Isolation of a Human Intestinal Bacterium That Transforms Mangiferin to Norathyriol and Inducibility of the Enzyme That Cleaves a *C*-Glucosyl Bond

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The *C*-glucosyl bond of *C*-glucosides generally tolerates acid and enzymatic hydrolysis. Many *C*-glucosides are cleaved by human intestinal bacteria. We isolated the specific bacterium involved in the metabolism of mangiferin (2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone), *C*-glucosyl xanthone, from a mixture of human fecal bacteria. The anaerobic *Bacteroides* species named MANG, transformed mangiferin to the aglycone, norathyriol, suggesting cleavage of a *C*-glucosyl bond. However, *B*. sp. MANG cleaved *C*-glucosyl in a dose- and time-dependent manner only when cultivated in the presence of mangiferin. Cleavage was abolished by inhibitors of RNA and protein syntheses, such as rifampicin and chloramphenicol, respectively, indicating that the enzyme that cleaves *C*-glucosyl is induced by mangiferin. In contrast, mangiferin did not affect bacterial α- and β-glucosidase activities under any conditions. The *C*-glucosyl-cleavage in cell-free extracts was not altered by potent glucosidase inhibitors such as 1-deoxynojirimycin and gluconolactone. Therefore, the *C*-glucosyl-cleaving enzyme substantially differs from known glucosidases that cleave *O*-glucosides. This is the first description of a specific intestinal bacterium that is involved in the metabolism of mangiferin and which produces a novel and inducible *C*-glucosyl-cleaving enzyme.

Key words human intestinal bacteria; *C*-glucoside; mangiferin; *Bacteroides*; *C*-glucosyl-cleaving enzyme

C-Glucosides, which are widely distributed among plants, are more resistant towards acid, alkaline and enzyme hydrolysis than *O*-glucosides. Even prolonged exposure to acid does not cleave the glucosyl bond of *C*-glucosides, in which glucose is attached by a carbon–carbon bond to the aglycone, to give the aglycone.[1] However, intestinal bacteria from human feces can cleave *C*-glucosyl bonds in various *C*-glucosides such as mangiferin, abrusin 2″-*O*-β-D-apioside, aloeresin A, aloesin, barbaloin, bergenin, homoorientin, puerarin and safflor yellow B.[2—8]

Mangiferin (2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone), was originally isolated from *Mangifera indica* L. (Anacardiaceae). It is distributed among at least sixteen plant families including Anacardiaceae, Iridaceae and Gentianaceae. Studies *in vitro* have shown that mangiferin has antiviral activity against herpes simplex virus[9] as well as antioxidant,[10] and anti-HIV activity.[11] Mangiferin orally administered to rats or mice exerts biological antitumor,[11] antidiabetic,[12] antioxidant,[13] hepatoprotective[14] and immunomodulative[15] activities. However, the pharmacokinetic studies in rats given oral mangiferin have failed to detect mangiferin in plasma,[16] suggesting poor absorption. On the other hand, appreciable amounts of euxanthone (1,7-dihydroxyxanthone) and its glucuronides are excreted into the urine of rabbits given oral mangiferin,[17] indicating a unique metabolic transformation that involves elimination of a *C*-glucosyl chain and two phenolic hydroxyl groups. The transformation of mangiferin to norathyriol by human fecal flora *in vitro* (Fig. 1)[5] indicates an important role of mangiferin-metabolizing bacteria in the disposition of norathyriol and its subsequent metabolites *in vivo*, as well as in the pharmacological activities of mangiferin. Norathyriol also exerts anti-inflammatory,[18,19] vasorelaxation[20] and antiplatelet[21] activities. Therefore, the biotransformation of mangiferin should be thoroughly investigated.

Here, we isolated and characterized a bacterium that transforms mangiferin to norathyriol. Furthermore, the novel enzyme that cleaved *C*-glucosyl was selectively inducible with mangiferin and apparently differs from α- and β-glucosidases.

MATERIALS AND METHODS

Chemical Mangiferin was purchased from Sigma-Aldrich Co. (U.S.A.). Chloramphenicol, rifampicin, *p*-nitrophenyl α-D-glucopyranoside (*p*NP-α-G) and *p*-nitrophenyl β-D-glucopyranoside (*p*NP-β-G) were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Diaphorase derived from *Clostridium kluyveri* was from Oriental Yeast Co. (Osaka, Japan) and NADH was purchased from Wako Pure Chemical Co. (Osaka, Japan). All other chemicals were of analytical reagent grade.

