Journal of Microbiology (2020) Vol. 58, No. 6, pp. 445–455
DOI 10.1007/s12275-020-9596-3

eISSN 1976-3794
pISSN 1225-8873

Comparative genomics of *Lactobacillus* species as bee symbionts and description of *Lactobacillus bombintestini* sp. nov., isolated from the gut of *Bombus ignitus*[§]

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(Received Dec 13, 2019 / Revised Feb 20, 2020 / Accepted Feb 21, 2020)

The *Lactobacillus* genus is widely used for fermentation of plant materials and dairy products. These species are typically found in highly specialized environments, with the bee gut serving as one of the niche locations in which *Lactobacillus* is detected. *Lactobacillus* species isolated from the bee gut and bee-related habitats were phylogenetically classified into three distinct groups, *Lactobacillus kunkeei*, Firm-4, and Firm-5. The *L. kunkeei* group was clearly differentiated from other members of the *Lactobacillus buchneri* group isolated from non-bee habitats. In comparison with non-bee members of the *L. buchneri* group, three bee-symbiotic *Lactobacillus* groups had a small-sized genome with low G + C content and showed a sharp reduction in the number of genes involved in energy production, carbohydrate transport and metabolism, and amino acid transport and metabolism. In addition, all three groups lacked the *mutY* gene, which encodes A/G-specific adenine glycosylase. The phylogenetic dendrogram based on the presence or absence of 1,199 functional genes indicated that these bee-symbiotic groups experienced convergent evolution. The occurrence of convergent evolution is thought to stem from the three bee-symbiotic groups sharing a similar habitat, i.e., the bee gut. The causative factor underlying genomic reduction was postulated to be *mutY*, which was absent in all three groups. Here, a novel strain, BHWM-4^T, isolated from the gut of *Bombus ignites* was studied using polyphasic taxonomy and classified as a new member of the *L. kunkeei* group. The strain was Gram-positive, facultative anaerobic, and rod-shaped. The 16S ribosomal RNA gene sequence and genome analysis revealed that strain BHWM-4^T was clustered into the *L. kunkeei* group, forming a compact cluster with *L. kunkeei* and *Lactobacillus apinorum*. Biochemical, chemotaxonomic, and genotypic data of strain BHWM-4^T supports the proposal of a novel species, *Lactobacillus bombintestini* sp. nov., whose type strain is BHWM-4^T (= KACC 19317^T = NBRC 113067^T).

***Keywords*:** *Lactobacillus bombintestini*, *Bombus ignitus*, bee gut, *mutY*

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[§]Supplemental material for this article may be found at http://www.springerlink.com/content/120956.
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Introduction

The genus *Lactobacillus* has been widely used for fermentation of plant materials, dairy products, and meat (Canchaya *et al*., 2006). It is comprised of more than 230 species, with *Lactobacillus delbrueckii* as the type species (http://www.bacterio.net/lactobacillus.html; accessed on 1 Feb 2019). Many *Lactobacillus* species are found in highly specialized niches (Falsen *et al*., 1999; Germond *et al*., 2003; Siezen *et al*., 2010). Each distinct niche consists of specific environmental factors. Species differentiation to unique niches can occur through the adaptive process (Hutchinson, 1957). The bee gut and bee-related environments are one of the many niches *Lactobacillus* species are found in, and *Lactobacillus* species were identified to be a core microbiota component (Martinson *et al*., 2011; Ahn *et al*., 2012; Vásquez *et al*., 2012; Kwong and Moran, 2016; Ellegaard and Engel, 2019). Bacteria that are associated with the bee gut and bee-related habitats were phylogenetically divided into three groups (the *Lactobacillus kunkeei* group, Firm-4, and Firm-5) (Martinson *et al*., 2011; Ahn *et al*., 2012; Vásquez *et al*., 2012). Here, we tentatively named the "*Lactobacillus kunkeei* group" as a group of six *Lactobacillus* species that includes *L. kunkeei* and the closely related species *Lactobacillus ozensis* (Kawasaki *et al*., 2011), *Lactobacillus kosoi* (Chiou *et al*., 2018), *Lactobacillus micheneri*, *Lactobacillus timberlakei*, and *Lactobacillus quenuiae* (McFrederick *et al*., 2018). The "*L. kunkeei* group" was classified as a branch within the *Lactobacillus buchneri* group but is clearly separable from other branches of the *L. buchneri* group, based on phylogenetic analysis. *L. kunkeei* was recognized as a member of fructophilic lactic acid bacteria (FLAB) and has distinct genomic characteristics that distinguish them from other members of the *L. buchneri* group (Endo *et al*., 2009; Endo, 2012; Maeno *et al*., 2016). Each of Firm-4 and Firm-5 formed an independent monophyletic clade comprising species isolated only from the bee gut. There are three species, *Lactobacillus melis*, *Lactobacillus melifer* (Olofsson *et al*., 2014), and *Lactobacillus bombi* (Killer *et al*., 2014b), within the Firm-4 group, and seven species, *Lactobacillus apis* (Killer *et al*., 2014a), *Lactobacillus helsingborgensis*, *Lactobacillus melliventris*, *Lactobacillus kimbladii*, *Lactobacillus kullabergensis* (Olofsson *et al*., 2014), *Lactobacillus panisapium* (Wang *et al*.,

2018), and *Lactobacillus bombicola* (Praet *et al.*, 2015), within the Firm-5 group.

On the basis of genomic and comparative genomic analysis, three groups isolated from bees or bee-related environments had a small genome size and a small number of genes involved in carbohydrate metabolism when compared with other *Lactobacillus* species (Edwards *et al.*, 1998; Tamarit *et al.*, 2015; Maeno *et al.*, 2016, 2017; Vuong and McFrederick, 2019). Such genomic properties were considered to be influenced by the distinct niche in which the *Lactobacillus* species dwell.

The aim of this study was to implement a comparative genome analysis between *Lactobacillus* species isolated from the bee gut and related habitats, as well as *Lactobacillus* species classified into the *L. buchneri* group, and to study the evolutionary pathways and genetic mechanisms underlying genome evolution for those *Lactobacillus* species. In addition, a novel *Lactobacillus* species, *Lactobacillus bombintestini* sp. nov., isolated from the gut of an adult worker bee (*Bombus ignites*) is described and proposed on the basis of its phenotypic and genotypic characterizations.

