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Aerobic bioconversion of C-glycoside mangiferin into its aglycone norathyriol by an isolated mouse intestinal bacterium

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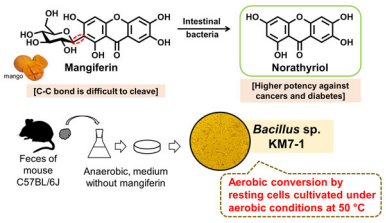
ABSTRACT

Norathyriol is an aglycone of a xanthonoid C-glycoside mangiferin that possesses different bioactive properties useful for humans compared to mangiferin. Mangiferin is more readily available in nature than norathyriol; thus, efficient mangiferin conversion into norathyriol is desirable. There are a few reports regarding mangiferin C-deglycosylation because of the C–C bond resistance toward acid, alkaline, and enzyme hydrolysis. In this study, we isolated a mangiferin-deglycosylating bacterium strain KM7-1 from the mouse intestine. 16S rDNA sequencing indicated that KM7-1 belongs to the *Bacillus* genus. Compared to the taxonomically similar bacteria, the growth characteristic of facultative anaerobic and thermophilic resembled, yet only *Bacillus* sp. KM7-1 was able to convert mangiferin into norathyriol. Resting cells of *Bacillus* sp. KM7-1 obtained from aerobic cultivation at 50 °C showed high norathyriol formation from 1 mM of mangiferin. Norathyriol formation can be conducted either under aerobic or anaerobic conditions, and the reaction depended on time and bacterial amount.

Graphical Abstract



C-glycoside mangiferin was converted into norathyriol under aerobic conditions by *Bacillus* sp. KM7-1 isolated from mouse feces.

Keywords: *Bacillus*, deglycosylation, C-glycoside, mangiferin, norathyriol

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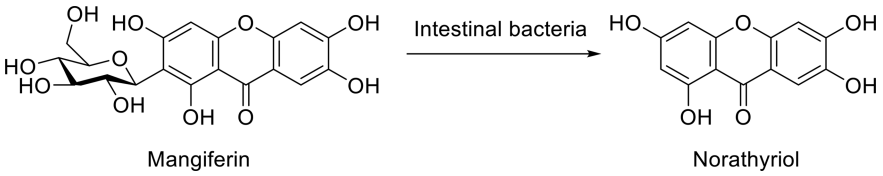

Figure 1. Bioconversion of mangiferin into norathyriol by intestinal bacteria.

Polyphenols are abundant and contribute to human health through the consumption of fruits, vegetables, and plant products as part of a daily diet. Mangiferin (2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone; Figure 1, left side), a xanthonoid C-glycoside belonging to polyphenols, was first isolated as coloring matter from mango (*Mangifera indica* Linn.) (Iseda 1957). Mangiferin was reported to possess bioactive properties such as anti-inflammatory (Wang *et al.* 2015), antiobesity (Subash-babu and Alshatwi 2015), and angiogenesis (Daud *et al.* 2010). The aglycone of mangiferin, norathyriol (Figure 1, right side), reportedly has different bioactive properties. Compared to mangiferin, norathyriol has more potency as an antidiabetic (Shi *et al.* 2017; Gu *et al.* 2019) and against cancer cells (breast (Wilkinson *et al.* 2015), colon, and lung (Souza *et al.* 2020)). Moreover, some reports stated that norathyriol as a mangiferin metabolite was responsible for antidiabetic activity (Wang *et al.* 2014), acted as an antihyperuricaemic agent (Niu *et al.* 2016), and worked against hepatic lipid metabolic disorder (Li *et al.* 2018).

Mangiferin is available in several plant families, including Anacardiaceae, Gentianaceae, and Iridaceae (Sanugul *et al.* 2005). Nevertheless, the C–C bond in C-glycosides is more resistant to acid, alkaline, and enzyme hydrolysis than the C–O bond in O-glycosides. Human intestinal bacteria are already reported to convert mangiferin into norathyriol (Hattori *et al.* 1989; Sanugul *et al.* 2005; Huang *et al.* 2011; Souza *et al.* 2020), as illustrated in Figure 1. To our knowledge, there were only 2 bacterial isolates capable of converting mangiferin into norathyriol. Both of *Bacteroides* sp. MANG (Sanugul *et al.* 2005) and *Lachnospiraceae* strain CG19-1 (Braune and Blaut 2011) (later announced as *Catenibacillus scindens* (Braune and Blaut 2018)) were isolated from the human intestine and were strictly anaerobic. Besides humans, the intestinal bacteria of rats were reported to deglycosylate mangiferin as well (Liu *et al.* 2011, 2012; Tian *et al.* 2016), yet no isolated bacterium has been reported at present.

This study reported the isolation and initial characterization of a mouse intestinal bacterium capable of deglycosylating mangiferin into norathyriol. The bacterium was unique as it was a facultative anaerobic bacterium that deglycosylated mangiferin even though it was cultivated in a medium that did not contain mangiferin. The bacterium was also able to produce norathyriol in a state of resting cells under both aerobic and anaerobic conditions.

Materials and methods

Materials

Gifu anaerobic medium (GAM) and nutrient broth (NB) medium used in this study were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Mangiferin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Puerarin was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Norathyriol and daidzein used as references were obtained from Tokyo Chemical Industry Co., Ltd. and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. Water for liquid chromatography (LC-MS grade) and methanol (gradient grade for liquid chromatography) were purchased from Merck KGaA (Darmstadt, Germany). DMSO and TFA were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Bacterial strains of *Bacillus hisashii* N-11 (NBRC 110226) and N-21 (NBRC 110227), and *Bacillus thermoamylovorans* LMG 18084 were obtained from National Bioresource Center, NITE (Osaka, Japan) and Belgian Co-ordinated Collections of Micro-organisms (Gent, Belgium), respectively.