Medium Peptone yeast extract-Fildes solution (PYF) broth, in a liter consisting of 10 g of Trypticase, 5 g of yeast extract, 40 ml of Fildes solution, 40 ml of salts solution (0.2 g of $CaCl_2$, 0.2 g of $MgSO_4$, 1 g of KH_2PO_4, 1 g of K_2HPO_4, 10 g of $NaHCO_3$, and 2 g of NaCl in 1 l), and 0.5 g of L-cys-

Mangiferin Norathyriol

Fig. 1. Conversion of Mangiferin to Norathyriol

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teine–HCl, was prepared as described by Mitsuoka.[22] Deoxycholate-hydrogen sulfaite-lactose (DHL) agar was purchased from Eiken Chemical Co., Ltd., Tokyo, Japan. General anaerobic medium (GAM) and glucose blood liver (BL) agar were purchased from Nissui Seiyaku Co. (Tokyo, Japan).

Bacterial Strains *Bacteroides gracilis* JCM 8538 (*Campylobacter gracilis*), *B. multiacidus* JCM 2054 (*Mistuokella multacida*), *B. ovatus* JCM 5824, *B. thetaiotaomicron* JCM 5827 and *B. vulgatus* JCM 5826 were purchased from the Japan Collection of Microorganisms (The Institute of Physical and Chemical Research, Saitama, Japan). *Bifidobacterium adolescentis*, *B. bifidum* a E319, *B. breve* S-2 kz 1287, *B. longum* IV-55, *B. pseudolongum* PNC-2-9-G, *Clostridium butyricum*, *C. innocuum* KZ-633, *C. perfringens* T0-23, *Enterococcus faecalis* II-136, *Escherichia coli* 0-127, *Fusobacterium nucleatum* G-470, *Gaffkya anaerobia* G-0608, *Klebsiella pneumoniae* ATCC 13883, *Lactobacillus acidophilus* ATCC 4356, *L. xylosus* ATCC 155775, *L. plantarum* ATCC 14917, *Peptostreptococcus anaerobius* 0240, and *Veillonella parvula* ss *parvula* ATCC 10790 were provided by Prof. T. Mitsuoka (Tokyo University, Japan). *Eubacterium* sp. SDG-2, *Peptostreptococcus* sp. SDG-1 and *Ruminococcus* sp. PO1-3 were isolated in our laboratory.[23,24]

Screening for Bacterial Strains That Metabolize Mangiferin Bacterial strains ($n=26$) cultured in GAM broth at 37 °C for 24 h in an anaerobic chamber (Tabai EAN-140) were suspended in 50 mM phosphate buffer (pH 7.3) and 100 μl suspensions were incubated with 1 mM mangiferin for 48 h under the same conditions. Reactions were stopped with 100 μl of acidified 1-butanol (saturated with distilled water and acidified with 0.1% acetic acid), and then mangiferin and norathyriol in the butanol layer were determined by TLC as described below.

Isolation of a Bacterium That Can Metabolize Mangiferin A bacterial suspension of fresh feces from a healthy volunteer was repeatedly cultured in 1 ml of PYF broth containing 0.5 mM mangiferin under anaerobic conditions at 37 °C and then metabolic activity was determined as described above. A portion of the bacterial suspension was simultaneously inoculated on BL and DHL agar plates. The ability of colonies on the agar plates to metabolize mangiferin was screened as described above.

Bacterial Identification Isolated bacterium was identified according to *Bergey's Manual of Systematic Bacteriology*,[25] supplemented with the methods described by Mitsuoka.[22,26] The motility test was conducted on sulfide indole motility (SIM) medium (Nissui, Japan) at 24, 48 and 72 h.

Bacterially produced fermentation products, volatile (acetic, propionic, butyric, isobutyric, valeric and isovaleric) and non-volatile (lactic and succinic) acids, were separated according to the method of Rizzo.[27] Bacterium was anaerobically grown in reduced peptone-yeast extract broth containing 0.1% of glucose at 37 °C for 48 h. The reduced broth contained L-cysteine 0.5 g/l. The culture medium was clarified by centrifugation at 1650 g for 10 min at 4 °C. To determine volatile acids, 24 ml of the supernatant was saturated with 6 g of NaCl and then acidified with 360 μl of 5 N HCl. The sample was cooled before extracting with 1 ml of diethyl ether. Non-volatile acids were determined in a portion of the supernatant as follows. Polypeptides were precipitated with 48 ml of methanol and placed at −25 °C for 45 min. After centrifugation at 1650 g for 10 min, the upper part was esterified by heating with 2 ml of 50% sulfuric acid at 80 °C for 30 min. Distilled water (24 ml) was added and the methyl esters of the non-volatile acids were extracted with 3 ml of chloroform. With the exception of formic acid, the volatile acids and the methyl esters of non-volatile acids were determined by gas chromatography using a GC-12A flame ionization detector (Shimadzu Co., Japan) and a DB-WAX column (0.25 mm by 30 m) (J&W Scientific, U.S.A.) under the following conditions: column temperature, 50 °C for 4 min followed by 10 °C/min increments to 220 °C; injection block temperature, 250 °C; helium pressure, 2 kg/cm^2; hydrogen pressure, 0.7 kg/cm^2; air pressure, 0.5 kg/cm^2.

