Dietary isoflavones such as puerarin and daidzein have been extensively investigated because of their potential health benefits.1—7) Puerarin, isoflavone C-glucoside, is isolated from Pueraria thunbergiana BENTH. abundantly. It was reported that puerarin was metabolized to daidzein by human intestinal flora, and that this compound exhibited more potent antioxidant, cytotoxic, and estrogenic effects8,9) than the parent compound. Daidzein occurs in high contents in leguminous plants such as soybeans. After ingestion from diet, it is converted by intestinal bacteria to O-desmethylangolensin and equol as end products (Fig. 1).10,11) Equol has recently aroused interest particularly because of its biological activities such as estrogenic properties, having affinity for both estrogen receptors, ERα and ERβ,12) and strong antioxidant activity.13) Moreover, equol is easily absorbed through the colon wall, but has a slower plasma clearance rate than daidzein, and is metabolically inert.12) However, only 30—50% of individuals in the general population are capable of producing equol from daidzein.14,15) Since equol is not of plant origin and is an exclusive product of the intestinal bacterial metabolism from isoflavones, its production may depend on the existence of bacterial species capable of metabolizing daidzein.12,16) Thus there have been several attempts to isolate bacterial species involved in degradation of daidzein.

Clostridium sp. strain HGH617) and Lactobacillus sp. Niu-O16,18) capable of reducing daidzein to dihydrodaidzein, were isolated from bovine rumen and human feces, respectively. However, these bacteria did not convert dihydrodaidzein to equol. Wang et al. isolated Eggerthella sp. Julong 732, capable of converting dihydrodaidzein to equol, from human feces,19) and transformation of daidzein to equol was recently reported by using a mixed culture of two strains, Eggerthella sp. Julong 732 and Lactobacillus sp. Niu-O16.20,21) Moreover, a Gram positive rod-shaped bacterium and Lactococcus 20—92, capable of converting daidzein to equol, were isolated from rat intestine and human feces, respectively.20,21)

In the present study, we attempted isolation of a single equol-producing bacterium from human feces using daidzein as a substrate. Furthermore, we tried to isolate a bacterium capable of transforming puerarin to daidzein, and examined production of equol from puerarin by a mixed culture of two isolated bacteria.

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 by DIP-360 automatic polarimeter (Jasco Co., Tokyo, Japan), CD spectra by JASCO J 805 spectropolarimeter (Jasco Co.), UV spectra by UV-2200 UV-VIS recording spectrophotometer (Shimadzu Co., Kyoto, Japan), melting points by Yanagimoto micro hot-stage melting point apparatus (Yanagimoto Co., Tokyo, Japan), and electron impact mass spectra (EI-MS) by JMS-GC mate mass spectrom-
eter 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 (HMOC), and heteronuclear multiple bond coherence (HMBC) were taken on Varian UNITY 500 ($^{1}$H: 500 MHz, $^{13}$C: 125 MHz) and Varian Gemini 300 ($^{1}$H: 300 MHz, $^{13}$C: 75 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 exposing I$_{2}$ vapor. Silica gel BW-820 MH (Fuji Silysis, Aichi, Japan) was used for column chromatography. The DNA sequences were aligned using DNASIS version 3.0 (Hitachi Software Engineering Co., Tokyo, Japan).

### Chemicals and Media
General anaerobic medium (GAM) was purchased from Nissui Co. (Tokyo, Japan). Puerarin was isolated from Pueraria lobata (Willd.) Ohwi (Tochimoto Tenkaido Co., Osaka, Japan). Daidzein and genistein were purchased from TCI (Tokyo, Japan). Dihydrodaidzein and equol were prepared by modified methods of Wang et al.$^{19}$ from daidzein.

### Bacterial Incubation and Sample Preparation for HPLC
Each bacterium was picked up from GAM agar plates and inoculated into 2 ml of GAM broth. When turbidity (O.D.) (strain PUE) and 0.30 vacuo MeOH solution was filtered through a 0.2-μm filter. Colonies were re-peatedly screened for their activity of transforming puerarin. Such a procedure was repeated until a single puerarin-metabolizing strain was isolated.

