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ORIGINAL PAPER

Characterization of an *O*-desmethylangolensin-producing bacterium isolated from human feces

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Abstract A bacterium that converted daidzein to *O*-desmethylangolensin was isolated from the feces of healthy humans. It was an obligately anaerobic, nonsporeforming, nonmotile and Gram-positive rod. The isolate used glucose, sucrose, raffinose, maltose, and fructose as carbon sources. It did not hydrolyze gelatin, esculin, or starch. The strain was urease, acid phosphatase, and arginine dihydrolase positive. It was catalase, oxidase, H_2S, and indole negative. The major products of glucose fermentation were butyrate and lactate. Its mol% G+C was 51.2. The major cellular fatty acids were $C_{16:0}$ DMA, $C_{16:0}$, and $C_{16:0}$ aldehyde. The structural type of cell wall peptidoglycan was suggested to be A1γ. The isolate was susceptible to β-lactam, cefem, and macrolide antibiotics and resistant to aminoglycoside and quinolone antibiotics. The bacterium was related to *Eubacterium ramulus* ATCC29099^T, *Eubacterium rectale* ATCC33656^T, and species of the genus *Roseburia*, but the highest 16S rRNA gene similarity to these described species was only 94.4%, consistent with its being classified as a novel genus. Based on the above, the isolate, named strain SY8519, was identified as belonging to a novel genus in the *Clostridium* rRNA cluster XIVa.

Keywords *O*-desmethylangolensin · Strain SY8519 · Isolate from human feces · Taxonomical property · *Clostridium* rRNA cluster XIVa · Antimicrobial susceptibility

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Introduction

Soybeans, which are a popular food material in Asia, contain high levels of isoflavonoids. The daidzein metabolites that are derived by intestinal bacteria from isoflavonoids are considered to have beneficial effects on human health. In particular, equol is known to exhibit greater estrogenic (Brienholt and Larsen 1998; Kuiper et al. 1998) and antioxidative behavior (Setchell et al. 2002) than daidzein. Equol has been shown to be beneficial in the prevention of breast cancer (Duncan et al. 2000; Ingram et al. 1997), prostate cancer (Akaza et al. 2002; Ozasa et al. 2004), osteoporosis (Lydeking-Olsen et al. 2002), and menopausal symptoms (Ueno et al. 2002). Recently, several equol-producing bacteria belonging to the family *Coriobacteriaceae* (Maruo et al. 2008; Minamida et al. 2006; Wang et al. 2005; Yokoyama and Suzuki 2008) were isolated from the mammalian intestine. These bacteria were assigned to new genera, including *Adlercreutzia equolifaciens* (Maruo et al. 2008) and *Asaccharobacter celatus* (Minamida et al. 2008).

O-desmethylangolensin (*O*-DMA) is a major metabolite of daidzein from intestinal bacteria and is produced in about 80–90% of humans (Arai et al. 2000; Frankenfeld et al. 2004a; Kelly et al. 1995). Several studies have reported the biochemical activity of *O*-DMA, including its genotoxic activity (Schmitt et al. 2003), stimulatory effect on the growth of MCF-7 cells (Kinjo et al. 2004), and inhibition of cancer cell proliferation (Schmitt et al. 2001). It has also been reported to correlate with lowering plasma triglyceride levels (Howes et al. 2000), elevating mammographic density (Frankenfeld et al. 2004a), and elevating follicle-stimulating hormone levels (Frankenfeld et al. 2004b) in epidemiological case studies. Nevertheless, the physiological roles of *O*-DMA are comparatively less well known than that of equol.

Little is known about *O*-DMA-producing bacteria. Only two bacteria, *Eubacterium ramulus* (Schoefer et al. 2002) and *Clostridium* sp. HGH 136 (Hur et al. 2002), have been described as producing *O*-DMA from daidzein. In our previous study, we screened for equol-producing bacterium (Yokoyama and Suzuki 2008), and a novel strain producing *O*-DMA was isolated from the feces of healthy humans. In the present study, we characterize the taxonomical properties of this strain and determine whether it constitutes a novel genus.