Formic acid was assayed by direct spectrophotometry using formate dehydrogenase and NAD as described by Hopner.[28] The reaction mixture (1 ml) in 40 mM phosphate buffer contained 1 mM NAD, 400 mU of formate dehydrogenase and an appropriate amount of sample. After mixing at the room temperature for 4 min, NADH formation was measured from the absorbance at 340 nm.

We tested the antimicrobial susceptibility of the bacterium. The minimal inhibitory concentration (MIC) of antibiotics (gentamycin, neomycin, paramomycin, ampicillin, penicillin G, lincomycin, chloramphenicol and tetracycline) that allows no visible growth after an 18 h incubation was determined by serial broth dilution method according to Koneman *et al*.[29]

Sequencing the Bacterial 16S rRNA Gene After incubating at 37 °C for 1 d, 5 ml culture of the bacterium was collected by centrifugation at 10000 g for 30 min. Total DNA was prepared using the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The 16S rRNA gene was amplified by PCR with two forward and reverse primer sets based on those of various *Bacteroides* strains from the database: Bac 1F (AGAGTTTGATCCTGGCTCAG) and Bac 1R (CCGTATTACCGCGGCTGCTG); and Bac 3F (TAACTACGTGCCAGCAGCCG) and Bac 3R (CCCGGGAACGTATTCACCG). Amplification proceeded in a reaction mixture containing 1 U of KOD-Plus DNA polymerase (Toyobo, Japan), 1×buffer mix, 0.8 mM dNTP mix (0.2 mM each), 1 mM $MgSO_4$, 0.3 μM of each primer, and 10—200 ng template DNA. The PCR conditions were as follows: a hot start at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s and elongation at 68 °C for 45 s, and a final elongation at 68 °C for 5 min. The PCR products were purified using the QIA Quick PCR Purification Kit (Qiagen, Germany), and directly sequenced using the Bigdye Terminator Cycle Sequencing Kit (Applied Biosystems, CA, U.S.A.), the primers (Bac 1F, Bac 1R, Bac 3F, and Bac 3R) and an ABI PRISM 310 (Applied Biosystems, CA, U.S.A.).

Bacterial Metabolism The novel bacterium was cultured with 0.5 mM mangiferin in GAM broth under anaerobic conditions for 24 h. The metabolic product was extracted from 50 ml of the cultured broth with 50 ml of butanol 3 times. The extract was dried under vacuum, dissolved in 50% methanol, and then applied to preparative HPLC to purify the metabolite under the following conditions: Cosmosil 5C18-AR-II (20×250 mm) column (Nacalai Tesque, Kyoto, Japan); flow rate, 4 ml/min; detection, 260 nm; solvent system, 20—80% linear gradient of acetonitrile in 0.1% trifluoroacetic

acid. The metabolite was obtained as yellow amorphous powder which showed 98% purity as a single peak on HPLC chromatogram; UV λ_{max}^{MeOH} (ε) nm: 237 (24000), 254 (31000), 312 (15000), 362 (13000); EI-MS *m/z*: 260 $[M]^+$; ^{1}H-NMR (500 MHz, CD_3OD) δ: 6.14 (1H, d, J=2 Hz, 2-H), 6.28 (1H, d, J=2 Hz, 4-H), 6.80 (1H, s, 5-H), 7.42 (1H, s, 8-H). The compound was identified as norathyriol by comparing with its reported data.[5)]

Bacterial suspension (100 μl) precultured in GAM broth was cultivated in 5 ml of PYF broth containing 0.4 mM of mangiferin at 37 °C under anaerobic conditions. Two 100 μl portions were removed every 6 h. Bacterial growth was measured in one portion at 540 nm (absorbance) and mangiferin and norathyriol were quantified in the other by HPLC as described below. The acidified butanol extract (5 μl) of a 100 μl portion was dried in a Speed Vac SC 110 (Savant Instruments, NY, U.S.A.) and then dissolved in 100 μl of 50% methanol for HPLC application.

