Characterization of *Phascolarctobacterium succinatutens* sp. nov., an Asaccharolytic, Succinate-Utilizing Bacterium Isolated from Human Feces

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Isolation, cultivation, and characterization of the intestinal microorganisms are important for understanding the comprehensive physiology of the human gastrointestinal (GI) tract microbiota. Here, we isolated two novel bacterial strains, YIT 12067[T] and YIT 12068, from the feces of healthy human adults. Phylogenetic analysis indicated that they belonged to the same species and were most closely related to *Phascolarctobacterium faecium* ACM 3679[T], with 91.4% to 91.5% 16S rRNA gene sequence similarities, respectively. Substrate availability tests revealed that the isolates used only succinate; they did not ferment any other short-chain fatty acids or carbohydrates tested. When these strains were cocultured with the xylan-utilizing and succinate-producing bacterium *Paraprevotella xylaniphila* YIT 11841[T], in medium supplemented with xylan but not succinate, their cell numbers became 2 to 3 orders of magnitude higher than those of the monoculture; succinate became undetectable, and propionate was formed. Database analysis revealed that over 200 uncultured bacterial clones from the feces of humans and other mammals showed high sequence identity (>98.7%) to YIT 12067[T]. Real-time PCR analysis also revealed that YIT 12067[T]-like bacteria were present in 21% of human fecal samples, at an average level of 3.34×10^8 cells/g feces. These results indicate that YIT 12067[T]-like bacteria are distributed broadly in the GI tract as subdominant members that may adapt to the intestinal environment by specializing to utilize the succinate generated by other bacterial species. The phylogenetic and physiological properties of YIT 12067[T] and YIT 12068 suggest that these strains represent a novel species, which we have designated *Phascolarctobacterium succinatutens* sp. nov.

Advances in culture-independent molecular techniques have allowed a more complete and accurate assessment of the biodiversity of the ecosystem of the human gastrointestinal (GI) tract microbiota (10, 52) and have revealed that most of the phylotypes detected are uncultured (42). For a better understanding of the physiology of the human GI tract microbiota, it is important to isolate, cultivate, and characterize the intestinal microorganisms. Such undertakings provide information on the ecology and physiology of the GI tract microbiota that cannot be acquired from gene sequence information alone. For example, a study that used an isolated strain in the gnotobiotic mouse model revealed that one of the bacteria most frequently detected from human feces, *Bacteroides thetaiotaomicron*, modulates the expression of host genes involved in nutrient absorption, mucosal barrier fortification, xenobiotic metabolism, angiogenesis, and postnatal intestinal maturation (19). Therefore, cultivation of previously uncultured bacteria will also contribute to a more comprehensive understanding of the human GI tract microbiota through not only the phenotypic characterization of these species but also the sequencing of their whole genomes as a reference for metagenomic studies (53).

For these reasons, we have attempted to isolate uncultured bacteria from human feces. To date, we have successfully isolated 17 species, including 4 new genera (33–40, 43, 56). Here, we isolated another novel species. Many uncultured bacterial clones found in the feces of humans and other mammals show high levels of identity in their 16S rRNA gene sequences to those of our isolates, implying that these isolates are common among GI tract microbiota. A substrate availability test (described below) revealed that these isolates did not use any of the carbohydrates we tested. On the other hand, for human resident gut microbiota,

dietary carbohydrates that resist degradation in the upper intestinal tract are important energy sources (7), and most of the phylotypes frequently detected in the human GI tract seem to be closely related to species that have functions in carbohydrate catabolism (48). Therefore, it was not clear what kind of substrate was used by these novel isolates and why they were prevalent in the GI tract. To address these questions, we analyzed the biochemical properties and energy source acquisition of these isolates. We also investigated the distribution of isolate-like bacteria in human feces by using real-time PCR analysis with species-specific primers, in addition to carrying out a database search.

MATERIALS AND METHODS

Isolation of strains YIT 12067[T] and YIT 12068. Fecal samples were collected from two healthy Japanese males (subjects S and O, 37 and 40 years old, respectively) and immediately transferred into an anaerobic glove box that contained 88% N_2, 7% H_2, and 5% CO_2. Each sample was weighed and diluted with prereduced 0.1 M phosphate-buffered saline (0.145 M NaCl, 0.15 M sodium phosphate; pH 7.0), and 100 μl of each was spread on modified Gifu anaerobic medium (GAM) agar plates (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with bile (2%, 4%, 6%, and 8% Bacto oxgall; Difco Laboratories, Detroit, MI), NaCl (1%, 3%, 6%, or 10%), or antibiotics (1 of 12 different antibiotics at three concentrations) in an attempt to suppress species that grew well and may there-

fore have inhibited our ability to isolate objective species. The composition of the modified GAM was as follows (per liter): peptone, 5.0 g; soy peptone, 3.0 g; proteose peptone, 5.0 g; serum powder, 10.0 g; yeast extract, 2.5 g; beef extract powder, 2.2 g; liver extract powder, 1.2 g; glucose, 0.5 g; soluble starch, 5.0 g; L-cysteine monohydrochloride, 0.3 g; sodium thioglycolate, 0.3 g; potassium dihydrogen phosphate, 2.5 g; sodium chloride, 3.0 g; L-tryptophan, 0.2 g; L-arginine, 1.0 g; vitamin K_1, 5 mg; and hemin, 10 mg. Plates were incubated at 37°C for 3 days in an anaerobic glove box, as described above. Single colonies showing different morphologies were picked out and grown on modified GAM agar plates. This step was repeated until pure cultures were obtained. Strain YIT 12067^T was isolated from a modified GAM agar plate supplemented with vancomycin (20 μg ml^{-1}) and inoculated with a 10^{-5} serially diluted fecal sample from subject S. Strain YIT 12068 was isolated from a modified GAM agar plate supplemented with oxacillin (4 μg ml^{-1}) and inoculated with a 10^{-5} serially diluted fecal sample from subject O. Both strains formed small pinpoint colonies on modified GAM agar plates.

