Enantioselective Dehydroxylation of Enterodiol and Enterolactone Precursors by Human Intestinal Bacteria

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During the course of experiments on the transformation of lignans to phytoestrogenic substances, such as enterodiol (END) and enterolactone (ENL), a previously isolated bacterium, Eubacterium (E.) sp. strain SDG-2, capable of phenolic p-dehydroxylation in the biotransformation of secoisolariciresinol diglucoside to END and ENL, was concluded to be Eggerthella (Eg.) lenta (Eg. sp. SDG-2) on the basis of 16S rRNA gene sequence analysis. The bacterium could transform (+)-dihydroxyenterodiol (DHEND, 3a) to (+)-END (1a), but not for (−)-DHEND (3b) to (−)-END (1b) under anaerobic conditions. By incubation of a mixture of (+) and (−)-dihydroxyenterolactone (DHENL, 4a and 4b) with Eg. sp. SDG-2, only (−)-DHENL (4b) was converted to (−)-ENL (2b), selectively. On the other hand, we isolated a different bacterium, strain ARC-1, capable of dehydroxylation (−)-DHEND (3b) to (−)-END (1b) from human feces. Strain ARC-1 could transform not only (−)-DHEND (3b) to (−)-END (1b), but also (+)-DHENL (4a) to (+)-ENL (2b). However, the bacterium could not transform (+)-DHEND (3a) and (−)-DHENL (4b). Both bacterial strains demonstrated different enantioselective dehydroxylation.

Key words human intestinal bacteria; lignan; enantioselectivity; dehydroxylation

Phytoestrogens are defined as plants-derived compounds with estrogen-like activities. Isoflavones and lignans have been categorized as phytoestrogens, based on their chemical structures and activities.1,2) Phytoestrogens have been explored in the field of metabolic degradation by intestinal bacteria.3–9) In particular, the biotransformation of plant lignans, such as secoisolariciresinol diglucoside (SDG), pinoresinol diglucoside (PDG), arctiin, and matairesinol to mammalian lignans, enterodiol (END, 1) and enterolactone (ENL, 2) have extensively been studied. The metabolic processes by intestinal bacteria include deglucosylation, ring cleavage, demethylation, dehydroxylation and oxidation. Mammalian lignans lacking of phenolic hydroxyl groups at the para position of aromatic rings are different from plant lignans,5) and dehydroxylation is an essential process on the metabolic conversion from plant lignans to mammalian lignans by intestinal bacteria.

Eubacterium (E.) sp. strain SDG-2 was isolated by Wang et al. as a bacterium capable of reductive dehydroxylation in the processes of SDG to END (1) and ENL (2).10) E. sp. strain SDG-2 was similar in characteristics to E. lentum, which was reclassified to Eggerthella (Eg.) lenta by phylogenetic identification.11) In this study, we determined phylogenetic affiliation of E. sp. SDG-2 with 16S rRNA gene-based identification using polymerase chain reaction with proper primers.

END (1) and ENL (2) have two enantiomeric, mirror image forms (1a and 1b; 2a and 2b) (Fig. 1). Secoisolariciresinol (SECO), prepared by incubation of PDG with intestinal bacteria, was a (−)-form.9) On the other hand, SECO, aglycone of SDG isolated from flaxseed, was a (+)-form.8) Although there were a few reports on the biotransformation of these lignans by intestinal bacteria,12–14) enantioselective conversion was not well confirmed. Therefore, metabolic specificity between enantiomers raised our interest in investigation.

We prepared two sets of enantiomers, such as (+)- and (−)-dihydroxyenterodiol (DHEND, 3a and 3b, respectively),
and (+)- and (−)-dihydroxyenterolactone (DHENL, 4a and 4b, respectively) from plant lignans by chemical and biological methods for investigating substrate specificity of E. sp. SDG-2.

Since (−)-pinoresinol, aglycone of PDG, was transformed to (−)-END (1b) through (−)-SECO (7b) by human intestinal microflora,19 isolation of bacterial species capable of dehydroxylating (−)-DHEND (3b) to (−)-END (1b) was attempted using (−)-DHEND (3b) as a substrate.

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), 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). 1H-NMR spectra were taken on Varian UNITY 500 (1H: 500 MHz) and Varian Gemini 300 (1H: 300 MHz). 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 by exposing I2 vapor. Silica gel BW-820 MH (Fuji Silesia, Aichi, Japan) was used for column chromatography. The DNA sequences were aligned using DNASIS, version 3.0 (Hitachi Software Engineering Co., Tokyo).