The ability to metabolize other natural *C*-glucosides was determined by incubating the novel bacterium in an anaerobic environment in 50 mM phosphate buffer (pH 7.3) with 0.5 mM with flavone *C*-glucosides (abrusin and precatorin I), anthrone *C*-glucoside (barbaloin), chromone *C*-glucoside (aloesin), and quinochalcone *C*-glucoside (hydroxysafflor yellow A) for one week. Metabolic products were extracted and determined by TLC as described.[2,3,7,8,30)]

Stimulation of Bacterial *C*-Glucosyl-Cleavage Activity After anaerobic cultivation at 37 °C for 12 h, the bacterium was harvested from 1 l of cultured GAM broth and suspended in 50 ml of 50 mM phosphate buffer (pH 7.3) containing 0.2 mM mangiferin. The bacterial suspension was anaerobically incubated at 37 °C and 200 μl of the incubation mixture was removed at various times (0, 0.5, 1, 2, 4, 8, 12 h). Another portion of the bacterial suspension was incubated with various concentrations of mangiferin (0, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2 mM) for 4 h and then *C*-glucosyl-cleavage was analyzed as described below.

To examine the effects of chloramphenicol and rifampicin on the stimulation of *C*-glucosyl-cleavage ability of mangiferin, bacterium was suspended in 50 mM phosphate buffer containing 0.2 mM mangiferin and anaerobically incubated with additives at the indicated concentrations at 37 °C. After 9 h, the bacterial cells were harvested and the activity was determined as described below.

We measured the appearance of *C*-glucosyl-cleavage activity of mangiferin-related xanthone derivatives (gerontoxanthone A, gerontoxanthone B, gerontoxanthone I and cudraniaxanthone), *O*-glucosides (swertiamarin and salicin), *C*-glucosides (abrusine, abrusine 2″-*O*-β-apioside, barbaloin, isobarbaloin, carminic acid and puerarin), a mangiferin derivative (octa-acetylmangiferin) and norathyriol. Each compound was added to a final concentration of 0.2 mM to the bacterial suspension. After anaerobic incubation at 37 °C for 9 h, the cells were harvested and the *C*-glucosyl-cleavage of mangiferin was assayed.

Preparation of a Cell-free Extract The *B*. sp. MANG was cultured under anaerobic conditions at 37 °C for 12 h in GAM broth containing 0.2 mM mangiferin, harvested and then suspended in 50 mM phosphate buffer (pH 7.3). The bacterial cells were disrupted by 10 sonic bursts of 30 s each (Branson Sonifier 250, Branson Unltrasonics Corporation, Danbury, CT, U.S.A.) on ice in a cold room, and then centrifuged at 100000 ***g*** for 60 min (Ultracentrifuge Beckman Optima XL-70, Beckman Instruments, Fullerton, CA, U.S.A.) at 4 °C to separate the cell-free extract (supernatant).

***C*-Glucosyl-Cleaving Activity Assay for Mangiferin** Bacterial Activity Assay: We incubated *B*. sp. MANG prepared as described above in 200 μl of 50 mM phosphate buffer (pH 7.3) containing 1 mM mangiferin in an anaerobic incubator at 37 °C for 30 min. The reaction was stopped with 200 μl of acidified butanol. Portions of the butanol extract (10 μl) were dried using a Speed Vac SC 110 and the residue was dissolved in 200 μl of 50% methanol. The norathyriol product was determined by HPLC.

Assay of Cell-free Extract Activity: The reaction mixture containing 20 mM NADH, 25 units of diaphorase, which probably consume oxygen, 20 mM dithiothreitol, 1 mM mangiferin and 30 μl of cell-free extract in a final volume of 100 μl, was anaerobically incubated at 37 °C for 2 h. The reaction was stopped by adding 100 μl of acidified butanol and then processed as described above.

Glucosidase Activity Assay Alpha and beta-glucosidase activities were measured using *p*NP-α-G and *p*NP-β-G as substrates, respectively, as described.[31)] The reaction mixture (1 ml) in 50 mM phosphate buffer (pH 6.0) contained a bacterial suspension or cell-free extract and 1.5 mM of each substrate. After incubation at 37 °C for the indicated periods, the reaction was stopped with 250 μl of Na_2CO_3. Liberated *p*-nitrophenol was measured from the absorbance at 405 nm.

