**Bacteroides uniformis** as the dominant bacterial species involved in activation of ginseng protopanaxadiol saponins in human intestines

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Human intestinal bacteria hydrolyze ginsenosides, triterpenoid glycosides of *Panax ginseng* C. A. MEYER (Araliaceae) to the active metabolites. M1 (20S-protopanaxadiol 20-O-β-D-glucopyranoside) is the final metabolite of protopanaxadiol-type ginsenosides. In the present study, we explored the dominant bacterial species involved in metabolism of ginsenosides by means of M1-producing activity assay and terminal restriction fragment length polymorphism (T-RFLP) analysis. Results from M1-producing activity assay of fecal specimens from 17 adults revealed remarkable individual differences in the activity. T-RFLP patterns of 16S ribosomal DNA (rDNA) PCR products from 5 of the 17 specimens, which showed the ability to produce M1, showed that the genus Bacteroides spp. were present among M1-producers. Then, type strains of the genus Bacteroides spp. including *B. acidofaciens*, *B. caccae*, *B. fragilis*, *B. intestinalis*, *B. ovatus*, *B. stercoris*, *B. thetaotaomicron*, *B. uniformis* and *B. vulgatus* were assayed for M1-producing activity. All the strains tested, except *B. uniformis* JCM 5825 T, produced no or less M1. Moreover, 11 reference strains of *B. uniformis* also produced M1. These results suggest that *B. uniformis* may act as the dominant bacterial species capable of producing M1 in human intestines.

**Key words** *Panax ginseng*, ginsenoside, intestinal bacteria, hydrolysis, protopanaxadiol, *Bacteroides uniformis*.

**Abbreviations** M1, 20S-protopanaxadiol 20-O-β-D-glucopyranoside; T-RFLP, terminal restriction fragment length polymorphism.

**Introduction**

Ginseng (the roots of *Panax ginseng* C. A. MEYER, Araliaceae) has been used as one of the most valuable traditional medicines in the Orient for over 2000 years. The main ingredients of ginseng are ginsenosides, glycosides containing an aglycone (protopanaxadiol or protopanaxatriol) with a dammarane skeleton. So far, numerous researchers have contributed to the accumulation of evidence that ginsenosides are responsible for the pharmacological effects of ginseng. Ginseng is orally ingested, in general. Therefore, its ingredients must meet gastric juice, digestive and bacterial enzymes in the intestines. Orally ingested ginsenoside passes through the stomach and small intestine without decomposition by either gastric juice or digestive enzymes into the large intestine, where ginsenoside is hydrolyzed (deglycosylated) by colonic bacteria followed by transit to the circulation: Colonic bacteria cleave the oligosaccharide connected to the aglycone stepwise from the terminal sugar to afford the major metabolites, 20S-protopanaxadiol 20-O-β-D-glucopyranoside (M1) and 20S-protopanaxatriol (M4). M1 is gradually hydrolyzed to the aglycone, 20S-protopanaxadiol (M12). 20S-Protopanaxatriol 20-O-β-D-glucopyranoside (M11) is the intermediate metabolite of M4 (Fig. 1). Many kinds of bacteria including *Eubacterium A-44*, 1) *Bifidobacterium K506*, 2) *Bacteroides JY6*, 3) and

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Fusobacterium K-60\textsuperscript{2} seem to cooperatively metabolize ginsenoside. Accumulating evidence strongly suggests that the metabolites are the real active molecules in the body.\textsuperscript{3}

We have been so far interested in the relatedness of ginseng efficacy with metabolite-producing potentials. Intestinal microbiota is well known to be very changeable in dependence on host conditions (diet, health, and even stress). In fact, we have observed the individual differences in metabolite-producing potentials of 58 human subjects.\textsuperscript{4} Furthermore, we have found that the anti-metastatic activities of orally ingested ginsenoside are correlated with the metabolite-producing potentials of mice.\textsuperscript{5} Therefore, intestinal microbiota is suspected of affecting ginseng efficacy.

In this study, we attempted to clarify the predominant bacterial species capable of producing M1 by means of hydrolyzing activity assay and terminal restriction fragment length polymorphism (T-RFLP) analysis.

Materials and Methods

Bacteria. Type strains of Bacteroides species (B. acidofaciens, B. caccae, B. fragilis, B. intestinalis, B. ovatus, B. stercoris, B. thetaiotaomicron, B. uniformis and B. vulgatus) and reference strains of B. uniformis were obtained from the Japan Collection of Microorganisms (JCM), as shown in Table 1. B. uniformis S10, S11, S12, S13, 21-42, 22-76, 22-80 and 23-18 were from our private collection.

