC 8486^{T*} (100%, M59120)	0.03 ± 0.02	7.13 ± 0.07	2.14 ± 1.16	2.72 ± 0.60	ND	1.06 ± 0.48	1.31 ± 0.13	Rainey (2009)
35Y21B	*Anaerofustis stercorihominis* DSM 17244^{T*} (99.72, ABIL02000006)	0.03 ± 0.02	7.11 ± 0.01	0.03 ± 0.11	17.22 ± 2.77	ND	0.63 ± 0.16	2.49 ± 1.55	Li *et al.* (2016a)
30A7	*Odoribacter splanchnicus* DSM 20712^T (99.57%, CP002544)	0.22 ± 0.18	7.35 ± 0.15	ND	27.02 ± 2.80	ND	19.22 ± 5.36	ND	Li *et al.* (2016a)
30A1	*Butyricimonas faecihominis* 180-3^T (99.72%, AB916501)	0.39 ± 0.25	5.86 ± 0.13	0.93 ± 0.18	10.49 ± 5.88	ND	14.4 ± 3.71	ND	Sakamoto *et al.* (2014)
6A29	*Bacteroides caccae* ATCC 43185^T (99.93%, AAVM02000012)	0.40 ± 0.49	5.76 ± 0.00	0.42 ± 0.05	19.89 ± 0.13	ND	1.2 ± 0.64	1.85 ± 1.27	Sakurazawa and Ohkusa (2005)
36Y5	***Sutterella stercoricanis* CCUG 47620^T (92.34%, AJ566849)**	**0.03 ± 0.00**	**7.38 ± 0.00**	**ND**	**ND**	**ND**	**ND**	**3.16 ± 1.61**	**< 97%**

Species name and identity of all isolates were identified based on the EzTaxon database.
Isolates presented are representative of their OTUs, with more than 97% homology with the 16S rRNA gene sequence of their closest valid named neighbors.
Isolates with less than 97% homology with the 16S rRNA gene sequence of their closest valid named neighbors are in bold.
The concentration of each short-chain fatty acid (SCFA) was shown after subtracting the concentration of SCFA in the culture from the concentration of SCFA in GAM media.
*, These isolates were cultivated in GAM broth for 96 h at 37°C under anaerobic conditions before use.
ND, not detectable.

with ultraviolet (UV) absorbance detection.

Quantification of short-chain fatty acids

Concentrations and identities of select SCFAs were determined by HPLC-UV. They included formic acid, acetic acid, lactic acid, propionic acid, butyric acid, and valeric acid (Eeckhaut *et al.*, 2011). Isolates were re-cultivated in GAM broth for 48 h except for three strains that required four days to grow (as indicated in Table 1) at 37°C under anaerobic conditions. The optical density (OD) of each strain was measured at a wavelength of 620 nm using a microplate reader (Bio-Rad) and the OD_{620} value of the un-inoculated medium was subtracted. Next, the culture was centrifuged at 1,400 × *g*, at 4°C, for 20 min. The cell pellet was used for DNA extraction and the supernatant was collected for SCFA quantification. The pH of the supernatant was measured with a pH meter (F-52, Horiba) and then adjusted to pH 2.0 using 6 N HCl. In the next step, the acidified supernatant was centrifuged under the same conditions as described above and passed through a 0.2 µm filter. SCFAs were extracted from the protein-containing cell-free supernatant with ethyl acetate (Sigma-Aldrich) as described (García-Villalba *et al.*, 2012), with a minor modification: 2 ml of the supernatant were mixed with 4 ml of ethyl acetate and left to stand undisturbed for 15 min, after which the upper organic layer was transferred into a new tube. This extraction step was repeated three times. Afterwards, the pooled organic solution was evaporated to dryness in a centrifugal evaporator (Sakuma). Finally, 1 ml of HPLC grade water was added and the extract was subjected to HPLC analysis. Standards were formic acid, butyric acid (Wako), acetic acid, propionic acid (Nacalai Tesque), lactic acid, and valeric acid (Sigma Aldrich). A calibration curve for each standard was generated and used to quantify the concentration of each of the corresponding compounds in the samples.

The HPLC-UV system consisted of a system controller (SCL-10A, Shimadzu), a degassing device (ERC-3115a, ERC Inc.), an HPLC pump (LC-10AD, Shimadzu), a column oven (CTO-10AC, Shimadzu) and a detector (SPD-20A, Shimadzu). SCFAs in 100 µl of each sample extract was separated at 30°C on a YMC Pack ODS-AM column, 4.6 mm inner diameter × 250 mm length, with 5 µm particles, 120 Å pore size. The mobile phase A contained 5% (v/v) acetonitrile (Nacalai Tesque) and 0.05% (v/v) trifluoroacetic acid (Wako) in HPLC grade water, and phase B contained 90% (v/v) acetonitrile and 0.05% (v/v) trifluoroacetic acid in HPLC grade water. The flow rate was 1 ml/min. The solvent gradient after injection was 5 min at 0% B, to 100% B at 40 min, and kept at 100% B until 45 min. Finally, the column was equilibrated back to 0% B by 50 min. The detector wavelength was set to 220 nm. HPLC chromatograms were analyzed with the Chromato-PRO software version 3.0 (Runtime Instrument). Each strain was tested twice and in duplicate HPLC analyses.