### Isolation of a Human Intestinal Bacterial Strain Capable of Converting Daidzein to Equol
Simi-larly, a bacterial suspension was repeatedly cultured in 2 ml of GAM broth containing 0.1 mm daidzein 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 at 37°C for 72 h. Colonies were repeatedly screened for their activity of transforming daidzein to obtain a single daidzein-metabolizing bacterium.

### Sequencing the Bacterial 16S ribosomal RNA (rRNA) Gene
The bacteria were 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, Hilde, 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 (AGAGTTTTGATCCTGCTCAG) and Bac 1R (CCGTATTACCGCCGCTGCTG); and Bac 3F (TAACATACTGACCAGCGCC) and Bac 3R (CCCCGGGACGTA- TTCACC). 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), 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, and Bac 3R) and an ABI PRISM 310 (Applied Biosystems).

### Preparation of Equol from Puerarin by a Mixed Culture of Two Anaerobic Bacteria
Each precultured bacterial suspension (5 ml) containing strains PUE or DZE was inoculated to 11 of GAM broth containing puerarin (300 mg) and incubated at 37°C in an anaerobic incubator for 114 h. The reaction mixture was then extracted three times with 400 ml of ethyl acetate. The organic layer was evaporated under reduced pressure to give a residue (382 mg). The residue was applied to a column of silica gel, which was eluted with a solvent system, CHCl$_{3}$–MeOH (20 : 1 (v/v)).

### Isolation of a Human Intestinal Bacterial Strain Capable of Converting Puerarin to Daidzein
A bacterial suspension from fresh feces of a healthy volunteer was repeatedly cultured in 2 ml of GAM broth containing 0.5 mm puerarin 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 at 37°C for 24 h. Colonies were picked up and screened for their activity of transforming puerarin. Such a procedure was repeated until a single puerarin-metabolizing strain was isolated.

### Isolation of a Human Intestinal Bacterial Strain Capable of Converting Daidzein to Equol
Similarly, a bacterial suspension was repeatedly cultured in 2 ml of GAM broth containing 0.1 mm daidzein 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 at 37°C for 72 h. Colonies were repeatedly screened for their activity of transforming daidzein to obtain a single daidzein-metabolizing bacterium.

### Sequencing the Bacterial 16S ribosomal RNA (rRNA) Gene
The bacteria were 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, Hilde, 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 (AGAGTTTTGATCCTGCTCAG) and Bac 1R (CCGTATTACCGCCGCTGCTG); and Bac 3F (TAACATACTGACCAGCGCC) and Bac 3R (CCCCGGGACGTA-TTCACC). 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), 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, and Bac 3R) and an ABI PRISM 310 (Applied Biosystems).

### Preparation of Equol from Puerarin by a Mixed Culture of Two Anaerobic Bacteria
Each precultured bacterial suspension (5 ml) containing strains PUE or DZE was inoculated to 11 of GAM broth containing puerarin (300 mg) and incubated at 37°C in an anaerobic incubator for 114 h. The reaction mixture was then extracted three times with 400 ml of ethyl acetate. The organic layer was evaporated under reduced pressure to give a residue (382 mg). The residue was applied to a column of silica gel, which was eluted with a solvent system, CHCl$_{3}$–MeOH (20 : 1 → 5 : 1 → 10 : 1), to give fractions A–D. (3S)-Equol (63 mg) was obtained with fraction A by crystallization from CHCl$_{3}$. Purity of the isolated compound was checked by HPLC under the aforementioned conditions.