<table>
<thead>
<tr>
<th>Bacteroides sp.</th>
<th>Strain</th>
<th>M1-producing activity</th>
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<tbody>
<tr>
<td>Type species strains of the genus Bacteroides</td>
<td></td>
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<tr>
<td>B. acidofaciens</td>
<td>JCM 10556\textsuperscript{7}</td>
<td>-</td>
</tr>
<tr>
<td>B. caccae</td>
<td>JCM 9498\textsuperscript{7}</td>
<td>w</td>
</tr>
<tr>
<td>B. fragilis</td>
<td>JCM 11019\textsuperscript{7}</td>
<td>-</td>
</tr>
<tr>
<td>B. intestinalis</td>
<td>JCM 13265\textsuperscript{7}</td>
<td>-</td>
</tr>
<tr>
<td>B. ovatus</td>
<td>JCM 5824\textsuperscript{7}</td>
<td>w</td>
</tr>
<tr>
<td>B. stercoris</td>
<td>JCM 9496\textsuperscript{7}</td>
<td>w</td>
</tr>
<tr>
<td>B. thetaiotaomicron</td>
<td>JCM 5827\textsuperscript{7}</td>
<td>-</td>
</tr>
<tr>
<td>B. uniformis</td>
<td>JCM 5828\textsuperscript{7}</td>
<td>+</td>
</tr>
<tr>
<td>B. vulgatus</td>
<td>JCM 5826\textsuperscript{7}</td>
<td>-</td>
</tr>
</tbody>
</table>

Reference strains of B. uniformis

| B. uniformis | JCM 13286 | + |
| B. uniformis | JCM 13287 | + |
| B. uniformis | JCM 13288 | + |
| B. uniformis | S10 | + |
| B. uniformis | S11 | + |
| B. uniformis | S12 | + |
| B. uniformis | S13 | + |
| B. uniformis | 21-42 | + |
| B. uniformis | 22-76 | + |
| B. uniformis | 22-80 | + |
| B. uniformis | 23-18 | + |

Symbols: +, positive reaction; -, negative reaction; w, weak reaction.

Fecal specimens. After informed consent, fecal specimens were collected from 17 healthy volunteers 29 to 53 years of age. The study was performed under the approval of RIKEN ethics committees.

Metabolite-producing activity assay. Metabolite-producing activity was determined by the following TLC method. Fecal specimens were taken into 3 ml GAM semi-solid without dextrose "Nissui" (Nissui Pharmaceutical, Japan) with 0.5% ginsenoside fraction, and anaerobically incubated at 37°C for 48 h. After incubation, a part (0.3 ml) of cultures was extracted with water-saturated n-ButOH (0.2 ml) and centrifuged (15,000 rpm, 3 min). Aliquots (2 μl) of the n-ButOH layer were analyzed by TLC: plates, silicagel 70 F\textsubscript{254}; developing solvents, CHCl\textsubscript{3}-MeOH-H\textsubscript{2}O (65:35:10 v/v, lower phase) and CHCl\textsubscript{3}-EtOH (8:1); detection of spots, spraying 8% vanillin in MeOH-72% H\textsubscript{2}SO\textsubscript{4} (1:5 v/v) followed by heating (140°C, 3-4 min). Positive reaction of metabolite-producing activity means that spots of the same Rf values as reference metabolites appear on TLC.

16S rRNA sequencing. One bacterial colony was scraped from the agar plate, suspended in 50 μl TE buffer with 10% Triton, and heated at 95°C for 5 min. This raw extracted DNA was used as template DNA for PCR. PCR mixture contained 10x Ex Taq buffer (10 μl), a mixture of dNTPs (2.5 mM) (8 μl), 10 μl of each of the primers (10 pmol/μl) (27F, 5′-AGA GTT TGA TCC TGG CTC AG-3′; 1492R, 5′-GTT TAC CTT GGT ACG ACT T-3′), 5 μl raw template DNA, and 3U Ex Taq (Takara). Distilled water was added to make 100 μl in a 200 μl micro tube. 16S rRNA gene was amplified using a PCR machine (Biometra, Germany) according the following program: 95°C for 3 min; followed by 30 cycles consisting of 95°C for 30 s, 60°C for 30 s, and 72°C for 90 s, with a final extension period at 72°C for 10 min. Amplified DNA was verified by 1.5% agarose gel electrophoresis staining with ethidium bromide (1 μg/ml). The 16S rRNA gene sequence was determined by direct PCR sequencing using an ABI PRISM BigDye Terminator Cycle Sequencing ready reaction kit (Applied Biosystems) and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), following the instructions provided by the manufacturer. Almost complete (1488 bp) 16S rRNA sequence was determined for each of the M1-producing strains and deposited in the GenBank database. The sequences were compared with similar sequences of the reference organisms by BLAST\textsuperscript{7} and FASTA\textsuperscript{8} searches. The 16 rRNA sequences of other bacteria were available in GenBank, EMBL and DDBJ. Levels of sequence similarity were calculated and used to produce an unrooted phylogenetic tree by the neighbour-joining method.\textsuperscript{9} The alignment and the stability of relationships were assessed by bootstrapping using CLUSTAL X (Version 1.8).\textsuperscript{10}

