Isolation and Characterization of a Human Intestinal Bacterium, *Eubacterium* sp. ARC-2, Capable of Demethylating Arctigenin, in the Essential Metabolic Process to Enterolactone

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Plant lignans, such as pinoresinol diglucoside, secoisolariciresinol diglucoside and arctiin, are metabolized to mammalian lignans, enterolactone or enterodiol, by human intestinal bacteria. Their metabolic processes include deglucosylation, ring cleavage, demethylation, dehydroxylation and oxidation. Here we isolated an intestinal bacterium capable of demethylating arctigenin, an aglycone of arctiin, to 2,3-bis(3,4-dihydroxybenzyl)butyrolactone (1) from human feces, and identified as an *Eubacterium* species (*E.* sp. ARC-2), which is similar to *Eubacterium limosum* on the basis of morphological and biochemical properties and 16S rRNA gene sequencing. By incubating with *E.* sp. ARC-2, arctigenin was converted to 1 through stepwise demethylation. Demethylation of arctigenin by *E.* sp. ARC-2 was tetrahydrofolate- and ATP-dependent, indicating that the reaction was catalyzed by methyltransferase. Moreover, *E.* sp. ARC-2 transformed secoisolariciresinol to 2,3-bis(3,4-dihydroxybenzyl)-1,4-butanediol by demethylation.

Key words human intestinal bacteria; plant lignan; arctigenin; *Eubacterium*; demethylation

Phytoestrogens are estrogen-like compounds found in many edible plants and are generally categorized as isoflavones and lignans according to their chemical structures.[1,2] Experimental and epidemiological studies that phytoestrogens have potential health benefits in hormone-dependent diseases such as breast and prostate cancers, have led to intense interest in their transformation and absorption.[3—5]

Transformation and absorption of the plant lignans have been studied[6,7] since enterolactone (ENL) and enterodiol (END) were first isolated from urine of humans and animal species in 1980. ENL and END are usually termed mammalian lignans because they possess phenolic hydroxyl groups only in the *meta* position of aromatic rings unlike plant lignans.[8] ENL and END are transformed from the plant lignans by bacteria in the intestinal tract and absorbed into the body. They exhibit biological ability such as estrogenic and antiestrogenic acivities[9—11] as well as antioxidative activity.[12]

The transformations of plant lignans like secoisolariciresinol diglucoside, pinoresinol diglucoside, arctiin, and matairesinol, to ENL and END by intestinal bacteria include a series of reaction such as deglucosylation, ring cleavage, demethylation, dehydroxylation, and oxidation. Wang *et al.*[13] isolated two bacteria responsible for transforming secoisolariciresinol diglucoside to ENL and END. *Peptostreptococcus* sp. strain SDG-1, one of these bacterial strains, capable of demethylation, however, did not transform tetra-*O*-methylsecoisolariciresinol. This observation suggested that an adjacent hydroxyl group to the methoxy group was necessary for demethylation by *P.* sp. SDG-1.

On the other hand, Xie *et al.*[14] reported that arctigenin was transformed to ENL, indicating the presence of bacteria capable of demethylating vicinal dimethoxy groups to yield free hydroxyl group(s).

In the present paper, we report the isolation and characterization of a bacterial strain, *Eubacterium* (*E.*) sp. ARC-2, capable of demethylating in lignans. The structural requirement of demethylation catalized by this bacterium was quite different from that previously isolated by Wang *et al.*[13]

There are many studies of microbial *O*-demethylation systems. Some reports showed *O*-demethylation by oxygenase or by tetrahydrofolate (THF)-dependent methyltransferase.[15—21] The importance of THF and ATP in the anaerobic *O*-demethylation was reported.[22] Here, we investigated effects of THF and ATP on demethylation of arctigenin by *E.* sp. ARC-2, a strictly anaerobic bacterium.

Moreover, we examined the biotransformation of (+)-secoisolariciresinol and (2*R*,3*R*)-2-(4-hydroxy-3-methoxybenzyl)-3-(3,4-dimethoxybenzyl)-1,4-butanediol [(−)-secoisolariciresinol 4″-*O*-methyl ether] by *E.* sp. ARC-2.

MATERIALS AND METHODS

General An anaerobic incubator, EAN-140 (Tabai Co., Osaka, Japan), was used for incubation of fecal suspensions and intestinal bacteria. Optical rotations were measured in MeOH solutions with a DIP-360 automatic polarimeter (Jasco Co., Tokyo, Japan), UV spectra with a UV-2200 UV–VIS recording spectrophotometer (Shimadzu Co., Kyoto, Japan), melting points with a Yanagimoto micro hot-stage melting point apparatus (Yanagimoto Co., Tokyo, Japan), and electron impact mass spectra (EI-MS) with a JMS-GC mate mass spectrometer at an ionization voltage of 70 eV (JEOL Co., Akishima, Japan). ^{1}H- and ^{13}C-NMR, ^{1}H–^{1}H-correlated spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond coherence (HMBC), and nuclear Overhauser effect spectroscopy (NOESY) experiment were taken on Varian UNITY 500 (^{1}H: 500 MHz, ^{13}C: 125 MHz) and Varian Gem-

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ini 300 (^{1}H: 300 MHz, ^{13}C: 75 MHz). High performance liquid chromatography (HPLC) was performed on a CCPM-II (Tosoh, Tokyo, Japan) equipped with a Tosoh UV-8020 spectrometer and a Shimadzu C-R8A chromatopac (Shimadzu, Kyoto, Japan) under the following conditions: column, TSK-gel ODS-80Ts (Tosoh Co., Tokyo, Japan, 4.6 mm×150 mm); mobile phase, H_2O (solvent system A) and CH_3CN (solvent system B) in a gradient mode (B from 25 to 26.2% in 50 min); flow rate, 1.0 ml/min; detection, UV 280 nm; temperature, room temperature. Thin-layer chromatography (TLC) was carried out on silica gel pre-coated 60 F_{254} plates (0.25 mm, Merck Co., Darmstadt, Germany) and spots were detected under a UV lamp or exposing I_2 vapor. Silica gel BW-820 MH (Fuji silysia, Aichi, Japan) was used for column chromatography. The DNA sequences were aligned using 'DNASIS' version 3.0 (Hitachi Software Engineering Co., Tokyo, Japan).