(3S)-Equol: Colorless prism, mp 189.5—191.5°C EI-MS $m/z$: 242 [M$^{+}$]. [α]$^{D}_{D}$ = −18.5° (c = 0.252, MeOH). This compound was identified by comparing the $^{1}$H- and $^{13}$C-NMR spectra, mass spectra, and [α]$^{D}$ value with those published.$^{19}$

### Preparation of 5-Hydroxyequol by Strain DZE
A bacterial suspension (50 ml) of strain DZE was inoculated to 0.81 GAM broth containing genistein (50 mg) and incubated at 37°C in an anaerobic incubator for 120 h. The reaction mixture was then extracted three times with ethyl acetate. The organic layer was evaporated under reduced pressure to give a residue. The residue was applied to a column of silica
gel, which was eluted with a solvent system, CHCl₃–MeOH (20:1), to give 5-hydroxyequol (24 mg, 50% in yield).

5-Hydroxyequol: Amorphous powder. EI-MS m/z: 258 [M⁺], [α]D²³ = -9.0° (c=0.256, MeOH). This compound was identified by comparing the ¹H-NMR spectrum with that published.²¹

RESULTS

Isolation of a Human Intestinal Bacterial Strain Capable of Converting Puerarin to Daidzein  A single bacterium (Strain PUE) was isolated through repeated culture of a bacterial mixture of human feces in GAM broth containing 0.5 mM puerarin. Strain PUE was strictly anaerobic. High viscosity of the colony on GAM agar plates was observed. The 16S rRNA gene sequence of strain PUE (Genbank accession no. EU377662) showed 97% similarity with that of uncultured bacterium N404 (AY975239) (Fig. 2A). Strain PUE had 92% similarity with Ruminococcus gnavus (X94967) and Dorea longicatena (AJ132842). Biotransformation of puerarin to daidzein by strain PUE was confirmed by LC/MS analysis. The retention time of puerarin, daidzein, and equol was 4.8, 12.9, and 16.6 min, respectively. When incubated with strain PUE, puerarin was converted to daidzein (Fig. 3A). Because of low solubility in water, precipitations of daidzein appeared during the incubation. When puerarin (200 µM) was anaerobically incubated with strain PUE for 120 h, 189.7±22.1 µM (mean±S.D.) of daidzein was produced (94.9% in yield).

Isolation of a Human Intestinal Bacterial Strain Capable of Converting Daidzein to Equol  Through repeated incubation, a single bacterium (strain DZE) having daidzein-metabolizing activity was isolated. The growth of strain DZE in GAM broth was very poor, but appreciably increased upon addition of 1% arginine-HCl. The 16S rRNA gene sequence of strain DZE (EU377663) had 89% and 88% similarity with Slackia faecicanis sp. CCUG 48399 (AJ608686) and Slackia exigua (AF101240), respectively (Fig. 2B). Moreover, strain DZE showed 87% similarity with Slackia heliotrinireducens (AF101241). Metabolic activity of strain DZE was confirmed by LC/MS analysis. Daidzein was converted to equol by strain DZE (Fig. 3B). When daidzein (150 µM) was anaerobically incubated with strain DZE for 120 h, 128.4±14.1 µM (mean±S.D.) of equol was produced (85.6% in yield).

Transformation of Puerarin to S-Equol by a Mixed Culture of Strains PUE and DZE  When puerarin was anaerobically incubated in GAM broth with a mixed culture of two anaerobic bacterial strains, PUE and DZE, equol was produced as a major metabolite (Fig. 3C). To determine the optical characteristics of isolated equol, several methods were used. The optical characteristics of isolated equol were determined using HPLC and NMR spectroscopy. The optical rotation of equol was measured at 23 °C in MeOH.

Fig. 2. Dendrograms of Phylogenic Affiliation of Strains PUE (A) and DZE (B)

Each 16S ribosomal RNA gene sequence of strains PUE and DZE was compared with those from Genbank database with ClustalW program.