Materials and methods

Organic chemical analysis

Daidzein and dihydrodaidzein were purchased from LKT Laboratories (West St. Paul, NM, USA) and Toronto Research Chemicals (North York, ON, Canada), respectively. *O*-DMA was synthesized in-house (manuscript under preparation). A silica gel 60 F_{254} thin layer chromatography (TLC) plate was obtained from Merck KGaA (Darmstadt, Germany). TLC analysis for daidzein and its metabolite, without β-glucuronidase treatment, has been described elsewhere (Yokoyama and Kuzuguchi 2007).

A test culture medium (500 ml scale) containing 50 μM daidzein (see Culture conditions) was extracted with ether, and the metabolites were separated by TLC using Merck precoated glass plates (silica gel PF_{254}; 1.0 mm thick, Merck KGaA). Metabolites were then eluted from the scraped spots using a solvent mixture of *n*-hexane/EtOAc (1:1). The ^{1}H NMR spectra were measured by a Bruker ARX400 NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). The compound was dissolved in deuterated acetone, and the chemical shifts were reported as δ-values using tetramethylsilane as the internal standard.

Culture conditions

BL agar and GAM broth (Nissui Pharmaceuticals, Tokyo, Japan) were used for culture media. The GAM culture broth was supplemented with daidzein (final concentration, 50 μM) and used as the test medium to investigate daidzein metabolism. Ten microliters of bacterial culture (OD_{600} = 0.15) was inoculated into a 24-well Multiwell™ plate (Becton–Dickinson) containing 1 ml of the test medium. The culture plates were incubated at 37°C for 3 days using the AnaeroPack®/Kenki culture system (disposable O_2 absorbing and CO_2 generating agent, Mitsubishi Gas Chemical, Tokyo, Japan).

Bacterial isolation

Fecal samples from healthy volunteers were obtained from the authors' relatives. Two grams of freshly voided fecal sample was homogenized and suspended in 5 ml of growth medium. Serial dilutions in sterile saline were spread on GAM agar plates and incubated at 37°C for 72 h. Separated colonies were transferred to a 96-well Multiwell™ plates (Becton–Dickinson) containing 200 μl of the medium supplemented with daidzein and incubated under anaerobic conditions. After 72 h of incubation at 37°C, samples were taken for TLC analysis.

Morphological and biochemical analysis

The isolated bacterium was grown on BL agar plates with and without 2% bile salt to observe its growth and colony morphology. Gram-staining was performed using a Favor G Nissui kit (Nissui Pharmaceuticals), and the cells were observed by light microscopy (Olympus model BX50F4, Tokyo, Japan). The Gram classification was further estimated by the KOH reaction (Ryu 1940). The biochemical features of the isolate were evaluated using an API system 20A, API ZYM, and API Rapid ID32A kit (bioMérieux, Lyon, France). To investigate substrate utilization, some supplementary carbohydrates were added to make a final concentration of 0.5% in the GAM medium without dextrose (Nissui Pharmaceuticals). Cellular fatty acid methyl esters were prepared from the cells grown on the PYG agar plate at 37°C for 48 h according to the operating manual (version 6), and the profiles were examined using the Sherlock® microbial identification system (version 5.0) (MIDI Inc., Newark, DE, USA). A cell wall peptidoglycan was prepared and hydrolyzed by the method as described by Kawamoto et al. (1981), and the amino acid composition was analyzed with the ACQUITY UPLC system (Nihon Waters, Tokyo, Japan).

Genetic analysis

Genomic DNA was extracted from the isolate using a Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA). The following oligonucleotides were synthesized as primers for amplification of the bacterial 16S rRNA gene: 9F, 5′-GAG TTT GAT CCT GGC TCA G-3′; and 1510R, 5′-GGC TAC CTT GTT ACG A-3′. Sequencing of the 16S rDNA fragments was performed using the ABI PRISM 3100 Genetic Analyzer System (Applied Biosystems, Foster City, CA, USA). The homology search of 16S rRNA gene sequence was performed by the BLAST program (Altschul et al. 1997) at the Ribosomal Database Project (RDP, http://rdp.cme.msu.edu/). A phylogenetic tree was constructed using the neighbor-joining method using the CLUSTAL W program (Thompson et al. 1994) and MEGA (ver 3.1) software (Kumar et al. 2004).