Cultivation of mouse intestinal bacteria

Feces were collected from diet-induced obesity model mice C57BL/6J. The animals were housed at a constant temperature (22 ± 1 °C) with a 12-h dark/light cycle and had access to tap water and food. All procedures were approved by the Animal Care and Use Committee, Okayama University (OKU-2016239) and were conducted in accordance with the Policy on the Care and Use of the Laboratory Animals, Okayama University. Fresh feces (0.1 g) were inoculated into 30 mL of GAM broth and incubated for 5 d at 37 °C under anaerobic conditions (AnaeroPack System, Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan). Resting cells were prepared by centrifugation (KUBOTA6500, Kubota Corporation, Osaka, Japan) of the bacterial suspension at 15 490 *g*, 4 °C for 10 min, and then the cells were washed with 0.85% NaCl. Fresh, wet resting cells were subjected to norathyriol formation activity assay.

Isolation of active strain

Part of the cultivated cells was preserved in glycerol (20% v/v) and kept in a −70 °C deep freezer for the next isolation step. After the activity assay, the −70 °C preserved culture containing active bacteria were cultured in GAM broth. The liquid culture obtained was diluted up to 10^{-7} in 0.85% NaCl before cultivation onto GAM agar plate. The plates were incubated at 37 °C under anaerobic conditions for 1-6 d. Resting cells were prepared by cell collection using 0.85% NaCl, followed by centrifugation (KUBOTA3740, Kubota Corporation) at 10 000 *g*, 4 °C for 10 min. Isolation based on norathyriol formation activity preceded by cultivation in liquid medium and plate culture dilution was repeatedly conducted until single colonies picked up. The single colonies were streaked onto the GAM agar plate, and the cells obtained were subjected to activity assay.

Identification of isolated bacterium

The isolated bacterium was cultivated on GAM agar plate at 37 °C for 4 d under anaerobic conditions. Identification was performed by TechnoSuruga Laboratory Co., Ltd (Shizuoka, Japan) on 16S rDNA. A comparison of nucleotide sequences was performed by BLAST homology search (Altschul *et al.* 1997). The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987), with the base substitution model

referred to Kimura-2-parameter (Kimura 1980), and the reliability of tree shape was evaluated using the bootstrap method (1000 replications) (Felnstein 1985). ENKI software (TechnoSuruga Laboratory) was used for homology search and phylogenetic analysis.

Cultivation of isolated bacterium

Resting cells used in the norathyriol formation activity assay of the isolated bacterium were obtained from various cultivation methods as follows: (1) under anaerobic or aerobic conditions, (2) in GAM or NB medium, (3) in an Erlenmeyer flask or test tube or an agar plate, (4) in shaking or static culture, (5) at several cultivation temperatures, and (6) for various cultivation times. The anaerobic cultivation condition was obtained using the AnaeroPack System. Static incubations were conducted in a Cool Incubator CN-40A and CN-25C (Mitsubishi Electric, Tokyo, Japan), and Bioshaker MBR-022UP and BR-43FL (Taitec Co., Ltd., Saitama, Japan). Shaking incubations were conducted at 180 strokes per min in an RTS-600R (Sanki Seiki Co., Ltd., Osaka, Japan) for the Erlenmeyer flask and test tube simultaneously.

Norathyriol formation activity assay

Resting cells were suspended in 20 µL of 250 mM sodium phosphate buffer (pH 7.3) and 75 µL of ion-exchange water. The norathyriol formation activity assay was started by the addition of 5 µL of 20 mM mangiferin in DMSO (final 1 mM of mangiferin in 100 µL suspension). The conversion reactions were performed at 37 °C under various conditions as follows: (1) under anaerobic or aerobic conditions, (2) using different resting cell amounts, and (3) observed at various times (mainly at 24 h except for another condition as explained). The anaerobic reaction condition was obtained by using the AnaeroPack System. The aerobic condition was an opened cap of conversion reaction tube and sealing paper (MAF710 AeraSeal Breathable Sterile Sealing Film; Gel Company, San Francisco, CA, USA) on the tube opening. The sealing paper was used to provide aeration while preventing contamination. A control without bacterial cells was incubated as well. After conversion, reaction mixtures were extracted 3 times using 100 µL of water-saturated 1-butanol containing 0.1% acetic acid. The butanol layer was then analyzed by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) as mentioned below.

Puerarin conversion activity assay of isolated bacterium

Puerarin conversion activity was measured according to the method of norathyriol formation activity described above, except that puerarin was used instead of mangiferin as the substrate. Resting cells used were obtained from the growth of isolated bacterium at 37 °C on GAM agar and in NB for 4 d under anaerobic and aerobic conditions, respectively. The conversion reaction was performed at 37 °C under anaerobic conditions for 24 h. As a comparison, mangiferin conversion activity assay by using the same cultivated cells was performed simultaneously.