Plant Materials The seeds of *Arctium lappa* L. were purchased from Tochimoto Tenkaido Co. (Osaka, Japan).

Chemicals and Media General anaerobic medium (GAM) broth was purchased from Nissui Co. (Tokyo, Japan). Arctigenin (**8**) was isolated from the seeds of *A. lappa* as follows: The softened and grinded seeds (1 kg) were extracted two times by immersing in MeOH (5 l) overnight. The combined solutions were concentrated to 450 ml under vacuum, to which 50 ml of water was added. The mixture was extracted with 300 ml of hexane and the residual solution was hydrolyzed with 2 N HCl at 80 °C for 2 h. The mixture was extracted with ethyl acetate and the ethyl acetate-soluble fraction was evaporated to yield a residue (46.2 g), which was chromatographed on a silica gel column (8 cm×36 cm) eluting with $CHCl_3$–MeOH (100 : 1→20 : 1). Then, (−)-arctigenin (15.0 g) [mp 103—104 °C, lit.[14] mp 100.5—101.5 °C; $[\alpha]_D^{23}$ −20.3° (c=0.054, MeOH), lit.[14] $[\alpha]_D^{23}$ −25.8° (MeOH), lit.[23] $[\alpha]_D$ −27.5° (MeOH)] was obtained by crystallization from MeOH–ether. Secoisolariciresinol diglucoside (SDG) was provided by Suntory Co. (Osaka, Japan) and purified by repeated column chromatography in our laboratory. (+)-Secoisolariciresinol was obtained as follows: SDG (96 mg) was dissolved in acetate buffer (pH 4.5) and incubated with 100 mg of naringinase at 37 °C overnight. The mixture was extracted with ethyl acetate and the ethyl acetate soluble fraction was evaporated to yield a residue, which was chromatographed on a silica gel column eluting with $CHCl_3$–MeOH (20 : 1→10 : 1). Then, (+)-secoisolariciresinol (43 mg) was obtained by crystallization from ethyl acetate. (−)-Secoisolariciresinol 4″-*O*-methyl ether, (+)-dihydroxyenterodiol and (−)-dihydroxyenterodiol were obtained by modified methods of Makela *et al.*[24] from arctigenin, (+)-secoisolariciresinol and (−)-secoisolariciresinol 4″-*O*-methyl ether, respectively.

(+)-Secoisolariciresinol: Colorless prism. mp 102—104 °C. $[\alpha]_D^{23}$ +41° (c=0.2, MeOH). This compound was identified by comparing the ^{1}H- and ^{13}C-NMR spectra with that published.[13]

(2*R*,3*R*)-2-(4-Hydroxy-3-methoxybenzyl)-3-(3,4-dimethoxybenzyl)-1,4-butanediol [(−)-Secoisolariciresinol 4″-*O*-Methyl Ether]: Colorless prisms. mp 100—101 °C. $[\alpha]_D^{23}$ −14.2° (c=0.077, MeOH). This compound was identified by comparing the ^{1}H- and ^{13}C-NMR spectra with that published.[29]

(+)-Dihydroxyenterodiol: Amorphorus powder. $[\alpha]_D^{23}$ +19.0° (c=0.11, MeOH). This compound was identified by comparing the ^{1}H- and ^{13}C-NMR spectra with that published.[13]

(−)-Dihydroxyenterodiol [(2*R*,3*R*)-2,3-Bis(dihydroxybenzyl)-1,4-butanediol]: Amorphorus powder. $[\alpha]_D^{23}$ −5.7° (c=0.15, MeOH). This compound was identified by comparing the ^{1}H- and ^{13}C-NMR spectra with that published.[27]

Isolation of an Human Intestinal Bacterial Strain Capable of Demethylating 8 to Yield 1 A bacterial suspension from fresh feces of a healthy volunteer was repeatedly cultured in 2 ml of GAM broth containing 0.5 mM arctigenin as a substrate at 37 °C in an anaerobic incubator. A portion of the culture, possessing metabolic activity, was seeded on GAM agar plates and anaerobically incubated for 24 h at 37 °C. Colonies were picked up and screened for their activity of transforming **8** to **1**. Such a procedure was repeated until a single strain having demethylation acitivity was isolated. Characterization of a bacterial strain was carried out according to the methods of Mitsuoka[25] and the Bergey's manual.[26]