Phylogenetic tree analyses based on sequences of the 16S rRNA gene

A phylogenetic tree was constructed to evaluate the relationship of isolates in this study with confirmed butyrate-roducing reference strains. Reference sequences of Type strains were downloaded from the EzTaxon database (Yoon *et al.*, 2017). All sequences were aligned and equalized with MEGA7 (Kumar *et al.*, 2016) using the neighbor-joining method (Saitou and Nei, 1987) with a bootstrap test (1,000 times) (Felsenstein, 1985), and pairwise gap deletion (Nei and Kumar, 2000; Eeckhaut *et al.*, 2011). *Escherichia coli* NCTC-9001^{T} (LN831047) was used as an outgroup.

Bacterial DNA extraction for butyrogenic gene detection

The pellets of butyrate-producing bacteria were rinsed twice with TE buffer (pH 8) (1 M Tris-HCl, 0.5 M EDTA, Invitrogen), suspended in TE buffer and incubated at 37°C for 1 h with 15 mg/ml lysozyme (Sigma-Aldrich). Next, purified achromopeptidase (Wako) was added to the suspension to obtain 2,000 units/ml and incubated at 37°C for 30 min. The enzymatic treatment was continued by adding 1 mg/ml proteinase K (Merck) and 100 µl of 10% sodium dodecyl sulfate (Nacalai Tesque) and kept at 55°C for 1 h. The bacterial DNA was separated with phenol:chloroform:isoamyl alcohol (25:24:1) (Nacalai Tesque) and precipitated with 99.5% isopropanol (Wako) and sodium acetate 3 M. The DNA pellet was obtained by washing twice with 75% ethanol, dried and dissolved in TE buffer overnight. RNA was removed by incubating the solution with 1 µl RNase A (Novagen) at 37°C for 1 h. The genomic DNA was recovered by precipitation with 26% PEG (Polyethylene glycol, Nacalai Tesque) in 1.6 M NaCl (Nacalai Tesque) on ice for 30 min and followed by centrifugation at 15,000 × *g* for 15 min at 4°C. The pellet was rinsed with 75% ethanol, dried and dissolved in 10 mM Tris-HCl buffer (pH 8) (Invitrogen), and stored at -20°C until subjected to identification of genes involved in butyrate formation (Morita *et al.*, 2007).

Identification of genes encoding for butyrate production in bacteria

The presence of four functional genes involved in butyrate biosynthesis in suspected isolates was analyzed by using four pairs of degenerate primers. They included *buk*, *but*, CoA-transferase-related, and propionate CoA-transferase genes (Eeckhaut *et al.*, 2011). Purified genomic DNA of all butyrate-producing bacteria was used as a PCR template. Primers PTBfor2 and BUKrev1 were designed to amplify the butyrate kinase operon in clostridia through the ramped annealing approach (Louis *et al.*, 2004). The *but* gene was amplified with BCoATscrF and BCoATscrR degenerate primers (Louis and Flint, 2007). Primers CoATDF1 and CoATDR2 were used to amplify a broad range of CoA-transferase-related sequences (Charrier *et al.*, 2006). Primers PCTfor1 and PCTrev2 were designed to detect the propionate CoA-transferase gene from *Clostridium propionicum* (Charrier *et al.*, 2006). Sequences of all primers are listed in Supplementary data Table S1. PCR amplicons in bands of expected sizes were purified using MagExtractor-PCR & Gel Clean-Up kit (Toyobo). The genes were sequenced with the same method as described above for sequencing of 16S rRNA genes. Contigs were created using GeneStudio and the exported sequences were translated into deduced amino acids sequences using the ExPASy translate tool (https://web.expasy.org/translate/) (Gasteiger *et al.*, 2003). These deduced amino acids were blasted against the GenBank