UPLC-MS analysis condition

Determination of compounds in reaction mixtures was conducted by Acquity H-Class UPLC-MS (Waters Corp., Milford, MA, USA) system. The UPLC was equipped with an H-Class QSM pump, a Sample Manager FTN, a PDA detector, and a QDa detector (all of Waters Corp.). A total of 0.2 µL of butanol extract of the reaction mixture was injected by an autosampler and separated in a Waters HSS T3 column, 1.8 µm diameter, 2.1 × 50 mm. The column temperature was maintained at 30 °C. Solvent A was water for liquid chromatography with 0.1% TFA, and solvent B was methanol with 0.1% TFA. The UPLC eluent condition was as follows: 0-0.79 min, 30% solvent B; 0.79-1.28 min, a gradient of 30%-75% solvent B; 1.28-2.32 min, 75% solvent B; 2.32-2.81 min, a gradient of 75%-30% solvent B; and 2.81-4.35 min, 30% solvent B. The sample was analyzed at a flow rate of 0.6 mL/min. PDA detection was continued with single quadrupole mass spectrometry detection in electrospray positive ionization mode. Mass spectra were generated under conditions as follows: capillary voltage, 0.8 kV; cone voltage, 15 V; desolvation temperature, 600 °C; and mass range, 100-500 Da. Product was identified based on the comparison of the retention time, maximum absorbance value, and mass spectra of the peak in the sample with the reference compound. The retention time of mangiferin, norathyriol, puerarin, and daidzein were 0.65, 1.93, 0.63, and 1.89 min, respectively. Quantification of mangiferin and norathyriol in the reaction mixture was made using respective reference compound calibration curves (chromatogram of reference compound is shown in Figure 2d). Software Empower 3 was used for instrument control and data acquisition.

Results

Detection of conversion of mangiferin to norathyriol by mouse intestinal bacteria

Mangiferin-deglycosylating activity was detected in 4 of 21 cultured mouse intestinal bacteria. The product formed has the same retention time and UV spectrum (254, 314, and 363 nm) to reference compound of norathyriol, and confirmed by positive electrospray mass spectrum which showed an $[M + H]^+$ ion at m/z 260.9. This result revealed that the bacteria converting mangiferin into norathyriol were found not only in the intestine of humans (Hattori *et al.* 1989; Sanugul *et al.* 2005; Huang *et al.* 2011; Souza *et al.* 2020) and rats (Liu *et al.* 2011; Liu *et al.* 2012; Tian *et al.* 2016) but also in mouse, and the bacteria were culturable. Although conversion by cultured mouse intestinal bacteria resulted in a decrease of mangiferin concentration, the norathyriol formation was only exhibited in 4 culturable bacterial mixtures, and the activity varied from very low (5%) to high (≥50%).

Isolation of mangiferin-deglycosylating mouse intestinal bacteria

Resting cells from 1 liquid culture obtained from mouse intestine (27 weeks old, regular feed, added preservative and mushroom extract for the drink) showed high mangiferin–deglycosylation activity, indicated by high norathyriol formation. Cultivations of the bacterial mixture up to the fourth generation continuously exhibited mangiferin deglycosylation. The activity was stably retained through generations; hence, isolation of the strains which exhibits conversion activity of mangiferin into norathyriol was conducted by using the dilution plate method. From a total of 24 picked-up colonies, 4 isolates coded KM7-1, KM2, KM4, and UH-8 showed the ability to deglycosylate mangiferin into norathyriol. From the 4 isolates, KM7-1 was selected for further characterization due to its stability in mangiferin–deglycosylation activity. UPLC chromatograms related to mangiferin deglycosylation into norathyriol are shown in Figure 2.

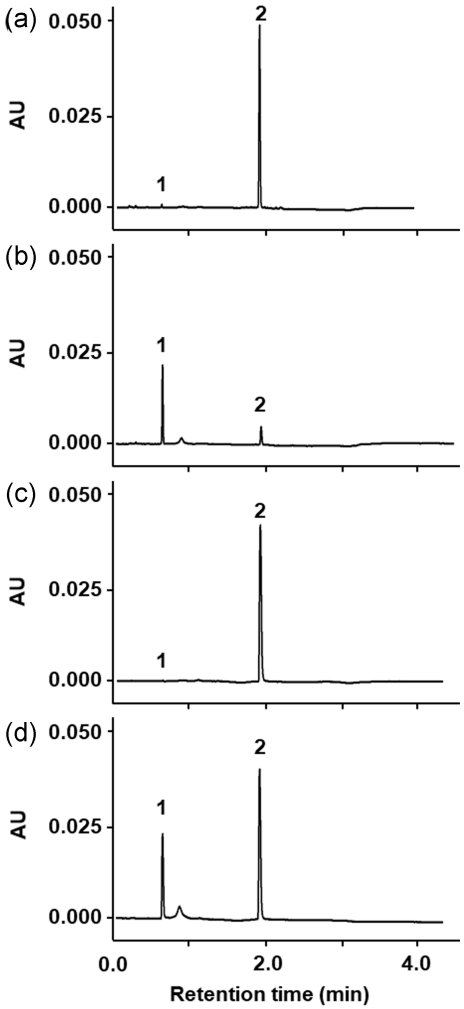

Figure 2. UPLC chromatograms related to mangiferin conversion (detection at 310 nm). (a) reaction by second generation of mouse intestinal bacterial mixture, (b) reaction by first generation of isolated bacterium KM7-1 cultivated under anaerobic conditions, (c) reaction by isolated bacterium KM7-1 cultivated under aerobic conditions, (d) reference compound, 1: mangiferin; 2: norathyriol.