Qualitative Assays of Mangiferin and Norathyriol by TLC and HPLC The butanol extract was separated by TLC (Silica gel RP-18 F254 S, Merck, Darmstadt, Germany) using $CH_3OH : H_2O : CH_3COOH$ (5 : 5 : 0.2) as the solvent, and then mangiferin and norathyriol were visualized under UV light (Spectroline CM-10, Spectronics Corporation, NY, U.S.A.).

The reversed phase HPLC (Shimadzu Co., Japan) conditions were as follows: recorder, C-R6A Chromatopac; pump, LC-6A; system controller, SCL-6B; monitor, SPD-6A; injector, SIL-9A; column, YMC-Pack ODS-AP AP-302 (4.6×150 mm) (YMC Co., Kyoto, Japan); flow rate, 1 ml/min; detection, 260 nm; solvent system, 10—40% acetonitrile linear gradient in 0.1% trifluoroacetic acid. The retention time of mangiferin and norathyriol were 7.5 and 14.5 min, respectively.

RESULTS

Isolation and Characterization of a Bacterium That Transforms Mangiferin to Norathyriol None of the 21 human bacterial strains screened including *Bifidobacterium*, *Clostridium*, *Enterococcus*, *Escherichia*, *Eubacterium*, *Fusobacterium*, *Gaffkya*, *Klebsiella*, *Lactobacillus*, *Peptostreptococcus*, *Ruminococcus* and *Veillonella*, metabolized mangiferin. A colony possessing potent ability to metabolize mangiferin was isolated from human feces and NMR spectroscopy identified the product as norathyriol. The isolated strain was a strict gram-negative anaerobic rod belonging to the Bacteroidaceae family. The organism was non-motile and fermented glucose to a mixture of volatile and non-volatile acids including lactic acid as a major product together with moderate amounts of acetic, butyric, formic, isovaleric and

succinic acids and trace levels of isobutyric and propionic acids. We therefore concluded that the organism was a member of the genus *Bacteroides*, and we named it *Bacteroides* sp. MANG.

The isolated bacterium was resistant to the aminoglycosides such as gentamycin, neomycin and paramomycin with MICs of 50, 200 and 250 μg/ml, respectively, which are features of *Bacteroides* species.[32] Furthermore, the MANG strain was susceptible to β-lactam type antibiotics (ampicillin and penicillin G), lincomycin, chloramphenicol and tetracycline with MICs in the range of 2—15μg/ml.

Lactic acid and H_2S production, nitrate reduction, as well as starch and esculin hydrolysis suggested that the strain was related to *Bacteroides multiacidus*. The sugars were fermented by the strain MANG in comparison with *B. multiacidus* as shown in Table 1.

We tested mangiferin metabolism in *Bacteroides* species such as *B. gracilis*, *B. multiacidus*, *B. ovatus*, *B. thetaiotaomicron* and *B. vulgatus*, which might be related to *B*. sp. MANG. However, we did not identify such metabolic activities (Table 2).

Table 1. Comparative Fermentation Reactions of *B*. sp. MANG and *B. multiacidus*

Compound	*B*. sp. MANG	*B. multiacidus* [a,b]
Amygdalin	−	+−
Arabinose	+	+
Cellobiose	+	+
Dextrin	−	+
Esculin	−	+−
Fructose	+	+
Galactose	+	+
Glucose	+	+
Glycerol	−	−
Glycogen	−	−
Inositol	−	+
Inulin	+	−
Lactose	+	+
Maltose	+	+
Mannitol	−	+−
Melibiose	+	+
Melezitose	+	+
Mannose	+	+
Rhamnose	+	−+
Ribose	+	+−
Raffinose	+	+
Starch	−	+
Salicin	+	+
Sorbitol	−	v
Sucrose	+	+
Trehalose	+	+−
Xylose	+	+

a) Bergey's Manual of Systematic Bacteriology, 1984.[25] *b*) Mitsuoka T., Intestinal Bacteriology, 1990.[22] Symbols: +, positive reaction (below pH 5.5); −, negative reaction (pH 6.0 or above); v, sometimes positive, sometimes negative; +−(−+), almost positive (negative) but exhibit a negative (positive) reaction.