Phylogenetic analysis. DNA extraction, PCR, and sequencing of the 16S rRNA genes were performed as described previously (43). Closely related sequences were retrieved from the EMBL-EBI database (http://www.ebi.ac.uk/tools/sss/fasta/nucleotide.html) by using the FASTA program. Pairwise sequence similarity values for strains YIT 12067^T and YIT 12068 and for the other species derived from the database search were calculated by using EzTaxon (5). Sequences were aligned by using ClustalX software (version 2.0) (50). Alignments were corrected manually by using the BioEdit program (16), and a phylogenetic tree was then produced by using the neighbor-joining (NJ) method with ClustalX. The topology of the tree was evaluated by a bootstrap analysis with 1,000 replicates. The tree was visualized by using the TreeView program (version 1.6.6) (41). Maximum-parsimony (MP) and maximum-likelihood (ML) methods were used to confirm the phylogenetic placement of the aligned sequences. MP analysis was performed by using the MEGA4 software package (47). The ML tree was calculated with the SEQBOOT and DNAML programs in the PHYLIP package (13). Fluorometric DNA-DNA hybridization in microdilution wells was performed as described previously (11). PCR-based random amplified polymorphic DNA (RAPD) fingerprinting was performed with the primers 5′-CCGCAGCC AA-3′ and 5′-AACGCGCAAC-3′ (2). Each 50-μl reaction mixture contained each deoxynucleoside triphosphate at a concentration of 200 μM, 80 pmol of primer, 3 mM MgCl$_2$, 1× final concentration of *Taq* polymerase reaction buffer, 2 U of TaKaRa ExTaq polymerase Hot Start version (Takara Bio, Otsu, Japan), and genomic DNA (50 to 100 ng). PCR conditions were as follows: (i) 94°C for 2 min; (ii) 5 cycles consisting of 94°C for 30 s, 36°C for 60 s, and 72°C for 90 s; (iii) 29 cycles consisting of 94°C for 20 s, 36°C for 30 s, and 72°C for 90 s; (iv) 72°C for 3 min; and (v) hold at 4°C. The PCR products were subjected to agarose gel electrophoresis.

Substrate availability test. Substrate availability for strains YIT 12067^T and YIT 12068 was examined by using prereduced, anaerobically sterilized peptone-yeast extract (PY) (18) broth tubes with the addition of different carbohydrates and short-chain fatty acids (SCFA). The carbohydrates and SCFA tested were esculin, L-arabinose, D-cellobiose, glycerol, lactose, D-maltose, D-mannitol, D-mannose, D-raffinose, rhamnose, salicin, D-sorbitol, sucrose, D-trehalose, D-xylose, glucose, xylan, acetate, citrate, lactate, pyruvate, and succinate. Each carbohydrate was added at 0.5% or 1.0% (wt/wt), and the SCFA were supplemented to a final concentration of 20 mM. Growth was determined by observing visible turbidity.

Other phenotypic characterization. Strains YIT 12067^T and YIT 12068 underwent further phenotypic characterization. *Phascolarctobacterium faecium* ACM3679^T, purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, served as a reference strain throughout. Growth in anaerobic and microaerophilic conditions was tested on PY agar plates supplemented with 80 mM sodium succinate (PYS). Addition of 80 mM succinate to PY medium was found to be better than 20 mM on the basis of a growth test using PY medium supplemented with 10,

20, 80, or 160 mM succinate. Anaerobic and microaerophilic atmospheres were created by using Anaeropack (Mitsubishi Gas Chemical, Tokyo, Japan) and CampyPak Plus microaerophilic system envelopes with a palladium catalyst (BBL, Sparks, MD), respectively. The morphology of the cells after a 2-day culture in the PYS medium was observed by using phase-contrast microscopy (BX51 system; Olympus, Tokyo, Japan) and scanning electron microscopy (SEM) (S-3400 system; Hitachi High-Technologies, Tokyo, Japan). For sample preparation for SEM, cells were placed on Sempore (JEOL Datum, Tokyo, Japan), fixed with glutaraldehyde and OsO$_4$, critical point dried, and coated with osmium plasma. Spore formation by isolates grown on PYS agar plates for 2, 4, and 9 days was examined by using the spore-staining method (44). Sensitivity to bile was determined by comparing rates of growth of the strains on modified GAM agar with and without 2% Bacto oxgall after a 5-day incubation. The biochemical characteristics of strains YIT 12067^T and YIT 12068 were determined by using the API 20A, API Rapid ID 32A, and APIZYM systems (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions, except that PYS agar plates were used for cell growth. All API tests were performed in duplicate. The end products from the isolates grown in prereduced PYS and PY medium supplemented with succinate were analyzed by using high-pressure liquid chromatography (HPLC), as described previously (4). Oxidase activity was examined by using oxidase test strips (Eiken Chemical Co., Ltd., Tokyo, Japan). Cellular fatty acid methyl esters (FAMEs) were obtained from lyophilized cells grown in PYS medium at 37°C for 2 days by saponification, methylation, and extraction, with minor modifications (24) of a method described previously (32). FAMEs were determined by using the MIDI system with MOORE5 5.00 of the MIS Standard Libraries. The DNA G+C contents were determined by hydrolyzing the DNA enzymatically and quantifying the nucleosides by using HPLC, as previously described (12).