Materials and Methods

Isolation of strain BHWM-4^T

Strain BHWM-4^T was isolated from the gut of an adult worker bee of the species *Bombus ignitus*, which was raised at the National Institute of Agricultural Sciences, Wanju-gun, Republic of Korea. The bee was washed in 95% ethanol, and the gut was extracted from the body. It was dissected with sterile forceps and homogenized in PBS by bead-beating. The homogenates were plated on de Man Rogosa Sharpe (MRS) medium (BD) (De Man *et al.*, 1960) and incubated at 28°C. Several bacterial strains were isolated and subcultured onto the MRS medium. Among them, one bacterial strain, BHWM-4^T, was identified as a novel genomic species on the basis of 16S ribosomal RNA (rRNA) gene sequence analysis. The reference strains, *L. kunkeei* KACC 19371^T, *L. apinorum* KACC 19372^T, *L. ozensis* KACC 19373^T, *L. micheneri* KACC 21189^T, *L. timberlakei* KACC 21190^T, and *L. quenuiae* KACC 21191^T, for the taxonomic study of strain BHWM-4^T were obtained from the Korean Agricultural Culture Collection (Republic of Korea).

16S rRNA gene sequencing and phylogeny

The 16S rRNA gene was PCR amplified using the universal primers 8F and 1512R (Felske *et al.*, 1997). The resulting PCR product was sequenced by the Sanger method using primers 27F (5′-GAGTTTGATCCTGGCTCAG-3′), 1492R (5′-GGTTACCTTGTTACGACTT-3′), 518R (5′-GTATTACC GCGGCTGCTGG-3′), and 785F (5′-GGATTAGATACCC TGGTA-3′) by Genotech. Sequence similarity values were calculated between BHWM-4^T and closely related species using EzBioCloud blast (http://www.ezbiocloud.net/identify) (Yoon *et al.*, 2017). Sequence alignment was conducted using the SILVA Incremental Aligner (Pruesse *et al.*, 2012), and the phylogenetic trees were reconstructed by neighbor-joining (Saitou and Nei, 1987), maximum-parsimony (Fitch, 1971), and maximum-likelihood (Felsenstein, 1981) algorithms using the software package MEGA (version 7) (Kumar *et al.*, 2016). The tree topology was evaluated by the bootstrap test (Felsenstein, 1981) with 1,000 replicates for each analysis.

Phenotypic and chemotaxonomic characterization

Bacterial morphology was observed using a light microscope (Axio Imager A1, Carl Zeiss) and a transmission electron microscope (LEO 912AB; LEO Electron) after 2 days of cultivation of the cells on MRS plates. Gram staining was examined using heat-fixed liquid cultures and a Gram staining kit (Sigma), according to the manufacturer's instructions. The appropriate pH range for growth was determined in MRS broth, for which the pH was adjusted with 20 mM citrate/phosphate buffer and 1 M HCl (pH 2.0), 20 mM citrate/phosphate buffer (pH 3.0–7.0), 20 mM Tris/hydrochloride buffer (pH 8.0–9.0), and 20 mM carbonate/bicarbonate buffer (pH 10.0–11.0). Growth under anaerobic conditions was assessed by incubating in a Gas Pak anaerobic system on MRS agar plates at 28°C for 5 days. Oxidase activity was observed by using 1% (w/v) tetramethyl-p-phenylenediamine, and catalase activity was determined by using 3% (w/v) H_2O_2 by observing the bubble production. Hydrolysis of casein, CM-cellulose, hypoxanthine, starch, Tween 80, tyrosine, and xanthine was tested using MRS agar medium supplemented with casein (1%, w/v), CM-cellulose (0.5%, w/v), hypoxanthine (0.5%, w/v), starch (1%, w/v), Tween 80 (1%, v/v), tyrosine (0.3%, w/v), or xanthine (0.5%, w/v) (Gerhardt *et al.*, 1994). Other physiological and enzymatic activities were evaluated using API 20N, API 20NE, and API ZYM test strips (bioMérieux). Acid production from carbohydrates was evaluated using the API 50CHL (bioMérieux) fermentation kit according to the manufacturer's instructions. API 20E, API 20NE, and API 50CHL were observed at 28°C for two weeks, and API ZYM was read at 28°C after 5 h.

Cellular fatty acid methyl esters (FAMEs) were analyzed using the Sherlock Microbial Identification System (MIDI, Microbial ID). All strains were cultivated on MRS agar medium at 28°C for ca. 1 day to exponential phase. Cells were collected and fatty acids were saponified, methylated, and extracted by following the standard protocol (Sasser, 1990). The fatty acid names and percentages were determined by the MIS Standard Software (Microbial ID, Sherlock version 6.1, database TSBA6 6.10). Polar lipids were extracted from freeze-dried cells according to Minnikin *et al.* (1984) and separated by two-dimensional silica gel TLC. The first direction was developed in chloroform/methanol/water (65:25:4, by vol.) and the second direction in chloroform/methanol/acetic acid/water (80:12:15:4, by vol.). Total polar lipids were detected by spraying with molybdophosphoric acid, and specific functional groups containing lipids were detected with the following spraying reagents: ninhydrin for free amino groups, molybdenum blue for phosphorus-containing lipids, α-naphthol for sugars, and Dragendorff's solution for quaternary nitrogen.

Genome sequencing and annotation

Whole-genome sequencing of strain BHWM-4^T was performed using PacBio RS II (Pacific Biosciences) at the Mac-

rogen. The sequences were assembled *de novo* using RS HGAP assembly version 3.0 (Chin *et al*., 2013), and the genome annotation was conducted using the NCBI prokaryotic genome annotation pipeline (Tatusova *et al*., 2016). The genome sequence was submitted to the GenBank database (http://www.ncbi.nlm.nih.gov/). To compare the average genome size and G + C content of *Lactobacillus*, the genomes of 182 type strains were obtained from the NCBI database. In addition, all of the genome sequences were re-annotated using Prodigal (Hyatt *et al*., 2010) and rapid annotation using subsystem technology v2.0 (RAST), which provides information regarding subsystems and metabolic pathways (Overbeek *et al*., 2014).

Comparative genomic analysis

Genome sequences of strain BHWM-4^T and 20 reference strains obtained from NCBI are listed in Table 1. OrthoANI values were calculated using EzBioCloud (http://www.ezbiocloud.net/tools/ani) and the unweighted pair group method with arithmetic mean (UPGMA) to construct the dendrogram (Lee *et al*., 2016). The pan-genome orthologous groups (POGs) and eggNOG categories were determined by re-annotation using Prodigal at the BIOiPLUG (https://www.bioiplug.com/). The presence or absence of the 1199 functional genes were identified and categorized using RAST subsystems, and an UPGMA dendrogram was constructed on the basis of the presence or absence of those genes using MEGA version 7 (Kumar *et al*., 2016).