Sequencing the 16S rRNA Gene We determined about 1353 bases of the 16S rRNA gene sequence from *B*. sp. MANG and deposited in the GenBank database under accession number AY764158. A comparison with 16S rRNA genes from various *Bacteroides* from a GenBank database in ClustalW program (European Bioinformatics Institute, U.K.), showed 71—74% sequence similarity to those of all *Bacteroides* species examined: *B. acidifaciens* (GenBank accession no. AB021156), *B. eggerthii* (AB050107), *B. vulgatus* (AB050111), *B. fragilis* (AB050106), *B. gracilis* (L04320), *B. ovatus* (AB050108), *B. putredinis* (L16497), *B. thetaiotaomicron* (AB050109), *B. uniformis* (AB050110) and *B. ureolyticus* (L04321). Phylogenetic analyses of the 16S rRNA sequences indicated the relationships among these bacteria (Fig. 2).

Metabolic Conversion of Mangiferin to Norathyriol by *B*. sp. MANG Figure 3 shows the time course of transformation of mangiferin to norathyriol by *B*. sp. MANG. Mangiferin was transformed to norathyriol in direct proportion to the bacterial growth in PYF broth, suggesting cleavage of the *C*-glucosyl bond of mangiferin. After 12 h culture, growth and norathyriol production reached the maximum.

However, *B*. sp. MANG did not metabolize any other *C*-glucosides such as abrusin, precatorin I, barbaloin, aloesin and hydroxysafflor yellow A.

Induction of *C*-Glucosyl-Cleaving Enzyme in *B*. sp. MANG by Mangiferin In the absence of mangiferin, *B*. sp. MANG did not have any ability to cleave *C*-glucosyl bond. However, *C*-glucosyl-cleavage appeared time-dependently in the presence of 0.2 mM mangiferin (Fig. 4). Figure 5 shows the dose-dependence of *C*-glucosyl-cleaving activity upon mangiferin.

The appearance and stimulation of the mangiferin *C*-glucosyl-cleavage in *B*. sp. MANG by mangiferin was inhibited by inhibitors of protein and RNA syntheses, such as chloramphenicol and rifampicin at 0.062 and 0.12 mM, respectively

Table 2. Mangiferin *C*-Glucosyl-Cleaving Activity of Various *Bacteroides* Species

Bacteroides species	Yield of norathyriol (%)
B. gracilis	ND
B. mutiacidus	ND
B. ovatus	ND
B. thetiotaomicron	ND
B. vulgatus	ND
B. sp. MANG	73.8

Each bacterium cell was anaerobically cultured in 50 mM phosphate buffer containing 1 mM mangiferin at 37 °C for 48 h, and then analyzed for norathyriol by HPLC. ND, not detected.

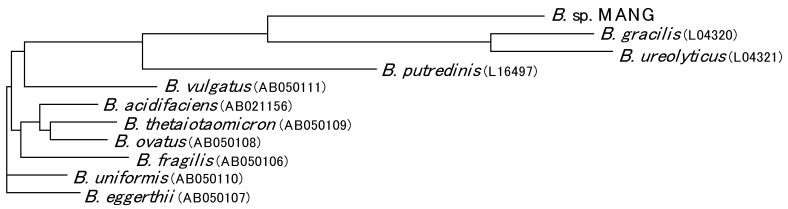

Fig. 2. Phylogenic Tree of *Bacteroides* sp. MANG Compared with *Bacteroides* spp. by Neighbour Joining

The 16S rRNA genes of *Bacteroides* spp. from database in ClustalW program.

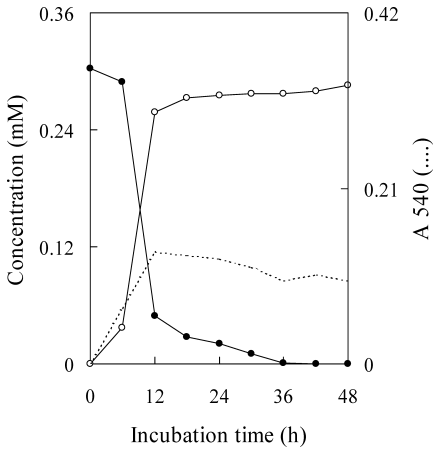

Fig. 3. Time Course of Conversion of Mangiferin (●) to Norathyriol (○) by *B*. sp. MANG in PYF Broth

Bacterial growth (····) was monitored by measuring absorbance at 540 nm.

Table 3. Effects of Antibiotics on Stimulation of *C*-Glucosyl-Cleaving Activity in *B*. sp. MANG

Additives	Concentration (mM)	Relative activity (%)
None		100
Chloramphenicol	0.062	0
	0.62	0
Rifampicin	0.012	10.1
	0.12	0

Each antibiotic was added during stimulation period before *C*-glucosyl-cleaving activity was determined.

