

## Isolation and Characterization of a Novel Equol-Producing Bacterium from Human Feces

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An equol-producing bacterium was newly isolated from the feces of healthy humans and its morphological and biochemical properties were characterized. The cells were obligate anaerobes. They were non-sporulating, non-motile, gram-positive bacilliform bacteria with a pleomorphic morphology. The strain was catalase-positive, and oxidase-, urease-, and indole-negative. The only other sugar utilized by the strain was glycerin. The strain also degraded gelatin, but not esculin. It was most closely related to *Eggerthella hongkongensis* HKU10, with 93.3% 16S rDNA nucleotide sequence homology. Based on these features, the isolate was identified as a novel species of the genus *Eggerthella*. It was named *Eggerthella* sp. YY7918. Strain YY7918 converted substrates daidzein and dihydrodaidzein into *S*-equol, but did not convert daidzin, glysitein, genistein, or formononetin into it. An antimicrobial susceptibility assay indicated that strain YY7918 was susceptible to aminoglycoside-, tetracycline-, and new quinolone-antibiotics.

**Key words:** *Eggerthella* sp. YY7918; isolate from human feces; *S*-equol; substrate specificity; antimicrobial susceptibility

Equol, a daidzein metabolite produced by intestinal microflora, is known to have higher estrogenic<sup>1,2</sup> and antioxidative<sup>3</sup> activities than other major soy isoflavonoids, such as daidzein and genistein. Epidemiological studies suggest that it might be beneficial in the prevention of breast cancer,<sup>4,5</sup> prostate cancer,<sup>6,7</sup> osteoporosis,<sup>8</sup> and menopausal syndromes.<sup>9</sup> Consequently, there is growing interest in the physiological effects of equol on human health. However, not everybody is able to produce equol. It is reported that only 20–60% of healthy adults can produce it.<sup>9–14</sup> It is thought that certain bacteria in the intestinal microflora are greatly involved in equol bioconversion. Some

workers have reported that a bacterial consortium comprising *Bacteroides ovatus*, *Ruminococcus productus* and *Streptococcus intermedius*,<sup>9</sup> or *Lactobacillus mucosae*, *Enterococcus faecium*, *Fingoldia magna* and *Veillonella* sp.<sup>15</sup> produces it from daidzein. An anaerobic gram-positive strain HGH-6,<sup>16</sup> an anaerobic bacterium Niu-O16,<sup>17</sup> the *Clostridium*-like bacterium TM-40,<sup>18</sup> which converts daidzein to dihydrodaidzein, and the strain SNU-Julong 732 which converts dihydrodaidzein to equol,<sup>19</sup> have also been isolated individually from humans. Recently, bacteria that convert daidzein to equol were isolated from rat cecal contents<sup>20</sup> and human feces<sup>21</sup> respectively. Little is known about equol-producing bacteria. In this study, we isolated a novel species of equol-producing bacterium from the feces of healthy humans and characterized the morphological and biochemical properties of the species.

### Materials and Methods

**Materials.** The culture broths used in this study were as follows: Brain heart infusion (BHI), heart infusion (HI), Bacto™ tryptic soy, and Difco™ Lactobacilli MRS from Becton-Dickinson (Sparks, MD, USA); and GAM from Nissui Pharmaceuticals (Tokyo). The following reagents were used as standard substances: Equol was purchased from LC Laboratories (Woburn, MA, USA), daidzein from Tokyo Chemical (Tokyo), daidzin from Wako Pure Chemical (Osaka), formononetin from EMD Biosciences (La Jolla, CA, USA), genistein from Sigma (St. Louis, MO, USA), and dihydrodaidzein from Toronto Research Chemicals (North York, Canada). A silica gel 60 F<sub>254</sub> thin layer chromatography (TLC) plate was obtained from Merck KGaA (Darmstadt, Germany).

**Culture conditions.** The culture broth was supplemented with daidzein or other isoflavonoids (final

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**Abbreviations:** GAM, Gifu anaerobic medium; CD, circular dichroism; BHI, brain heart infusion; HI, heart infusion; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; PDA, photodiode array; MIC, minimal inhibitory concentration

concentration, 50  $\mu\text{M}$ ) and used as a growth medium. 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. Fifty microliters of the fecal homogenate or 10  $\mu\text{l}$  of bacterial culture ( $\text{OD}_{600} = 0.02$ ) was inoculated into a 24-well Multiwell™ plate (Becton-Dickinson) containing 1 ml of growth medium. The plates were incubated at 37 °C for 72 h using the AnaeroPack®/Kenki culture system (disposable O<sub>2</sub> absorbing and CO<sub>2</sub> generating agent, Mitsubishi Gas Chemical, Tokyo). After the addition of 20% (v/v) glycerol, culture samples were stored at –80 °C.