Analysis of fermentation products

For quantitative determination of the fermentation products, the cells were cultured in GAM broth for 5 days, and fermentation products were examined as described by Richardson et al. (1989). Samples were analyzed by gas chromatography (model GC-2014; Shimadzu, Kyoto, Japan) equipped with an InertCap 1 capillary column (df = 0.25 μm, 4.6 mm ID × 250 mm, GL Science, Tokyo, Japan). The tests were repeated in triplicate.

Determination of the G+C content

DNA G+C content was examined using HPLC (model LC-10; Shimadzu, Kyoto, Japan) after enzymatic digestion of DNA to deoxyribonucleotides using nuclease P1 (Katayama-Fujimura et al. 1984). An equimolar mixture of four deoxyribonucleotides from a GC kit (Seikagaku Kogyo, Tokyo, Japan) was used as the quantitative standard.

Antimicrobial susceptibility test

The antimicrobial susceptibility of the isolated bacterium was estimated using an Optopanel MP (OP-1) kit from Kyokuto Pharmaceuticals (Tokyo, Japan). Briefly, 0.05 ml of bacterial preculture (OD_{600} = 0.154–0.176) was mixed with 12 ml of GAM broth, and 0.1 ml of the broth was applied to the OP-1 kit. The minimum inhibitory concentration (MIC) was determined after incubation for 3 days under anaerobic conditions. The test was performed in duplicate with freshly prepared media on separate occasions.

Results

Isolation of a bacterium producing unique metabolite from daidzein

In the previous report, a unique spot (R_f = 0.52) different from commercial isoflavones was found from one male volunteer's urine (Yokoyama and Kuzuguchi 2007). A bacterium that produces the metabolite was isolated from the volunteer's feces by the screening trial and designated strain SY8519.

Identification of the bacterial metabolite from daidzein

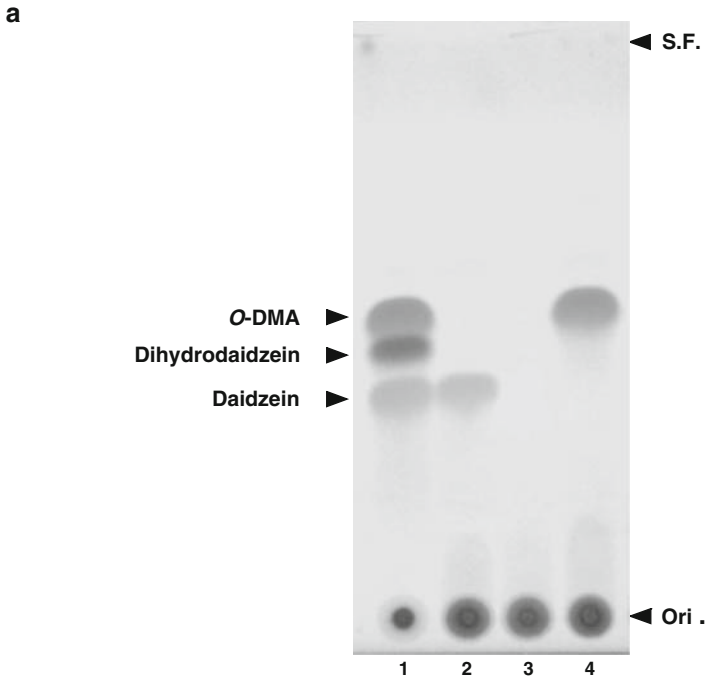
The bacterium completely converted 50 μM daidzein within 3 days of cultivation (Fig. 1, panel A, lane 4). In the TLC analysis, R_f value was corresponding to chemically synthesized *O*-DMA (R_f = 0.52). In order to confirm the fact that the compound in the spot was *O*-DMA, the TLC-purified spot was further analyzed with ^{1}H NMR spectroscopy analysis. The NMR data showed a characteristic duplet peak at δ 1.43 and quadruplet peak at δ 4.76 ppm, which corresponded to protons 3 and 2, respectively, of *O*-DMA (Fig. 1, panel B) and were consistent with the report of Salakka and Wähälä (1999). Based on these analyses, it is concluded that the isolated bacterium produced *O*-DMA from daidzein.