Sequencing the Bacterial 16S rRNA Gene The bacterium was incubated at 37 °C in an anaerobic incubator for 2 d and collected by centrifugation at 10000×***g*** for 10 min. Total DNAs were extracted with a DNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's protocol. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) with two forward and reverse primer sets based on those of various strains from the database: Bac 1F (AGAGTTTGATCCTGGCTCAG) and Bac 1R (CCGTATTACCGCGGCTGCTG); and Bac 3F (TAACTACGTGCCAGCAGCCG) and Bac 3R (CCCGGGAACGTATTCACCG). Amplification proceeded in a reaction mixture containing 1 U of KOD-Plus DNA polymerase (Toyobo, Osaka, Japan), 1×PCR buffer mix, 0.8 mM dNTP mix (0.2 mM each), 1 mM $MgSO_4$, 0.3 μM of each primer, and 100 ng of template DNA. The PCR program was as follows: 94 °C for 2 min, 30 cycles of 94 °C for 14 s, 55 °C for 30 s, 68 °C for 45 s, and finally 68 °C for 5 min. The PCR products were purified using a QIA Quick PCR Purification Kit (Qiagen, Germany), and directly sequenced using a Bigdye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster city, CA, U.S.A.) wth primers (Bac 1F, Bac 1R, Bac 3F, and Bac 3R) and an ABI PRISM 310 (Applied Biosystems, Foster city, CA, U.S.A.).

Incubation of Arctigenin (8) with *E.* sp. ARC-2 and Isolation of Metabolites (−)-Arctigenin (1 g in 10 ml MeOH) and a bacterial suspension (50 ml) of *E.* sp. ARC-2 were added to 1 l of GAM broth and incubated at 37 °C in an anaerobic incubator for 62 h. The reaction mixture was then extracted three times with ethyl acetate. The organic layer was evaporated under reduced pressure to give a residue (1.726 g). The residue was applied to a column of silica gel, which was eluted with a solvent system, $CHCl_3$–MeOH (50 : 1) to give fractions A—E. Fraction E gave **1** (63 mg). Fractions B—D were chromatographed on a silica gel column ($CHCl_3$: MeOH=100 : 1), followed by HPLC (22 or 24% of $CHCN_3$ in H_2O) to yield compounds **5** (16.3 mg), **6** (14.6 mg), **7** (8.7 mg) from fraction B; compound **6** (20 mg) from fraction C; compounds **2** (5.4 mg), **3** (2.7 mg), **4** (63.5 mg) from fraction D. An original compound of arcti-

genin (121 mg) was recovered from the reaction mixture.

(2*R*,3*R*)-2,3-Bis(3,4-dihydroxy)butyrolactone [(−)-Dihydroxyenterolactone] (**1**): Colorless oil. $[\alpha]_D^{23}$ −25.9° (*c*=0.159, MeOH) [lit.[27]) $[\alpha]_D^{25}$ −14° (MeOH)]. This compound was identified by comparing the ^{1}H-NMR spectrum with that published.[27])

(2*R*,3*R*)-2-(4-Hydroxy-3-methoxybenzyl)-3-(3,4-dihydroxybenzyl)butyrolactone (**2**): Amorphorus powder. $[\alpha]_D^{23}$ −22.8° (*c*=0.114, MeOH) [lit.[27]) $[\alpha]_D^{25}$ −20° (MeOH)]. This compound was identified by comparing the ^{1}H-NMR spectrum with that published.[27])

(2*R*,3*R*)-2-(3,4-Dihydroxybenzyl)-3-(4-hydroxy-3-methoxybenzyl)butyrolactone (**3**): Amorphorus powder. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 2.45—2.64 (4H, m, H-2, 3, 7″), 2.85 (2H, d, *J*=6.5 Hz, H-7′), 3.83 (3H, s, –OC$\underline{H}_3$), 3.88 (1H, dd, *J*=8, 8.8 Hz, H_a-4), 4.12 (1H, dd, *J*=7, 8.8 Hz, H_b-4), 6.45 (1H, d, *J*=2 Hz, H-2″), 6.51—6.53 (2H, m, H-6′, 6″), 6.60 (1H, d, *J*=1.5 Hz, H-2′), 6.75 (1H, d, *J*=7.5 Hz, H-5′), 6.80 (1H, d, *J*=8 Hz, H-5″) (the numbering of protons is indicated in Fig. 3). UV λ_{max} (MeOH) nm (ε): 221 (12200), 281 (6100). EI-MS (rel. int.) *m*/*z*: 123 (73), 137 (100), 344 $[M]^+$ (48). $[\alpha]_D^{23}$ −24.0° (*c*=0.075, MeOH).

(2*R*,3*R*)-2-(3,4-Dihydroxybenzyl)-3-(3-hydroxy-4-methoxybenzyl)butyrolactone (**4**): Amorphorus powder. ^{1}H-NMR (CD_3OD, 500 MHz): δ 2.33—2.59 (4H, m, H-2, 3, 7″), 2.74 (1H, dd, *J*=7, 14.5 Hz, H_a-7′), 2.81 (1H, dd, *J*=5.5, 14.5 Hz, H_b-7′), 3.78 (3H, s, –OC$\underline{H}_3$), 3.84 (1H, t, *J*=8.5 Hz, H_a-4), 4.01 (1H, dd, *J*=7.5, 8.5 Hz, H_b-4), 6.47 (2H, dd, *J*=2, 8 Hz, H-6′, 6″), 6.53 (1H, d, *J*=2 Hz, H-2″), 6.64 (1H, d, *J*=2 Hz, H-2′), 6.69 (1H, d, *J*=8 Hz, H-5′), 6.77 (1H, d, *J*=8 Hz, H-5″) (the numbering of protons is indicated in Fig. 3). UV λ_{max} (MeOH) nm (ε): 220 (12200), 281 (5900). EI-MS (rel. int.) *m*/*z*: 123 (68), 137 (100), 344 $[M]^+$ (49). $[\alpha]_D^{23}$ −32.5° (*c*=0.52, MeOH).

