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NOTES

Production of Equol from Daidzein by Gram-Positive Rod-Shaped Bacterium Isolated from Rat Intestine

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Isoflavones (mainly daidzein and genistin) belong to the flavonoid group of compounds and are classified as phytoestrogens. In the intestine, daidzin is converted to daidzein by β-glucosidase, and then daidzein is converted to *O*-desmethylangolensin (*O*-DMA) or equol via dihydrodaidzein by enzymes of intestinal bacteria. We isolated, for the first time, an anaerobic gram-positive rod-shaped strain capable of producing equol from daidzein. Its 16S rDNA gene sequence (1428 bp) showed 99% similarity with that of the human intestinal bacterium SNU-Julong 732 (AY310748) and 93% similarity with that of *Eggerthella lenta* ATCC 25559^{T} (AF292375). This strain converted daidzein to equol via dihydrodaidzein in an equol-assay medium anaerobically. The addition of butyric acid and arginine increased the conversion ratio of daidzein to equol 4.7- and 4.5-fold, respectively.

Isoflavones are flavonoids present in various plants, particularly in soybean germ. They are classified as phytoestrogens because their structures resemble that of estrogen and they have a weak affinity for the estrogen receptor. Epidemiologic and experimental studies showed that they had preventive effects on breast cancer, prostate cancer, cardiovascular disease, osteoporosis and menopausal symptoms (1, 2). Isoflavones exist as glycosides in some plants, mainly as daidzin and genistin. In the intestine, daidzin is converted to daidzein by β-glucosidase. Then, daidzein is converted via dihydrodaidzein to *O*-desmethylangolensin (*O*-DMA) or equol by enzymes of intestinal bacteria (3; Fig. 1). Equol has a stronger estrogenic activity than daidzein and *O*-DMA (4, 5). Humans capable of producing equol from daidzein (equol producers) have a lower risk of developing breast and prostate cancers than non-equol producers (6, 7). Several animals, particularly rodents, produce adequate concentrations of equol. However, in humans, only 30-50% of the population can produce equol owing to differences in intestinal microbiota among individuals (8, 9). Daidzein-metabolizing phenotypes are stable in individuals over time (7) because the intestinal microbiota of such individuals are stable. Therefore, non-equol producers excrete no equol, even when they ingest soy protein powder (34 g/d) for one month (10).

There are few reports on daidzein-metabolizing intestinal bacteria. An anaerobic

gram-positive strain HGH 6 that converts daidzein to dihydrodaidzein (11), and a *Clostridium* sp. strain HGH 136 (12) and *Eubacterium ramulus* (13) that converts daidzein to *O*-DMA were isolated from humans. The human intestinal bacterium SNU-Julong 732 that converts dihydrodaidzein to equol was also isolated from humans (14). A mixture of *Bacteroides ovatus*, *Ruminococcus productus* and *Streptococcus intermedius* (15) or *Lactobacillus mucosae*, *Enterococcus faecium*, *Finegoldia magna* and *Veillonella* sp. (16) produces equol from daidzein. However, no bacterium that produces equol from daidzein alone (an equol-producing bacterium) has yet been found. Therefore, we intended to isolate an equol-producing bacterium from rat cecal contents, because rats are good producers of equol.

Standards for daidzein and equol were purchased from LC Laboratories (Woburn, MA, USA). Dihydrodaidzein was purchased from Toronto Research Chemicals. (North York, ON, Canada). The equol-assay medium contained 29.5 g of GAM broth (Nissui Pharmaceutical, Tokyo), 10 g of $CaCO_3$ and 2 g of Fujiflavone P10 (Fujicco, Kobe) per liter of distilled water. After autoclaving, the medium was stored in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA) under an 85% N_2, 10% CO_2 and 5% H_2 atmosphere. A frozen glycerol stock of the cecal content of a male Sprague-Dawley rat (SLC Japan, Tokyo) fed a casein diet for 3 weeks according to the