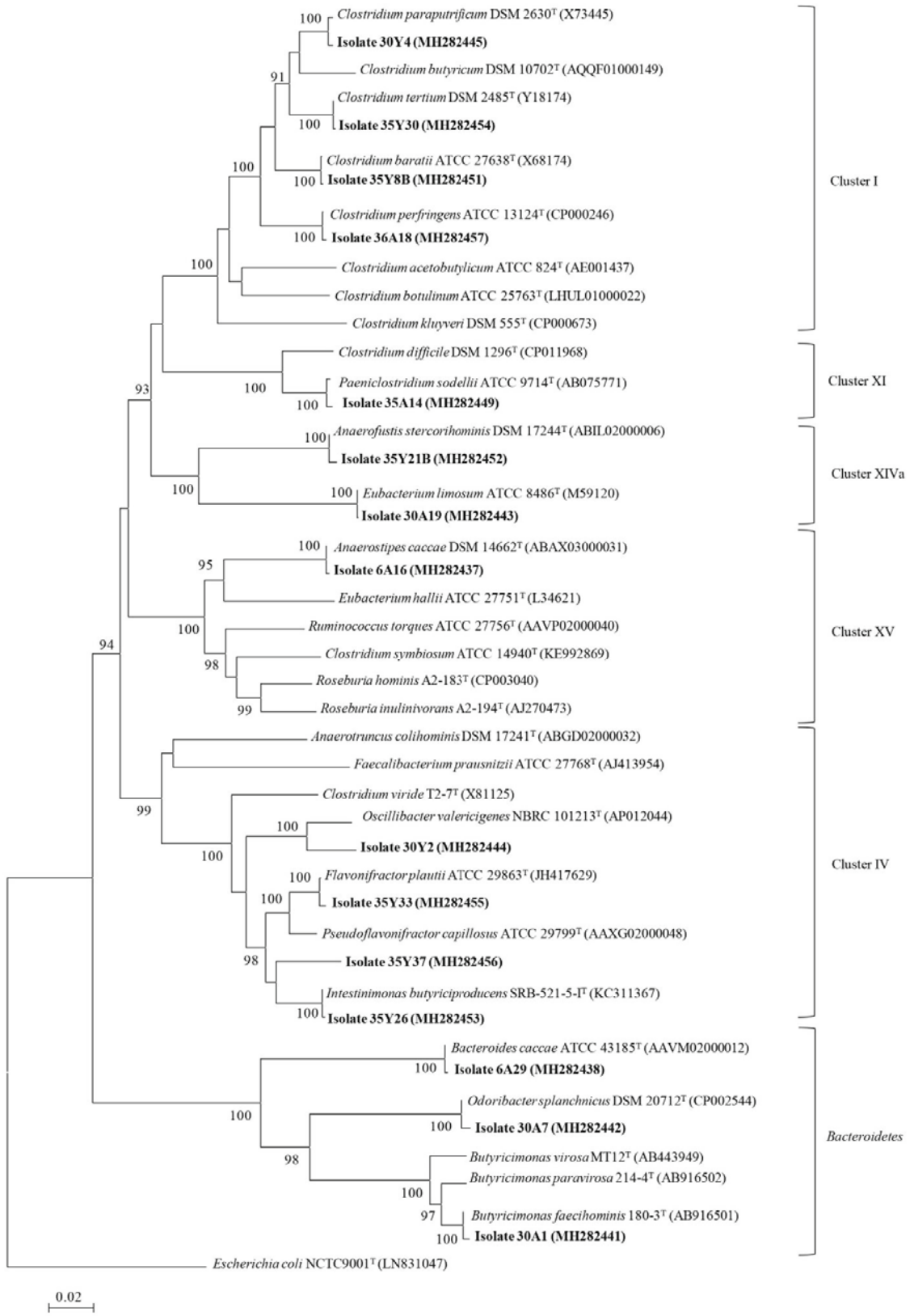

Fig. 1. 16S rRNA gene sequence-based phylogenetic tree of butyrate-producing isolates (in bold) and of known reference. Roman numerals indicate clostridial clusters. Accession numbers of Type strains are from the EzTaxon database (in parentheses). Bootstrap values are shown at the nodes of the tree (values under 90% were removed). 16S rRNA gene sequences of *Escherichia coli* NCTC9001^{T} (LN831047) was used as an outgroup at the root of the tree. Scale bar: 0.02 substitutions per nucleotide position.

database using BLASTP with a Reference Proteins database (Altschu *et al.*, 1990). Amino acid sequences from isolates in this study and of corresponding reference genes were used to create phylogenetic trees using the same methods and parameters as those used for reconstructing for 16S rRNA gene-based phylogenetic tree (Zuckerkandl and Pauling, 1965; Felsenstein, 1985; Nei and Kumar, 2000; Kumar *et al.*, 2016).

Nucleotide sequence accession numbers

All sequences were deposited into the GenBank database. Accession numbers of the 16S rRNA gene sequences (MH282437-MH282438, MH282441-MH282445, MH282449, and MH282451-MH282457) matching with each isolate are shown in Fig. 1. And the accession numbers of functional gene sequences (MH390321-MH390337) corresponding to these isolates are described in Fig. 2A, B, and C.

Results

Isolation, identification, and validation of isolates of butyrate-producing bacteria

From a total of 226 colonies, randomly picked from both media, we identified four isolates as distinct OTUs and grouped