T-RFLP analysis. T-RFLP analysis of fecal microbiota was done according to the method described previously.\textsuperscript{11} The analytical study was performed under the approval of RIKEN ethics committees.
Results and Discussion

Individual differences in metabolite-producing activities. Seventeen randomly selected volunteers were assayed for metabolite-producing activity. As shown in Fig. 2, remarkable differences were observed among individuals: Volunteers C and J showed M11-, M1-, M4-, and M12-producing activities. Volunteer I showed M11-, M1-, and M12-producing activities. Volunteer L showed M11-, M1-, and M4-producing activities. Volunteer M showed M11- and M1-producing activities. Volunteer O showed M11- and M12-producing activities. Volunteers E, F, and Q showed just M4-producing activity. Volunteers C, I, J, L, and M showed the ability to produce M1. The latest 5 volunteers were selected as M1-producers. The results are consistent with those found in the previous study. These data provide evidence that hydrolyzing activities differ among individuals.

T-RFLP analysis of M1-producers. Five M1-producers (Volunteers C, I, J, L, and M) were analyzed for T-RFLP patterns of 16S ribosomal DNA (rDNA) PCR products. Figure 3 demonstrates that the Hha I-derived T-RF of 98 bp, corresponding to the Bacteroides spp., was observed among the M1-producers tested.

M1-producing activity of Bacteroides species. To investigate which species of the Bacteroides was involved in M1-producing activity, type strains of B. acidofaciens, B. caccae, B. fragilis, B. intestinatis, B. ovatus, B. stercoris, B. thetaiotaomicron, B. uniformis and B. vulgatus were assayed. As shown in Table 1, all the type strains tested, except B. uniformis JCM 5825^T, produced or less M1. Concerning B. uniformis, the 11 reference strains tested all produced M1 (Table 1), suggesting that M1-producing activity is characteristic to B. uniformis. In the phylogenetic tree (Fig. 4), the M1-producing strains of B. uniformis formed one cluster at high bootstrap levels more than 99.7% sequence similarity. As for M1-producing bacteria in the genus Bacteroides, B. stercoris strain HJ-15 is shown to produce less M1 from ginsenoside^3^ in agreement with our observation. Although Bacteroides species strain JY-6 is reported to produce more M1 than B. stercoris strain HJ-15,^2^ the species assignment is obscure.

Collectively, our data suggest that B. uniformis may act as the dominant bacterial species capable of producing M1 in human intestines. Design of specific primers for detecting B. uniformis in fecal specimens is in progress.

![Fig. 2 Individual differences in metabolite-producing potentials of 17 volunteers](image)

![Fig. 4 Phylogenetic tree showing the positions of the M1-producing bacteria within the Bacteroides cluster. The tree was constructed by the neighbour-joining method based on 16S rRNA sequences. The scale bar represents 0.01 substitutions per nucleotide position. The numbers at the nodes of the tree indicate bootstrap values (branch lengths) for each node out of 100 bootstrap resamplings. The strain of superscript (T) represents the type strain of species.](image)
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References


Japanese abstract

漢方処方を構成する生薬には様々な配糖体が有効成分をと

含まれている。配糖体は加水分解されてから吸収され、薬効を

発揮する。この加水分解には、腸内細菌が深く関与している。 

腸内細菌叢の構成は個体差が大きく、配糖体を加水分解する

能力を有する腸内細菌の有無が、薬効を引き起こす一因にな

ることが懸念される。そこで著者らは、人参 (Panax ginseng C. A. MEYER) を例にとり、人参配糖体（サポニン）

を加水分解する能力にどの程度個体差があるか、配糖体の

加水分解に関わる腸内細菌を解析した。まず無作為に抽出し

た被験者17名の配糖体分解活性を測定した結果、配糖体分解

活性に著しい個体差が認められ、ジオール系サポニンとトリ

オール系サポニンで分解の様相が異なり、分解に関与する

腸内細菌が異なることが示唆された。そこで、配糖体の加水

分解に関わる腸内細菌を特定する目的で、ジオール系サポニ

ンの代謝物であるM1を生成する配糖体分解活性を有保した

被験者5名の糞便細菌収をT-RFLP法で解析した結果、バ

クテリオデス属細菌が共通して検出された。さらに、

Bacteroides属細菌9種の配糖体分解活性を測定し、代謝物

M1を生成する腸内細菌種として Bacteroides uniformis を

特定した。

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