AIN-93G formulation (17) was added to the equol-assay medium and incubated anaerobically at 37°C for 2 d. The culture broth was spread on a plate containing 14.75 g of GAM broth, 2 g of Fujiflavone P10 and 15 g of agar per liter of distilled water, and then incubated anaerobically at 37°C for 2 d. A number of colonies that developed on the plate were selected and inoculated into the equol-assay medium, incubated anaerobically at 37°C for 2 d, extracted and analyzed by HPLC as described below. After isolating an equol-producing bacterium, a precultured GAM broth containing 1% L-arginine at 37°C for 28 h was added to an equol-assay medium for quantitative determination containing 59 g of GAM broth and daidzein (final concentration: 200 μM) per liter of distilled water. Then, the medium was incubated anaerobically at 37°C, extracted and analyzed by HPLC as described below. To investigate the effects of arginine and butyric acid on equol production, 1% arginine and/or butyric acid (final concentration: 50 mM) was added to the equol-assay medium (arginine, before autoclaving; butyric acid, after autoclaving) and the resulting solutions were analyzed similarly. The pH of the medium containing 1% arginine was adjusted to 7.0 before autoclaving, whereas the pH of the arginine-free medium was not adjusted (approximately pH 7.0). Absorbance at 600 nm (OD_{600}) was measured using Spectronic 20D+ spectrophotometer (Thermo Electron, Waltham, MA, USA) and culture broth pH

was measured using an ISFET pH meter KS-701 (Shindengen Electric, Tokyo).

Aliquots of the assay media were extracted three times with ethyl acetate of 1.5 volume of the media and evaporated using a rotary evaporator. Then, the aliquots were dissolved in methanol and filtered using a 0.45-μm filter (Millex-LH; Millipore, Tokyo). Each sample was injected into HPLC (Jasco, Tokyo) equipped with a Mightysil RP-18 GP 250-3.0 column (3.0×250 mm; 3 μm; Kanto Chemical; Tokyo) and a UV detector (280 nm; Jasco). The mobile phase was a solution of water : acetonitrile : acetic acid, 75 : 25 : 0.1 (V/V/V), the flow rate was 0.4 ml/min and the column temperature was 60°C. Metabolites were identified by comparing their retention times with those of standards.

Cell morphology after anaerobic cultivation for 2 d at 37 ^{o}C in GAM broth was examined using phase-contrast microscopy (ECLIPSE E600; Nikon, Tokyo). The Gram straining solution used was neo-B&M Wako (Wako, Osaka). The isolated bacterium was identified by 16S rDNA gene sequence analysis (18). Homology searches were performed in the GenBank database using the BLAST search program. Some 16S rDNA sequences were retrieved from the DDBJ, EMBL and GenBank databases for comparison in the phylogenetic analysis. Sequence data were aligned with the CLUSTAL X package program and corrected by manual inspection. Nucleotide

substitution rates were calculated, and a phylogenetic tree was constructed using the neighbor-joining method.

An anaerobic gram-positive rod-shaped strain capable of producing equol was isolated from a rat cecal content. This strain is referred to as the Gram-positive bacterium do03 (AB266102). Its 16S rDNA gene sequence (1428 bp) showed 99% similarity with the human intestinal bacterium SNU-Julong 732 (AY310748), 94% similarity with *Eggerthella sinensis* HKU14 (AY321958), 94% similarity with *Eggerthella hongkongenesis* HKU10 (AY288517) and 93% similarity with *Eggerthella lenta* ATCC 25559^{T} (AF292375). The phylogenetic tree showed that the isolated strain does not belong to the genus *Eggerthella* (Fig. 2). The strains do03 and Julong 732 occupy the same cluster. Therefore, these strains may belong to a new genus. Moreover, the strain Julong 732 was isolated from a fecal sample of a healthy female human and the strain do03 was isolated from a rat cecal content. Hence, these strains are indigenous intestinal bacteria.

The strain do03 converted 200 μM daidzein to equol via dihydrodaidzein for 4 d at 37 ^{o}C anaerobically (Fig. 3). For 2 and 4 d, distinct peaks were observed at 4 min. There have been no reports on equol being metabolized by intestinal bacteria. Despite the fact that different amounts of equol were produced, the areas of the peaks were about the

same (data not shown). Therefore, these peaks do not correspond to equol split products but to other metabolic products.