**Screening method.** Serial dilutions in sterile saline were spread on GAM agar plates and incubated at 37 °C for 96 h. Separated colonies were transferred to a 96-well Multiwell™ plate (Becton-Dickinson) containing 200  $\mu\text{l}$  of growth medium incubated under anaerobic conditions. After 72 h of incubation at 37 °C, samples were taken for TLC analysis.

TLC analysis for isoflavonoids, without  $\beta$ -glucuronidase treatment, has been described by Yokoyama *et al.*<sup>22)</sup> Some modifications were made for the screening assay. Briefly, culture supernatant was transferred onto MultiScreen™-BV (pore size, 1.2  $\mu\text{m}$ , Millipore, Bedford, MA, USA), and filled with 80  $\mu\text{l}$  of Wakogel® 50C18 (38–63  $\mu\text{m}$  in diameter). The columns were washed with 200  $\mu\text{l}$  of water and 200  $\mu\text{l}$  of 30% methanol together, and then eluted with 200  $\mu\text{l}$  of methanol. The eluate was vacuum dried. The samples were dissolved in 10  $\mu\text{l}$  of methanol and analyzed by normal phase TLC with a solvent system of toluene:acetone (2:1). The isoflavonoids developed on TLC were visualized with an UV transilluminator (312 nm) and/or iodine vapor.

**Morphological and biochemical analysis.** The isolated bacterium was grown on GAM agar plates, and its colony form was observed. It was stained using Favor G Nissui (Nissui Pharmaceutical) and observed by light microscopy (Olympus model BX50F4, Tokyo). The biochemical features of the isolate were estimated using an API system 20A Kit (bioMérieux, Lyon, France).

**Genetic analysis.** Genomic DNA of the isolate was extracted 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). Analysis of 16S RNA gene sequences and sequence homology searches were completed with standard DNA sequencing programs and the BLAST server of the National Center for

Biotechnology Information using the BLAST algorithm<sup>23)</sup> and the BLASTN program for comparison of a nucleotide query sequence against a nucleotide sequence database. A phylogenetic tree was constructed according to the neighbor-joining method with the CLUSTAL W program<sup>24)</sup> and visualized using the FigTree program.

**Chiral column high performance liquid chromatography (HPLC) analysis.** After preparative TLC, the silica gel at the position that corresponded to the R<sub>f</sub> value of equol was scratched up and eluted with methanol. Then the eluate was analyzed with a HPLC-photodiode array (PDA) detection system. The assay method of Wang *et al.*<sup>19)</sup> was adopted for analysis of enantiomers. One milligram of the lyophilized sample was dissolved in 500  $\mu\text{l}$  of H<sub>2</sub>O/acetonitrile (60:40), and then diluted 10-fold with the mobile buffer. Then aliquots (last dosage, 2  $\mu\text{g}$ /sample) were applied to a Sumichiral OA-7000 column (4.6 mm ID  $\times$  250 mm, Sumika Chemicals, Osaka) and eluted with 40% acetonitrile in 20 mM potassium phosphate buffer (pH 3.0) at 40 °C. The flow rate was 1 ml/min, and the UV spectra of the peaks were recorded from 200 to 400 nm. The lyophilized sample was also dissolved in ethanol (0.5 mg/ml), and the optical circular dichroism (CD) spectrum was recorded on a J-820 CD spectrometer (Jasco, Tokyo).

**Antimicrobial susceptibility test.** The antimicrobial susceptibility of the isolated bacterium was estimated using an Optopanel MP (OP-1) Kit from Kyokuto Pharmaceuticals (Tokyo). Briefly, 0.05 ml of bacterial preculture ( $\text{OD}_{600} = 0.161$ – $0.164$ ) was mixed with 12 ml of GAM broth, and a 0.1-ml aliquot of the broth was applied to the OP-1 Kit. The minimal inhibitory concentration (MIC) was determined after 5 d of incubation under anaerobic conditions. The test was confirmed in duplicate with freshly prepared media on separate occasions.