Coculture experiments with succinate-producing bacteria. To investigate the potential cross-feeding of succinate to the novel isolates from the succinate producer *Paraprevotella xylaniphila* YIT 11841^T (34), we conducted coculture experiments in test tubes containing 3 ml of PY medium supplemented with 1% birchwood xylan (PYX). Strains YIT 12067^T and YIT 12068 were grown at 37°C in PYS medium, and *P. xylaniphila* YIT 11841^T was grown at 37°C in PYX medium. After incubation for 2 days under anaerobic conditions, strains YIT 12067^T and YIT 12068 were cocultured with *P. xylaniphila* YIT 11841^T in PYX medium. The inoculum size was 2% (vol/vol). The inoculated tubes were incubated at 37°C for 72 h. Growth was followed by plating the diluted inocula on the appropriate selective agar plates. Strains YIT 12067^T and YIT 12068 were enumerated on PYS agar plates and *P. xylaniphila* YIT 11841^T was enumerated on PY agar plates with 1% glucose and 25 μg/ml streptomycin. The plates were then incubated for 48 h at 37°C under anaerobic conditions, as described above. Triplicate experiments were performed using the same inocula. The concentrations of SCFA (succinate, propionate, acetate, formate, lactate, butyrate, isobutylate, valerate, and isovalerate) were determined by using HPLC, as described above. The coculture experiments were performed in triplicate.

Quantification of YIT 12067^T-like bacteria in human feces by using real-time PCR. Fecal numbers of strain YIT 12067^T-like bacteria were quantified by using real-time PCR with 16S rRNA gene-targeted oligonucleotide primers. To design the species-specific primers, we obtained the sequences of the 16S rRNA genes of YIT 12067^T, YIT 12068, and other closely related bacteria from the Ribosomal Database Project (6) and aligned them by using the ClustalX program; specific regions for both strains YIT 12067^T and YIT 12068 were detected. Primers were designed by using the NCBI Primer-BLAST program (http://www.ncbi.nlm.nih .gov/tools/primer-blast/). The primers, PhasF (5′-AGCAATCTCGCATG AGGATGCTGT-3′) and PhasR (5′-GCCGTGGCTTATTCGTTTACTA CCG-3′), provided a 341-bp amplicon. Primer specificity was verified by using the Primer-BLAST and ProbeCheck programs (http://131.130.66 .200/cgi-bin/probecheck/content.pl?id=home) (28). Specificity was further confirmed by use of DNA extracted from bacterial cells at values
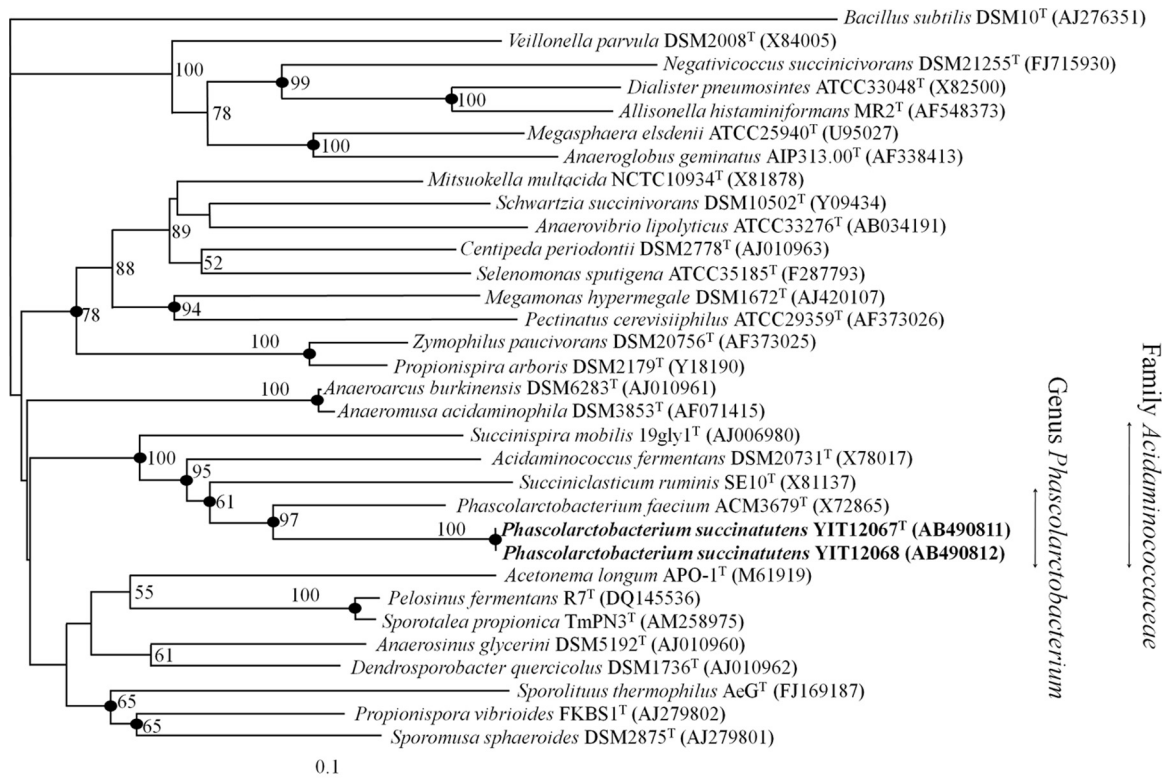
FIG 1 Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic position of strains YIT 12067^T and YIT 12068 among related taxa. Bootstrap values (>50%) are given at the branch nodes. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony and maximum-likelihood algorithms. Bar, 0.1 substitutions per nucleotide position.