In GAM broth (control), the conversion ratio of daidzein to equol (equol ratio: amount of equol production/amount of supplemented daizein) was 0.15±0.01. In the medium containing butyric acid, the equol ratio increased to 0.71±0.03, although OD_{600} and culture broth pH did not change compared with those of the control. In the medium containing arginine, the equol ratio increased to 0.67±0.01 with increases in OD_{600} and culture broth pH. In the medium containing butyric acid and arginine, the equol ratio increased to 0.58±0.01 with a slight increase in culture broth pH (Table 1). Because butyric acid stimulates equol production (16), the equol ratio in the medium containing butyric acid was considered to have increased. Moreover, for the growth of some bacteria such as *E. lentum,* arginine is required because they obtain energy for growth using the arginine dihydrolase pathway (19). The bacterial metabolism of arginine produces NH_3, which caused the increase in culture broth pH. Arginine supplementation increased OD_{600}; thus, the strain do03 uses arginine for growth. Therefore, the increase in equol ratio may be attributed to an increase in the number of do03 cells. The supplementation of butyric acid and arginine decreased the equol ratio by approximately 10%. Because culture broth pH increased more than that of the

control, the strain do03 seemed to use arginine; however, OD_{600} did not increase. Butyric acid supplementation caused a decrease in OD_{600}. The mechanism of equol production stimulated by butyric acid supplementation has not yet been reported. Antagonist action seemed to occur by the supplementation of butyric acid and arginine.

In the human intestine, when the strains HGH 6 and Julong 732 are present, the microbial community is able to produce equol from daidzein (16). However, the strain do03 converted daidzein to equol via dihydrodaidzein without any other strains (Fig. 3).

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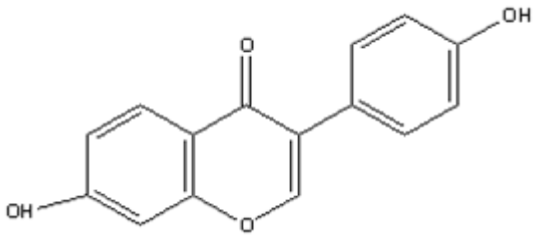
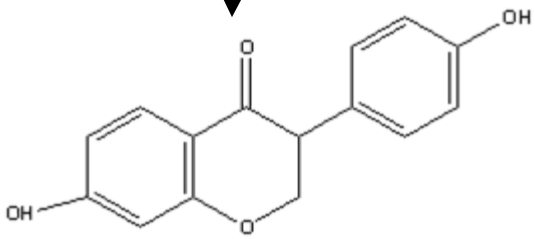
FIG. 1. Metabolism of daidzein to equol in intestine.

FIG. 2. 16S rDNA-based phylogenetic tree showing relationship between newly isolated strain do03 and other closely related species. The numbers are bootstrap values calculated from 1000 trees. GenBank accession numbers are shown in parentheses.

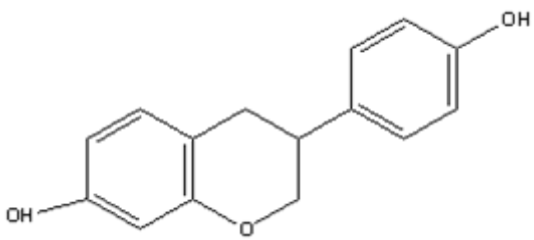
FIG. 3. HPLC elution profiles of the supernatant of daidzein conversion by newly isolated strain do03 at 0, 2 and 4 d under anaerobic condition in equol-assay medium containing 50 mM butyric acid. The concentration of daidzein was 192.7 μM at 0 d; those of daidzein, dihydrodaidzein and equol were 62.3, 45.3 and 63.7 μM, respectively, at 2 d; and that of equol was 138.7 μM at 4 d.