## Results

### *Isolation of equol-producing bacteria*

Initially we examined five culture media to select a suitable one for isolation of equol-producing bacteria. BHI, HI, Tryptic soy, MRS, and GAM broth were supplemented with 50  $\mu\text{M}$  daidzein. No daidzein metabolism was observed during the entire incubation period when the fecal sample was incubated with MRS or HI. Hence we decided to use GAM broth as a basic growth medium in the studies that followed. By selecting colonies from GAM agar plates on which serial dilutions from the equol-producing fecal sample had grown, one colony was retrieved that produced equol upon subculturing. After several transfers to fresh growth medium, the culture maintained its ability to convert daidzein into equol.

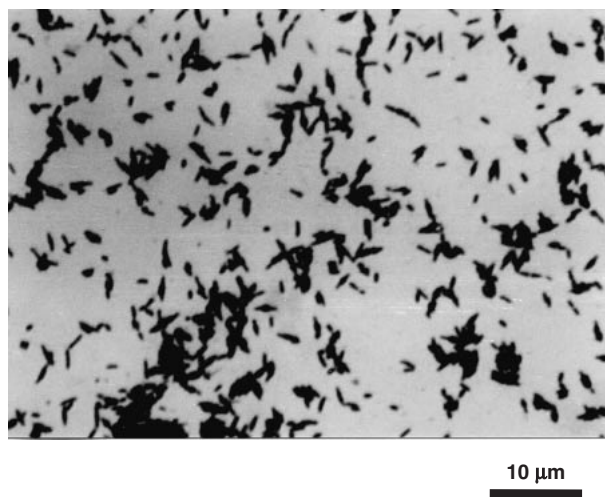
**Table 1.** Morphological and Biochemical Properties of the Isolated Bacterium

Cell shape		Pleomorphic bacilli (0.7–0.8 × 2.0–4.0 μm)
Gram staining		+
Spore		–
Motility		–
Colony form	Growth rate	Scanty
	Diameter	<1.0 mm
	Color	Cream
	Shape	Round
	Uplift condition	Lens
	Marginal	Entire
	Surface	Smooth
	Transparency	Opaque
Growth temp. (°C)	Property	Butyrous
	30	+
45	+	
Catalase		+
Oxidase		–
Gelatin		+
Esculin		–
Urease		–
Glycerin oxidation		+
Glucose oxidation		–
O/F test	Mannitol, Lactose, Saccharose, Maltose, Salicin, D-Xylose, L-Arabinose, Cellobiose, D-Mannose, Melezitose, Raffinose, D-Sorbitol, L-Rhamnose, D-Trehalose	–

\*These estimations were performed using GAM medium.

#### Morphological and biochemical properties of the isolated equol-producing bacterium

The morphological and biochemical properties of the isolated equol-producing bacterium are summarized in Table 1. The strain grew on GAM agar plates as cream-colored colonies with a smooth surface less than 1 mm in diameter after 7 d of incubation at 37°C in an anaerobic environment. Light microscopic observations revealed that the cells were gram-positive pleomorphic bacilli arranged in several chains (Fig. 1). The cells were non-sporulating, non-motile obligate anaerobes. The strain was catalase-positive, oxidase-negative, urease-negative, and indole-negative, and utilized glycerin.



**Fig. 1.** Microscopic Image of the Equol-Producing Bacterium Isolated from Human Feces.

After gram-staining, the isolated bacterium was observed under a light microscope (magnification, ×2,000). The bold line below the photograph indicates the size bar.