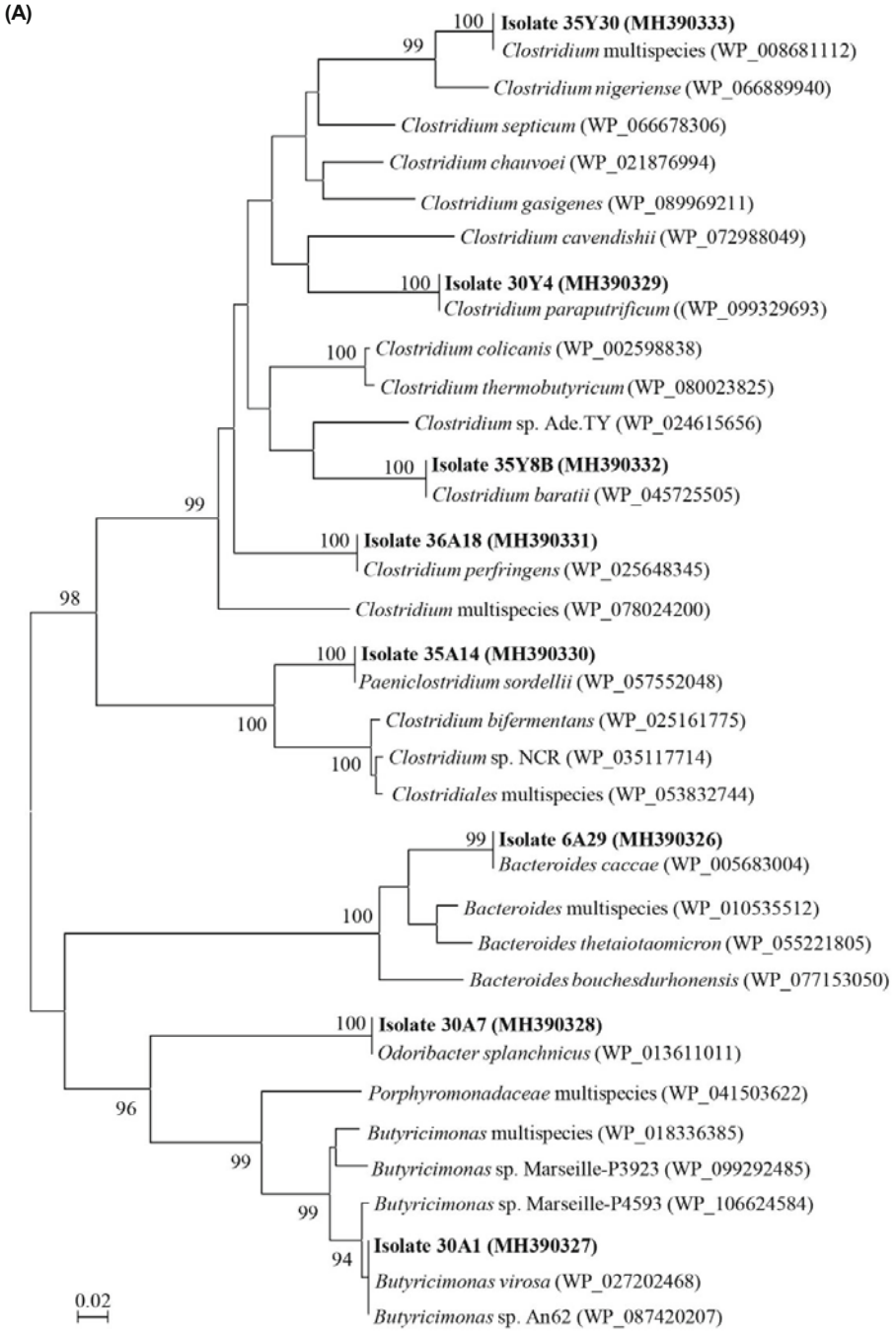

Fig. 2. Phylogenetic tree based on deduced proteins of isolates from this study and of reference strains. Buk proteins (2A), But proteins (2B), and 4-Hbt proteins (2C). Accession numbers are given in parentheses. Bootstrap values are indicated at the nodes of the tree (values under 90% were removed). Scale bar: 0.02 substitutions per nucleotide position.