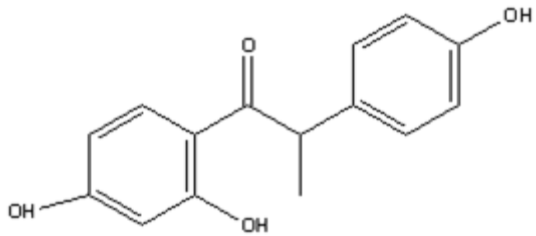
Daidzein



Dihydrodaidzein



Equol



O-Desmethylangolensin

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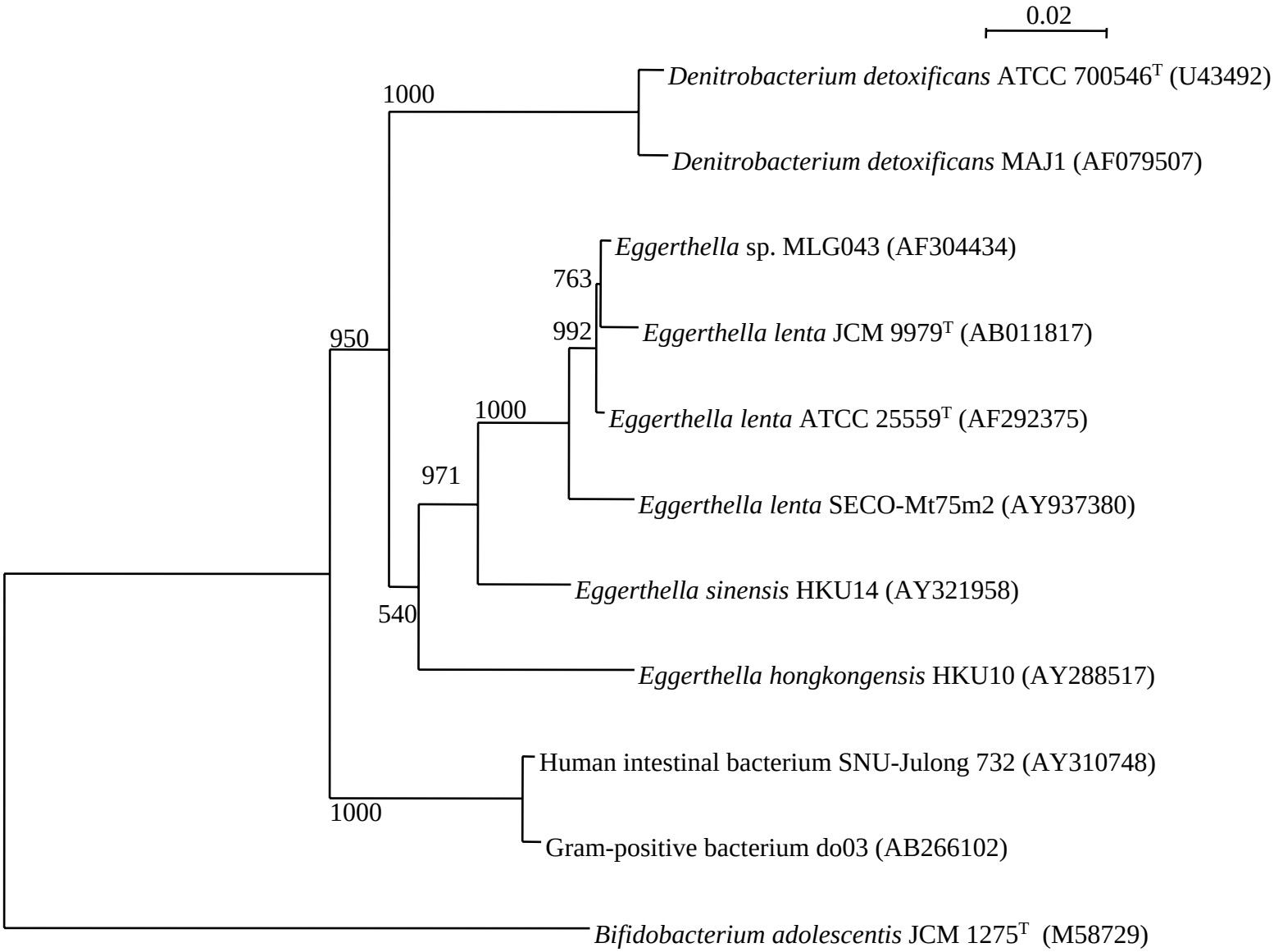