(2*R*,3*R*)-2,3-Bis(4-hydroxy-3-methoxybenzyl)butyrolactone (**5**): Amorphorus powder. $[\alpha]_D^{23}$ −23.6° (*c*=0.057, MeOH) [lit.[23]) $[\alpha]_D$ −50.0° (MeOH)]. This compound was identified by comparing the ^{1}H-NMR spectrum with that published.[23])

(2*R*,3*R*)-2-(3,4-Dihydroxybenzyl)-3-(3,4-dimethoxybenzyl)butyrolactone (**6**): Amorphorus powder. $[\alpha]_D^{23}$ −40.3° (*c*=0.197, MeOH) [lit.[14]) $[\alpha]_D^{25}$ −42.8° (MeOH)]. This compound was identified by comparing the ^{1}H-NMR spectrum with that published.[14])

(2*R*,3*R*)-2-(4-Hydroxy-3-methoxybenzyl)-3-(3-hydroxy-4-methoxybenzyl)butyrolactone (**7**): Amorphorus powder. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 2.43—2.62 (4H, m, H-2, 3, 7″), 2.92 (2H, m, H-7′), 3.82—3.86 (7H, m, H_a-4, –OC$\underline{H}_3$×2), 4.09 (1H, dd, *J*=7, 9 Hz, H_b-4), 6.47 (1H, dd, *J*=2.3, 8 Hz, H-6″), 6.58 (1H, d, *J*=2.3, H-2″), 6.63 (1H, dd, *J*=2, 8 Hz, H-6′), 6.67 (1H, d, *J*=2, H-2′), 6.72 (1H, d, *J*=8 Hz, H-5″), 6.83 (1H, d, *J*=8 Hz, H-5′) (the numbering of protons is indicated in Fig. 3). UV λ_{max} (MeOH) nm (ε): 223 (12900), 281 (6300). EI-MS (rel. int.) *m*/*z*: 137 (100), 358 $[M]^+$ (34). $[\alpha]_D^{23}$ −31.0° (*c*=0.087, MeOH).

Time Course for the Transformation of Arctigenin (8) by *E.* sp. Strain ARC-2 GAM broth (3 ml) containing arctigenin (a final concentration of 0.6 mM) was incubated with a bacterial suspension of *E.* sp. ARC-2 (150 μl) at 37 °C under anaerobic conditions. A 100 μl aliquot was taked out at intervals of 1 h and extracted with ethyl acetate. After evaporation of ethyl acetate *in vacuo*, the residue was dissolved in 0.3 ml of MeOH. The MeOH solution was filtered through a 0.2 μm membrane filter, and a 10 μl portion was injected to a column for HPLC analysis. Concentrations of arctigenin (**8**) and its metabolites were calculated from the calibration curves of the respective authentic samples.

Incubation of Compounds 5, 6, and 7 with *E.* sp. ARC-2 A 100 μl portion of precultured *E.* sp. ARC-2 was inoculated to 2 ml of GAM broth containing 0.5 mM arctigenin (for induction of methyltransferase) and anaerobically incubated for 24 h. After confirming of complete transformation to dihydroxyenterolactone (**1**) by HPLC, compounds **5**, **6** and **7** were separately added to a bacterial suspension and incubated anaerobically for 1 h. A 100 μl aliquot was then taken out and analyzed as described above.

Preparation of Cell-Free Extracts *E.* sp. ARC-2 was cultured in 500 ml of GAM broth containing 0.1 mM arctigenin (inducer) under anaerobic conditions at 37 °C for 11 h. The cells were harvested and then suspended in 100 mM phosphated buffer (pH 7.3). The cell suspensions were sonicated by 10 sonic bursts of 30 s each (Branson Sonifier 250, Branson Ultrasonics Corporation, Danbury, CT, U.S.A.) on ice, and then centrifuged at 100000×***g*** for 60 min (Ultracentrifuge Beckman Optima XL-70, Beckman Instruments, Fullerton, CA, U.S.A.) at 4 °C. The supernatants were filtered with 0.45 μm microfilter and then used as cell-free extracts.

Measurement of Demethylation Activity The cell-free extracts containing 0.6 mM arctigenin, 4 mM THF, and 4 mM ATP were anaerobically incubated at 37 °C for 24 h. A 100 μl aliquot was taked out and extracted with ethyl acetate. After evaporation of the ethyl acetate *in vacuo*, the residue was dissolved in 0.3 ml of MeOH. The MeOH solution was filtered through a 0.2 μm membrane filter, and a 10 μl portion was injected to a column for HPLC analysis. Concentrations of arctigenin (**8**) and its metabolites were calculated from the calibration curves of the respective authentic samples.

Incubation of (+)-Secoisolariciresinol and (−)-Secoisolariciresinol 4″-*O*-Methyl Ether with *E.* sp. ARC-2 A 100 μl portion of precultured *E.* sp. ARC-2 was inoculated to 2 ml of GAM broth containing 0.3 mM (+)-secoisolariciresinol or (−)-secoisolariciresinol 4″-*O*-methyl ether and anaerobically incubated for 72 h. A 100 μl aliquot was taken out and extracted with ethyl acetate. After evaporation of the ethyl acetate *in vacuo*, the residue was dissolved in 0.3 ml of MeOH. The MeOH solution was filtered through a 0.2 μm membrane filter, and a 10 μl portion was injected to a column for chiral HPLC analysis under the following conditions: column, chiral CD-Ph (Shiseido, Tokyo, Japan, 4.6 mm×250 mm); mobile phase, 0.1% TFA (solvent system A) and CH_3CN (solvent system B) in a gradient mode (B from 25 to 31% for 18 min, B from 31 to 42% from 18 to 40 min); flow rate, 0.5 ml/min; detection, UV 280 nm; temperature, 30 °C. The retention times of (+)-dihydroxyenterodiol, (−)-dihydroxyenterodiol, (+)-secoisolariciresinol and (−)-secoisolariciresinol 4″-*O*-methyl ether were 14.3, 15.1, 23.8 and 31.5 min, respectively.