Multilocus sequence analysis (MLSA) was conducted with 19 housekeeping genes (*argS*, *atpd*, *cdsA*, *clpC1*, *cysS*, *dnaK*, *dnaN*, *gapA*, *glnA*, *gyrA*, *gyrB*, *hisS*, *pheS*, *recA*, *rpoA*, *rpoB*, *serS*, *topA*, and *xtp*). All genes were extracted from 22 genome sequences including *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293^T as the reference strain and aligned using CLUSTAL W. The phylogenetic tree was constructed using MEGA version 7 (Kumar *et al*., 2016). The neighbor-joining (Saitou and Nei, 1987), maximum-parsimony (Fitch, 1971), and maximum-likelihood (Felsenstein, 1981) algorithms were used, with the bootstrap test (Felsenstein, 1985), following 1000 replicates for each analysis.

Statistics

Statistical data were analyzed using SPSS software version 20 (IBM Corp). Average values are expressed as Mean ± SD. Differences in the number of genes between groups (as shown in Fig. 4) were evaluated by one-way analysis of variance (ANOVA) and Duncan's test at $P < 0.05$.

Results

Characterization of *Lactobacillus bombintestini* sp. nov., using the polyphasic taxonomic approach

Strain BHWM-4^T showed the highest 16S rRNA gene sequence similarity with *L. kunkeei* YH-15^T (97.7%), *L. apinorum* Fhon13N^T (97.7%), *L. kosoi* NBRC 113063^T (97.3%), *L. micheneri* Hlig3^T (97.3%), and *L. timberlakei* HV_12^T (97.1%),

Table 1. ***Lactobacillus bombintestini* sp. nov. BHWM-4^T and *Lactobacillus* reference species used in this study**

Species	Strain	Source of isolation	Country	Genome accession number
'*Lactobacillus kunkeei* group'				
Lactobacillus bombintestini	BHWM-4^T = NBRC 113067^T = KACC 19317^T	Bumble bee	South Korea	CP032626
Lactobacillus kunkeei	YH-15^T = DSM 12361^T = KACC 19371^T	Wine	Unite State	JXDB01000000
Lactobacillus kunkeei	Fhon2	Western honey bee	Sweden	JXCU01000000
Lactobacillus kunkeei	LMbe	Stingless bee	Mexico	JXDE01000000
Lactobacillus apinorum	Fhon13N^T = DSM 26257^T = KACC 19372^T	Western honey bee	Sweden	JXCT01000000
Lactobacillus kosoi	NBRC 113063^T	Fermented beverage	Japan	BEXE01000000
Lactobacillus micheneri	Hlig3^T	Sweet bee	Unite State	POSO01000000
Lactobacillus timberlakei	HV_12^T	Wild bee (sweet bee)	Unite State	POST01000000
Lactobacillus quenuiae	HV_6^T	Wild bee (sweet bee)	Unite State	POSN01000000
Lactobacillus ozensis	DSM 23829^T = KACC 19373^T	Flower (Inular)	Japan	AYYQ01000000
Firm-4				
Lactobacillus mellifer	Bin4^T	Western honey bee	Sweden	JXJQ01000000
Lactobacillus mellis	Hon2^T	Western honey bee	Sweden	JXBZ01000000
Firm-5				
Lactobacillus kimbladii	Hma2N^T	Western honey bee	Sweden	JXLH01000000
Lactobacillus kullabergensis	Biut2N^T	Western honey bee	Sweden	JXBY01000000
Lactobacillus melliventris	Hma8^T	Western honey bee	Sweden	JXLI01000000
Lactobacillus helsingborgensis	Bma5N^T	Western honey bee	Sweden	JXJR01000000
Lactobacillus apis	Hma11^T	Western honey bee	Sweden	JXLG01000000
***Lactobacillus buchneri* group**				
Lactobacillus otakiensis	DSM 19908^T	Pickle	Japan	BASH01000000
Lactobacillus sunkii	DSM 19904^T	Pickle	Japan	AZEA01000000
Lactobacillus buchneri	DSM 20057^T	Tomato pulp	Japan	AZDM01000000
Lactobacillus iners				
Lactobacillus iners	DSM 13335^T	Urine (Human)	Sweden	ACLN01000000

revealing similarity values less than 97% to the other *Lactobacillus* species. The sequence similarity values were much lower than the cut-off value of 98.7% used to define a new bacterial species (Chun *et al.*, 2018), strongly supporting the classification of strain BHWM-4^{T} as a novel bacterial species. According to the maximum-likelihood phylogenetic tree (Fig. 1), strain BHWM-4^{T} formed a robust cluster with *L. kunkeei* and *L. apinorum* strains, which were isolated from wine, honey, flowers, and the guts of bees. This group formed a larger cluster that included three species (*L. micheneri*, *L. timberlakei*, and *L. quenuiae*), which were isolated from the bee gut, and two other species (*L. kosoi* and *L. ozensis*) that were isolated from sugar-vegetable fermented beverages and flowers, respectively. The phylogenetic topology was also supported by neighbor-joining and maximum-parsimony trees (Fig. 1). The whole-genome sequence of strain BHWM-4^{T} was assembled as one circular chromosome. The genome of strain BHWM-4^{T} was 1,289,211 bp in length. It had 1,237 genes, of which 1,152 genes were annotated as protein-coding genes (CDS), 15 genes as rRNA, 61 genes as tRNA, and 3 genes as noncoding RNA (Supplementary data Table S1). The annotation using Prodigal and RAST revealed that the number of