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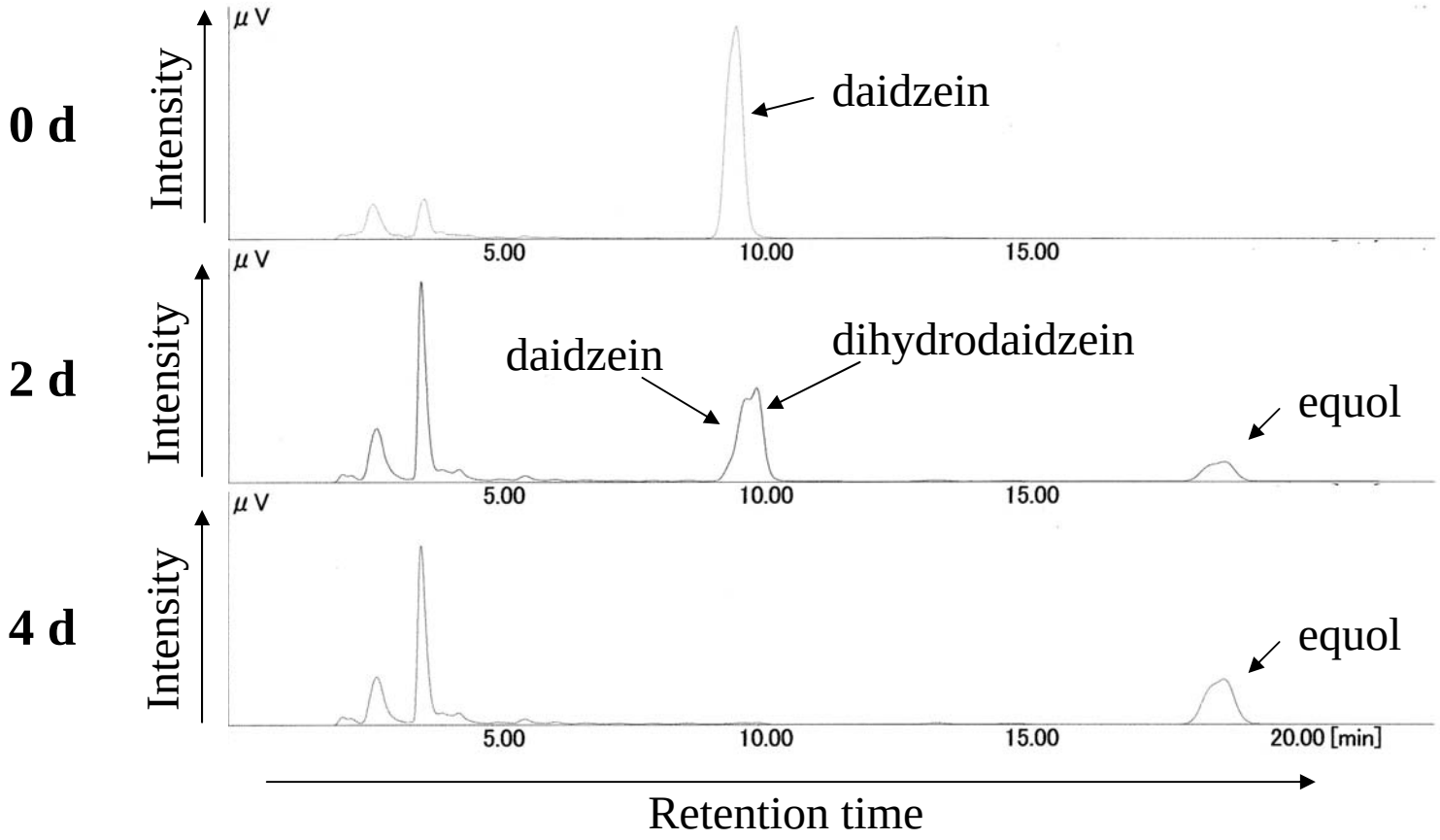

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TABLE 1. Effects of arginine and butyric acid on equol production

	GAM (control)	GAM + butyric acid	GAM+ arginine	GAM + butyric acid and arginine
Conversion ratio of daidzein to equol	0.15±0.01	0.71±0.03	0.67±0.01	0.58±0.01
OD_{600}	0.277±0.005	0.222±0.003	0.431±0.004	0.203±0.004
pH	7.2	7.2	7.9	7.5

Values are means±SD.