corresponding to 5 × 10^6 cells of the YIT 12067^T strain and 96 different species, comprising five phyla: *Actinobacteria* (including 7 species of *Bifidobacterium*, 3 each of *Atopobium* and *Collinsella*, and 1 of *Eggerthella*), *Bacteroidetes* (including 8 species of *Bacteroides* and 3 of *Prevotella*), *Firmicutes* (including 18 species of *Clostridium*, 10 of *Lactobacillus*, 8 of *Eubacterium*, 5 of *Ruminococcus*, 4 of *Roseburia*, 3 of *Veillonella*, 2 of *Peptostreptococcus*, and 1 each of *Anaerostipes*, *Coprococcus*, *Faecalibacterium*, *Megasphaera*, *Phascolarctobacterium*, and *Pseudobutyrivibrio*), *Fusobacteria* (including 5 species of *Fusobacterium*), and *Proteobacteria* (including 2 species each of *Desulfovibrio*, *Enterobacter*, and *Enterococcus* and 1 each of *Citrobacter*, *Escherichia*, and *Proteus*). Bacterial cell counts were determined by using the 4′,6-diamidino-2-phenylindole (DAPI) staining method (20). Real-time PCR using each DNA extract of the 96 reference strains revealed that no amplified signal was detected under the conditions described below.

Real-time PCR amplification and detection were performed in a 10-μl volume with the ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA) and SYBR Premix EX *Taq* II (Takara Bio, Otsu, Japan), according to the manufacturers' instructions, with a final concentration of 0.4 μM for each primer. The amplification program consisted of 1 cycle at 95°C for 30 s, then 40 cycles at 95°C for 5 s and 60°C for 34 s. The fluorescent product was detected in the last step of each cycle. To distinguish the target PCR product from nontarget product, melting curve analysis was performed after amplification using the default settings of the ABI software from 60°C to 95°C.

A standard curve was created by using serial 10-fold dilutions of strain YIT 12067^T pure culture DNA corresponding to 10^6 to 10^1 cells per reaction, as determined microscopically by using the DAPI staining method. The bacterial concentration of each sample was calculated by comparing the threshold cycle (C_T) values obtained from the standard curve. All samples were analyzed in triplicate. A linear relationship between the cell

counts and the C_T values ($r^2 = 0.9975$) was observed when the number of cells per reaction mixture was between 10^6 and 10^1.

Thirty-three healthy volunteers (30 males and 3 females; ages, 20 to 64 years [average, 39.4 ± 10.3 years]) provided fresh fecal samples. DNA extraction from fecal samples was performed according to the method using glass beads and phenol described previously (30).

Nucleotide sequence accession numbers. Sequences for the 16S rRNA gene of strains YIT 12067^T and YIT 12068 have been deposited in the GenBank/EMBL/DDBJ database under accession no. AB490811 and AB490812, respectively. Accession numbers for sequences used as references are indicated in the phylogenetic tree.

RESULTS

Phylogenetic analysis of strains YIT 12067^T and YIT 12068. We assessed 1,610 bp and 1,611 bp of the 16S rRNA genes of strains YIT 12067^T and YIT 12068, respectively, and found that they had 99.9% sequence identity. Although the isolates could be discriminated by RAPD fingerprinting (data not shown), they had 93% DNA-DNA relatedness. Database searches revealed that the strain with the highest 16S rRNA gene sequence relatedness to strains YIT 12067^T and YIT 12068 was *Phascolarctobacterium faecium* ACM 3679^T, with 91.4% and 91.5% identity, respectively. The 16S rRNA gene sequences of strains YIT 12067^T and YIT 12068 and those of related species of the family *Acidaminococcaceae* in the order *Selenomonadales* were aligned, and a phylogenetic tree was constructed by using *Bacillus subtilis* as an outgroup (Fig. 1). The phylogenetic tree shows that the new isolates belonged to the family *Acidaminococcaceae*. The positions of the strains were phylogenetically distinct, and the specific lineages were robust and sup-
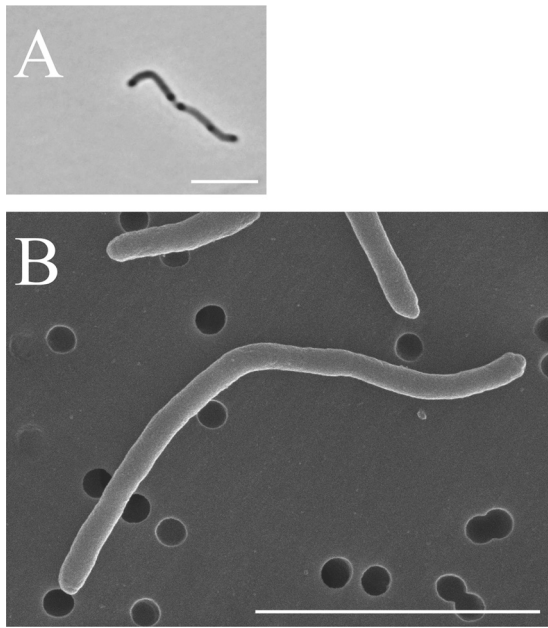
FIG 2 Phase-contrast (A) and scanning electron (B) micrographs of strain YIT 12067^T grown in PYS broth for 2 days. Bars, 5 μm.