Chemicals (−)-Arctigenin was obtained by hydrolysis of arctin from the seeds of Arctium lappa L. which was purchased from Tochimoto Tenkaido Co. (Osaka).15 (+)-Secoisolariciresinol (5a) was prepared by hydrolysis of SDG, which was provided by Suntory Co. (Osaka, Japan). 15) (−)-Secoisolariciresinol 4-ethyl ether (6b) was obtained by a modified method of Makela et al.16) (−)-DHENL (4b) and (−)-DHEND (3b) were prepared from (−)-arctigenin and (−)-seciosolariciresinol 4-ethyl ether (6b), respectively, and (−)-DHEND (3a) was obtained from (−)-seciosolariciresinol according to the modified method of Makela et al.16) (−)-DHENL (4b), (−)-DHEND (3b), and (−)-DHEND (3a) were identified by comparing the spectral data with those published, and these compounds were chromatographically pure monitored by analytical HPLC with a chiral column.9,10) (−)-END (1b) was prepared by anaerobic incubation of (−)-seciosolariciresinol 4-ethyl ether (6b) with a human intestinal bacterial (HIB) mixture. (−)-END (1a) was obtained from SDG by incubation with an HIB mixture.10) A racemic mixture of ENL (2a + 2b) was purchased from Cayman Chemical Company (Ann Arbor, Michigan, U.S.A.). (+)-DHENL (4a) was prepared from styrraxlignolides C, D and E17) as follows: a bacterial suspension (10 ml) of E. sp. ARC-215) was inoculated to 200 ml of GAM broth containing styrraxlignolides C (7 mg), D (7 mg) and E (6 mg), and incubated at 37 °C in an anaerobic incubator for 48 h. The reaction mixture was then extracted three times with 200 ml of ethyl acetate. The organic layer was evaporated under reduced pressure to give a residue (76 mg). The residue was applied to a column of silica gel, which was then eluted with a solvent system of CHCl3–MeOH (30:1) to yield (−)-DHENL (4a, 4 mg): amorphous powder. 1H-NMR (CD3OD, 300 MHz): δ 2.32—2.66 (4H, m, H-2, 3, 7°), 2.81 (2H, t, J=5.7 Hz, H-7°), 3.86 (1H, t, J=8.3 Hz, H4-4), 4.04 (1H, t, J=8.3 Hz, H5-4), 6.39 (1H, dd, J=2.1, 7.7 Hz, H6°), 6.47—6.52 (2H, m, H-2°, 6°), 6.64—6.71 (3H, m, H-2°, 3°, 5°). EIMS m/z: 330 [M]+. [α]D23 +32.2° (ε=0.180, MeOH). (−)-Hydroxyenterol (HEND) (5b) was prepared from (−)-DHEND (3b) as follows: two milliliters of precultured strain ARC-1 was inoculated to 300 ml of GAM broth containing (−)-DHEND (3b, 30 mg) and arginine-HCl (0.06%), and anaerobically incubated for 36 h at 37 °C. The reaction mixture was then extracted three times with 300 ml of ethyl acetate. The organic layer was evaporated under reduced pressure to give a residue (76 mg). The residue was applied to a column of silica gel, which was then eluted with a solvent system of CHCl3–MeOH (20:1) to yield (−)-HEND (5b), 7 mg) [α]D24−12.8° (ε=0.447, MeOH).

LC/MS Analysis HPLC/MS was performed on an Agilent 1100 system (Agilent Technologies, Waldbronn, Germany) with a photodiode array detector and an Agilent 1100 series binary pump, and an Esquire 3000plus mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) coupled with an ESI interface and an ion trap mass analyzer, under the following conditions: column, chiral CD-Ph (Shiseido, Tokyo, 4.6 mm ×250 mm); mobile phase, 0.1% TFA (solvent system A) and CH3CN (solvent system B) in gradient modes [(−)-DHEND (3a), (−)-DHEND (3b), (−)-END (1a) and (−)-END (1b) analysis, B from 25 to 38% for 39 min, (+)-DHENL (4a), (−)-DHENL (4b), (+)-END (2a) and (−)-ENL (2b) analysis, B from 25 to 37% in 30 min, B from 37 to 45% from 30 to 46 min]; flow rate, 0.5 ml/min; detection, UV at 280 nm; temperature, 30 °C. High-purity nitrogen was used as dry gas at a flow rate at 10 l/min, temperature at 360 °C. Helium was used as nebulizer at 50 psi. The ESI interface and mass spectrometric parameter were optimized to obtain maximum sensitivity.