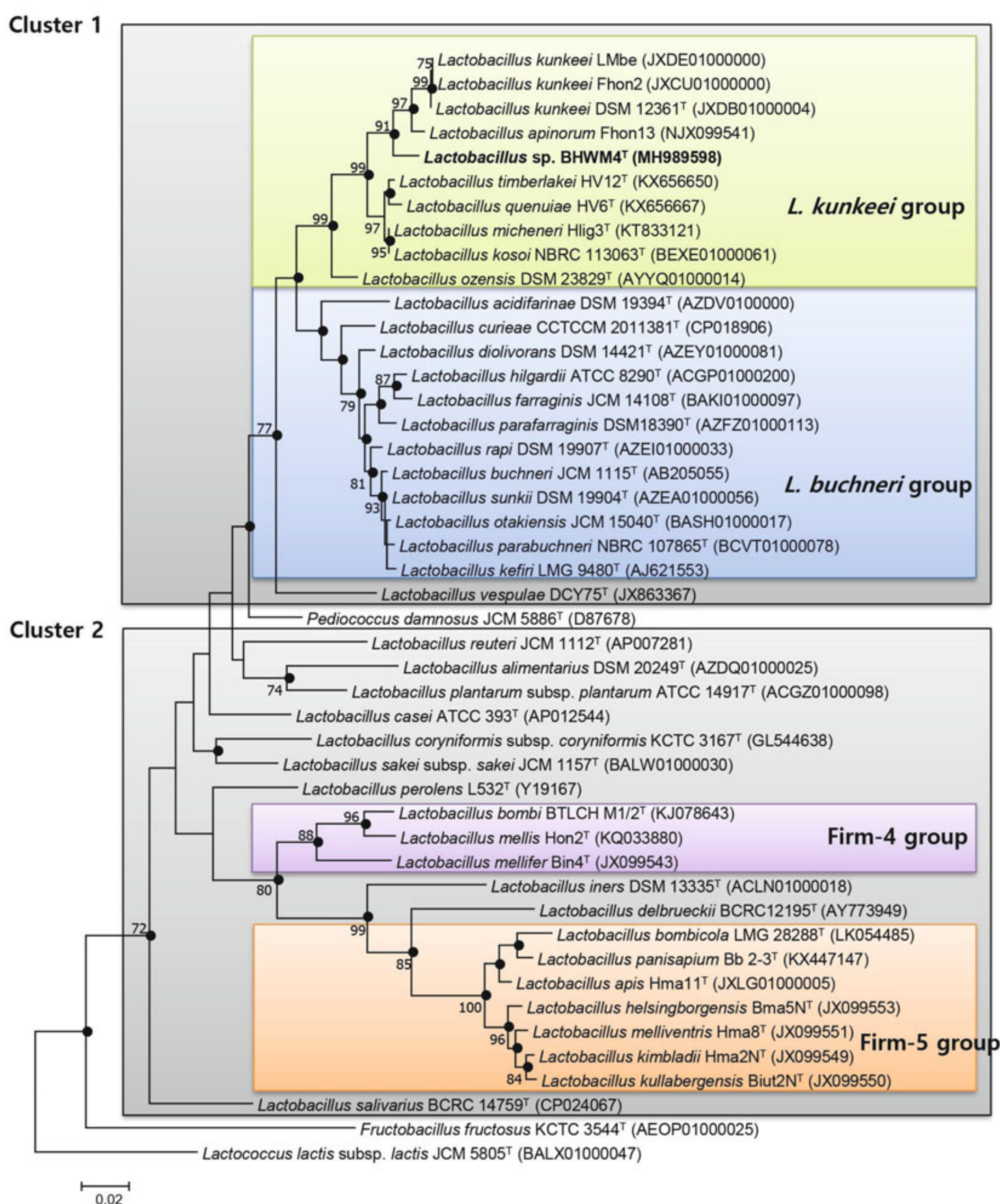

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences by using the maximum-likelihood method. Filled circles indicate branches that were also recovered both in the neighbor-joining and maximum-parsimony algorithm. Only bootstrap values more than 70% were shown at branching points. Bar, 0.02 substitutions per nucleotide position.

CDS is 1,161 and 1,170, respectively. Genomic G + C content of strain BHWM-4^T was 34.1 mol%.

To determine whether strain BHWM-4^T should be classified as a new species, ANI and dHH values were determined. ANI values were calculated via OrthoANI using EZbiocloud (http://www.ezbiocloud.net/tools/ani); the algorithm of which was described by Yoon *et al.* (2017). dDDH values were calculated using formula 2 of Genome-to-Genome Distance Calculation (GGDC) version 2.1 (http://ggdc.dsmz.de/distcalc2.php), originally described in Auch *et al.* (2010) and updated in Meier-Kolthoff *et al.* (2013). The OrthoANI values of strain BHWM-4^T with *L. kunkeei* YH-15^T, *L. apinorum* Fhon13N^T, *L. kosoi* NBRC 113063^T, *L. micheneri* Hlig3^T, and *L. timberlakei* HV_12^T were 77.5, 77.7, 75.7, 75.8, and 75.2%, respectively. The dDDH values estimated by the GGDC of strain BHWM-4^T with *L. kunkeei* YH-15^T, *L. apinorum* Fhon13N^T, *L. kosoi* NBRC 113063^T, *L. micheneri* Hlig3^T, and *L. timberlakei* HV_12^T were 20.3, 20.2, 19.2, 19.4, and 19.0%, respectively. Considering that the proposed and generally accepted species boundaries for ANI and dDDH values are 95–96% and 70%, respectively (Chun *et al.*, 2018), strain BHWM-4^T should be considered as a new genomic species.

Phenotypically, strain BHWM-4^T was Gram-positive, facultative anaerobic, rod-shaped, 0.6–0.8 in width and 1.4–2.0 in length (Supplementary data Fig. S1), and catalase- and oxidase-negative. The strain grew at temperatures of 20–37°C and pH values of 2.0–8.0. It was negative for nitrate reduction, indole production, and hydrolysis of urea and gelatin, but positive for aesculin hydrolysis. It could utilize D-glucose, D-fructose, D-sorbitol, and D-trehalose. Strain BHWM-4^T and closely related *Lactobacillus* species can utilize only a limited number of carbohydrates, including D-glucose and D-fructose. All BHWM-4^T strains could utilize D-sorbitol and D-trehalose, which could not be used by other *Lactobacillus* species (Table 2). Characteristically, the Voges-Proskauer test can be used to differentiate among BHWM-4^T strains and closely related *Lactobacillus* species (Table 2).

Fatty acids from strain BHWM-4^T consisted of $C_{16:0}$ (34.1%), $C_{18:1}$ $\omega9c$ (31.1%), summed feature 8 (including $C_{18:1}$ $\omega6c$ and/or $C_{18:1}$ $\omega7c$; 13.7%), $C_{19:0}$ cyclo $\omega8c$ (9.6%), summed feature 3 ($C_{16:1}$ $\omega6c$ and/or $C_{16:1}$ $\omega7c$; 4.5%), and summed feature 7 (unknown fatty acid ECL 18.846, $C_{19:1}$ $\omega6c$ and/or $C_{19:0}$ cyclo $\omega10c$; 3.4%) (Table 3). The fatty acid composition of strain BHWM-4^T was similar to those of closely related *Lactobacillus* species; however, it contained a relatively high $C_{18:1}$ $\omega9c$ content and low levels of $C_{19:0}$ cyclo $\omega8c$ (Table 3). The main polar lipids in strain BHWM-4^T were diphosphatidylglycerol, phosphatidylglycerol, an aminophospholipid, and two glycolipids (Supplementary data Fig. S2). The polar lipid patterns of *Lactobacillus* species were examined for a few species, of which the polar lipid patterns were quite different from species to species. The polar lipid pattern of BHWM-4^T was quite similar to that of *L. nuiruki* SYF10-1a^T (Heo *et al.*, 2018).