RESULTS

Isolation of a Bacterial Strain Capable of Transforming (−)-Arctigenin (8) to (−)-2,3-Bis(3,4-dihydroxybenzyl)bu-

tyrolactone (Dihydroxyenterolactone) (1) from a Human Fecal Suspension Through repeated culture of a bacterial mixture of human feces in GAM broth containing 0.5 mM arctigenin (**8**), and screening of colonies on GAM agar plates for demethylation activity, a bacterial strain (strain ARC-2) capable of transforming **8** to **1** was isolated. Strain ARC-2 was strictly anaerobic, Gram-positive, and regular rods (Table 1). Based on these characteristics, strain ARC-2 was considered to belong to the genus *Eubacterium*. The strain was not able to produce indole and nitrite, and hydrolyzed esculin but not starch. In addition, it produced butyric acid from glucose predominantly and fermented erythritol, fructose, glucose, mannitol, and ribose (Table 2). Therefore, the strain was concluded to be an *Eubacterium* sp. with characteristics similar to those of *E. limosum* (Genbank accession no. M59120), and was named *E.* sp. ARC-2 (EF413640). Furthermore, the 16S rRNA gene sequence had 99% similarity to that of *E. limosum* (Fig. 1).

Transformation of (−)-Arctigenin (8) by *E.* sp. ARC-2 After anaerobic incubation of arctigenin (**8**) with *E.* sp. ARC-2 for 12 h, seven metabolites together with compound **1** were detected in the culture by HPLC analysis (Fig. 2). For isolating the metabolites, a large amount of arctigenin (**8**) was cultured with *E.* sp. ARC-2 under the similar conditions. The culture was extracted with ethyl acetate and the extract was subject to silica gel column chromatography and HPLC. Seven metabolites (**1**—**7**) (Fig. 3) were isolated and the structures of these metabolites were determined by ^{1}H-, ^{13}H-NMR (Table 3), 2D-NMR and MS spectroscopy.

Compounds **5**—**7** and **2**—**4** showed molecular ion peaks at *m*/*z* 358 $[M]^+$ and 344 $[M]^+$, respectively, in the ESI-MS spectra. The formers were 14 mass units less than the molecular ion of (−)-arctigenin (**8**), suggesting that **5**—**7** were dimethyl derivatives of dihyroxyenterolactone, while the latters were 28 mass units less, suggesting that **2**—**4** were monomethyl derivatives of dihydroxyenterolactone. This was confirmed by the presence of only two or one signal(s) for *O*-methyl proton(s) in comparison with three siglet signals of arctigenin in the ^{1}H-NMR spectra.

For determining the location of methoxy groups of compounds **5**—**7**, heteronuclear multiple-bond coherence (HMBC) experiments were undertaken to see HMBC correlations between signals of methoxy protons and aromatic car-

Table 1. Biochemical Characteristics of *E.* sp. ARC-2 and *E. limosum*

Characterisitic	*E.* sp. ARC-2	*E. limosum*[a)]
Gram stain	+	+
Aerobic growth	−	−
Motility	−	−
Esculin hydrolysis	+	+
Indole production	−	−
Starch hydrolysis	−	−+
Nitrate reduction	−	−

a) Data of "Bergey's Manual of Systematic Bacteriology."[26] Symbols: +, positive; −, negative.

Table 2. Comparative Fermentation Reactions of *E.* sp. ARC-2 and *E. limosum* (ATCC8486)

Carbohydrate	*E.* sp. ARC-2	*E. limosum*[a)]
Amygdalin	−	−
Arabinose	−	w
Cellobinose	−	−
Dextrin	−	ND
Erythritol	+	+ w
Esculin	−	−
Fructose	+	+
Glucose	+	+
Glycerol	−	− w
Glycogen	−	−
Inositol	−	−
Inulin	−	−
Lactose	−	−
Maltose	−	− w
Mannitol	+	+ w
Mannose	−	− w
Melbiose	−	−
Melezitose	−	−
Raffinose	−	−
Rhamnose	−	−
Ribose	+	+ w
Salicin	−	−
Sorbitol	−	−
Starch	−	−
Sucrose	−	−
Trehalose	−	−
Xylose	−	d

a) Data of "A Color Atlas of Anaerobic Bacteria,"[25] "Bergey's Manual of Systematic Bacteriology."[26] Symbols: +, positive (pH below 5.5); −, negative (pH upper 5.9); w, weak reaction (pH 5.5—5.9); d, 40—60% of strains positive; ND, no data.