Despite the reports of the ability of the intestinal bacteria of rats to deglycosylate mangiferin (Liu *et al.* 2011, 2012; Tian *et al.* 2016), no isolated bacterium has been reported at present. KM7-1 was the first mangiferin-deglycosylating intestinal bacterium to be isolated from a mouse, and the third of all isolated bacteria reported to possess such activity.

Identification of isolated bacterium

The base sequence of 500 bp on the 5′ end side of 16S rDNA of KM7-1 was sequenced for bacterial identification, and the phylogenetic affiliation of the bacterium was determined by comparing the gene sequence to that in the database. The partial 16S rDNA sequence of KM7-1 (500 bp) showed 99.2% identity to *Bacillus hisashii* N-11 (accession no. AB618491) and 99.0% to *Bacillus thermoamylovorans* LMG 18084 (accession no. FN666255) (Figure 3). The corresponding sequences of KM2 and KM4 were identical to KM7-1. Based on the sequence similarity, we named the isolated strain *Bacillus* sp. KM7-1. The partial sequence of the 16S rDNA has been deposited in DDBJ/GenBank/EMBL under accession number LC589309.

Growth and mangiferin conversion activity of *Bacillus* sp. KM7-1 in various cultivation conditions

B. hisashii and *B. thermoamylovorans*, the taxonomically similar *Bacillus* species to *Bacillus* sp. KM7-1, were both facultative anaerobic, thermophilic, and grew optimally at 50 °C (Combet-Blanc *et al.* 1995; Nishida *et al.* 2015). *B. hisashii* was isolated from the caeca of mice which were fed a high temperature-fermented feed of marine products, and it can grow at 25-60 °C (Miyamoto *et al.* 2013). *B. thermoamylovorans* was isolated from an African palm wine (tropical alcoholic beverage) sampled in Senegal, and it can grow at temperatures up to 58 °C (Combet-Blanc *et al.* 1995). Based on these information, we cultivated the *Bacillus* sp. KM7-1 in various temperatures and under aerobic conditions and then examined whether the resting cell still exhibited the ability to deglycosylate mangiferin.

Cultivation of *Bacillus* sp. KM7-1 at 37 °C for 4 d resulted in better growth under aerobic conditions (in NB) than under anaerobic conditions (in GAM broth). By using the wet cell weight, the resting cells of *Bacillus* sp. KM7-1 cultivated under aerobic conditions in NB (reaction under aerobic conditions) exhibited higher mangiferin conversion activity into norathyriol compared to the cells cultivated under anaerobic conditions in GAM broth (reaction under anaerobic conditions) (Table 1). *Bacillus* sp. KM7-1 cultivated under aerobic conditions converted mangiferin into norathyriol at the same level, either under anaerobic or aerobic conversion conditions (Table 2). Although *Bacillus* sp. KM7-1 was isolated under anaerobic conditions at 37 °C, it grew better and still exhibited mangiferin deglycosylation under aerobic conditions.

Cultivation of *Bacillus* sp. KM7-1 at higher temperatures (45, 50, 55, and 60 °C) showed that *Bacillus* sp. KM7-1 grows at all of the tested temperatures, with optimal growth at 50 °C. Cultivation at 55 and 60 °C still exhibited good growth and cells grown at these temperatures showed the same level of norathyriol formation activity (data not shown). A more detailed examination of growth at 50 °C under aerobic conditions is shown in Figure 4. At the same incubation duration, a static culture of *Bacillus* sp. KM7-1 resulted in higher cell growth than shaking culture. *Bacillus* sp. KM7-1 grew faster in an Erlenmeyer flask than in a test tube. Although aerobic conditions resulted in better growth of *Bacillus* sp. KM7-1 than anaerobic conditions, too much aeration as in the test tube or shaking conditions resulted in slower growth instead. The optimum growth condition of *Bacillus* sp. KM7-1 was obtained in 10 mL of NB in a 20 mL of Erlenmeyer flask at 50 °C under aerobic, static culture for 3 d of incubation.

Considering the ability of *Bacillus* sp. KM7-1 to grow at higher temperatures, norathyriol formation activity assay was conducted using 35 mg of resting cells from cultivation at 37, 45, and 50 °C from 4 d static, aerobic incubations. The result showed that higher cultivation temperature tends to increase norathyriol formation (data not shown). Related to the various cultivation methods at 50 °C under aerobic conditions as shown in Figure 4, Figure 5 shows that it did not affect the high level of norathyriol production, with the exception on resting cells from shaking cultivation in a 20 mL of Erlenmeyer flask for 3 and 4 d of incubation. Instead of using a rotary-shaking incubator, the shaking incubations of Erlenmeyer flask was conducted simultaneously with the test tube in a reciprocal-shaking incubator, and it might affect the norathyriol formation activity. Lower wet cell weight (15 mg), obtained from the optimum growth condition described above, was completely converted mangiferin into norathyriol within 24 h of conversion reaction (data not shown). The optimum conversion condition of 1 mM of mangiferin by