($>$10%) cellular fatty acids of strains YIT 12067^T and YIT 12068 were C$_{14:0}$ dimethyl acetal and summed feature 4, comprising C$_{15:2}$ and/or C$_{15:1}\omega$8c and/or an unknown fatty acid of ECL 14.762. The DNA G+C contents of strains YIT 12067^T and YIT 12068 and *P. faecium* ACM3679^T, as determined by HPLC, were 46.5, 47.3, and 45.4 mol%, respectively. The value for strain YIT 12067^T was also determined by genome sequence analysis and found to be 47 mol%. In the phenotypic analyses with the API systems (API 20A, API Rapid ID32A, and API ZYM), the novel isolates and *P. faecium* ACM3679^T showed no positive reactions.

Effect of coculture with a succinate producer. Succinate is not a major fermentation product in human feces (51); however, it is produced by many of the microorganisms that colonize the human GI tract, such as members of the *Bacteroides* and *Prevotella* genera (45). Therefore, to create a nutritional situation analogous to what takes place *in vivo*, coculture experiments with the succinate producer *P. xylaniphila* YIT 11841^T were performed. *Paraprevotella xylaniphila* YIT 11841^T can use xylan as a growth substrate, producing mainly succinate (34), whereas strains YIT 12067^T and YIT 12068 do not ferment xylan. Xylan is one of the most abundant components of plant fibers, which are important sources of substrates for the resident gut microorganisms in humans. After 72 h of coculture with *P. xylaniphila* YIT 11841^T, the cell numbers of the strains YIT 12067^T and YIT 12068 became

ported by high bootstrap values. These results were supported by those obtained with the MP and ML methods (data not shown).

Carbohydrate and SCFA utilization. Dietary carbohydrates that escape digestion in the upper gastrointestinal tract are the most important energy sources for microbial growth in the large intestine (7). Carbohydrate utilization tests using PY broth tubes showed that strains YIT 12067^T and YIT 12068 did not utilize any carbohydrate tested. We then tested whether these strains used SCFA, which are major end products of the bacterial breakdown of carbohydrates and also serve as important energy sources for GI tract microbiota (51). PY broth tube tests indicated that strains YIT 12067^T and YIT 12068 grew well only in PY medium supplemented with succinate. HPLC analysis showed that succinate was quantitatively converted to propionate. These results indicate that succinate decarboxylation stimulated the growth of strains YIT 12067^T and YIT 12068. Addition of succinate to modified GAM or PY agar plates at a final concentration of 80 mM resulted in the formation of larger colonies (up to 1.2 mm in diameter) than the pinpoint colonies on these media without succinate.

Phenotypic characteristics. Strains YIT 12067^T and YIT 12068 grew under strict anaerobic conditions, but not in aerobic or microaerobic atmospheres. The cells of both strains were Gram negative, nonmotile, non-spore-forming, and long rod shaped. The cells had several darker regions, like nodes, after 2 days of incubation in PYS medium (Fig. 2A). Strain YIT 12067^T cells were 0.4 to 0.5 μm wide and 2.4 to 22.3 μm long (Fig. 2B); strain YIT 12068 cells were nearly identical in size to those of strain YIT 12067^T. Both strains were oxidase and catalase negative. Growth of the isolates was completely suppressed by the addition of bile to the PYS agar. In contrast, *P. faecium* formed colonies on PYS agar supplemented with bile, although there were fewer colonies than on normal PYS agar. The cellular fatty acid compositions of strains YIT 12067^T and YIT 12068 and that of *P. faecium* ACM3679^T as the closest related reference strain are shown in Table 1. The major

TABLE 1 Cellular fatty acid compositions of strains YIT 12067^T and YIT 12068 and *Phascolarctobacterium faecium* ACM3679^T