(Table 3). These results, together with the findings that both antibiotics did not appreciably affect the enzymatic assay in the cell-free extract (data not shown), indicated that the *C*-glucosyl-cleavage enzyme was induced by mangiferin.

We examined induction of the *C*-glucosyl-cleaving enzyme using *C*-glucosides and mangiferin-related compounds such as abrusine, abrusine 2″-*O*-*β*-apioside, barbaloin, isobarbaloin, carminic acid, puerarin, gerontoxanthone A, gerontoxanthone B, gerontoxanthone I, cudraniaxanthone, octa-acetylmangiferin and norathyriol. None of these compounds stimulated the *C*-glucosyl-cleaving activity of mangiferin. Some *O*-glucosides such as swertiamarin and salicin also had no affect. Thus, the *C*-glucosyl-cleavage enzyme in *B*. sp. MANG that was induced by mangiferin, was not affected by related compounds.

Distinction of *C*-Glucosyl-Cleavage Enzyme from *O*-Glucosidases Alpha and beta-glucosidase activities in the bacterial cells were not related to the *C*-glucosyl-cleavage enzyme under any tested conditions. The activities of *α*- and *β*-glucosidase activities remained high in the bacterium even when it was not preincubated with mangiferin, and no *C*-glucosyl-cleaving activity was evident (Figs. 4, 5). The activities of both glucosidases were not appreciably affected by mangiferin compared with that of the enzyme that cleaved *C*-glucosyl bond.

Gluconolactone and 1-deoxynojirimycin, specific inhibitors of glucosidases, did not inhibit the *C*-glucosyl-cleavage activity in cell-free extracts of *B*. sp. MANG, but potently inhibited the activities of *α*- and *β*-glucosidases (Table 4).

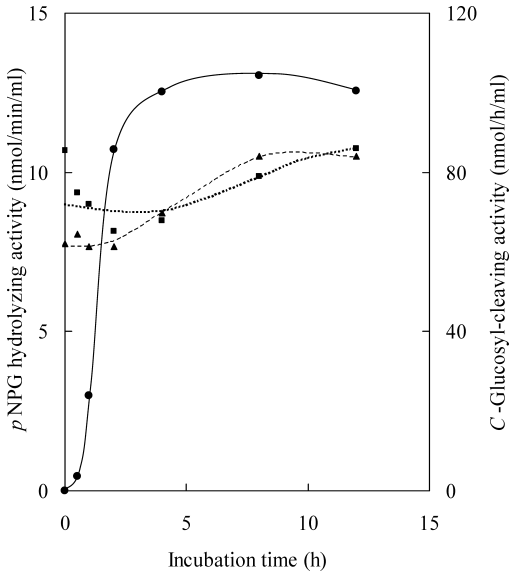

Fig. 4. Time Course of *C*-Glucosyl-Cleavage Activity of *B*. sp. MANG Induced by 0.2 mM Mangiferin at 37 °C

C-Glucosyl-cleavage activity (●) compared with *p*NP-*α*-G- (▲) and *p*NP-*β*-G- (■) hydrolyzing activities under the same conditions.

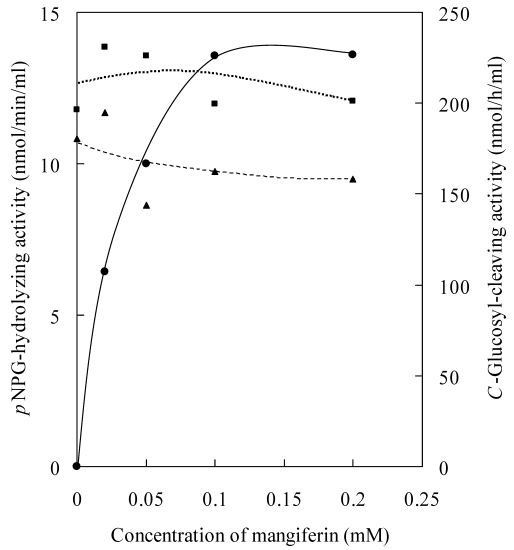

Fig. 5. Effect of Mangiferin Concentrations on the Stimulation of *C*-Glucosyl-Cleaving Activity of *B*. sp. MANG at 37 °C for 4 h

C-Glucosyl-cleaving activity (●) was determined together with *p*NP-*α*-G- (▲) and pNP-*β*-G- (■) hydrolyzing activities.