Sequencing of the Bacterial 16S rRNA Gene A 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, Hilden, Germany) following the manufacturer’s protocol. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) with three forward and reverse primer sets based on those of various strains from the database: Bac 1F (AGAGTTTTGATCCTGCGTCAG) and Bac 1R (CCTACTACGGGAGGTGCTG); Bac 3F (TAATACGACTCACTATAGGG) and Bac 3R (CCCTGAGAAGTCGACTAG); and Bac 6F (CAGACTCTACGGGAGGCAG) and Bac 6R (CAGGTTACACTAATCTGTTCC). Amplification proceeded in a reaction mixture containing 1 U of KOD-Plus DNA polymerase (Toyobo, Osaka), 1×PCR buffer mix, 0.8 mM dNTP mix (0.2 mM each), 1 mM MgSO4, 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, Hilde, Germany), and directly sequenced using a Bigdye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster city, CA, U.S.A.) with primers (Bac 1F, Bac 1R, Bac 3F, Bac 3R, Bac 6F, Bac 6R) and an ABI PRISM 310 (Applied Biosystems, Foster city, CA, U.S.A.).
A bacterial suspension from fresh feces of a healthy volunteer was repeatedly cultured in 2 ml of GAM broth containing 0.2 mM (+)-DHEND (3b) at 37 °C in an anaerobic incubator. A portion of the culture, possessing metabolic activity, was seeded on GAM and BL agar plates, and anaerobically incubated for 48 h at 37 °C. Colonies were separately picked up and screened for the activity of transforming (+)-DHEND (3b) to (+)-END (1b) by thin layer chromatography. Such a procedure was repeated until a single strain was isolated.

Incubation of DHEND or DHENL with a Bacterium, and Sample Preparation for HPLC

A 100 ml portion of precultured Eg. sp. SDG-2 or strain ARC-1 was inoculated to 2 ml of GAM broth containing 0.1% L(+)-arginine hydrochloride with 0.2 mM (+)-DHEND (3a) and (+)-DHEND (3b) and the mixture was anaerobically incubated at 37 °C. (+)-DHENL (4a) and (+)-DHENL (4b) were incubated in a similar way. A 100 μl aliquot was taken out and extracted three times with 100 μl of 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 chiral HPLC analysis under the conditions described above.

RESULTS

Isolation of a Bacterial Strain Capable of Transforming (+)-DHEND (3b) to (+)-END (1b) from a Human Fecal Suspension

A single bacterium (strain ARC-1) was isolated by repeated incubation of a bacterial mixture of human feces in GAM broth containing 0.2 mM (+)-DHEND (3b) after screening of colonies on GAM and BL agar plates. Strain ARC-1 was strictly anaerobic, Gram-positive, and regular rods. The growth of strain ARC-1 in GAM broth was very poor, but remarkable proliferation was observed by the addition of 1% of arginine-HCl (data not shown).

Identification of Strains SDG-2 and ARC-1 by 16S rRNA Gene Analysis

The 16S rRNA gene sequence of strain SDG-2, which was previously assigned as E. lentum (syn. Eg. lenta) or a closely related bacterium, (9) showed only 79% and 78% similarity with those of E. limosum (Genbank accession no. M59120) and E. callenderi (X96961), respectively (Fig. 2A). In the present experiment on the 16S rRNA gene, we found that the 16S rRNA gene sequence of the strain SDG-2 had 99% similarity with that of Eg. lenta (AF292375) and 97% similarity with that of Eg. MLG043 (AF304434). Furthermore, similarity in sequence with those of other Eg. species was over 90% showing that the strain may belong to Eg. species in 16S rRNA gene sequence, and strain SDG-2 was tentatively named Eg. sp. SDG-2 (EF413638) since E. lentum was reclassified into Eg. lenta.

Strain ARC-1 (EF413639), newly isolated in the present experiment, showed 95% similarity with Denitrobacterium CCUG45665 (AJ518870) in 16S rRNA gene sequence (Fig. 2B). Moreover, the 16S rRNA gene sequence of strain ARC-1 had 92% similarity with that of Eg. sp. SDG-2.