Fatty acid	% total fatty acids^a for:		
	YIT 12067^T	YIT 12068	*P. faecium*
Saturated straight chain			
C$_{11:0}$	8.23	8.38	5.11
C$_{12:0}$	1.54	4.76	5.47
C$_{13:0}$	9.92	7.03	2.18
C$_{14:0}$			1.47
C$_{15:0}$	8.99	7.54	14.21
C$_{16:0}$	2.64	2.50	5.23
C$_{18:0}$	1.12	1.26	
Unsaturated straight chain			
C$_{16:1}\omega$9c		3.12	12.90
C$_{16:1}\omega$7c	5.31	3.83	5.09
C$_{18:1}\omega$9c	4.21	3.98	4.51
C$_{18:2}\omega$6,9c	1.42	1.36	1.46
Hydroxy acid			
C$_{15:0}$ 3-OH	1.92	1.86	4.05
Dimethyl acetal			
C$_{14:0}$ DMA	22.43	23.99	15.53
C$_{16:0}$ DMA	1.33	2.02	
C$_{16:1}\omega$7c DMA	2.77	3.20	1.07
Summed features^b			
4	18.27	11.89	4.22
5	3.53	5.28	8.60
6		1.18	2.21
7			1.75

^{a} Only those fatty acids that make up $>$1% of the total are shown.
^{b} Summed feature compositions are as follows: 4, C$_{15:2}$ and/or C$_{15:1}\omega$8c and/or an unknown fatty acid of ECL 14.762; 5, C$_{15:0}$ DMA and/or C$_{14:0}$ 3-OH; 6, anteiso-C$_{15:0}$ 3-OH and/or C$_{16:1}\omega$9cDMA; 7, C$_{17:1}\omega$7c and/or an unknown fatty acid of ECL 16.760.
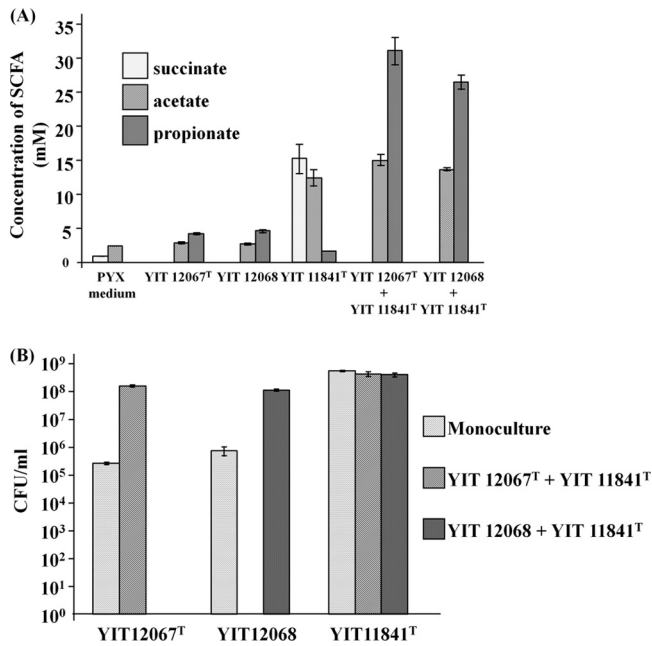
FIG 3 Coculture of the succinate-producing bacterium *P. xylaniphila* YIT 11841^T and strain YIT 12067^T or YIT 12068 after 72 h of growth in PY medium supplemented with 1% birchwood xylan. (A) Concentrations of succinate, acetate, and propionate, as determined by using HPLC. Triplicate experiments were performed. (B) CFU of strains YIT 12067^T and YIT 12068 and *P. xylaniphila* YIT 11841^T. Diluted inocula were plated on the appropriate selective agar plates. Strains YIT 12067^T and YIT 12068 were enumerated on PYS agar plates, and *P. xylaniphila* YIT 11841^T was enumerated on PY agar plates with 1% glucose and 25 μg/ml of streptomycin. Plates were incubated for 48 h at 37°C under anaerobic conditions. Triplicate experiments were performed. The initial cell numbers of strains YIT 12067^T and 12068 and of *P. xylaniphila* YIT 11841^T were 3.85×10^3, 8.44×10^4, and 7.67×10^5 CFU ml^{-1}, respectively.

approximately 2 to 3 orders of magnitude higher than those of the monoculture; succinate became undetectable, and propionate was formed (Fig. 3). Growth of *P. xylaniphila* YIT 11841^T was not affected by the presence of strains YIT 12067^T and YIT 12068.

Database search for YIT 12067^T-like uncultured bacterial clones. Recently improved sequencing technology has led to the deposition of a large number of uncultured bacterial clones in the GenBank/EMBL/DDBJ public databases. To obtain a broader estimate of the redundancy of these isolates, we searched for uncultured bacterial clones that were closely related to the sequence of strain YIT 12067^T. With 98.7% identity as the threshold (this is the % identity proposed for distinguishing species 46), we found 210 published uncultured bacterial clones by using the FASTA program (Table 2). These clones were isolated from fecal samples from humans and other mammals, especially primates. Similar sequences were also detected from the feces of other mammals, namely, rats, beef cattle, and swine. These results indicate that YIT 12067^T-like bacteria are broadly distributed in the intestines of humans and other mammals, especially primates.

Quantification of strain YIT 12067^T-like bacteria in human feces. To determine the actual prevalence and proportion of strain YIT 12067^T-like bacteria in human feces, we performed real-time PCR with species-specific primers. Application of real-time PCR to fecal samples from 33 volunteers showed that strain YIT 12067^T-like bacteria were detected in 7 of the 33 fecal samples (21%), at an average level of 3.34×10^8 cells/g of feces (Table 3).