Comparative genomic analysis of bee-symbiotic groups and the *L. buchneri* group

From the genome sequence data of 183 type strains of the genus *Lactobacillus* retrieved from the NCBI database (retrieved as of July 2019), the genome sizes and DNA G + C contents were calculated to be in the range of 1.3–3.6 Mbp (average ± SD, 2.3 ± 0.6 Mbp) and 30.3–57.0% (average ± SD, 41.5 ± 6.1), respectively. The genome size of *Lactobacillus iners* DSM 13335^T was the smallest (1.3 Mbp), whereas that of *Lactobacillus collinoides* DSM 20515^T was the largest (3.6 Mbp). The G + C content of *Lactobacillus quenuiae* HV_6 was the lowest (30.3 mol%), whereas that of *Lactobacillus nasuensis* JCM 17158^T was the highest (57.0 mol%). Among them, the genome size and G + C content of bee-symbiotic or bee-related *Lactobacillus* species were 1.3–1.6 Mbp and 30.3–36.5%, respectively (Supplementary data Table S1). The genome sizes and DNA G + C content for these species were smaller and lower than the average values for the *Lactobacillus* genus (Supplementary data Fig. S3).

The 16S rRNA gene phylogenetic tree was reconstructed using bee-symbiotic *Lactobacillus* species, *Lactobacillus* species isolated from bee-related habitats, and *Lactobacillus* species from other habitats. This tree clearly showed four separate

Table 2. Differential phenotypic properties shown among strain BHWM-4^T and the closely related species
Strain: 1, *Lactobacillus bombintestini* BHWM-4^T; 2, *Lactobacillus apinorum* Fhon13N^T; 3, *Lactobacillus kunkeei* YH-15^T; 4, *Lactobacillus ozensis* Mizu2-1^T; 5, *Lactobacillus micheneri* Hlig3^T; 6, *Lactobacillus timberlakei* HV_12^T; 7, *Lactobacillus quenuiae* HV_6^T. All strains produce acid from D-glucose and D-fructose, and do not produce acid from glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-galactose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-lactose, D-melibiose, inulin, D-melezitose, D-raffinose, amidon, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2- ketogluconate or potassium 5-ketogluconate.

Characteristics	1	2	3	4	5	6	7
Voges Proskauer	-	+	+	-	-	-	-
Acid production from:							
D-Mannitol	-	+	+	-	+	-	-
D-Sorbitol	+	-	-	-	-	-	-
D-Maltose	-	-	-	+	-	-	-
D-Saccharose	-	+	+	-	+	+	+
D-Trehalose	+	-	-	-	-	-	-

Table 3. Fatty acid composition of strain BHWM-4^T and the closely related species
Strains: 1, *Lactobacillus bombintestini* sp. nov. BHWM-4^T; 2, *Lactobacillus apinorum* KACC 19372^T; 3, *Lactobacillus kunkeei* KACC 19371^T; 4, *Lactobacillus ozensis* KACC 19373^T. -, None or less than 0.5% of the total fatty acids.

Fatty acids	1	2	3	4
$C_{12:0}$	-	-	-	0.9
$C_{14:0}$	0.9	0.5	1.5	7.0
$C_{14:0}$ 2-OH	0.6	0.6	0.7	1.2
$C_{16:0}$	**34.1**	**37.3**	**38.7**	**35.2**
$C_{18:1}$ $\omega9c$	**31.1**	**15.2**	**11.3**	**27.8**
$C_{18:0}$	1.1	1.9	0.8	1.3
$C_{19:0}$ cyclo $\omega8c$	9.6	**23.8**	**23.4**	5.2
$C_{20:1}$ $\omega9c$	-	0.5	-	
Summed feature*				
3	4.5	2.1	5.2	7.4
7	3.4	6.3	5.9	9.0
8	**13.7**	**11.4**	**11.4**	4.5

* Summed feature 3 contained $C_{16:1}$ $\omega6c$ and/or $C_{16:1}$ $\omega7c$; summed feature 7 contained unknown 18.846, $C_{19:1}$ $\omega6c$ and/or $C_{19:0}$ cyclo $\omega10c$; summed feature 8 contained $C_{18:1}$ $\omega6c$ and/or $C_{18:1}$ $\omega7c$.

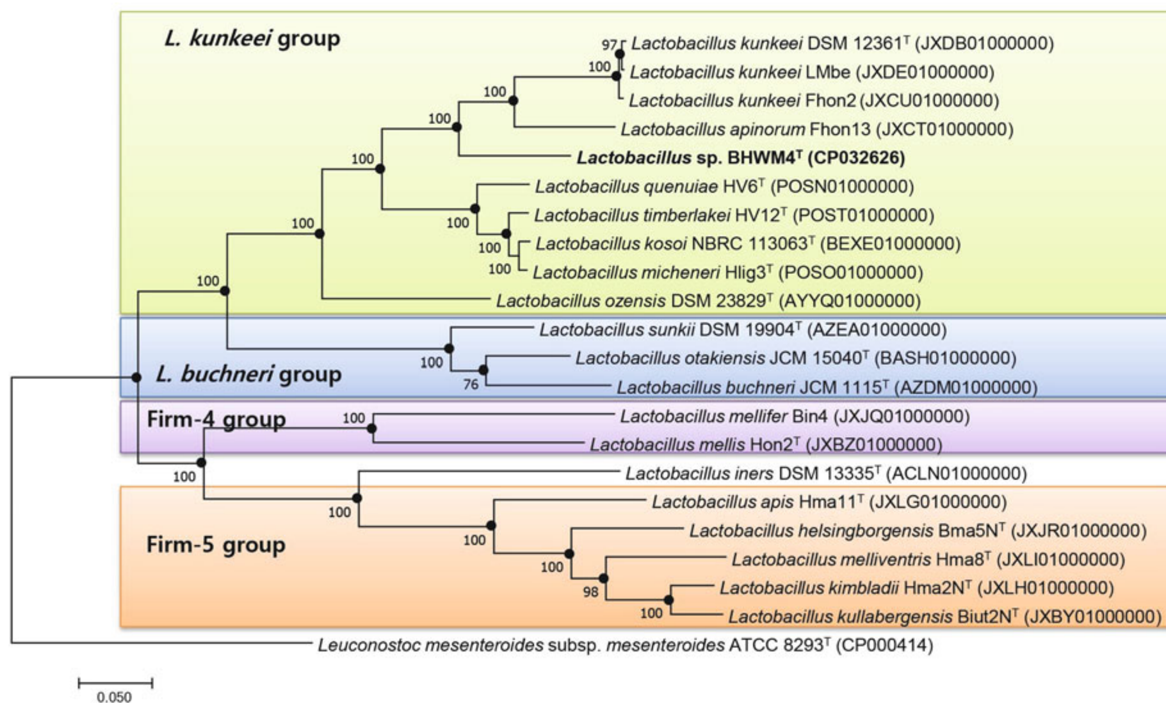

Fig. 2. Phylogenetic tree based on 19 housekeeping genes by using the maximum-likelihood method. Filled circles indicate branches that were also recovered both in the neighbor-joining and maximum-parsimony algorithm. Only bootstrap values more than 70% were shown at branching points. Bar, 0.05 substitutions per nucleotide position.