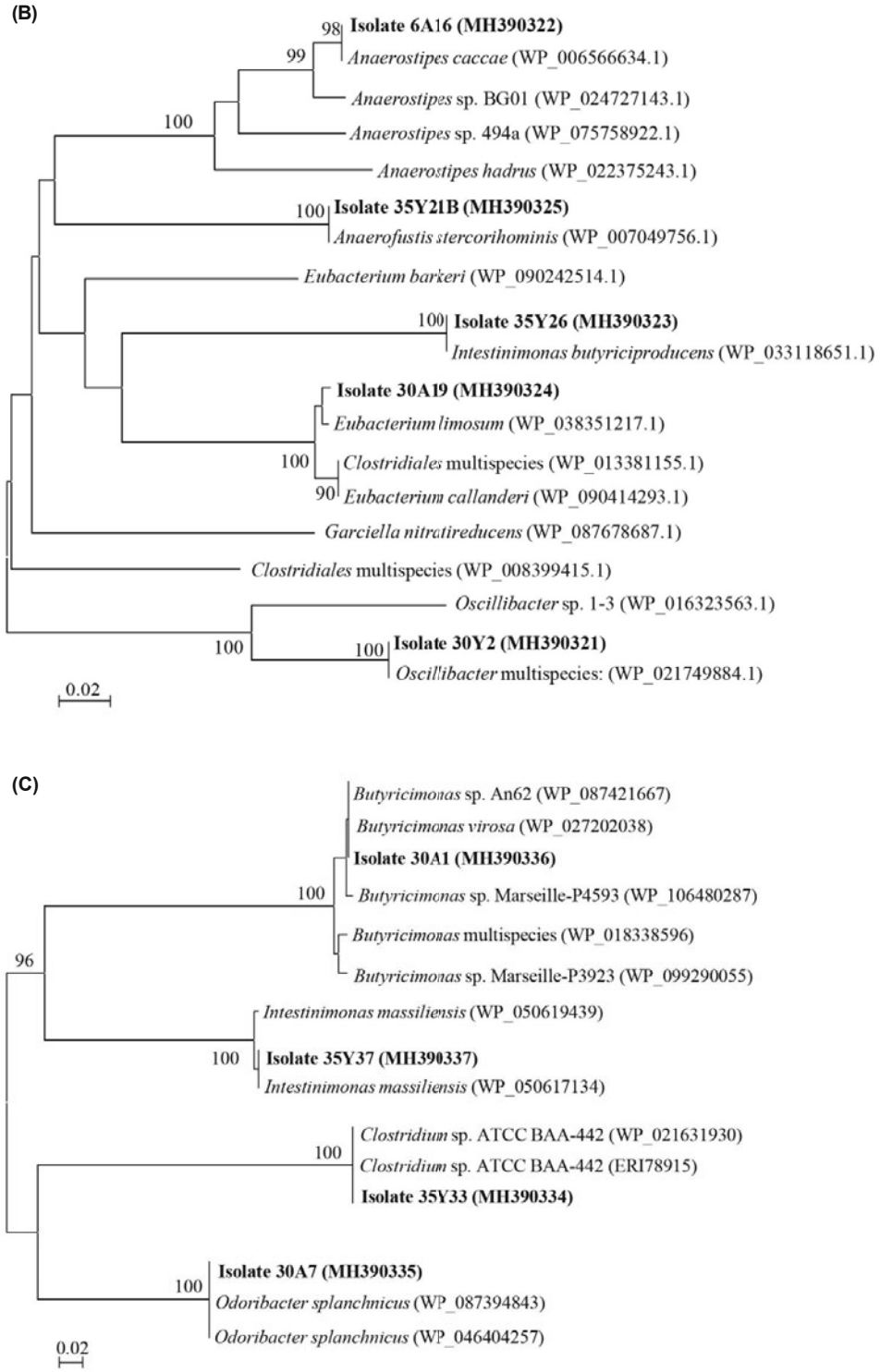

Fig. 2. Continued.

222 isolates into 56 OTUs (Supplementary data Table S2). The near-full-length 16S rRNA gene sequences of these combined 60 representative OTUs were again compared to existing sequence databases, including the 16S ribosomal RNA sequence, the RefSeq Genome, the EzTaxon, and the SILVA databases (Supplementary data Table S3).

In the high identity group, each isolate was found to have identical species name in the four reference databases, except for eight isolates 30A20, 35Y30, 35Y33, 36Y11, 6Y13, 30Y20, 30Y9, and 35A7, which did not. Isolate 30A20 matched with a *Bacteroides* sp.-related strain in the RefSeq Genome database but it was identified as *Bacteroides nordii*-related strain in the three remaining databases. Similarly, isolate 35Y30 and 35Y33 were identified as a *Clostridium* sp. and as a *Clostri-*

diales bacterium in the RefSeq Genome database, respectively. However, isolates 35Y30 and isolate 35Y33 were identified as *Clostridium tertium*-related and *Flavonifractor plautii*-related strains (formerly *Clostridium orbiscindens* [Carlier *et al.*, 2010]), respectively, based on the other three databases. Isolate 36Y11 was identified as a *Blautia wexlerae*-related strain in the EzTaxon and the SILVA databases. However, it matched with the entry *Blautia luti*-related strain in the 16S ribosomal RNA sequence database, and the entry *Blautia* sp.-related strain in the RefSeq Genome database. Other two isolates 6Y13 and 30Y20 were determined as *Escherichia fergusonii*-related strain and *Tyzzerella nexilis*-related strain (formerly *Clostridium nexile* [Holdeman and Moore, 1974; Yutin and Galperin, 2013]), respectively, in the 16S ribosomal RNA sequence and the EzTaxon databases. However, they were identified as an *Escherichia coli*-related strain and a *Tyzzerella* sp.-related strain in the two remaining databases, respectively. Finally, two isolates 30Y9 and 35A7 were identified as a *Terrisporobacter mayombei*-related strain and a *Terrisporobacter petrolearius*-related strain, respectively, in the 16S ribosomal RNA sequence and the EzTaxon databases. But they were identified as a *Terrisporobacter* sp.-related strain and an uncultured bacterium, respectively, in the SILVA database. Interestingly, both of them matched with *Terrisporobacter othiniensis*-related strains in comparison with entries in the RefSeq Genome database.

Out of the four isolates in the low identity group, three isolates (isolates 30Y2, 35Y37, and 36Y5) were identified based on the 16S ribosomal RNA sequences, the RefSeq Genome, and the EzTaxon databases while the one remaining (isolate 30A1) was identified with the SILVA database (Supplementary data Table S3). The first isolate 30Y2 was identified as an *Oscillibacter valericigenes*-related strain with 95.74–95.79% identity against the 16S ribosomal RNA sequence and the EzTaxon databases but it was classified as an *Oscillibacter* sp.-related strain with more than 98% identity in the two remaining databases. The second isolate 35Y37 was classified as an *Intestinimonas butyriciproducens*-related strain with 95.04–95.19% identity in the 16S ribosomal RNA sequences and EzTaxon databases. However, its species name matched an *Intestinimonas massiliensis*-related strain with more than 99% identity in the two other databases. The third isolate 36Y5 matched to a *Sutterella stercoricanis*-related strain with identity lower than 95% identity in the 16S ribosomal RNA sequence and EzTaxon databases. However, it was identified as an uncultured bacterium (94.78% identity) and a *Dakarella massiliensis*-related strain (99.71% identity) by matching with entries deposited in the SILVA and the RefSeq Genome database, respectively. The final isolate 30A1 matched to a *Butyricimonas faecihominis*-related strain in the 16S ribosomal RNA sequence and EzTaxon databases, with more than 99% identity. It was also identified as a *Butyricimonas faecihominis*-related strain but with only 95.50% identity in the SILVA database. However, it matched to *Butyricimonas* sp. An62 in the RefSeq Genome database with 97.89% identity. The identification analyses of each strain played an important role because they helped to determine encoding genes for butyrate synthesis in the corresponding bacteria.

