Weissella diestrammenae sp. nov., isolated from the gut of a camel cricket (*Diestrammena coreana*)

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A novel, Gram-stain-positive, non-motile, facultatively anaerobic, rod- or coccoid-shaped bacterium, designated strain ORY33^T, was isolated from the gut of a camel cricket (*Diestrammena coreana*). The 16S rRNA gene sequence analysis showed that strain ORY33^T belonged to the genus *Weissella*, with highest sequence similarity to *Weissella koreensis* S-5623^T (97.7 %). The strain grew optimally at 30 °C and pH 7 in the presence of 0 % (w/v) NaCl. Catalase and oxidase activities were negative. The genomic DNA G+C content of strain ORY33^T was 45.1 mol%. DNA–DNA hybridization values between strain ORY33^T and closely related members of the genus *Weissella* were less than 27 %. The major fatty acids of strain ORY33^T were $C_{18:1}\omega9c$, $C_{16:0}$ and $C_{14:0}$. Based on these phenotypic, phylogenetic and genotypic analyses, strain ORY33^T represents a novel species belonging to the genus *Weissella*, for which the name *Weissella diestrammenae* sp. nov. is proposed. The type strain is ORY33^T (=KACC 16890^T=JCM 18559^T).

Insects dominate terrestrial ecosystems; they are represented by many phylogenetically diverse species and also help to support human life (Vilmos & Kurucz, 1998). Because they bite, sting and transmit diseases, they also have profound negative effects on humans and domestic animals. Although the symbiotic relationships between insects and their gut bacterial communites, such as provision of nutrients and colonization resistance to non-indigenous pathogens, are well understood (Dillon & Dillon, 2004), most micro-organisms in the gut of insects have not been identified and also their functions are unknown.

The genus *Weissella* comprises a group of heterofermentative species, which belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales* and family *Leuconostocaceae* (Collins *et al.*, 1993). The taxonomic characterization of the novel isolate ORY33^T was performed using polyphasic analyses.

Strain ORY33^T, belonging to the genus *Weissella*, was isolated during an investigation of the microbial ecology of insects in Korea. Strain ORY33^T was isolated from the intestinal tract of a camel cricket (*Diestrammena coreana*) in South Korea. Homogenized tissue from the intestinal tract was diluted with sterile PBS (BIONEER) and then spread on MRS agar medium (MRSA; Difco). After incubation at 25 °C for 1 week, the isolate was transferred onto fresh MRSA to obtain a pure culture. The sequence of the 16S rRNA gene was amplified by colony PCR using a PCR pre-mix (iNtRon Biotechnology) and two universal bacteria-specific primers: 8F and 1492R (Lane, 1991). The amplification products were sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems), and the reaction mixture was analysed in an automated system (PRISM 3730XL DNA analyser; Applied Biosystems). The sequenced 16S rRNA gene fragments were assembled using SeqMan (DNASTAR) and a near-full-length 16S rRNA gene sequence (1451 bp) was compared with the 16S rRNA gene sequences of other type strains using the EzTaxon server (Chun *et al.*, 2007). The results of EzTaxon analysis indicated that strain ORY33^T should be allocated to the genus *Weissella* as it showed a 16S rRNA gene sequence similarity of 97.7 % with *Weissella koreensis* S-5623^T and 97.6 % with *Weissella kandleri* NRIC 1628^T. For phylogenetic analysis, the 16S rRNA gene sequence of the isolate was aligned with those of its nearest relatives using the multiple sequence alignment program CLUSTAL W (Thompson *et al.*, 1997). The aligned sequences were then examined manually using BioEdit software (Hall, 1999). The phylogenetic relationships between strain ORY33^T and closely related species were analysed using MEGA5 (Tamura

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Abbreviation: DDH, DNA–DNA hybridization.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *pheS* gene sequences of *Weissella dieastrammenae* ORY33^T are JQ646523 and KC470113, respectively.

One supplementary figure is available with the online version of this paper.

et al., 2007). Phylogenetic distances were determined by the neighbour-joining (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and maximum-likelihood (Felsenstein, 1981) algorithms with 1000 randomly chosen bootstrap replications. The phylogenetic trees based on the 16S rRNA gene sequences showed that the isolate formed a cluster with *W. koreensis* S-5623^T and *W. kandleri* NRIC 1628^T (Fig. 1 and Fig.S1 available in IJSEM Online). These two type strains were obtained from the Korean Agricultural Culture Collection for microbes (KACC 11853^T: *W. koreensis* S-5623^T) and the Korean Collection for Type Cultures (KCTC 3610^T: *W. kandleri* NRIC 1628^T) for comparative analysis. Sequence analysis of the housekeeping *pheS* genes was also investigated to determine phylogenetic status (De Bruyne *et al.*, 2007, Naser *et al.*, 2005). Comparison of the *pheS* gene sequence was carried out using NCBI BLAST confirming that the closest relative of strain ORY33^T was *Weissella halotolerans* LMG 9469^T (81 %). The neighbour-joining method (Saitou & Nei, 1987) based on *pheS* was performed similarly to the 16S rRNA gene sequence analysis. The result demonstrated that strain ORY33^T clustered with species of the genus *Weissella* (Fig. 2).