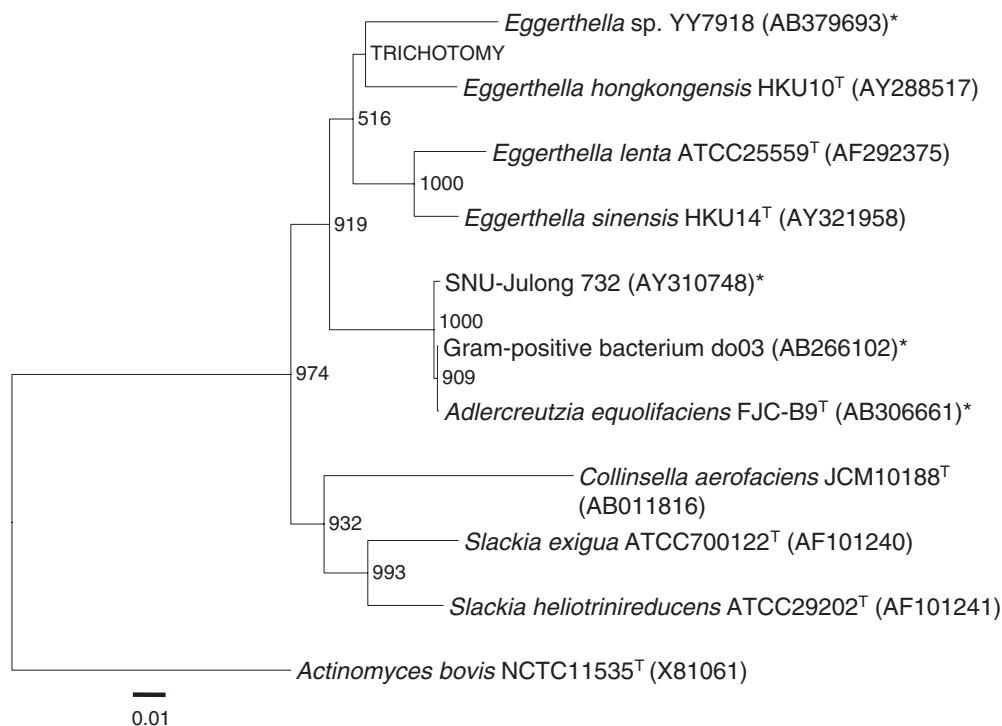
It did not utilize the other sugars tested, including mannitol, lactose, saccharose, maltose, salicin, D-xylose, L-arabinose, cellobiose, D-mannose, melezitose, raffinose, D-sorbitol, L-rhamnose, and D-trehalose. It also degraded gelatin, but not esculin.

#### Genetic properties of the isolated equol-producing bacterium

The 1,469-basepair sequence of the 16S rRNA gene (rDNA) of the isolated bacterium was sequenced. The 16S rDNA nucleotide sequence has been deposited in DDBJ/GenBank/EMBL under accession no. AB379693. The identity of the isolated strain was determined by comparing the 16S rDNA sequence with a database. The isolate had 93.3% sequence similarity with *Eggerthella hongkongensis* HKU10 (accession no. AY288517). The phylogenetic tree also indicated that the isolate was to be placed in the same clusters as *Eggerthella* species, but it was different from other equol-producing bacteria (Fig. 2). Hence it was named strain YY7918, as one species of the genus *Eggerthella*.

#### Enantiomeric character of the equol produced by strain YY7918

To determine the enantiomeric character of the biosynthesized equol, TLC-purified equol from the bacterial culture was analyzed with a chiral column HPLC-PDA detection system. The chiral column HPLC data showed that there was a mono peak at a retention time of 12.2 min at 270 nm (Fig. 3, panel B), while twin peaks, corresponding to the *S* and *R* forms respectively, were observed at retention times of 12.2 and 15.9 min with commercial equol (Fig. 3, panel A). In order to confirm the chirality, the purified equol was subjected to CD spectrum analysis. The CD spectrum data showed that positive (17 mdeg) and negative (–10 mdeg) peaks



**Fig. 2.** Comparison of 16S rDNA Sequence from Strain YY7918 with Other Bacteria in the Database.

The 16S rDNA sequence was analyzed by extracting the DNA, performing PCR with universal 16S primers, and sequencing with the same primers by standard methods. The resulting sequence of 1,469 bases was searched against Genbank (NCBI). The phylogenetic tree was constructed using the FigTree program. Super-imposed T and asterisk represent the type strain and the equol-producing bacterium respectively. Numbers at the branch points are bootstrap values based on 1,000 samplings. Parentheses indicate the accession numbers. *Actinomyces bovis* NCTC11535 was used as the outgroup. The scale bar represents genetic distance.

occurred at 240 and 280 nm respectively (Fig. 3, panel C), which was consistent with the report of Wang *et al.*<sup>19)</sup> Consequently, based on these analyses, YY7918 was found to produce only *S*-equol from daidzein.