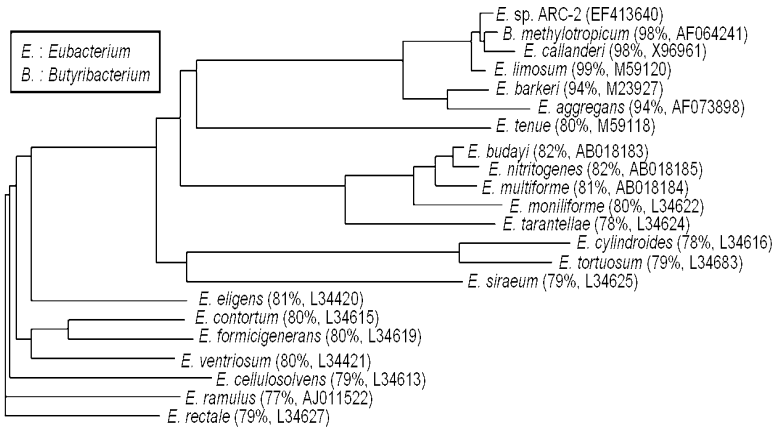

Fig. 1. Dendrogram of Phylogenic Affiliation of *E.* sp. ARC-2

A 16S rRNA gene sequence-based phylogenic tree shows relationship between newly isolated *E.* sp. ARC-2 (EF413640) and other closely related species. The sequence of *E.* sp. ARC-2 was compared to those from Genbank database with ClustalW program.

Table 3. ^{13}C-NMR Spectral Data of Compounds **1**—**8**

C	**1**[a,c]	**2**[a,d]	**3**[b,d]	**4**[a,c]	**5**[b,d]	**6**[b,d]	**7**[b,d]	**8**[a,c]
1	181.7	181.4	179.1	181.7	178.6	179.9	178.5	181.5
2	47.7	47.9	46.5	47.7	46.6	46.5	46.7	47.7
3	42.6	42.4	41.1	42.5	41.0	40.8	41.0	42.4
4	72.8	72.8	71.5	72.8	71.3	71.7	71.3	72.9
1′	130.8	130.5	130.1	130.7	129.4	129.8	129.4	130.7
2′	117.5	113.8	116.0	117.4	111.3	116.1	111.5	113.8
3′	146.3	148.8	143.6	146.3	146.5	144.0	146.5	149.0
4′	145.1	146.2	142.7	145.1	144.4	142.9	144.4	146.4
5′	116.4	116.0	115.1	116.4	114.0	115.2	114.2	116.1
6′	121.9	122.9	121.6	121.9	121.9	121.5	122.0	123.0
7′	35.1	35.2	34.2	35.1	34.6	34.0	34.6	35.4
1″	131.5	131.3	129.7	132.7	129.6	130.5	131.0	132.8
2″	116.8	116.7	111.0	116.7	110.8	111.7	114.6	113.5
3″	146.3	146.2	146.5	147.4	146.4	148.9	145.5	150.4
4″	144.9	144.8	144.2	147.6	144.2	147.7	145.2	149.1
5″	116.4	116.3	114.4	112.8	114.3	111.3	110.6	113.0
6″	121.0	120.9	121.3	121.0	121.2	120.7	120.0	122.0
7″	38.6	38.7	38.3	38.4	38.4	38.0	38.0	38.8
–Me		56.3	55.9	56.4	55.8	55.8	55.9	56.4
–Me					55.9	55.9	56.0	56.3×2

Measured in *a*) CD_3OD and *b*) $CDCl_3$. NMR data performed on spectrometers of *c*) 125 MHz and *d*) 75 MHz.

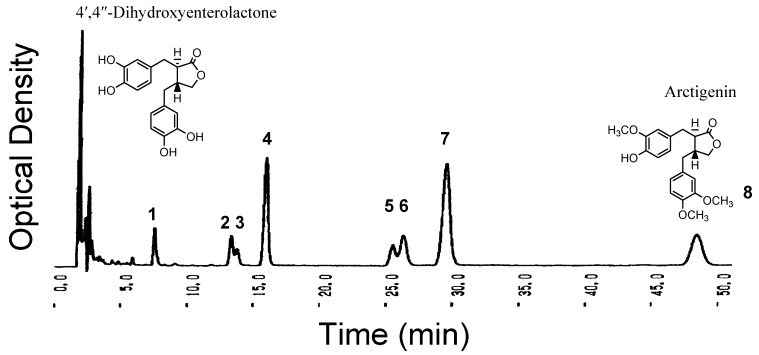

Fig. 2. HPLC Profile of Metabolites of Arctigenin by *E.* sp. ARC-2

A 100 μl portion of precultured *E.* sp. ARC-2 was inoculated to 2 ml of GAM broth containing 0.5 mM arctigenin, and anaerobically incubated for 12 h. A 100 μl aliquot was then taken out and analyzed under the following conditions: Column, TSK-gel ODS-80Ts (4.6 mm i.d.×150 mm, Tosoh); solvent, CH_3CN 25→26.2% (50 min); detector, UV at 280 nm; flow rate, 1 ml/min; temp., room temperature.

Compound	R_1	R_2	R_3
1	-H	-H	-H
2	-H	-H	$-CH_3$
3	$-CH_3$	-H	-H
4	-H	$-CH_3$	-H
5	$-CH_3$	-H	$-CH_3$
6	$-CH_3$	$-CH_3$	-H
7	-H	$-CH_3$	$-CH_3$
8	$-CH_3$	$-CH_3$	$-CH_3$

Fig. 3. Arctigenin and Its Metabolites by *E.* sp. ARC-2

bons. Compound **7** showed HMBC correlations between signals of methoxy protons and signals of carbons at C-3′ and C-4″. The structure of compound **7** was consequently determined to be 3″-desmethylarctigenin. Compound **6** showed HMBC correlations between signals of methoxy protons and carbons at C-3″ and C-4″, and compound **5** showed correlations between signals of methoxy protons and carbons at C-3′ and C-3″. Therefore, the structures of compounds **6** and **5** were concluded to be 3′-desmethylarctigenin, and 4″-desmethylarctigenin, respectively.