It was assumed that the four low identity isolates could produce butyrate. Based on various literature references (see Table 1), 12 OTUs were selected as putative butyrate-producers out of the 56 representative OTUs of high identity isolates. In total, these 16 frozen stocks were then recovered and cultured for quantification of short-chain fatty acid production and gene identification.

Quantification of short-chain fatty acids

Considered as the first study to isolate butyrate-producing bacteria in the human gut, Barcenilla and co-authors chose a cut-off value of 2 mM butyrate produced by bacteria in the culture at 24 h after inoculation under anaerobic conditions in M2GSC broth (Barcenilla *et al.*, 2000). They suggested that this value could be used to clearly distinguish butyrate produced by the bacteria from the original butyrate concentration of the broth (Barcenilla *et al.*, 2000). However, the initial concentrations of each SCFA in un-inoculated M2GSC broth was not shown in their study. Therefore, in our study, by subtracting the butyrate concentration of each inoculated culture from the initial butyrate concentration of the GAM broth, every strain tested here that measurably increased the original butyrate concentration was considered as a true butyrate producer.

The SCFAs data analysis showed that 15 out of the 16 cultivable strains could produce butyrate. The concentration of butyrate varied from 0.25 ± 0.05 mM to 19.22 ± 5.36 mM (Table 1). The *Sutterella stercoricanis*-like isolate 36Y5 did not produce butyrate. Two isolates of the family *Odoribacteraceae* within the phylum *Bacteroidetes* (isolates 30A1 and 30A7) exhibited a high ability to produce butyrate. OD values did not correlate with a strain's level of butyrate production. Some strains did not grow well in the GAM broth but their final butyrate concentration in the culture was higher than that of other better-growing bacteria and vice versa. Furthermore, the increase or decrease in the broth's pH after incubation of the cultures was also not associated with the final concentration of butyrate and other SCFAs. None of the tested butyrate-producing strains were found to produce propionate. Almost all bacterial strains consumed acetate in the medium for their growth, except for the *Anaerostipes caccae*-like isolate 6A29, which released acetate into the culture.

The 16S rRNA gene sequences of all cultivable butyrate-producing isolates and of other known butyrate-producing bacteria were used to construct a phylogenetic tree (Fig. 1). All isolates were distributed into two phyla, which included clostridial clusters I, IV, XI, XIVa, and XV of the phylum *Firmicutes* and families *Odoribacteraceae* and *Bacteroidaceae* of the phylum *Bacteroidetes.* Isolates that belonged to the clostridial clusters I and IV were more abundant than isolates of other clusters.

Identification of genes encoding for butyrate production in bacteria

Different encoding genes for butyrate production were identified in each isolate by using four sets of degenerate primers (Supplementary data Table S4). By using PTBfor2 and BUKrev1 primers, the *buk* gene was detected in 8 isolates (Fig. 2A), with an expected size of ~380 bp. They separated into two main branches of the phylogenetic tree, which was generated from their deduced amino acid sequences and

their references. One branch was populated by *Bacteroidetes* isolates 30A1, 30A7, and 6A29, while another one contained clostridial cluster I isolates 36A18, 30Y4, 35Y30 and 35Y8B, and the XI isolate 35A14 of the phylum *Firmicutes*. The tree was highly consistent with the 16S rRNA gene-based phylogenetic tree, except for isolates 30A1 and 35Y30. As described by their protein phylogenetic tree, the Buk protein of isolate 30A1 and 35Y30 indicated that they were related to *Butyricimonas* sp. An62 and *Butyricimonas virosa*, and multiple species of *Clostridia*, respectively, while not being closely related to *Butyricimonas faecihominis* and *Clostridium tertium* as identified by their 16S rRNA gene sequences. After considering their valid species name assignment, we concluded that isolate 30A1 was actually a *B. faecihominis*-related strain and isolate 35Y30 was a *C. tertium*-related strain.

However, the *but* gene segment had an expected size of ~510 bp and was detected in five isolates of the phylum *Firmicutes* by using the BCoATscrF and BCoATscrR primer pairs. They were distributed across clostridial clusters IV (isolates 30Y2 and 35Y26), XIVa (isolates 35Y21B and 30A19), and XV (isolate 29A16). The phylogeny was consistent between the 16S rRNA gene-based phylogenetic tree and the deduced but protein phylogenetic tree (Fig. 2B), with the exception of isolate 30Y2. The protein-based phylogeny of isolate 30Y2 indicated that it was related to *Oscillibacter* sp., not to *Oscillibacter valericigenes*. However, based on similar identification analyses as that of isolates 30A1 and 35Y30, we propose that isolate 30Y2 was an *O. valericigenes*-related strain.