Morphological properties of strain SY8519

The morphological properties of strain SY8519 are as follows. The strain grew on BL agar plates as smooth surfaced, light yellow-colored colonies with a diameter of 2.0–3.0 mm after 2 days incubation at 37°C in under anaerobic environment. They grew at temperatures ranging from 30 to 50°C and at pH ranging from 5 to 9. The isolate did not grow in BL agar plates supplied with 2% bile salt. Light microscopic observations revealed that the cells were Gram-variable bacilli and existed either individually or in pairs (Fig. 2). Because the KOH reaction was negative, the strain was characterized as Gram-positive. On BL agar plates, the cells were about 0.8–1.0 μm wide and 1.5–2.0 μm long. The isolated bacterium has been deposited in the Gifu Type Culture Collection of Bacteria (GTC) under the number GTC 14498.

Genetic properties of strain SY8519

The 1,493 base pair sequences of the 16S rRNA gene of the isolated bacterium were sequenced, and the nucleotide sequence has been registered in the DDBJ/GenBank/EMBL under accession no. AB477431. The phylogenetic affiliation of the isolated strain was determined by comparing the



b

Proton	δ (ppm)	J (Hz)
H-3	1.43	6.8
H-2	4.76	6.8
H-3'	6.30	2.4
H-5'	6.37	2.4, 8.6
H-3", 5"	6.78	8.6
H-2", 6"	7.20	8.5
H-6'	7.89	8.8

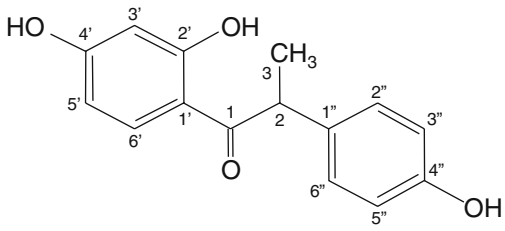


Fig. 1 Thin-layer chromatography (TLC) (**a**) and ^{1}H NMR spectrometric (**b**) analysis of the daidzein metabolite produced by strain SY8519. **a** The TLC plate was developed in toluene:acetone (2:1) and visualized under UV light at 312 nm. *Lane 1*, daidzein, dihydrodaidzein, and *O*-desmethylangolensin (*O*-DMA) standards (25 μg each); *lane 2*, culture medium containing daidzein but without bacterial incubation; *lane 3*, culture supernatant without daidzein; and *lane 4*, culture supernatant after incubation with daidzein. **b** The corresponding proton positions are numbered in the structural formula of *O*-DMA

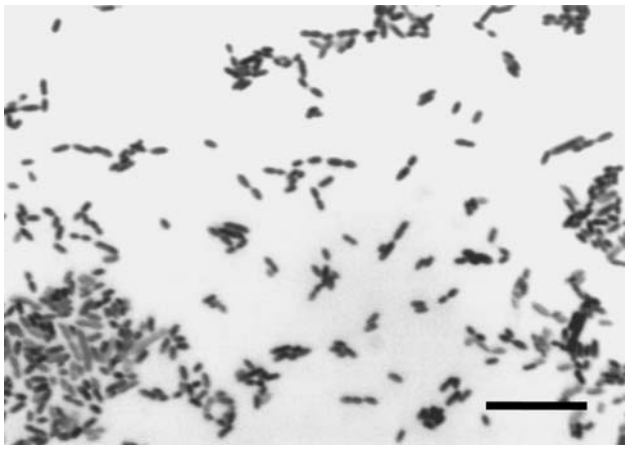


Fig. 2 Microscopic image of strain SY8519. After Gram-staining, the isolated bacteria were observed under a light microscope (magnification, ×2,000). The *bold line* in the *photograph* indicates the scale (10 μm in length)