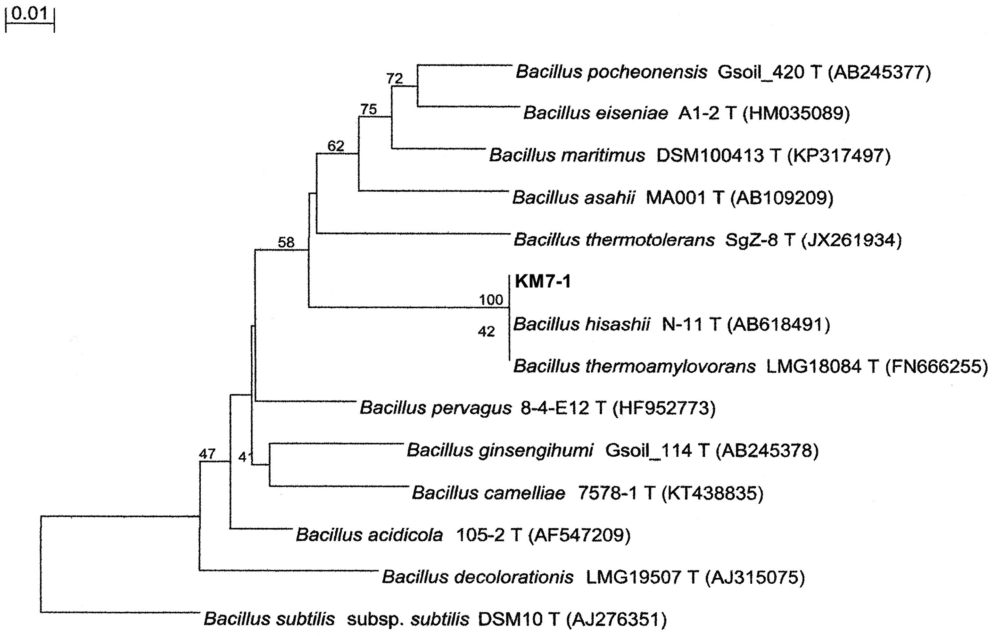

Figure 3. Phylogenetic tree of KM7-1 based on 16S rDNA partial nucleotide sequence. The tree was constructed using the neighbor-joining method (Saitou and Nei 1987). Upper left line is the scale bar, and bootstrap values are listed as percentages at the branching points.

Table 1. Growth of *Bacillus* sp. KM7-1 at 37 °C under anaerobic and aerobic conditions and norathyriol formation by using the obtained cells

Cultivation condition	Bacterial growth (mg)	Norathyriol formation (μM)
Anaerobic (in GAM broth)	95.0 ± 26.9	119.5 ± 19.1
Aerobic (in NB)	149.5 ± 9.2	736.0 ± 11.3

Cultivations were conducted in 30 mL of medium in 50 mL of Erlenmeyer flask in static condition. A total of 40 mg of bacteria from each cultivation was used for norathyriol formation activity assay at 37 °C under anaerobic and aerobic conditions for cells cultivated in GAM broth and NB, respectively. Values represent the means and standard deviations of duplicate experiments.

Table 2. Norathyriol formation under anaerobic and aerobic reaction conditions by 40 mg of resting cells of *Bacillus* sp. KM7-1 from cultivation under aerobic conditions.

Conversion reaction condition	Norathyriol formation (μM)
Anaerobic	774.0 ± 24.0
Aerobic	741.5 ± 78.5

Cultivation was conducted in 30 mL of medium in 50 mL of Erlenmeyer flask in static condition. Values represent the means and standard deviations of duplicate experiments.

resting cells of *Bacillus* sp. KM7-1 was by approximately 15 mg of wet cell weight from optimum cultivation condition, at 37 °C under aerobic conditions for 24 h. A typical UPLC chromatogram of mangiferin deglycosylation by *Bacillus* sp. KM7-1 in optimum conversion condition is shown in Figure 2c.

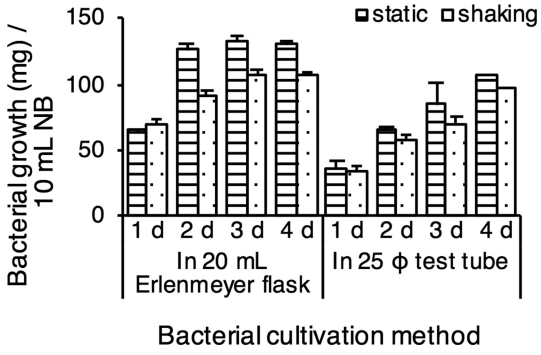

Figure 4. Growth of *Bacillus* sp. KM7-1 at 50 °C under aerobic conditions. Different variables were cultivation vessel, incubation day, static or shaking. Values represent the means and standard deviations of duplicate experiments.

Puerarin conversion activity of *Bacillus* sp. KM7-1

Lachnospiraceae CG19-1, a previously reported mangiferin-deglycosylating bacterium, was able to deglycosylate other C-glycosides such as puerarin (Braune and Blaut 2011). *Bacillus* sp. KM7-1 was examined for its ability to convert puerarin. Norathyriol formation from mangiferin was observed, but daidzein formation from puerarin was not observed in the reactions using either aerobic or anaerobic cultivated cells of *Bacillus* sp. KM7-1 (Figure 6). Aerobic cultivated cells exhibited higher norathyriol formation compared to anaerobic cultivated cells, as shown in Table 1. After the reaction with resting cells of *Bacillus* sp. KM7-1, puerarin remained in the reaction mixture at the same level as the control reaction. This indicated that *Bacillus* sp. KM7-1 did not have puerarin conversion activity and suggested that the C-deglycosylation ability was different from *Lachnospiraceae* CG19-1 (Braune and Blaut 2011).