#### Substrate specificity of strain YY7918

TLC analysis indicated that the bacterium converted equol ( $R_f = 0.49$ ) from daidzein and dihydrodaidzein with almost 100% efficiency (50  $\mu$ M equol/72 h of culture) (Fig. 4, lanes 2 and 4). However, daidzin, glysitein (a minor isoflavonoid of soy bean), genistein, and formononetin, which is abundant in red clover, were not metabolized by the bacterium (Fig. 4, lanes 3, 5, 6, and 7 respectively).

#### Antimicrobial susceptibility of strain YY7918

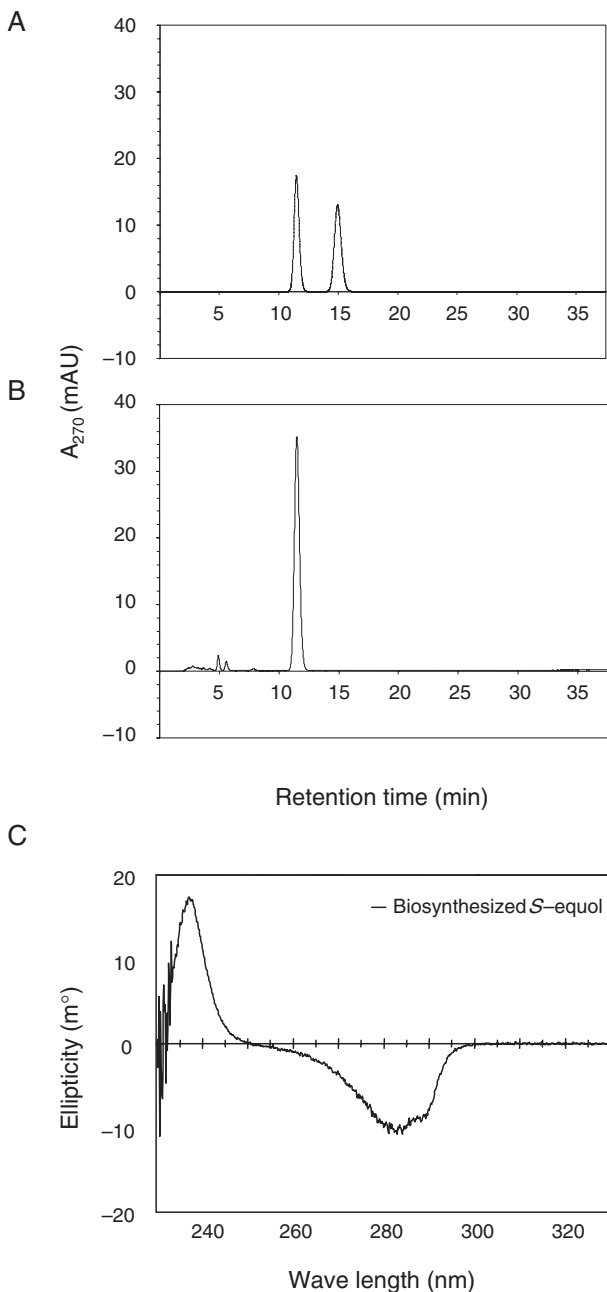
The antibiotic susceptibility patterns of strain YY7918 are shown in Table 2. The strain was susceptible to benzylpenicillin, ampicillin, piperacillin, imipenem, meropenem, gentamicin, minocycline, and levofloxacin. It was resistant to cefotiam, cefotaxime, cefoperazone/sulbactam, ceftazidime, ceftidoren, erythromycin, and clindamycin.

## Discussion

The 16S rDNA sequence of isolated strain YY7918 showed relatively high homology (92.9–93.3%) with

*Eggerthella* strains. This indicates that strain YY7918 is closely related to the genus *Eggerthella* (Fig. 2). The morphological and biochemical characteristics of the strain were also similar to that of genus *Eggerthella*,<sup>25)</sup> particularly the fact that no sugars were oxidized or fermented by the bacterium (Table 1). These results indicate that the bacterium belongs to the genus *Eggerthella*. Strain YY7918 showed highest sequence homology (93.3%) with *E. hongkongensis* HKU10 (Fig. 2). Generally, an object bacterial strain is identified as the same species as a known one when there is over 97% sequence homology on 16S rDNA sequence analysis, but no strain was found with 16S rDNA that was over 97% homologous to strain YY7918. This suggests that there is no reported species that is the same as strain YY7918. In addition, strain YY7918 was morphologically different from *E. hongkongensis*. The cells of strain YY7918 were pleomorphic bacilli (Fig. 1), while those of *E. hongkongensis* were coccobacilli.<sup>25)</sup> Considering the results of the 16S rDNA sequence homology search and the morphological characteristics of the strain together, strain YY7918 was identified as a novel species of the genus *Eggerthella*.