TABLE 2 Sources of uncultured bacterial clones that showed ≥98.7% 16S rRNA gene sequence identity to that of strain YIT 12067^T listed in the EMBL database

Source	Diet	No. of sequences retrieved[a]	Reference(s) of study(ies) in which 16S rRNA gene sequences were detected
Primates			
Human	Omnivore	132	10, 14, 15, 23, 26, 27, 29, 31, 52
Chimpanzee	Omnivore	12	26
Bonobo	Omnivore	4	26
East Angolan colobus	Herbivore	4	26
Francois' langur	Herbivore	4	26
Mongoose lemur	Omnivore	4	26
Eastern black and white colobus	Herbivore	2	26
Black lemur	Omnivore	1	26
Sumatran orangutan	Herbivore	1	26
Other mammals			
Rat	Omnivore	36	1, 3
Beef cattle	Herbivore	9	9
Swine	Omnivore	1	25

[a] Retrieved from published data.

The detection ratio and concentration of strain YIT 12067^T-like bacteria were similar to those of members of subdominant species of human GI tract microbiota, such as *Bifidobacterium bifidum* (detection ratio, 28%; concentration, 2.00×10^8 cells/g) and *Bifidobacterium breve* (17%, 2.00×10^7 cells/g) (30), indicating that strain YIT 12067^T-like bacteria are also subdominant members of the human GI microbiota.

DISCUSSION

The use of molecularly based methods has revealed that many of the phylotypes found in the human GI tract are detected only through the use of culture-independent methods (42). In this uncultured phylogenetic group, however, some species were isolated from other mammals or environmental samples. The genus *Phascolarctobacterium* had consisted of only one species, *P. faecium*, isolated from koala feces (8). Although uncultured clones

TABLE 3 Cell counts of strain YIT 12067^T-like bacteria in fecal samples from Japanese volunteers determined by using real-time PCR

	Count (cells/g feces) of:		
Subject[a]	Strain YIT 12067^T-like bacteria	Total cells[b]	% relative to the total no. of cells
E036	3.70×10^8	7.94×10^{10}	0.45
E038	2.20×10^7	1.26×10^{11}	0.02
E041	2.44×10^7	2.51×10^{10}	0.10
E046	1.19×10^7	1.58×10^{11}	0.01
E049	1.28×10^9	2.51×10^{11}	0.53
E059	4.74×10^8	1.26×10^{12}	0.41
E061	1.54×10^8	1.00×10^{11}	0.14
Mean	3.34×10^8	2.86×10^{11}	0.24

[a] Subjects from which strain YIT 12067^T-like bacteria were detected, among 33 volunteers.
[b] Total number of bacteria as determined by DAPI count.

that were closely related to *P. faecium* were frequently detected in samples from the human GI tract, there had been no isolates belonging to the genus *Phascolarctobacterium* from the human GI tract.

On the basis of the findings described here, we propose that strains YIT 12067^T and YIT 12068 belong to the genus *Phascolarctobacterium* within the family *Acidaminococcaceae*; these strains are strictly anaerobic, nonmotile, non-spore-forming, Gram negative, and asaccharolytic. They utilize succinate and produce propionate. In the API systems, they were unreactive. They had cellular fatty acid compositions and DNA G+C contents similar to those of *P. faecium* ACM3679^T. However, these novel isolates could be differentiated from *P. faecium* ACM3679^T on the basis of bile sensitivity and 16S rRNA gene sequence identity (91.4%). Although the isolates and *P. faecium* shared low sequence identity, these two species had quite similar phenotypic characteristics. Accordingly, we propose that strains YIT 12067^T and YIT 12068 represent a new species of the genus *Phascolarctobacterium*. To our knowledge, this is the first report of the isolation of members of the genus *Phascolarctobacterium* from the human GI microbiota.

Isolation of members of the genus *Phascolarctobacterium* might have been difficult because of the very narrow range of energy sources available. In our experiments, strains YIT 12067^T and YIT 12068 used only succinate as an energy source, as has been shown for *P. faecium* ACM3679^T. We isolated these novel strains by using modified GAM-based medium to which succinate had not been added. HPLC analysis revealed that the modified GAM contained a small quantity of succinate (0.7 mM), which may have allowed these strains to form very small pinpoint colonies. The isolation of bacteria highly specific for using succinate might have been caused by selective pressure that suppressed the growth of other bacterial strains forming large colonies on the basal agar. To this end, we have isolated 17 new species from human GI tract microbiota by using this strategy (33–40, 43, 56); the use of nutrient-rich basal agar plates supplemented with various selective pressures thus appears to be effective for isolating various uncultured species from the GI microbiota.