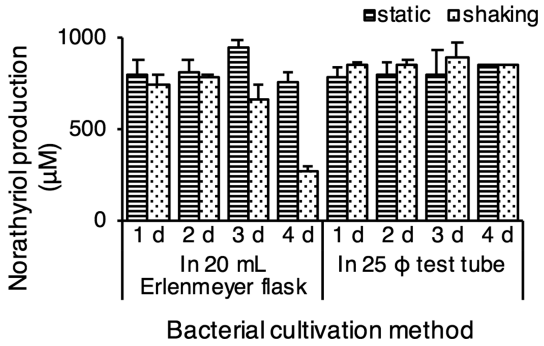

Figure 5. Norathyriol formation by 25 mg of resting cells of *Bacillus* sp. KM7-1 from cultivation aforementioned in Figure 4, conducted at 37 °C for 24 h under aerobic conditions. Values represent the means and standard deviations of duplicate experiments.

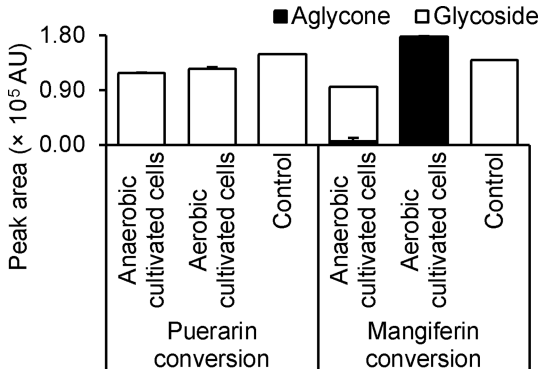

Figure 6. Puerarin and mangiferin conversion by 25 mg of resting cells of *Bacillus* sp. KM7-1 from cultivation under anaerobic and aerobic conditions. Glycosides were puerarin and mangiferin, and aglycones were daidzein and norathyriol, respectively. Values represent the means and standard deviations of duplicate experiments.

Comparison of mangiferin conversion activity into norathyriol between *Bacillus* sp. KM7-1 and the taxonomically similar bacteria

The experiments were performed to compare the growth and mangiferin conversion ability of *Bacillus* sp. KM7-1 with strains of the taxonomically similar species: *B. hisashii* N-11 (NBRC 110226) and N-21 (NBRC 110227), and *B. thermoamylovorans* LMG 18084. Bacterial strains were cultivated in 30 mL of NB in 50 mL of Erlenmeyer flask at 37 and 50 °C under aerobic, static conditions. The resting cells were subjected to norathyriol formation activity assay at 37 °C for 24 h under aerobic conditions. *Bacillus* sp. KM7-1, *B. hisashii* N-11, and *B. thermoamylovorans* showed better growth at 50 °C than 37 °C in NB under the aerobic conditions (Figure 7). Despite the similar growth characteristics between the examined bacteria, only *Bacillus* sp. KM7-1 converted mangiferin into norathyriol (Figure 7).

Time-course experiment of mangiferin conversion reaction by *Bacillus* sp. KM7-1

A time-course experiment was conducted using the resting cells of *Bacillus* sp. KM7-1 that was cultivated in optimum growth condition. Mangiferin conversion experiments were conducted at 37 °C under aerobic conditions using 8 and 16 mg of bacteria. Norathyriol formation was observed along with mangiferin consumption by time (Figure 8). Using 16 mg of cells, norathyriol

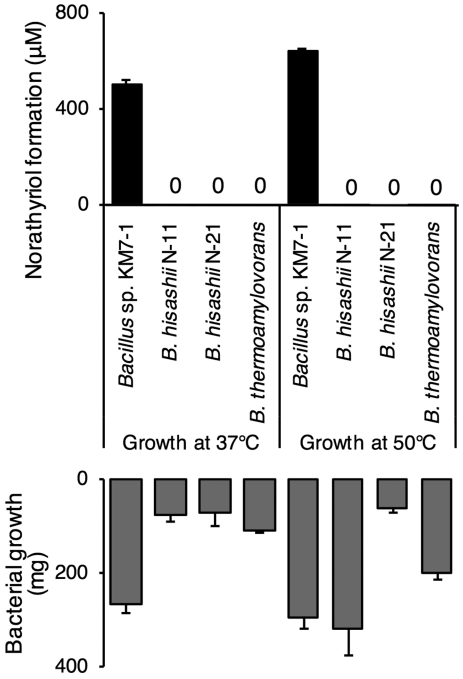

Figure 7. Bacterial growth of *Bacillus* sp. KM7-1 and taxonomically similar bacteria at 2 different temperatures under aerobic conditions and norathyriol formation using the obtained cells. Values represent the means and standard deviations of duplicate experiments.

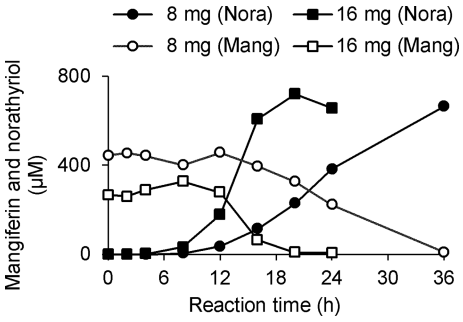

Figure 8. Time-course experiment of mangiferin conversion into norathyriol by *Bacillus* sp. KM7-1. Nora: norathyriol; Mang: mangiferin.

formation was started earlier (in 4 h) compared to using 8 mg of cells (in 8 h). The 16 mg of cells were observed to convert mangiferin into norathyriol in 20 h, while the 8 mg of cells needed more time, up to 36 h. Therefore, mangiferin conversion into norathyriol by *Bacillus* sp. KM7-1 was dependent on time and amount of bacteria.