16S rRNA gene sequence with RDP database. The isolate had 94.4 and 93.2% sequence similarity with *E. ramulus* ATCC29099^{T} (accession no. L34623) and *Eubacterium rectale* ATCC33656^{T} (L34627), respectively. The isolate also had 94.0, 93.7, 93.6, 93.2, and 92.9% sequence similarity with *Roseburia faecis* M72/1^{T} (AY305310), *Roseburia inulinivorans* A2-194^{T} (AJ270473), *Roseburia cecicola* ATCC33874^{T} (L14676), *Roseburia intestinalis* L1-82^{T} (AJ312385), and *Roseburia hominis* A2-183^{T} (AJ270482), respectively. The phylogenetic tree showed that the isolate, named strain SY8519, was a member of the *Clostridium* rRNA cluster XIVa (Fig. 3; Collins et al. 1994), as are the species named previously.

Biochemical characteristics of strain SY8519

The cells were nonsporulating, nonmotile obligate anaerobes. Nitrate reduction was positive, and H_2S production was negative. The strain utilized glucose, sucrose, raffinose, maltose, and fructose as carbon sources but did not utilize the other carbon sources tested, including mannitol, lactose, salicin, xylose, arabinose, cellobiose, mannose, melezitose, sorbitol, rhamnose, trehalose, ribose, galactose, melibiose, glycogen, inulin, inositol, amygdalin, glycerol, and starch.