Although three bacterial species (strains SNU-Julong 732 and do03, and *Adlercreutzia equolifaciens*) have been reported as equol-producing bacteria,<sup>19–21)</sup> the homologies of the 16S rDNA sequences from the newly isolated bacteria with 16S rDNA from these strains were



**Fig. 3.** Enantiomeric Analysis of Bacterial-Synthesized Equol. HPLC elution profiles on a Sumichiral OA-7000 column of commercial equol (racemic-mixture) (panel A) and equol purified from daidzein-containing GAM broth cultured with *Eggerthella* sp. YY7918 under anaerobic conditions (panel B). CD spectra of the bacterial synthesized equol (panel C).

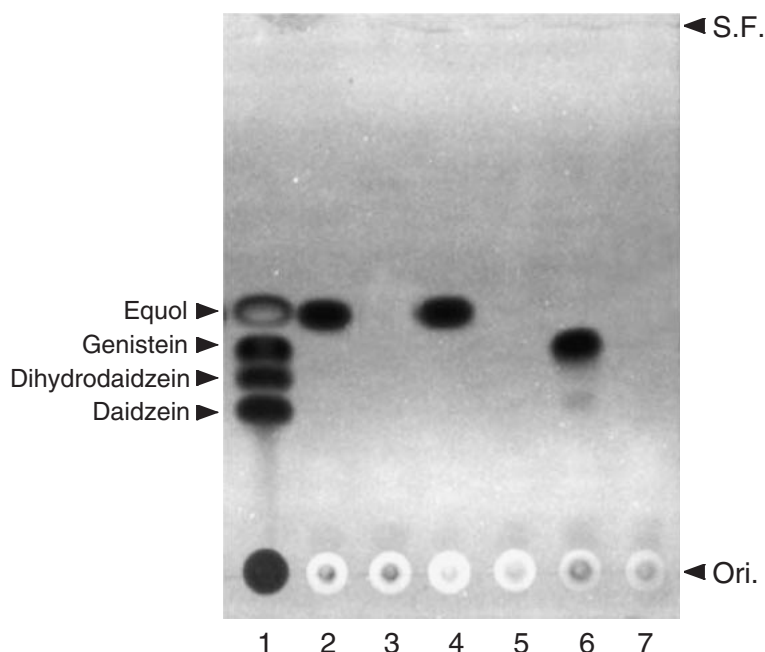
92.1% (accession no. AY310748), 92.0% (accession no. AB266102), and 92.2–92.4% (accession nos. AB306660–AB306663) respectively, lower than that of *E. hongkongensis* HKU10. In addition, strain do03 is an isolate from a non-human resource (rat cecum).<sup>20</sup> Strain SNU-Julong 732, which was isolated from human feces, required dihydrodaidzein as the single substrate for equol production, and no conversion was observed with daidzein.<sup>19</sup> *A. equolifaciens*, which was also isolated from human feces and converted daidzein to equol,

differs in cell shape (coccobacilli) from strain YY7918.<sup>21</sup> Thus *Eggerthella* sp. YY7918 can be distinguished from the other species that are already known as equol producers. Since these bacteria all belong to the family *Coriobacteriaceae*, this bacterial family might play a pivotal role in isoflavonoid metabolism within the mammalian intestine.

Several studies have indicated that equol has selective affinity for estrogen receptors. In particular, *S*-equol has a high affinity for estrogen receptor  $\beta$ , whereas *R*-equol is relatively inactive.<sup>26–29</sup> Since the enantiomeric character must relate to the bioavailability of the equol, it is very important to clarify the enantiomeric character of equol produced by these bacteria. In this study, strain YY7918 was found to produce only *S*-equol. Strain SNU-Julong 732 is known to produce *S*-equol from dihydrodaidzein.<sup>19</sup> Setchell *et al.* also established that *S*-equol is the exclusive product of mammalian intestinal bacterial synthesis from soy isoflavones using human and rat urine collected after ingestion of soy foods and a human fecal culture.<sup>29</sup> These items of information strongly suggest that only the *S*-form racemate of equol is produced by intestinal bacteria.