Bacterial Growth and Kinetics in Transformation of (+)-DHEND (3b) to (+)-END (1b) by Strain ARC-1

Figure 3 shows the kinetics in transformation of (+)-DHEND (3b) to (+)-END (1b) by strain ARC-1. Strain ARC-1 (EF413639), newly isolated in the present experiment, showed 95% similarity with Denitrobacterium CCUG45665 (AJ518870) in 16S rRNA gene sequence (Fig. 2B). Moreover, the 16S rRNA gene sequence of strain ARC-1 had 92% similarity with that of Eg. sp. SDG-2.
(5b) was observed 36 h after the start of incubation, and then the amount was gradually decreased, accompanied by a gradual increase in the amount of (−)-END (1b). (−)-END (1b) reached the maximum concentration at 51 h.

**Enantioselective Biotransformation of DHENL by *Eg. sp. SDG-2 or Strain ARC-1***

In the elution profile of analytical HPLC with a chiral column, a pair of enantiomers showed different retention times, but same mass spectral patterns, such as either (+)- and (−)-DHENL (4a, b) or (+)- and (−)-ENL (2a, b) in the ESI-MS spectra (Fig. 4). The retention times of (−)-DHENL (4b) and (+)-DHENL (4a) were 23.0 and 23.5 min, respectively, while those of (−)-ENL (2b) and (+)-ENL (2a) were 41.5 and 42.7 min, respectively. When incubated with a mixture of (+)- and (−)-DHENL (4a, b), *Eg. sp. SDG-2 converted (−)-DHENL (4b) to (−)-ENL (2b), but not for (+)-DHENL (4a) (Fig. 6). Similarly, by independent incubation of either (+)-DHENL (4a) or (−)-DHENL (4b), this bacterium converted only the latter to (−)-ENL (2b) (data not shown). On the other hand, when incubated with a mixture of (+)- and (−)-DHENL (4a, b), strain ARC-1 converted (+)-DHENL (4a) to (+)-ENL (2a) but not for (−)-DHENL (4b). The substrate specificity of this bacterium was further demonstrated by independent incubation with (+)-DHENL (4a) or (−)-DHENL (4b).

**Enantioselective Biotransformation of DHEND by *Eg. sp. SDG-2 or Strain ARC-1***

Similarly, retention times of
(+)-DHEND (3a), (−)-DHEND (3b), (+)-END (1a) and (−)-END (1b) were 13.8, 14.4, 30.9 and 34.4 min, respectively, when the compounds were analyzed by HPLC with a chiral column under the conditions described above (Fig. 5). When incubated with a mixture of (+)- and (−)-DHEND (3a, b), Eg. sp. SDG-2 converted (+)-DHEND (3a) to (+)-END (1a) (Fig. 7). Similarly, the substrate specificity was confirmed by independent incubation of the two enantiomers. On the other hand, strain ARC-1 converted only (−)-DHEND (3b) to (−)-END (1b) but not (+)-DHEND (3a). This was further confirmed by independent incubation of the two enantiomers. The bacterium converted only (−)-DHEND to (−)-END in contrast with Eg. sp. SDG-2 (data not shown).

DISCUSSION

Eggerthella (Eg.) sp. SDG-2 was isolated from a human
fetal suspension as a bacterium capable of dehydroxylation in the transformation of SDG to ENL. This bacterium was previously named Eurobacterium (E.) sp. strain SDG-2, due to the morphological and biochemical similarity to E. lenta (syn. *E. lenta* according to the new classification of bacteria). In the present study, we confirmed that the isolate was closely related to *E. lenta* (ATCC25559) on the basis of 16S rRNA gene sequencing, and that both strains had the same dehydroxylation activity for precursors of END and ENL.

PDG is a major antihypertensive compound of the bark of *Eucommia ulmoides* Oliv. [18] This compound was converted to (−)-ENL (2b) by human intestinal flora via (−)-SECO as a metabolic intermediate. [9] On the other hands, SDG from flaxseed was transformed to (+)-ENL (2a) via (+)-SECO. [10] Since naturally-occurring lignans possess optical properties in the most of cases, we investigated the metabolism of a variety of optical isomers by human intestinal bacteria.

In our course of studies on enantioselective biotransformation, we isolated two intestinal bacteria capable of oxidizing END to ENL. [19] *Ruminococcus* sp. END-1 converted (−)-END, but not (−)-END. On the other hands, strain END-2 converted only (−)-END to (−)-ENL, selectively. We also reported *E. sp.* ARC-2, capable of demethylating (−)-arctigenin to (−)-DHEND. [15] However, *E. sp.* ARC-2 could convert both (−)-secoisolariciresinol and (−)-secoisolariciresinol 4′-methyl ether to (−)-DHEND and (−)-DHENL, respectively, without enantioselectivity.