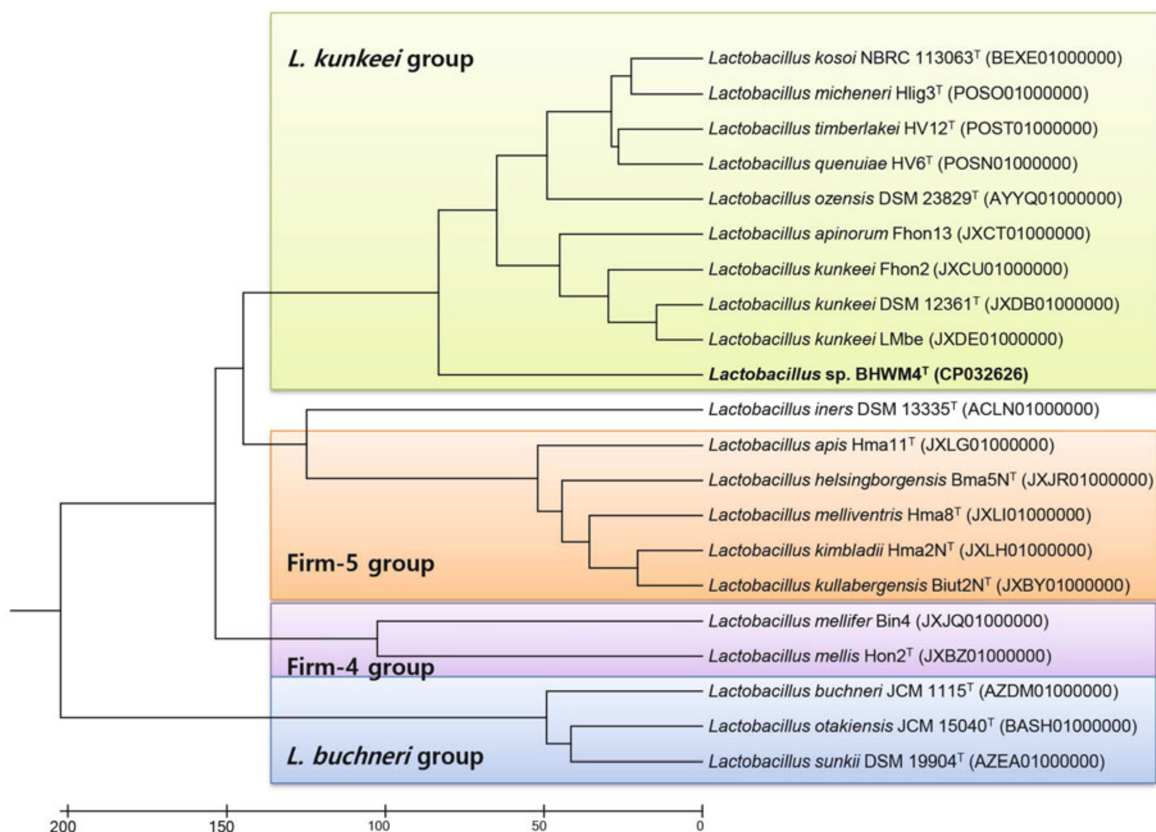

Fig. 3. Phylogenetic tree based on the presence or absence of genes categorized by RAST subsystem by using unweighted pair group method with arithmetic mean (UPGMA) dendrogram.

groups: *L. kunkeei*, *L. buchneri*, Firm-4, and Firm-5 (Fig. 1; Ellegaard *et al*., 2015). Members of the *L. kunkeei*, Firm-4, and Firm-5 groups originated from the bee gut or bee-related habitats; in contrast, some *L. buchneri* group species were isolated from habitats not associated with bees. Phylogenetic trees based on OrthoANI values and MLSA were constructed using the majority of *Lactobacillus* species isolated from the bee gut and the bee-related habitats, including flowers, and some species belonging to the *L. buchneri* group, which served as reference taxa. The topologies of the phylogenetic tree constructed from the genome data were identical to those from the 16S rRNA gene sequences (Fig. 2 and Supplementary data Fig. S4). Among all phylogenetic trees, the *L. kunkeei* group was closely related to the *L. buchneri* group but still clearly differentiated from it. The Firm-4 and Firm-5 groups also each formed a separate and independent group, which is consistent with previous studies (Killer *et al*., 2014a, 2014b; Olofsson *et al*., 2014; Praet *et al*., 2015; Wang *et al*., 2018). OrthoANI values and MLSA similarity values among members of the *L. kunkeei* group were in the range of 71.4–97.8% and 78.4–99.4%, respectively (Supplementary data Table S2). OrthoANI values and MLSA similarity values for the Firm-4 group (including *L. mellifer* and *L. mellis*) were 71.9 and 75.3%, respectively, and those values for the Firm-5 group (comprising five species) were from 75.8–93.4% and 65.4–93.9%, respectively. Moreover, the dendrogram, which was based on the presence or absence of 1,199 functional genes showed that the three groups isolated from the bee gut or bee-related habitats formed one large cluster that was separate from the *L. buchneri* group (Fig. 3). The three separate subclusters matched the *L. kunkeei*, Firm-4, and Firm-5 groups, based on the 16S rRNA gene sequence and genome data.

In the RAST subsystems, the number of average genes associated with carbohydrate and amino acid metabolism in the *L. kunkeei* group were 45.9 ± 4.9 and 54.7 ± 16.5, respectively; those in the Firm-4 group were 89.5 ± 16.3 and 57.5 ± 4.9, and Firm-5 group values were 78.4 ± 22.5 and 47.0 ± 2.9. In contrast, the average of these numbers for the *L. buchneri* group were more than double (111.3 ± 9.5 and 143 ± 7.8, respectively) (Fig. 4 and Supplementary data Table S3). The number of genes related to membrane transport for strain BHWM-4^{T} and *L. kunkeei* (7.8 genes) was smaller than that for other members of the *L. kunkeei* group (21.6 genes). The eggNOG categories also showed similar results (Supplementary data Table S4). The average number of genes associated with carbohydrate transport and metabolism (G), and amino acid transport and metabolism (E) in the *L. kunkeei* group were 64.8 ± 3.9 and 98.2 ± 12.6, respectively. By contrast, the average numbers for the *L. buchneri* group were