The degenerate primer set of CoATDF1 and CoATDR2, which was used for detection of a broad range of CoA transferase-related sequences, was able to amplify butyryl-CoA: 4-hydroxybutyrate CoA-transferase gene (*4-hbt*) in 2 isolates of the clostridial cluster IV (isolates 35Y37 and 35Y33) and in 2 isolates of the family *Odoribacteraceae*, phylum *Bacteroidetes* (isolates 30A1 and 30A7). Only the phylogeny of the *4-hbt* gene sequences of isolate 30A7 was in good agreement with the 16S rRNA gene-based phylogeny. Three out of four isolates did not correlate well between their *4-hbt* gene-based phylogeny and their 16S rRNA gene-based phylogeny, including isolates 30A1, 35Y33, and 35Y37. Isolate 35Y37 showed that it was closely related to *Intestinimonas massiliensis* in the protein phylogeny. This observation was consistent with entries of its 16S rRNA gene sequence in the SILVA and RefSeq Genome databases. However, the 16S rRNA gene sequence of isolate 35Y37 corresponded to an *Intestinimonas butyriciproducens*-related strain in the 16S ribosomal RNA sequence and EzTaxon databases as noted in the sections above. Therefore, with the support of protein evidence and the two former databases, isolate 35Y37 was confirmed as an *I. massiliensis*-related strain. Isolate 30A1, for which both the *buk* gene and the *4-hbt* gene were detected, showed consistent butyrate-gene-based phylogenetic trees, demonstrating that it was closely related to *Butyricimonas* sp. An62 and *Butyricimonas virosa*. Finally, the *4-hbt* gene of isolate 35Y33 named *Flavonifractor plautii* was closely related to *Clostridium* sp. as described in Fig. 2C. The 16S rRNA sequence of isolate 35Y33 corresponded with the entry titled *F. plautii*-related strain in the three databases namely, the SILVA, 16S rRNA sequence and EzTaxon databases whereas it was linked to an entry entitled "unclassified *Clostridiales* bacterium" in the RefSeq Genome database. Therefore, we concluded that isolate 35Y33 was *Flavonifractor plautii*-related strain. Finally, propionate CoA-transferase was not amplified in any isolates in this study when using the PCTfor1 and PCTrev2 primers.

Discussion

Identification of butyrate-producing bacteria

Based on 16S rRNA gene sequences, the identification of butyrate-producing bacteria isolated from the human fecal samples indicated that they are mainly comprised of Gram-positive *Firmicutes* bacteria (Louis and Flint, 2009). They are related to the class Clostridia, including clostridial clusters I, III, IV, XI, XIVa, XV, and XVI (Louis *et al.*, 2007) according to the classification by Collins and co-authors (Collins *et al.*, 1994). In our study, a remarkable variety amongst the small group of distinct butyrate-producing bacteria was noted. The phylogeny indicated that the isolated bacteria appeared in most of the clostridial clusters, except for clusters III and XVI. Clostridial cluster IV and XIVa are considered as major butyrate producers in the human gut (Pryde *et al.*, 2002; Louis and Flint, 2009). Here, isolates of cluster IV were *F. plautii*-like isolates (isolate 35Y33), *I. massiliensis*-like isolates (isolates 35Y37), *I. butyriciproducens*-like isolates (isolate 35Y26), and *O. valericigenes*-like isolates (isolate 30Y2). These bacteria synthesize butyrate via the protein-fed pathway (Vital *et al.*, 2017). The successful isolation of these strains demonstrated that although the dominant butyrate-producing bacteria of the human gut utilize carbohydrates as a major energy source, the butyrate-producing bacteria that consume proteins can also be isolated. Moreover, three OTUs related to Gram-negative *Bacteroidetes* bacteria were cultured and identified. Our finding provides evidence that cultivable butyrate producers in the human gut do not only belong to the *Firmicutes* phylum but that they also exist in another phylum such as *Bacteroidetes*. Consistently, similar findings were reported in a study which investigated cultivable butyrate-producing bacteria in the gut contents and feces of pigs by successful identification of some butyrate producers that belonged to the phyla of *Fusobacteria* and *Bacteroidetes* (Eeckhaut *et al.*, 2011).

The weak consistency between 16S rRNA gene-based phylogeny and functional gene-based phylogeny of some strains is likely due to insufficient gene annotations.

Genes encoding for butyrate production in bacteria

Butyrate is produced in the human gut from carbohydrates or proteins via four different synthesis pathways, including the acetyl-CoA pathway (Ac pathway), the glutarate, the lysine, and the 4-aminobutyrate/succinate pathways (Vital *et al.*, 2014). While the Ac pathway, which uses carbohydrates as its major fuel, plays a predominant and important role in butyrate metabolism, other pathways fed by proteins were considered as minor contributors (Louis *et al.*, 2004; Vital *et al.*, 2014). In the Ac pathway, butyrate can be produced from

butyryl-CoA via the catalysis of either terminal enzymes Buk or But (Louis *et al.*, 2004; Vital *et al.*, 2014). *buk* and *but* are also considered as terminal genes for the glutarate pathway due to the absence of the co-substrate for butyryl-CoA transferase beyond this pathway (Vital *et al.*, 2014).

Butyrate producers with the *buk* gene were characterized by lower abundance than other butyrate producers that contained the *but* gene. The *buk* gene was found only in few members of the clostridial clusters IV and XIVa (Louis *et al.*, 2004; Eeckhaut *et al.*, 2011). For example, only one out of 16 butyrate-producing bacteria isolated from the caecum of chickens (Eeckhaut *et al.*, 2011) and four out of 38 butyrate producers isolated from the healthy human gut (Louis *et al.*, 2004) carried this gene. However, in this study, isolates 30Y4, 36A18, 35Y8B, and 35Y30 belonging to clostridial cluster I, as well as isolates 35A14 of cluster XI, possessed this gene. More interestingly, *Bacteroidetes* bacteria (isolates 6A29, 30A7, and 30A1) also showed the ability to produce butyrate in this way. Although *but* gene-carrying butyrate-producing bacteria were more abundant than *buk* gene carriers, as described in the previous studies (Louis *et al.*, 2004; Eeckhaut *et al.*, 2011), the number of isolates in this study possessing the *but* gene was not high (5 out of 15 isolates).