In the substrate utility test, strains YIT 12067^T and YIT 12068 required succinate as an energy source. Although the free energy change of succinate decarboxylation is small compared with that of carbohydrates, amino acids, and other major SCFA (49), succinate decarboxylation to propionate is coupled with ATP synthesis (21), and the ability to decarboxylate succinate is found mainly among members of *Clostridium* cluster IX (e.g., *P. faecium* [8], *Succiniclasticum ruminis* [54], and *Schwartzia succinivorans* [55] as exclusively succinate utilizers and *Succinispira mobilis* [22] as a succinate and amino acid utilizer). Succinate is seldom detected as a major fermentation product in human feces (51), yet succinate-producing saccharolytic bacteria are abundant among the GI microbiota. Therefore, the specific fermentation of succinate may result in not only the acquisition of a constant energy source but also the ability to coexist with bacteria that use other high-energy-yielding substrates, such as resistant carbohydrates. In fact, our coculture experiments showed that the growth of strains YIT 12067^T and YIT 12068 was stimulated by the presence of succinate-producing bacteria, as succinate was converted to propionate (Fig. 3A and B). It seems likely that, to adapt to the GI tract environment with its variety of bacterial species, strains YIT 12067^T and YIT 12068 might have evolved to specialize in the utilization of succinate produced by other microorganisms. Anal-

ysis of the EMBL database library (Table 2) and real-time PCR analysis (Table 3) support the concept that strain YIT 12067^T-like bacteria have adapted to the GI tract and are prevalent in humans and other mammals as subdominant members. Harmsen et al. (17) reported that the detection ratio for the *Phascolarctobacterium* group, including the genera *Succiniclasticum* and *Acidaminococcus*, was 81% (8 of 11 samples) at an average level of 9.0×10^8 cells/g feces (dry weight). Thus, other members of this group with characteristics similar to those of strains YIT 12067^T and YIT 12068 are also likely to be persistent in the human GI tract.

Interestingly, the database search for uncultured bacterial clones with high levels of similarity in their 16S rRNA gene sequences to those of the novel isolates also revealed many clones in the feces of several kinds of primates and other mammals (Table 2). These host organisms were omnivorous or herbivorous. Ley et al. (26), through a network-based analysis of bacterial diversity from fecal samples of humans and 59 other mammalian species, suggested that both diet and phylogeny of the host influence GI bacterial diversity. Presumably, strain YIT 12067^T-like bacteria might have undergone widespread distribution in the GI tracts of herbivorous or omnivorous mammals—mainly primates, including humans.

Strain YIT 12067^T has been selected for inclusion in the catalog of reference genomes by the human microbiome project (53), and a high-quality draft sequence has been completed. The assembled and annotated genomic sequences of this bacterium have been submitted to the GenBank/EMBL/DDBJ databases (http://www.ncbi.nlm.nih.gov/genome/2893). These sequences contain 2,203 predicted genes in 2,122,261 bp, as read by the Genome Sequencing Center, Washington University School of Medicine. Further genomic research, such as exploring the genes involved in energy metabolism or comparing the genomic structure with those of other members of the human GI tract, may provide additional insights into the role of this succinate-specific utilizer in the GI tract or into its evolutionary strategy of adaptation for competition and survival in this complex microbial ecosystem.

The phylogenetic and phenotypic analysis indicated that strains YIT 12067^T and YIT 12068 belonged to the same species. Although these isolates could be differentiated from their nearest relative, *P. faecium*, in terms of 16S rRNA gene sequence similarity and bile resistance, they shared similar phenotypic characteristics. We therefore propose that strains YIT 12067^T and YIT 12068 represent a novel species of the genus *Phascolarctobacterium*, for which the name *Phascolarctobacterium succinatutens* sp. nov. is proposed.

Description of *Phascolarctobacterium succinatutens* sp. nov. *Phascolarctobacterium succinatutens* (suc.ci.nat.u′tens; N.L. n. *succinas -atis*, succinate; L. v. *utor*, make use of; N.L. part. adj. *sucinatutens*, using succinate). Cells are Gram negative, nonmotile, non-spore-forming, and long rod shaped. They are strictly anaerobic. Cells are 0.4 to 0.5 μm wide and 2.4 to 22.3 μm long. Colonies after 2 days of growth at 37°C on PY agar supplemented with 80 mM succinate are 0.8 to 1.2 mm in diameter, circular, and beige. Oxidase, catalase, and bile resistance are negative. API 20A tests are negative for acid production from L-arabinose, D-cellobiose, glucose, glycerol, lactose, maltose, D-mannitol, D-mannose, D-melezitose, D-raffinose, L-rhamnose, salicin, sucrose, D-sorbitol, D-trehalose, and D-xylose. API Rapid ID32A and API ZYM results are negative for nitrate reduction, urease, and

hydrolysis of esculin and gelatin. Indole is not produced. Negative reactions are obtained for alanine, arginine, cystine, glutamyl glutamic acid, glycine, histidine, leucine, phenylalanine, proline, pyroglutamic acid, serine, tyrosine, valine, and leucyl glycine arylamidase and for naphthol-AS-BI-phosphohydrolase, acid phosphatase, *N*-acetyl-β-glucosaminidase, alkaline phosphatase, arginine dihydrolase, α-arabinosidase, α-fucosidase, α- and β-galactosidase, 6-phospho-β-galactosidase, α- and β-glucosidase, β-glucuronidase, α-mannosidase, chymotrypsin, esterase (C_4), esterase lipase (C_8), glutamic acid decarboxylase, lipase (C_{14}), and trypsin. The major cellular fatty acids are $C_{14:0}$ dimethyl acetal and summed feature 4, comprising $C_{15:2}$ and/or $C_{15:1}\omega$8c and/or an unknown fatty acid of ECL 14.762. The DNA G+C content is 46.5% to 47.3%. The type strain, YIT 12067^T (DSM 22533^T or JCM 16074^T), was isolated from human feces. Strain YIT 12068 (DSM 22521 or JCM 16075) is also assigned to this species.

ACKNOWLEDGMENTS

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