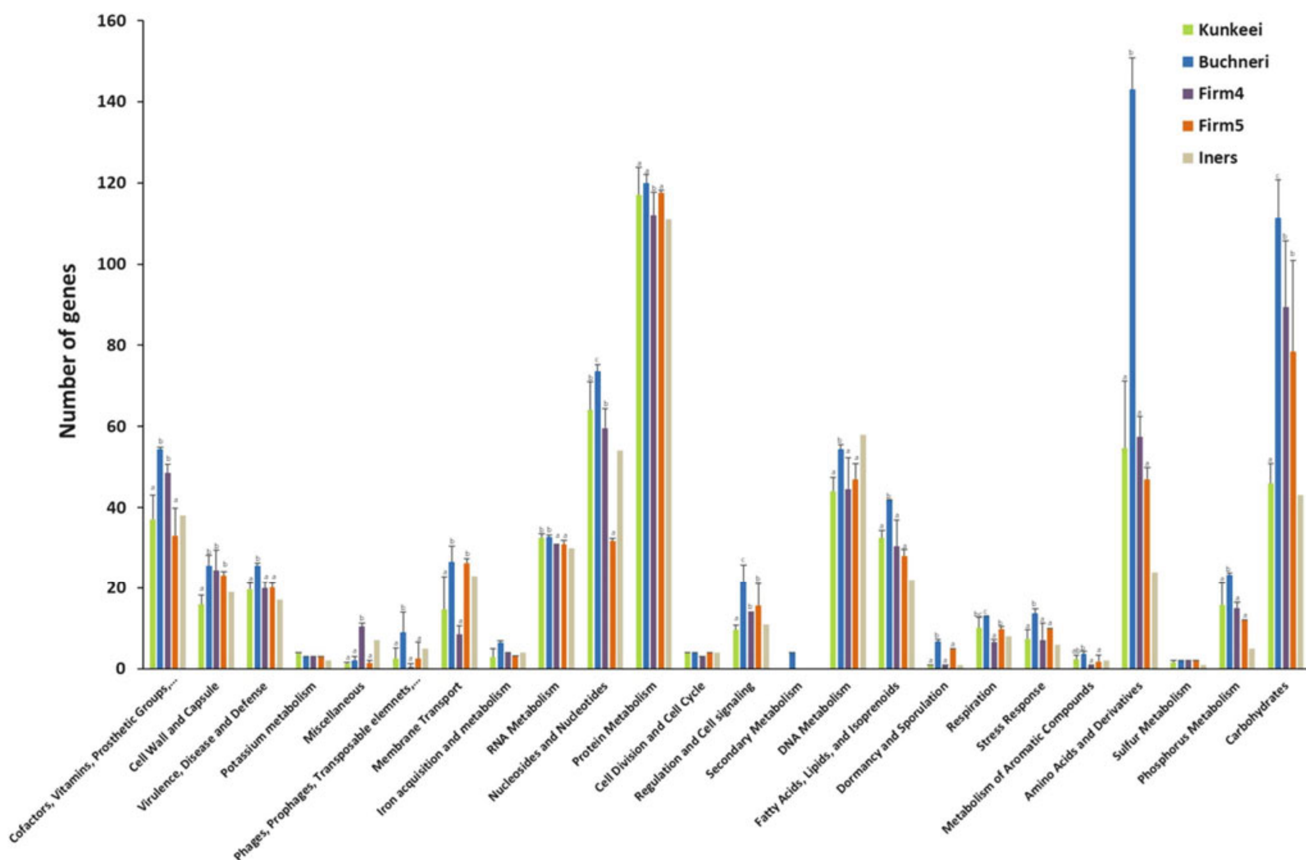

Fig. 4. Comparison of the number of genes annotated using RAST subsystem categorized by function and metabolism. The number of genes refers to the average value of each group (Green, '*Lactobacillus kunkeei* group'; Blue, *Lactobacillus buchneri* group; Purple, Firm-4 group; Orange, Firm-5 group; Grey, *Lactobacillus iners*). Means bearing different letters are significantly different at $P < 0.05$ according to the Duncan test.

Table 4. **Distribution of fructose utilization and DNA repairing genes**

Strain	Strain	Furctose utilization		DNA repairing			
		Fructokinase [EC 2.7.1.4]	Sucrose-6-phosphate hydrolase [EC 3.2.1.26]	MutY	RecA protein	MutL	XthA
'*L. kunkeei* group'							
Lactobacillus sp.	BHWM-4[T]	-	-	-	+	+	-
Lactobacillus kunkeei	DSM 12361[T]	+	+	-	+	+	-
Lactobacillus kunkeei	Fhon2	+	+	-	+	+	-
Lactobacillus kunkeei	LMbe	+	+	-	+	+	-
Lactobacillus apinorum	Fhon13N[T]	+	+	-	+	+	-
Lactobacillus kosoi	NBRC 113063[T]	+	+	-	+	+	-
Lactobacillus micheneri	Hlig3[T]	+	+	-	+	+	-
Lactobacillus timberlakei	HV_12[T]	+	+	-	+	+	-
Lactobacillus quenuiae	HV_6[T]	+	+	-	+	+	-
Lactobacillus ozensis	DSM 23829[T]	-	-	-	+	+	-
Firm-4							
Lactobacillus mellifer	Bin4[T]	-	-	-	+	+	-
Lactobacillus mellis	Hon2[T]	+	+	-	+	+	+
Firm-5							
Lactobacillus kimbladii	Hma2N[T]	+	+	-	+	+	+
Lactobacillus kullabergensis	Biut2N[T]	+	+	-	+	+	+
Lactobacillus melliventris	Hma8[T]	+	+	-	+	+	+
Lactobacillus helsingborgensis	Bma5N[T]	+	+	-	+	+	+
Lactobacillus apis	Hma11[T]	-	-	-	+	+	+
***L. buchneri* group**							
Lactobacillus otakiensis	DSM 19908[T]	-	-	+	+	+	+
Lactobacillus sunkii	DSM 19904[T]	+	-	+	+	+	+
Lactobacillus buchneri	DSM 20057[T]	+	-	+	+	+	+
L. iners							
Lactobacillus iners	DSM 13335[T]	-	-	-	+	+	+

more than twice as high (161.7 ± 19.0 and 202.7 ± 9.1, respectively).

Concerning carbohydrate-associated genes, the *L. kunkeei* group had only fructokinase [EC 2.7.1.4] and sucrose-6-phosphate hydrolase [EC 3.2.1.26] among the gene categories associated with di- and oligosaccharides metabolism. Among the genes involved in monosaccharide metabolism, genes related to metabolism of D-gluconate, ketogluconates, and D-ribose were present (Supplementary data Table S4). The *L. kunkeei* group revealed a loss of genes involved in the use of monosaccharides, such as deoxyribose, L-arabinose, and xylose, compared with that in the *L. buchneri* group.