Gram staining was performed using a Gram stain kit (bioMérieux) according to the manufacturer's instructions. Cell morphology was observed under a light microscope (ECLIPSE 50*i*; Nikon). The motility test was performed using MRS medium containing 0.4 % agar (Tittsler & Sandholzer, 1936). Growth under anaerobic conditions was determined by incubation for 7 days in an anaerobic chamber (N_2 : CO_2 : H_2, 90 : 5 : 5). Catalase activity was assessed with 3 % (v/v) hydrogen peroxide, and oxidase activity was confirmed using 1 % (w/v) tetramethyl-*p*-phenylenediamine (bioMérieux). To examine the optimal growth conditions for strain ORY33^T, the isolate was grown at various temperatures; NaCl concentrations and pH were also examined. All tests were performed in triplicate. The growth test was performed at 4, 15, 20, 25, 30, 37, 45, 55 and 65 °C, and in the presence of 0, 1, 2, 3, 4, 5, 8, 10, 12 and 15 % (w/v) NaCl in MRS broth (Difco). The pH was adjusted to 4–6 (buffered by 10 mM MES with HCl), 7 and 8 (buffered by 10 mM TAPS with NaOH) and 9 and 10 (buffered by 10 mM Na_2HPO_4 with NaOH). After incubation at 30 °C for 24 h, 48 h or 7 days, the OD_{600} of each culture was measured in a spectrophotometer (SYNERGY MX; BioTek). Production of gas from glucose and arginine hydrolysis were tested using the method of De Bruyne *et al.* (2008). Microscopy revealed that strain ORY33^T was Gram-positive, short rod- or coccoid-shaped and non-motile. Catalase and oxidase activity were not detected. Growth under anaerobic