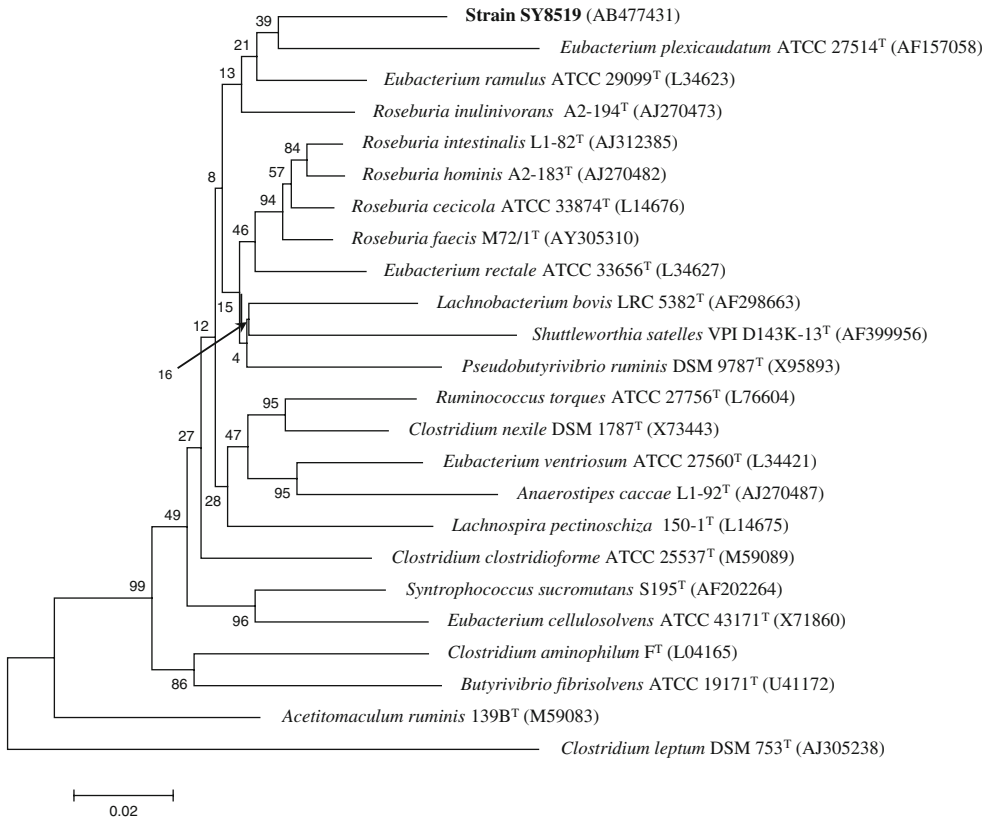


Fig. 3 Comparison of 16S rRNA gene sequence from strain SY8519 with other bacteria categorized in *Clostridium* rRNA cluster XIVa. The phylogenetic tree was constructed using the FigTree program. The superimposed *T* represents the type strain. Numbers at the branch points are bootstrap values based on 1,000 samples. *Parentheses* indicate the accession numbers. *Clostridium leptum* DSM 753^T was used as the out-group. The *scale bar* represents the genetic distance

Table 1 Antimicrobial susceptibility of strain SY8519

Antimicrobial agent	MIC[a] (ug/ml)
Benzylpenicillin	≦0.06
Ampicillin	≦0.25
Piperacillin	≦2
Ampicillin/Sulbactam	≦0.25/0.12
Cefotiam	0.5
Cefotaxime	≦0.5
Cefoperazone/Sulbactam	≦1/0.5
Ceftazidime	2
Cefditoren	0.25
Imipenem	0.5
Meropenem	≦0.12
Gentamicin	>32
Erythromycin	0.5
Clindamycin	≦0.12
Minocycline	2
Levofloxacin	>8

[a] Minimum inhibitory concentration

Furthermore, the strain did not hydrolyze gelatin, esculin, or starch. The strain was catalase, oxidase, and indole negative. The isolate produced acid phosphatase, urease, and arginine dihydrolase but did not produce other enzymes tested by API ZYM and API Rapid ID32A kits. The major end products of glucose fermentation were butyrate (20.3 ± 1.3 mM) and lactate (40.6 ± 1.9 mM) (n = 3). Succinic acid was also formed as a minor product (2.3 ± 0.1 mM). Its mol% G+C was 51.2. In the cellular fatty acid profile of the strain, the major fatty acids were $C_{16:0}$ DMA (43.4%), $C_{16:0}$ (38.4%), and $C_{16:0}$ aldehyde (9.3%). The profile showed no similarity with the MIDI database of MIS Standard Libraries (MOORE5). The cell wall peptidoglycan of the isolate contained glutamic acid, alanine, and *meso*-diaminopimelic acid at a molar ratio of 1.0:1.2:1.7. Therefore, the structural type was estimated to be A1γ, (L-Ala)-D-Glu-*m*-Dpm.

Antimicrobial susceptibility of strain SY8519

The antibiotic susceptibility patterns of strain SY8519 are listed in Table 1. The strain was susceptible to benzylpenicillin, ampicillin, piperacillin, cefotiam, cefotaxime, cefoperazone, ceftazidime, cefditoren, imipenem, and meropenem. It was resistant to gentamicin and levofloxacin and relatively resistant to erythromycin and minocycline.

Table 2 Characteristics that differentiate strain SY8519 from other closely related bacterial species

Characteristic	Strain SY8519	*E. plexicaudatum*	*E. ramulus*	*E. rectale*	Genus *Roseburia*	*P. ruminis*	*L. bovis*	*S. satelles*
Cell shape (rod)	Straight	Slightly curved	Straight	Slightly curved	Slightly curved	Curved	Straight	Slightly curved
Cell size (μm)	0.8–1.0 × 1.5–2.0	1.5–2.0 × 4.0–10.0	0.5–0.9 × 1.0–5.0	0.5 × 2.0–5.0	0.5 × 1.5–5.0	0.3–0.5 × 1.0–3.0	0.6–0.75 × 2.0–3.0	0.4–0.6 × 1.0–2.5
Gram reaction	+ (variable)	+	+	+	− (or variable)	+	+	+
Motility	−	+	−	+	+	+	±	−
Fermentation								
Arabinose	−	+	−	−	±	+	+	+
Cellobiose	−	+	+	+	+	+	+	+
Melibiose	−	+	+	+	+	ND	ND	+
Raffinose	−	+	+	+	+	ND	ND	+
Xylose	−	+	−	+	+	+	±	ND
End fermentation products	B, L, s	a, B, (s)	a, B, f, l	B, F, L	B, F or f, l or L	(a), B, F, L	(a, b), L	a, B, l
G+C content (mol%)	51.2	44.0	39.0	38.0	29.0–47.4	40.5	33.9	50–51
Habitat	Human feces	Mouse cecum	Human feces	Human feces	Human feces or cattle rumen and feces	Cow rumen	Cattle rumen and feces	Human oral cavity