According to an analysis of genes associated with DNA repair systems, such as *mutY*, *recA*, *mutL*, and *XthA* (Aravind *et al*., 1999; Lind and Andersson, 2008), *L. kunkeei*, Firm-4, and Firm-5 groups lacked the *mutY* gene, which is encoded by an A/G-specific adenine glycosylase, whereas the *L. buchneri* group retained the gene (Table 4).

Discussion

Maeno *et al.* (2016, 2017) suggested that *L. kunkeei* and *L. apinorum* which were recognized as members of fructophilic lactic acid bacteria (FLAB) experienced a genome reduction during their adaptation to fructose-rich environments, as observed in the *Fructobacillus* genera. Supporting their FLAB classification, he suggested that both *Lactobacillus* species shared the genes involved in fructose metabolism but also underwent deletion of redundant genes involved in carbohydrate transport and metabolism (Endo *et al*., 2015; Maeno *et al*., 2016, 2017). According to our study, however, the explanation for their evolution towards a small genome size is that the *Lactobacillus* species have a symbiotic relationship with the bee gut. Some members of the *L. kunkeei* group, including *L. bombintestini*, were found to lack fructose metabolism genes, including fructokinase and sucrose 6-phosphate hydrolase; thus, genome reduction cannot be explained on the basis of a fructose-rich habitat. Because *L. bombintestini* and *L. ozensis* were isolated from the gut of bumble bees, which do not accumulate honey like honeybees, or from mountain flowers, they could not be surrounded by fructose-rich environments. And so, their genome reduction cannot be explained by fructose-rich environments.

The group that includes *L. kunkeei* and closely related *Lactobacillus* species was defined as the *L. kunkeei* group, which is distinct from other members of the *L. buchneri* group, although the *L. kunkeei* group is a member of the *L. buchneri* group (Figs. 1, 2, 4). The *L. kunkeei* group was isolated from the bee gut or bee-gut related environments such as flowers, fruit, and wine (Edwards *et al*., 1998; Endo, 2012; Olofsson *et al*., 2014; Maeno *et al*., 2016). The common ancestor of the original *L. buchneri* group was supposed to have evolved into two groups, the *L. kunkeei* and *L. buchneri* groups (exclud-

ing the *L. kunkeei* group), depending on adaptation to the bee gut (Salvetti *et al*., 2018). Although some members of the *L. kunkeei* group were found in environments outside of the bee gut, they may temporarily inhabit those environments as a result of their association with the bee gut.

Our dendrogram based on the presence and absence of genes categorized by RAST showed that the *L. kunkeei* group was more closely related to Firm-4 and Firm-5 groups, which were found only in the bee gut. Since small genome size and "AT richness" are known to be common characteristics of symbiotic gut bacteria (Kikuchi *et al*., 2009; Nikoh *et al*., 2011), the *L. kunkeei* group can be considered to be a member of the bee-gut microbiota and could clearly be differentiated from other species of the *L. buchneri* group. Each of the three *Lactobacillus* groups (*L. kunkeei*, Firm-4, and Firm-5) evolved independently from each other; in particular, the *L. kunkeei* group was evolutionarily distant from the other two groups. Despite having different ancestors, the three groups may have undergone convergent evolution by sharing a common habitat: the bee gut. There have been several reports regarding the mechanism responsible for genome reduction (Dufresne *et al*., 2005; Toft and Andersson, 2010; Tamarit *et al*., 2015). Lind and Anderson (2008) suggested the loss of *mutY* gene function (coding A/G-specific adenine glycosylase) as a mechanism for genome size reduction. The deletion of the gene *mutY* leads to an increase in A + T content and a decrease in G + C content of the genome during the damaged DNA repair process. The increase in A + T content makes the genome unstable, and, as a result, it ends up with a sharp reduction in genome size (McCutcheon and Moran, 2012). According to our genome analysis, all members of the three bee-symbiotic groups (*L. kunkeei*, Firm-4, and Firm-5) lacked the *mutY* gene, while the *L. buchneri* group–the closest relatives to the *L. kunkeei* group, but not a bee symbiont–kept it. Thus, the decrease in the genome size of the three bee-symbiotic groups is suspected to be caused by the deletion of the gene *mutY*, because these groups shared a symbiotic interaction in the bee gut.

In conclusion, three bee-symbiotic groups originated from three different ancestors in the process of bee symbiosis and experienced convergent evolution, resulting in genome reduction and an increase in genomic A+T content. Moreover, on the basis of biochemical, chemotaxonomic, and genotypic data, we propose a new *Lactobacillus* species, *L. bombintestini*, which was isolated from the bee gut and can be classified into the *L. kunkeei* group.

Description of *Lactobacillus bombintestini* sp. nov.

Lactobacillus bombintestini (bomb.in.tes.ti'ni. N.L. masc. n. *Bombus* a genus of bumblebees; L. neut. n. *intestinum* intestine; N.L. gen. n. *bombintestini* of the intestine of bumblebees).

The cells are Gram-positive, facultative anaerobic, rod-shaped (0.6–0.8 × 1.4–2.0 μm), catalase- and oxidase-negative, and grow at temperatures of 20–37°C and pH of 2.0–8.0. Aesculin, casein, CM-cellulose, and tyrosine are hydrolyzed, but gelatin, hypoxanthine, starch, Tween 80, urea, and xanthine are not. It shows negative reactions for nitrate reduction, indole production, arginine dihydrolase, and the Voges-Proskauer test. Positive enzymatic activity is observed for leucine arylamidase and acid phosphatase, and weakly positive activity for valine arylamidase and naphthol-AS-BI-phosphohydrolase. Enzymatic activities of alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase are negative. Acid is produced from D-glucose, D-fructose, D-sorbitol, and D-trehalose but is not produced from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-galactose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, inulin, D-melezitose, D-raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, or potassium 5-ketogluconate. The major fatty acids are $C_{16:0}$, $C_{18:1}$ *ω9c* and summed feature 8 (including $C_{18:1}$ *ω6c* and/or $C_{18:1}$ *ω7c*). The polar lipids diphosphatidylglycerol, phosphatidylglycerol, an aminophospholipid and two glycolipids are present. The DNA G + C content is 34.1 mol%.

The type strain BHWM-4^T (= KACC 19317^T = NBRC 113067^T) was isolated from the gut of an adult worker bee of *Bombus ignites* in Wanju-gun, Republic of Korea.

Acknowledgments

We thank professor Bernhard Schink and Aharon Oren for advice on the etymology of the new names proposed.

This study was carried out with the support (PJ013549) of National Institute of Agricultural Sciences, Rural Development Administration, Republic of Korea.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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