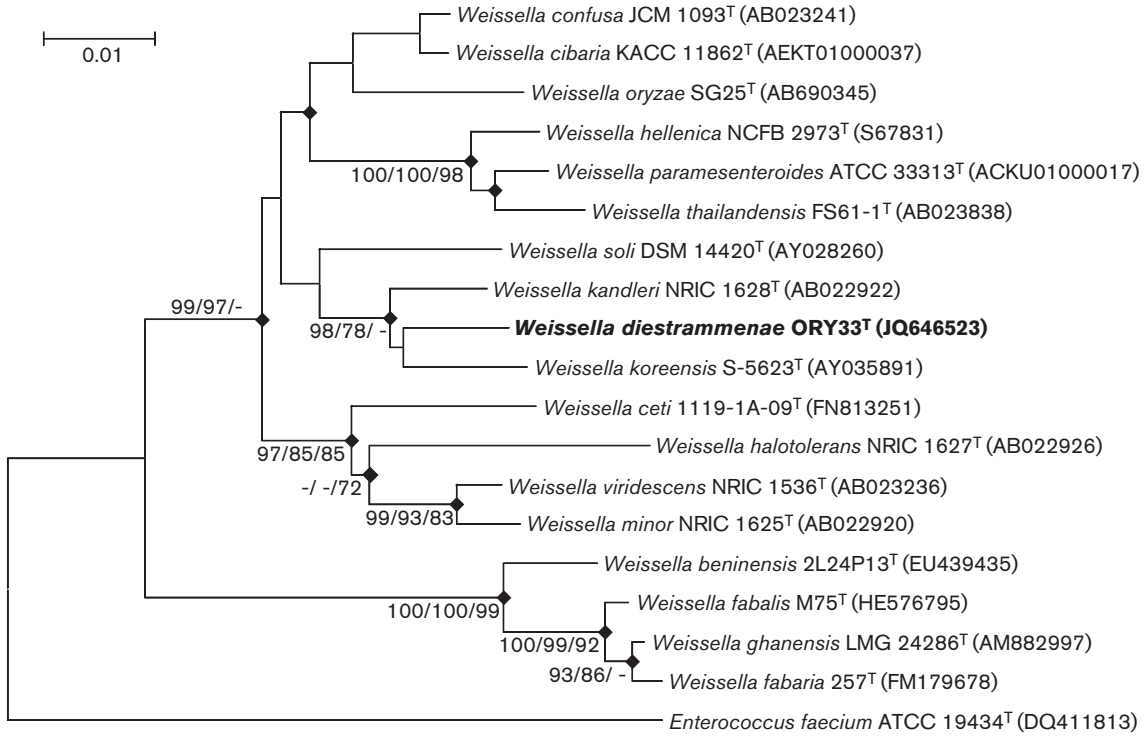

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequences generated using the neighbour-joining, maximum-parsimony and maximum-likelihood algorithms. Filled diamonds represent branches generated by the three different methods. The numbers at the nodes indicate bootstrap values (>70 %) as percentages of 1000 replicates. Bar, 0.01 substitutions per nucleotide.

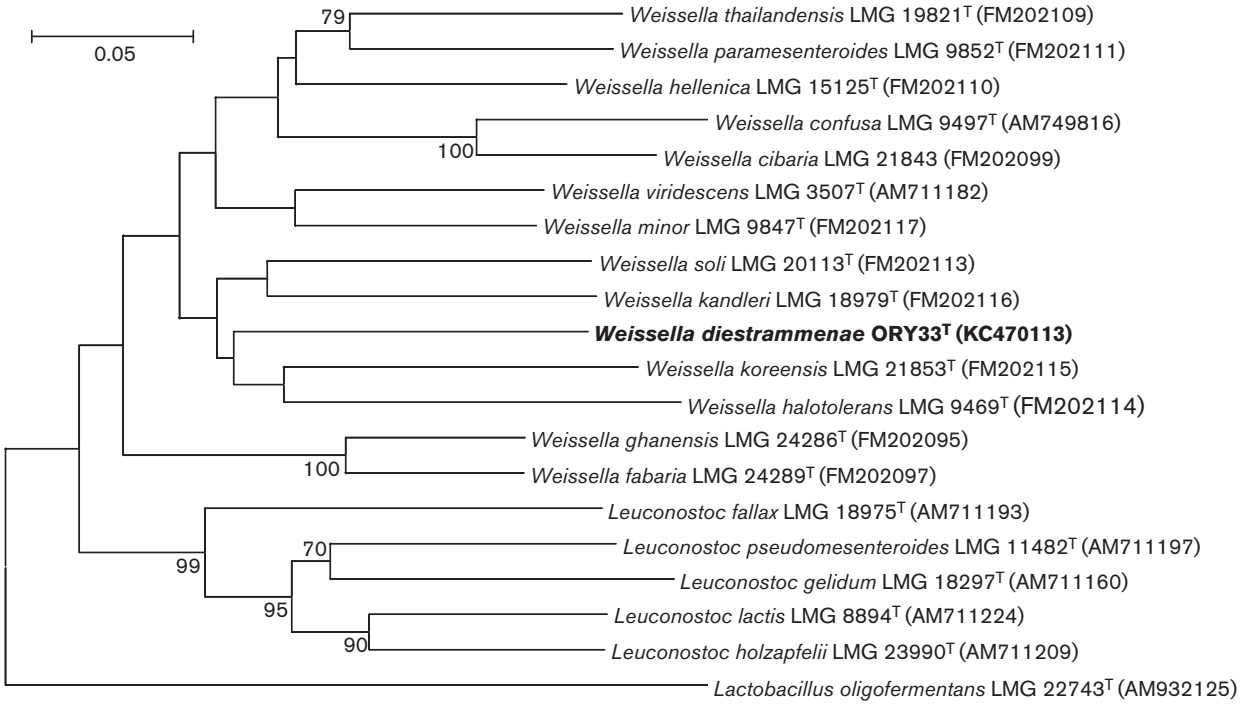

Fig. 2. Phylogenetic neighbour-joining tree based on *pheS* gene sequences showing the phylogenetic relationships of strain ORY33^T within the genus *Weissella*. Numbers at nodes represent bootstrap values (>70 %) based on 1000 replications. Bar, 0.05 accumulated changes per nucleotide.

conditions was observed. The growth tests showed that strain ORY33^T grew at 4–37 °C, pH 5–8 and in the presence of 0–4 % (w/v) NaCl. Optimal conditions for growth were 30 °C, pH 7 and 0 % (w/v) NaCl. Strain ORY33^T produced gas from glucose and hydrolysed arginine.