Data for reference taxa were taken from *Eubacterium plexicaudatum* (Wilkins et al. 1974), *Eubacterium ramulus* (Moore et al. 1976), *Eubacterium rectale* (Duncan and Flint 2008; Nakazawa and Hoshino 1994), genus *Roseburia* (Duncan et al. 2006; Duncan et al. 2002; Stanton and Savage 1983), *Pseudobutyrivibrio ruminis* (Gylswyk et al. 1996), *Lachnobacterium bovis* (Whitford et al. 2001), and *Shuttleworthia satelles* (Downes et al. 2002)

+, Positive; −, negative

ND not determined, *F* formate, *A* acetate, *B* butyrate, *L* lactate, *S* succinate, and () trace amounts detected. Capital letters indicate major products

Discussion

Strain SY8519 was isolated and characterized as producing *O*-DMA from daidzein. The 16S rRNA gene sequence of the strain showed high similarity values with some genus *Eubacterium* (including *E.ramulus* or *E. rectale*) and genus *Roseburia* (*R. inulinivorans*, *R. faecis*, *R. cecicola*, *R. hominis*, or *R. intestinalis*). Because these species are included in *Clostridium* rRNA cluster XIVa, a phylogenetic analysis was carried out with the genera included in the cluster. *Eubacterium plexicaudatum* ATCC27514^T with 91.4% similarity (accession no. AF157058) was included in the phylogenetically neighbor cluster with SY8519 with low bootstrap values (Fig. 3). The phylogenetic tree shows that strain SY8519 represents a novel lineage at the genus level in the *Clostridium* rRNA cluster XIVa (Fig. 3). The morphological and biochemical properties of the strains that are closely related with strain SY8519 in the phylogenetic tree are summarized in Table 2. While butyric acid is the common end product of fermentation from glucose, other properties differed between the strains, especially in the G+C content. These phenotypic data (Table 2) support the conclusion that strain SY8519 is a novel species.

While *E. ramulus* is taxonomically distinct from strain SY8519, *E. ramulus* strain wk1 in the same species is an *O*-DMA-producing bacterium (Schoefer et al. 2002) like strain SY8519. Although *Clostridium* sp. HGH 136 was also found to be *O*-DMA-producing (Hur et al. 2002), it has not been registered in the DDBJ/GenBank/EMBL database. Little is known about the taxonomical properties of *Clostridium* sp. HGH 136 except that it is capable of producing indole (compared to the indole-negative strain SY8519).

Recently, many kinds of polyphenols, such as plant-derived flavones or isoflavones, have been recognized as bioactive substances that show good effects on human health and are used as functional food materials included in popular dietary supplements. It is known that *E. ramulus* strain wk1 can degrade (or metabolize) not only daidzein but also other various kinds of flavonoids (Braune et al. 2001; Schneider and Blaut 2000; Schoefer et al. 2002). An intestinal bacterium with such metabolic capability may have an impact on the effects of orally taken polyphenols.

In conclusion, strain SY8519 is a novel bacterium belonging to *Clostridium* rRNA cluster XIVa. It is expected that strain SY8519 can also metabolize a variety of polyphenols because it belongs to the cluster phylogenetically neighboring that of *E. ramulus*. We are now conducting further study on this strain's polyphenols-metabolizing property. Strain SY8519's early taxonomical categorization must be established, as it is believed to take an important role in intestinal flora and food functions.

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