The only legume isoflavonoids that *Eggerthella* sp. YY7918 is able to metabolize are daidzein and dihydrodaidzein, the latter of which is thought to be the middle metabolite of daidzein in intestinal bacteria.<sup>30</sup> Interestingly, daidzin, a glycoside form of daidzein, was also scarcely converted to equol. These results suggest that the bacterium has no  $\beta$ -glucosidase activity, at least against daidzin. An examination of the biochemical data indicated that *Eggerthella* sp. YY7918 has very few sugar metabolism pathways. Although there has been no report on isoflavonoid metabolism, *E. hongkongensis* has been reported to retain  $\beta$ -glucosidase activity.<sup>25</sup>

In this study, *Eggerthella* sp. YY7918 was susceptible to  $\beta$ -lactam-, aminoglycoside-, tetracycline-, and new quinolone-antibiotics, and was resistant to cefem, erythromycin-, and clindamycin-antibiotics. These results are not contradicted by reports on *E. hongkongensis* and *E. sinensis*, which are sensitive to penicillin and metronidazole and resistant to cefotaxime.<sup>25</sup> It is possible that equol-producing activity is reduced or lost when a person takes  $\beta$ -lactam-, aminoglycoside-, tetracycline-, or new quinolone-antibiotics. Atkinson *et al.* report that when a human fecal culture is supplemented with kanamycin (aminoglycoside-antibiotics) or rifampicin, equol production is inhibited.<sup>31</sup> By contrast, kanamycin was virtually eliminated by equol production in the plasma of cynomolgus monkeys,<sup>32</sup> and hence its antimicrobial susceptibility effects might not be uniform. Because these effects were estimated in a complex system using natural or artificial (fecal culture) microflora (comprising many kinds of bacterial species), antibiotics might be degraded or modified by resistant bacteria in the flora. Equol production might also be affected by the constitution of microfloral bias caused by antibiotic prescription. Much more *in vivo* research is



**Fig. 4.** Bioconversion of Various Isoflavonoids by *Eggerthella* sp. YY7918.

Culture samples supplemented with various isoflavonoids (50  $\mu$ M) were treated as described in "Materials and Methods," developed in toluene:acetone (2:1), and exposed to iodine vapor. Lane 1, isoflavonoid standards; lane 2, daidzein; lane 3, daidzin; lane 4, dihydrodaidzein; lane 5, glycitein; lane 6, genistein; lane 7, formononetin.

**Table 2.** Antimicrobial Susceptibility of *Eggerthella* sp. YY7918

Antimicrobial agent	MIC* ( $\mu$ g/ml)
Benzylpenicillin	0.5
Ampicillin	$\leq 0.25$
Piperacillin	$\leq 2$
Ampicillin/Sulbactam	$\leq 0.25/0.12$
Cefotiam	$> 8$
Cefotaxime	16
Cefoperazone/Sulbactam	16/8
Ceftazidime	$> 16$
Cefditoren	1
Imipenem	4
Meropenem	1
Gentamicin	$\leq 1$
Erythromycin	$> 2$
Clindamycin	$> 4$
Minocycline	0.5
Levofloxacin	2

\*Minimal inhibitory concentration

required to determine the influence of antibiotic administration on equol-producing activity.

In conclusion, while the equol-producing bacterium isolated in this study was identified as a novel species, it belongs to the same family as other equol-producing bacteria reported previously.<sup>19–21</sup> Hence it is thought that these bacteria share common genotypic or phenotypic features in the equol production process. More detailed studies of strain YY7918 should yield much basic knowledge of the mechanisms of daidzein metabolism and the relationships between equol-producing bacteria, the host, and other bacterial communities within the

intestinal microflora. Since equol is considered to be beneficial in a variety of diseases, it might have some potential for use as a pharmaceutical agent. Because the genus *Eggerthella* (*E. lenta* is listed as a representative) belongs to biosafety level 1 according to the "Manual for Biosafety Relating to Pathogenic Bacteria" (Japanese Society for Bacteriology), strain YY7918 can be used in the industrial production of *S*-equol. Hence a study to determine ways of improving bacterial production of *S*-equol in the near future is desirable.

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