Table 4. Effects of Glucosidase Inhibitors on Glucosidase Activities (as Determined with *p*NP-*α*-G and *p*NP-*β*-G as the Substrates) and Mangiferin *C*-Glucosyl-Cleaving (CGC) Activity of a Cell-free Extract

Additives	Concentration (mM)	Relative activity (%)		
		*p*NP-*α*-G	*p*NP-*β*-G	CGC
None		100	100	100
1-Deoxynojirimycin	0.01	54.6	49.7	94.7
	0.1	20.4	18.8	102
	1	3.6	2.4	112
Gluconolactone	0.01	102	90.1	89.7
	0.1	62.8	39.1	91.6
	1	36.8	22.4	107

DISCUSSION

A bacterium responsible for the conversion of mangiferin, *C*-glucoside, to its aglycone, norathyriol, was isolated from human feces and identified as *Bacteroides* species MANG, by biochemical and 16S rRNA sequence analyses. In addition, twenty-six defined strains of human intestinal bacteria including five strains of *Bacteroides* closely related to *B*. sp. MANG, did not metabolize mangiferin.

The ability of *B*. sp. MANG to produce a mixture of fermentation products including lactate, acetate, butyrate, formate, succinate, isovalerate, isobutyrate and propionate from glucose and other fermentation characteristics (Table 1) indicated that this strain is close to *B. multiacidus*. On the other hand, a phylogenic tree based on a comparison of 16S rRNA sequences placed *B*. sp. MANG in a cluster containing *B. putredinis*, *B. gracilis* and *B. ureolyticus*. However, the 16S rRNA sequence of *B. multiacidus* has not yet been determined. Besides *B. gracilis*, *B. multiacidus*, *B. ovatus*, *B. thetaiotaomicron* and *B. vulgatus* did not show metabolize mangiferin, indicating that *B*. sp. MANG is a novel strain of the genus *Bacteroides*.

The stoichiometric conversion of mangiferin to norathyriol suggested that *B*. sp. MANG cleaved a C–C bond only between norathyriol and glucose. The *Eubacterium* sp. BAR transforms among *C*-glucoside, only barbaloin, to its aglycone.[33] These results suggest that specific bacterial strains in human feces metabolize various *C*-glucosides. This implies that the respective enzymes recognize the aglycone, and not the glucose moieties, of various *C*-glucosides. Similarly, specific factors might be required at the transcription or translation level to induce these enzymes, because only mangiferin and barbaloin induced their respective metabolic enzymes.

The *Bacteroides* genus has glucosidase activities towards various substrates that are substrate-induced.[34,35] Many results supported a distinction between the *C*-glucosyl-cleavage enzyme and glucosidases in *B*. sp. MANG. Potent α- and β-glucosidases activities were detected in the bacterium in the absence of mangiferin, when mangiferin-metabolizing activity was undetectable, whereas *C*-glucosyl-cleaving activity appeared and increased in the presence of mangiferin without affecting glucosidases activities (Figs. 4, 5). Furthermore, the glucosidases inhibitors, 1-deoxynojirimycin and gluconolactone, inhibited the activity of glucosidases, but not that of the *C*-glucosyl-cleaving activity (Table 4). Therefore, the *C*-glucosyl-cleavage enzyme for mangiferin does not associate with, and is quite different from the glucosidases. In fact, neither α-glucosidase from *Saccharomyces* (Wako Pure Chemical Co., Osaka, Japan) nor β-glucosidase from almonds (Sigma Aldrich Co., U.S.A.) had *C*-glucosyl-cleaving activity towards mangiferin (data not shown), a *C*-glucoside, as reported.[36]

Mangiferin and norathyriol have many pharmacological activities as noted in the Introduction. When orally administered, mangiferin has antitumor, antidiabetic, antioxidant, hepatoprotective and immunomodulative activities.[11—15] Owing to poor absorption from the gastrointestinal tract,[16] *B*. sp. MANG seems to play important roles in the pharmacological actions of mangiferin. After providing oral mangiferin to rabbits, euxanthone and its glucuronides are excreted into the urine,[17] suggesting further dehydroxylation of norathyriol to euxanthone. Since intestinal bacteria easily dehydroxylate the phenol ring[24] and *B*. sp. MANG did not further metabolize norathyriol, other intestinal bacteria must contribute to this process. Such metabolism by intestinal bacteria might be prerequisite for activation by mangiferin.

To our knowledge, this is the first reported of an enzyme that can cleave the C–C bond of mangiferin. Future studies will focus upon the purification and characterization of the *C*-glucosyl-cleavage enzyme.

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