Bacteria often carry multiple types of CoA-transferases in their genomes (Louis and Flint, 2017) and some bacteria in the human large intestine use it for butyrate production (Charrier *et al.*, 2006). In the previous studies in which the genes encoding butyrate formation in bacteria were detected, *but* gene amplicons were obtained using the CoATDF1 and CoATDR2 degenerate primers (Charrier *et al.*, 2006; Eeckhaut *et al.*, 2011). However, none of the strains possessing *but* gene in this study could be amplified with the CoATDF1 and CoATDR2 primers. This indicated that BcoATscrF and BcoATscrF degenerate primers which were used to identify the *but* gene had higher specificity to their target than the CoATDF1 and CoATDR2 degenerate primers (Eeckhaut *et al.*, 2011). However, identification of the expected sizes amplicons with CoATDF1 and CoATDR2 degenerate primers revealed the presence of the *4-hbt* gene. Butyrate-producing bacteria that possessed the *4-hbt* gene in this study were two isolates of clostridial IV, *I. massiliensis*-like and *C. tertium*-like strains, and two isolates of the family *Odoribacteraceae*, *B. faecihominis*-like and *O. splanchnicus*-like strains. Consistent with our observations, it was previously shown that the *Butyricimonas* and *Odoribacter* genera were able to produce butyrate via a 4-aminobutyrate pathway with the participation of the *4-hbt* gene as a terminal gene (Vital *et al.*, 2017).

The usage of degenerate primers PCTfor1 and PCTrev2 did not lead to any specific amplicons of the propionate CoA-transferase gene in the tested butyrate-producing bacteria. In the previous study on butyrate-producing bacteria isolated from chicken caecum, the propionate CoA-transferase gene could be amplified with these primers in some strains (Eeckhaut *et al.*, 2011). Their results indicated that bacteria carrying a propionate CoA-transferase gene took part in the butyrate synthesis process. There may be another reason for the involvement of propionate CoA-transferase in butyrate formation since this gene is located directly downstream of the butyrate central pathway genes in the butyrate-producing bacteria cluster XVI (Eeckhaut *et al.*, 2011). Members of clostridial cluster XVI were not isolated in this study. On the other hand, the absence of propionate CoA-transferase gene in all tested strains was supported by the evidence that no strains were able to produce propionate as their final metabolites.

In the 15 tested strains, either a strain carried the *buk* gene or the *but* gene but none of the strains possessed both genes. No isolates of the *Bacteroidetes* phylum carried the *but* gene but all of them possessed the *buk* gene. Interestingly, two strains carrying the *buk* gene also possessed the *4-hbt* gene, including isolates 30A1 and 30A7 of the phylum *Bacteroidetes*. This finding emphasized the flexibility in energy source usage by these strains when producing butyrate, and may hint towards a central role for the energy management of butyrate synthesis (Vital *et al.*, 2017).

Amongst the four pathways for butyrate synthesis, the glutarate-based and 4-aminobutyrate pathways were recorded as the least prevalent pathways (Vital *et al.*, 2017). The 4-aminobutyrate (γ-aminobutyrate, GABA) is a product of glutamate degradation in a number of gut bacteria under acid stress conditions. GABA is an inhibitory neurotransmitter and plays an important role in regulating our mood, cognition, and behavior (Feehily and Karatzas, 2013). An imbalance between the excitatory glutamate and GABA in the nervous system may contribute to neuronal disorders, leading to Alzheimer's disease, Huntington's disease, and schizophrenia (Li *et al.*, 2016b). The production or consumption of GABA by gut microbes may lead to changes in mood and behavior (Louis and Flint, 2017). In the brain of AD patients, the GABA level was lower than in healthy elderly people (Li *et al.*, 2016b). In the human gut microbiota, GABA is converted to 4-hydroxybutyrate, a substrate of *4-hbt* gene, by the enzymes 4-aminobutyrate aminotransferase and 4-hydroxybutyrate dehydrogenase, which is an important step in the 4-aminobutyrate pathways of butyrate metabolism (Vital *et al.*, 2014). Therefore, the existence of butyrate producers carrying the *4-hbt* gene in the gut of AD patients may provide leads for further study on this bacterial group in AD patients.

In this study, many butyrate-producing bacteria were identified and characterized for their butyrate-producing ability, SCFA production, and genotype. To our knowledge, this is the first study to use a brain-related disease sample to isolate butyrate-producing bacteria in the human gut. Interestingly, butyrate-producing bacteria are not totally absent in the gut of elderly Japanese patients diagnosed with Alzheimer's disease. For a deeper understanding of the role of butyrate-producing bacteria in these particular patients, a study related to their whole community in fecal samples as well as their butyrate synthesis pathways should be carried out in the future.

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Conflict of Interest

The authors declare that they have no competing interests.

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