In addition, compound **2** was identified as dihydroxyenterolactone 3′-*O*-methyl ether on the basis of the correlations between signals of methoxy protons and a carbon at C-3′ in the HMBC experiment and a correlation between signals of methoxy protons and an H-2′ proton in the NOESY experiment. As compound **3** showed HMBC correlations between signals of methoxy protons and a carbon at C-3″, the structure was determined to be dihydroxyenterolactone 3″-*O*-methyl ether. Compound **4** was concluded to be dihydroxyenterolactone 4″-*O*-methyl ether on the basis of a NOESY correlation between signals of methoxy protons and an H-5″ proton.

The metabolic processes were deduced by time course experiments and structures of isolated metabolites. A stepwise demethylation from arctigenin to dihydroxyenterolactone was proceeded by incubation with *E.* sp. ARC-2 (Fig. 4). Compounds **5**, **6** and **7** were independently incubated with *E.*

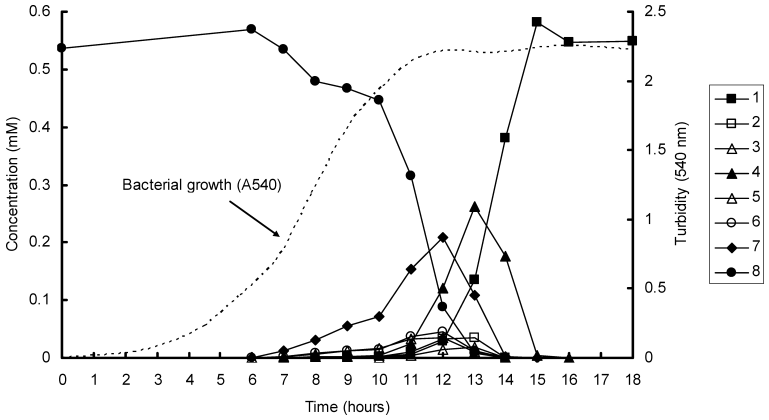

Fig. 4. Time Course of Transformation of (−)-Arctigenin (**8**) to (−)-Dihydroxyenterolactone (**1**) by *E.* sp. ARC-2

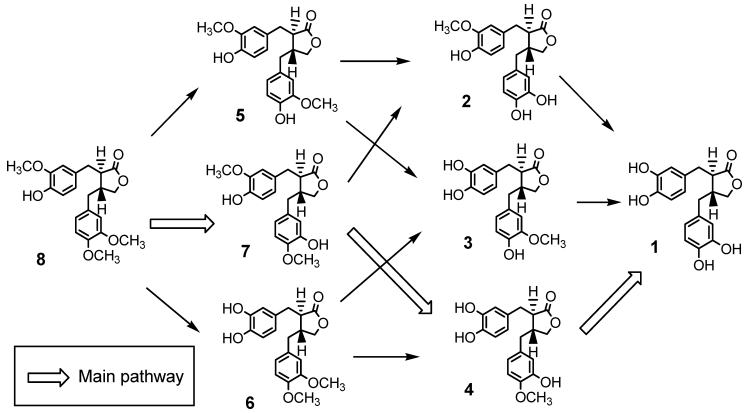

Fig. 5. Possible Metabolic Processes of Arctigenin by *E.* sp. ARC-2

sp. ARC-2 for confirming of transformation pathways to compounds **2**, **3** and **4**. The HPLC analysis of an incubation mixture showed that compound **5** was demethylated to yield compounds **2** and **3**, compound **6** was transformed to **3** and **4**, compound **7** to **2** and **4**, respectively (data not shown). Based on these findings, we drew metabolic pathways of demethylation of arctigenin (Fig. 5). Arctigenin (**8**) was transformated by *E.* sp. ARC-2 to yield a number of metabolites, but the main pathway seems to be **8**→**7**→**4**→**1** on the basis of the time course experiments of the respective metabolites and the structural consideration.

Effects of Tetrahydrofolate and ATP on Demethylation by a Cell-Free Extract of *E.* sp. ARC-2 When arctigenin was incubated with a cell-free extract of *E.* sp. ARC-2, only 36.4% for mono-desmethylarctigenins and 6.0% for di-desmethylarctigenins were produced and the rest was recovered as the strarting material (Tabel 4). However, addition of ATP (4 mM) increased the percentage of demethylated products, which accounted for 39.2% for mono-desmethylarctigenins and 9.6% for di-desmethylarctgenins. Addition of THF (4 mM) also increased the demethylated products up to 45.0% and 23.4% for mono-desmethylarctigenins, and di-desmethylarctigenins, respectively. Moreover, addition of both ATP and THF appreciably increased in the percentage of the demethylated products, which accounted for 39.5%, 40.7% and 7.2% for mono-, di-, and tri-desmethylarctigenins, respectively. The demethylation by the cell-free extract seems to proceed *via* **8**→**7**→**4**→**1** as that by *E.* sp. ARC-2 cells.

Substrate Specificity of *E.* sp. ARC-2 The substrate specificity of this bacterium was assayed using (+)-secoisolariciresinol and (−)-secoisolariciresinol 4″-*O*-methyl ether (Fig. 6). All methoxy groups of two compounds were eliminated to yield (+)-dihydroxyenterodiol and (−)-dihydroxyenterodiol, respectively, by incubation with *E.* sp. ARC-2.