Discussion

Mangiferin is the most reported xanthone among angiosperm plants (El-Seedi *et al.* 2009) and is the most common xanthone *C*-glycoside (Negi *et al.* 2013). Since the first discovery of mangiferin deglycosylation by human intestinal bacteria in 1989 (Hattori *et al.* 1989), only 2 bacteria that possess such ability have been isolated. Fewer reports of mangiferin bioconversion compared to the other *C*-glycosides suggest the difficulty in mangiferin deglycosylation. Only 1 bacterium, *Lachnospiraceae* CG19-1, that deglycosylates puerarin also deglycosylated mangiferin (Braune and Blaut 2011). Nevertheless, while deglycosylation

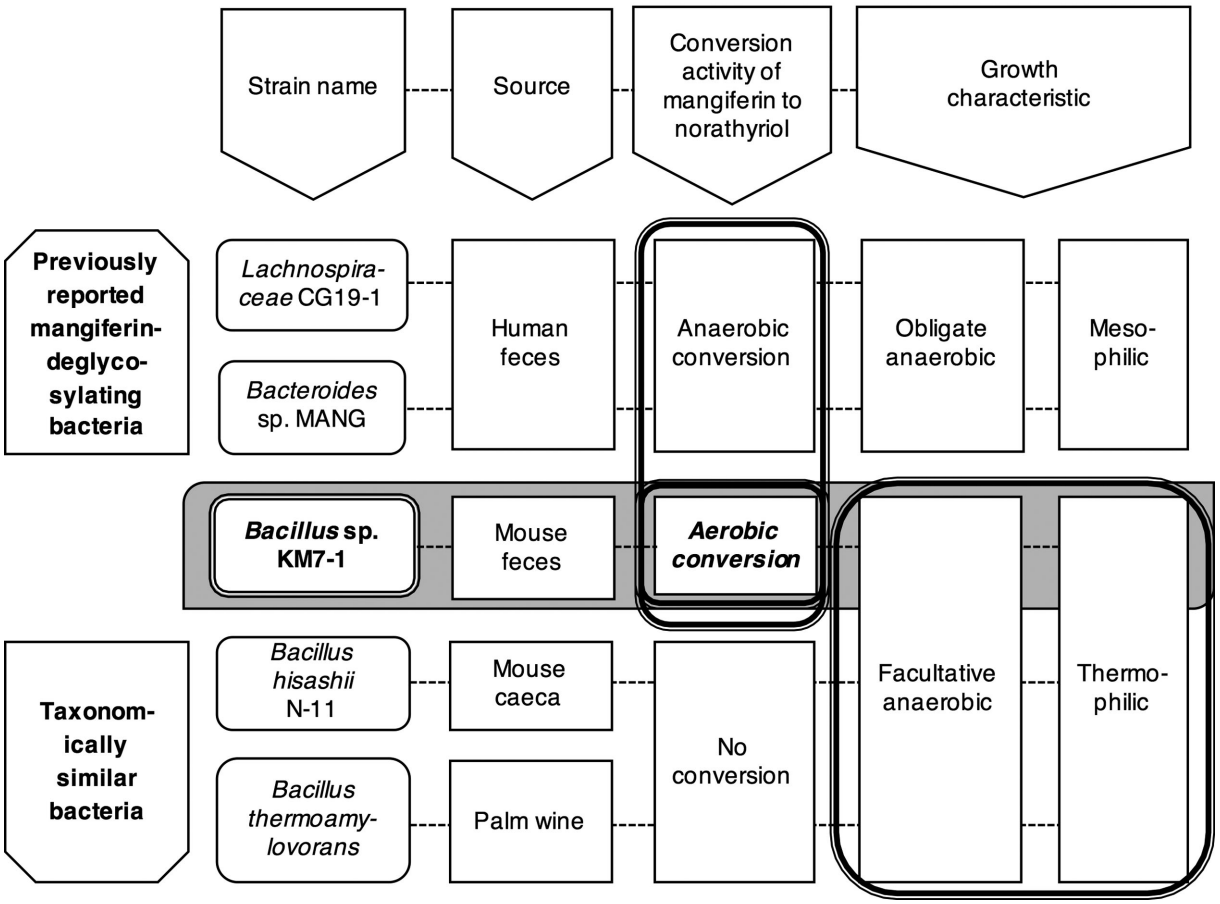

Figure 9. Comparison of *Bacillus* sp. KM7-1 with previously reported mangiferin-deglycosylating bacteria and taxonomically similar bacteria.

of other *O*- and *C*-glycosides by *Lachnospiraceae* CG19-1 was further studied, the detailed information of mangiferin deglycosylation was not available in the next report (Braune, Engst and Blaut 2016).