For investigation of enantioselective dehydroxylation in this study, we prepared four compounds, (±)(2S,3S)-DHENL (4a), (±)(2R,3R)-DHENL (4b), (±)(2S,3S)-DHEND (3a) and (±)(2R,3R)-DHEND (3b) from appropriate natural products by chemical and bacterial transformations. Only (−)-DHEND (4b) and (+)-DHEND (3a) were transformed to (−)-ENL (2b) and (+)-END (1a), respectively, by *E. sp.* SDG-2. That is, dehydroxylation by *E. sp.* SDG-2 proceeded for a (−)-form of the lactone compounds and a (+)-form of the diol compounds. On the other hand, (−)-pinoresinol was transformed to (−)-SECO (7b), and then to (−)-END (1b) by human intestinal flora. [9] Since *E. sp.* SDG-2 could not participate in these transformations based on our experiments, we tried to isolate a new bacterium having activity for transforming the respective enantiomeric substrates. A strain ARC-1 isolated from human feces, had ability to convert (−)-DHEND (4a) and (−)-DHEND (3b) to the corresponding dehydroxylated derivatives in the lactone and diol types, respectively. Table 1 shows enantioselective dehydroxylation of two bacteria, *E. sp.* SDG-2 and strain ARC-1.

Since strain ARC-1 has a metabolic activity to convert (−)-DHEND (3b) to (−)-END (1b), it may take part in the metabolic processes of (−)-SECO (7b). However, oxidized lactone type compounds such as (−)-DHENL (4b) were isolated during transformation of (−)-SECO (7b) by an intestinal bacterial suspension. [9] Once (−)-DHEND (3b) is oxidized to (−)-DHENL (4b), strain ARC-1 cannot take part in dehydroxylation. In the metabolic processes of (±)-SECO (5a) to (±)-ENL (2a), conversely, *E. sp.* SDG-2 (*E. lenta*) may not participate in dehydroxylation of (±)-DHENL (4a) but bacteria, such as strain ARC-1 may be responsible for dehydroxylation.

END and ENL are transformed from plant lignans by anaerobic bacteria in the intestinal tract and absorbed into the body fluid. Several biological activities like estrogenic and antiestrogenic activities, [20–22] antioxidant activity, [23] aromatase inhibition [24] as well as endogenous digitalis-like activity [25] were reported up to date. However, most of the researches have not been considered the stereochemistry of END and ENL. Enantioselective recognition of several chiral compounds by estrogen receptors has been reported. [26–28] From this viewpoint, chiral characteristics of END and ENL must be considered in the experiments of biological activities.

In a preparative purpose, both bacteria, *E. sp.* SDG-2 and strain ARC-1, can be used for preparation of (±)- or (−)-form of END and ENL from the respective racemic precursors. Saarinen et al. [29] confirmed that the absolute configurations at C-2 and C-3 of END and ENL were not changed during the microbial metabolism, as a result of the enantioselective dehydroxylation by intestinal bacteria, and we succeeded here to isolate two different bacteria responsible for enantioselective dehydroxylation. In connection with the metabolic processes of lignans to END and ENL, we already reported the isolation and characterization of a human intestinal bacterium, *E. sp.* ARC-2, capable of demethylating (−)-arctigenin to (−)-DHEND (4b). [15] This bacterium converted (±)-SECO (5a) and (−)-secoisolariciresinol 4′-methyl ether (6b) to (±)-DHEND (3a) and (−)-DHEND (3b), respectively, but the reaction proceeded without enantioselectivity.

Individual variations in human intestinal flora cannot be disregarded in the metabolism of plant lignans. [30] Individual diversity of END- and ENL-producing abilities may arise from not only numbers of the responsible bacteria, but also composition of intestinal bacterial species. The symbiotic and/or competitive relationship among bacteria, such as *E. sp.* SDG-2, strain ARC-1 and *E. sp.* ARC-2, seems to be extremely important to understanding the real metabolic processes of lignans to estrogenic substances in humans.

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### References


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**Table 1. Enantioselective Dehydroxylation by *Eg. sp.* SDG-2 and Strain ARC-1**

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Substrate</th>
<th>DHEND</th>
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<td></td>
<td>(+) 3a</td>
<td>(−) 3b</td>
<td>(+) 4a</td>
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<td><em>Eg. sp.</em> SDG-2</td>
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