DISCUSSION

Biotransformation of lignans by intestinal bacteria has been studied for a long time. Elimination of glucose, methoxy, and hydroxy groups is essential steps to yield END or ENL from precursors such as arctiin,[14] pinoresinol diglucoside,[27] and secoisolariciresinol diglucoside.[13] In the course of studies on the metabolic activation of herbal medicines, we isolated some bacterial strains involved in the metabolism of lignans, and investigated their characteristics and metabolites.

We isolated a bacterial strain, *E.* sp. ARC-2 as one of bacteria capable of demethylation. Since *E.* sp. ARC-2 was similar to *E. limosum* in characteristics and 16S rRNA gene sequence, we anaerobically incubated arctigenin (**8**) with either *E. limosum* (ATCC 8486) or *E.* sp. ARC-2 and obtained the same metabolite dihydroxyenterolactone. However, the shape and viscosity of colony in BL agar plates was different from each other. Especially, the high viscosity of *E. limosum* was apparent in contrast with that of *E.* sp. ARC-2.

Table 4. Effects of Tetrahydrofolate (THF) and ATP on Demethylation by a Cell-Free Extract (CFE) of *E.* sp. ARC-2

	Recovered	Dimethyl der.			Monomethyl der.			Free form
	8	**7**	**6**	**5**	**4**	**3**	**2**	**1**
CFE	57.4		36.4			6.0		0.2
		22.0	9.8	4.6	3.8	0.8	1.4	
CFE+ATP	51.2		39.2			9.6		0
		24.8	9.0	5.4	5.4	1.5	2.7	
CFE+THF	29.0		45.0			23.4		2.6
		28.3	11.4	5.3	16.4	2.7	4.3	
CFE+ATP+THF	12.6		39.5			40.7		7.2
		27.5	7.8	4.2	29.6	4.1	7.0	

Relative concentration (%).

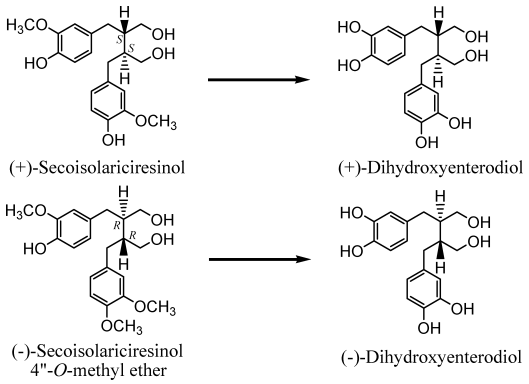

Fig. 6. Biotranformation of (+)-Secoisolariciresinol and (−)-Secoisolariciresinol 4″-*O*-Methyl Ether by *E.* sp. ARC-2

We isolated seven demethylated metabolites (**1**—**7**) of **8** by incubation with *E.* sp. ARC-2 although the production of these metabolites seems to vary depending on the bacterial constitutions in the gastro-intestinal flora of humans. Through the transformation experiments and the time course of intermediate and final metabolite formations, the metabolic pathway was speculated as shown in Fig. 5. In the present study, **8** was demonstrated to transform through the major processes of **8**→**7**→**4**→**1** by *E.* sp. ARC-2, although it was reported to proceed through a processes of **8**→**6** by a bacterial mixture of human feces.[14] This difference of metabolic processes between a single bacterium and bacterial flora is important to understand the actual event in the intestinal tract. Furthermore, when we added **8** to *E.* sp. ARC-2 already proliferated in GAM broth, the major process was **8**→(**6**, **7**)→**4**→**1** (data not shown). This means the pattern of biotransformation by intestinal bacteria may be altered due to the number of bacteria or the substrates present in the intestinal environment.

To investigate the mechanism of demethylation of *E.* sp. ARC-2, we prepared a cell-free extract (CFE), which showed demethylating activity of **8** and acceleration by adding ATP and/or tetrahydrofolate (THF). ATP and THF seem to be necessary for demethylating activity of *E.* sp. ARC-2, suggesting that demethylation is catalyzed by THF-dependent methyltransferase.[22]

For investigating substrate specificity, (+)-secoisolariciresinol and (−)-secoisolariciresinol 4″-*O*-methyl ether were anaerobically incubated with *E.* sp. ARC-2. Both of the compounds were tranformed to dihydroxyenterodiol, indicating that demethylation by *E.* sp. ARC-2 is taken place regardless of their (+)- or (−)-forms. Moreover, dihydroxyenterolactone was obtained by anaerobic incubation of arctiin with *E.* sp. ARC-2 in GAM broth at 37 °C (data not shown). Vanillic acid, isovanillic acid and 3,4-dimethoxybenzoic acid were transformed to protocatechuic acid by incubation with this bacterium. Syringic acid and 3,4,5-trimethoxybenzoic acid were transformed to gallic acid, too (data not shown). *E.* sp. ARC-2 showed low substrate specificity compared with *P.* sp. SDG-1, which requires a 4-hydroxy-3-methoxy phenyl ring system as a substrate.[13] The former is more preferable for modification of naturally occurring methylated compounds to free hydroxyl forms. As regards microbial demethylation, it was recently reported that *E. limosum* had metabolic activity capable of *O*-demethylation of biochanin A, formononetin, and glycitein.[28]

Since dehydroxylation in the metabolic processes of lignans to enterolactone or enterodiol takes place only in the presence of vicinal two hydroxy groups, the accumulation of metabolic intermediates having free 3,4-dihydroxy phenyl residues catalyzed by intestinal bacteria such as *E.* sp. ARC-2, may be preferable for the formation of phytoestrogenic substances.

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