We summarize the comparison of *Bacillus* sp. KM7-1 with the reported mangiferin-deglycosylating bacteria and the taxonomically similar bacteria in Figure 9. *Bacillus* sp. KM7-1 was isolated from a source other than human, which is different from the 2 previously reported bacteria, *Bacteroides* sp. MANG and *Lachnospiraceae* CG19-1. *Bacteroides* sp. MANG was isolated based on the bacterial ability to metabolize mangiferin. *Lachnospiraceae* CG19-1 was first screened and isolated based on the ability to metabolize puerarin; however, it deglycosylated *O*-glycosides and other *C*-glycosides, including mangiferin. *Bacteroides* sp. MANG and *Lachnospiraceae* CG19-1 were obligate anaerobic bacteria and reported to grow at 37 °C (Braune and Blaut 2011, 2018; Sanugul *et al.* 2005). Conversely, *Bacillus* sp. KM7-1 was a facultative anaerobic bacterium and showed better growth at high temperature (i.e. 50 °C). Mangiferin conversion into norathyriol by *Bacillus* sp. KM7-1 can be conducted under aerobic and anaerobic conditions and thus, distinguished from the 2 mangiferin-deglycosylating bacteria previously reported, which deglycosylated mangiferin only under anaerobic conditions (Braune and Blaut 2011; Sanugul *et al.* 2005). To the taxonomically similar bacteria, *B. hisashii* and *B. thermoamylovorans*, *Bacillus* sp. KM7-1 resembled the growth characteristics as facultative anaerobic and thermophilic; nevertheless, it can be discriminated by the ability of *Bacillus* sp. KM7-1 to convert mangiferin into norathyriol. The ability of *Bacillus* sp. KM7-1 to grow under aerobic conditions even at higher temperatures and converted mangiferin under aerobic conditions distinguished this bacterium from the previously reported mangiferin-deglycosylating bacteria and the taxonomically similar bacteria.

Mangiferin was reported to be deglycosylated by bacteria from genus *Bacteroides* and *Catenibacillus* (Braune and Blaut 2018; Sanugul *et al.* 2005). Moreover, deglycosylation of other *C*-glycosides such as isoflavonoid puerarin (Kim *et al.* 1998; Park *et al.* 2006; Jin *et al.* 2008; Braune and Blaut 2011; Kim, Lee and Han 2015; Gaya, Peirotén and Landete 2017) and flavones homoorientin, orientin, vitexin, isovitexin (Braune and Blaut 2011, 2012; Xu *et al.* 2014; Zheng *et al.* 2019) were reported by intestinal bacteria from *Lachnospiraceae*, *Enterococcaceae*, *Streptococcaceae*, or *Bifidobacteriaceae* families (Braune and Blaut 2016; Gaya, Peirotén and Landete 2017). The isolated *Bacillus* sp. KM7-1 belongs to the same Firmicutes phylum as *Lachnospiraceae*, *Enterococcaceae*, *Streptococcaceae*, but a different family, *Bacillaceae*.

In the isolation of *Bacillus* sp. KM7-1, since the beginning of the bacterial isolation, resting cells of the bacterial mixture exhibited norathyriol formation activity, although it was cultivated in the absence of mangiferin (Figure 2a). The bacterial mixture retained its activity through several generations, as well as the isolated *Bacillus* sp. KM7-1, so the *C*-deglycosylating enzyme might be constitutively expressed. As a comparison, isolation of *Bacteroides* sp. MANG was performed through cultivations in a medium containing mangiferin. The presence of mangiferin during cultivation was required for *Bacteroides* sp.

MANG to cleave mangiferin into norathyriol, either by using growing cells or resting cells (Sanugul *et al.* 2005). On the other hand, the time-course experiment by *Bacillus* sp. KM7-1 resulted in mangiferin conversion into norathyriol was time-dependent and bacterial amount-dependent (Figure 8). There was a lag time in the first several hours of mangiferin conversion into norathyriol, indicated by only a little change in either mangiferin or norathyriol concentration. The enzymatic reaction process may consist of multiple stages, and mangiferin may be converted into a reaction intermediate before converted into norathyriol in the first few hours. Other *C*-glycoside, puerarin, was deglycosylated into daidzein by Strain PUE in a multistep reaction via 3″-oxo-puerarin as an intermediate compound (Nakamura *et al.* 2019, 2020). Mechanism of mangiferin *C*-deglycosylation into norathyriol has not been reported yet and may occur in a similar mechanism reported in puerarin. The other possibility is that the presence of mangiferin in the reaction mixture induced the enzymatic conversion activity in a short period of time. These indicated the difference in expression of mangiferin-deglycosylating enzymes between *Bacillus* sp. KM7-1 and *Bacteroides* sp. MANG.

The conversion of *C*-glycoside to its aglycone under aerobic conditions has not been reported at present. To our knowledge, the conversion reaction of bacterial *C*-deglycosylation were all reported to be performed under anaerobic conditions (Kim *et al.* 1998; Sanugul *et al.* 2005; Park *et al.* 2006; Jin *et al.* 2008; Braune and Blaut 2011, 2012; Xu *et al.* 2014; Kim, Lee and Han 2015; Gaya, Peirotén and Landete 2017; Zheng *et al.* 2019). This was due to the similar conditions in the intestine as the isolation source of bacteria. Although the activity-guided isolation was conducted under anaerobic conditions, our isolated *Bacillus* sp. KM7-1 retained its high activity to convert mangiferin into norathyriol under aerobic conditions. Aerobic condition is more accessible due to the simplicity; no special apparatus needed as for anaerobic conditions.

In summary, *Bacillus* sp. KM7-1 was a mangiferin-deglycosylating bacterium isolated from mouse intestine that grew aerobically that was able to grow at high temperatures and still exhibited norathyriol formation. This report revealed that C–C bond cleavage by the bacterium can be conducted under aerobic conditions.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Author contribution

H.K. and T.N. designed the research. U.H. and K.M. performed the experiments and analyzed the data together with H.K. and T.N. U.H. wrote the draft of manuscript and H.K. supervised manuscript preparation. H.K., T.N., and U.H. reviewed the manuscript.

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Disclosure statement

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