Fig. 3. Transformation by Isolated Bacteria, Strains PUE and DZE, Analyzed by HPLC

(A) Transformation of puerarin to daidzein by strain PUE, (B) transformation of daidzein to equol by strain DZE, (C) transformation of puerarin to equol by a mixture of strain PUE and DZE. Other peaks except puerarin, daidzein, and equol were identified as ingredients of GAM broth through analysis of incubation samples in the absence of substrates. STD, standards.
were examined. The CD spectrum of produced equol in methanol showed a positive Cotton effect at 244 nm and a negative Cotton effect at 280 nm. Moreover, the optical rotation \([\alpha]_{D}^{24}\) was \(-18.5^\circ\) in methanol \([\alpha]_{D}^{248} = \sim 21.5^\circ\) in chloroform).\(^{23}\) When we analyzed chemically synthesized equol by LC/MS with a chiral column, two peaks were detected with different retention times of 33.8 and 34.4 min, but they showed the same mass spectral pattern (Fig. 4), while equol obtained by mixed cultivation showed only one peak under the same analytical conditions. On the basis of the above findings, the metabolite was concluded to be \((-)-(3S)\)-equol (\(S\)-equol). When puerarin (200 \(\mu M\)) was anaerobically incubated with strain PUE and DZE for 120 h, 170.0 \(\pm 10.7 \mu M\) (mean \(\pm\) S.D.) of equol was produced (85.0% in yield).

**DISCUSSION**

Phytoestrogen is defined as plants-derived compounds with estrogen-like activities. Isoflavones and lignans have been categorized as phytoestrogens, based on their chemical structures and activities.\(^{24,25}\) Particularly, equol (a metabolite of daidzein, daidzin, and puerarin) has been a matter of concern because of not only high consumption of its precursors in diet but also its biological activity. However, the conversion to equol shows individual variations in humans; even if precursors of equol are taken through diet, equol cannot be produced in some individuals. Moreover, equol was found to be produced exclusively by intestinal bacteria.\(^{26}\) Therefore extensive studies on the intestinal bacterial communities that produce equol are necessary.

In the present study, we isolated two bacteria that participate in transformation to equol from puerarin. One of them, strain PUE, showed metabolic activity that converts puerarin to daidzein. Several bacteria capable of converting to daidzein have been reported.\(^{9}\) However, 16S rRNA gene sequence had low similarity among them. Although elimination of a \(C\)-glucosyl bond is generally performed with an oxidative or reductive process in plants, the \(C\)-glucosyl bond of puerarin was reductively cleaved by bacteria to give an aglycone in high yield. This bacterium had high substrate specificity and did not convert other \(C\)-glucosides.

We also isolated strain DZE capable of transforming daidzein to equol from human feces. Though the growth of strain DZE in GAM broth was poor, we could maintain the strain by co-incubation with another bacterium such as strain PUE or the addition of 1% arginine-HCl. Equol has a chiral carbon atom at position C-3 of the dihydropyran ring. Therefore there are two enantiomeric forms, \(S\)-equol and \(R\)-equol. Equol obtained by strain DZE was identified as \(S\)-equol by several analyses. Our findings agree with reports that only \(S\)-equol was isolated as a metabolite.\(^{19,27,28}\)

Co-incubation of puerarin with strains PUE and DZE produced \(S\)-equol. Dihydrodaidzein was detected as a metabolic intermediate during the incubation and converted to equol by
and its conjugates were not yet detected in human urine.\textsuperscript{29} It genistein to 5-hydroxyequol as well, although this compound is a normal inhabitant of human intestinal flora. Continuous inoculation of a bacterial mixture altered not only bacterial composition but also equol production status in SHIME. Equol was produced stably even after stopping inoculation. This suggests that strain DZE can be applied to this system because strain DZE is a normal inhabitant of human intestinal flora. In substrate specificity experiments, strain DZE converted genisteen to 5-hydroxyequol as well, although this compound and its conjugates were not yet detected in human urine.\textsuperscript{29} It is of interest to investigate the formation, translocation, and excretion of 5-hydroxyequol and its physiological activity.

REFERENCES