API 50CH test strips (bioMérieux) with 50 CHL medium (bioMérieux) and API ZYM test strips (bioMérieux) were used to check the sugar-fermentation patterns and enzyme activity, respectively, of the isolate and reference species according to the manufacturer's instructions. Assimilation of sole carbon substrates was tested using GP2 MicroPlates (Biolog) according to the manufacturer's instructions. Lactic acid isomers were confirmed in cells grown at 30 °C for 3 days using the DL-lactate test kit (Roche). Strain ORY33^T produced acid from L-arabinose, D-ribose, D-glucose, D-mannose, *N*-acetylglucosamine, L-xylose, maltose and potassium gluconate by fermentation. D-Lactic acid was produced as an end product of glucose fermentation. The different characteristics of strain ORY33^T and the most closely related species are presented in Table 1.

Strain ORY33^T and the reference strains were cultured on MRSA at 30 °C for 48 h for analysis of their cellular fatty acid composition. The fatty acids were extracted according to the method described for the Sherlock Microbial Identification Systems (MIDI, 1999) and subsequently analysed by gas chromatography using an Agilent 7890 gas chromatograph (Agilent Technologies). Individual fatty acids were identified using the Microbial Identification software package (Sherlock version 4.0) (Sasser, 1990) based on the TSBA6 database. $C_{18:1}\omega 9c$ (58.5 %) and $C_{16:0}$ (19.7 %) were predominant in strain ORY33^T and in the reference strains; however, $C_{14:0}$ (9.8 %) was only detected in strain ORY33^T. The complete fatty acid composition of the isolate and those of the reference strains are presented in Table 2. The composition of the cell wall was determined by the method of Bousfield *et al.* (1985). Cell-wall peptidoglycan amino acids were purified to hydrolysates (6 M HCl, 121 °C, 15 min) and analysed using one-dimensional TLC on a cellulose TLC plate (Merck). Cell walls contained Lys–Ala–Ser.

Extraction of genomic DNA from the isolate and the reference species was carried out as described by Rochelle *et al.* (1992). DNA G+C content was determined using a fluorometric method with SYBR Gold I and a real-time PCR thermocycler (Gonzalez & Saiz-Jimenez, 2002). The genomic DNA of *Escherichia coli* K-12, *Ruegeria pomeroyi* DSS-3^T and *Ruminococcus obeum* ATCC 29174^T were used as calibration references (Sambrook *et al.*, 1989). The genomic DNA G+C content of strain ORY33^T was 45 mol%. This result was within the range of other species in the genus *Weissella* (Collins *et al.*, 1993). DNA–DNA hybridization (DDH) using genome-probing microarrays

Table 1. Different characteristics of ORY33^T and type strains of the most closely related species

Strains: 1, ORY33^T; 2, *W. koreensis* S-5623^T; 3, *W. kandleri* NRIC 1628^T. All data were obtained in the current study except where indicated. Data regarding carbon source assimilation, acid production from carbohydrates and enzyme activity were obtained using a GP2 MicroPlate (Biolog), API 50CHL and API ZYM, respectively. All strains produced acid from L-arabinose, D-ribose, D-glucose and potassium gluconate. All strains assimilated *N*-acetyl-D-glucosamine, D-fructose, α-D-glucose, D-mannose and D-ribose. +, Positive; −, negative; W, weakly positive; ND, no data available.

Characteristic	1	2	3
Temperature for growth (°C)			
Range	4–37	10–37*	ND
Optimum	30	25*	ND
pH			
Range	5–8	4–8*	ND
Optimum	7	6*	ND
Acid production from:			
D-Mannose	+	+	W
N-Acetylglucosamine	+	W	W
L-Xylose	+	+	−
Maltose	+	−	−
D-Fructose	−	+	W
D-Mannitol	−	W	−
Potassium 2-ketogluconate	−	−	W
D-Galactose	−	−	W
Assimilation of:			
Dextrin	+	W	W
Adenosine	+	W	+
Inosine	+	W	+
Uridine	+	W	+
L-Lactic acid	+	−	−
Maltose	+	−	−
L-Arabinose	−	+	+
D-Mannitol	−	+	−
D-Galactose	−	−	+
Enzyme activity			
Alkaline phosphatase	+	W	−
Leucine arylamidase	−	−	W
Acid phosphatase	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	W	W
β-glucuronidase	−	+	−
Hydrolysis of aesculin	+	+	+
NH_3 from arginine	+	+	+
Murein type	Lys–Ala–Ser	Lys–Ala–Ser*	Lys–Ala–Gly–Ala_2*

*Data from Lee *et al.* (2002).

(Bae *et al.*, 2005, Chang *et al.*, 2008) was performed to elucidate the genetic relatedness of strain ORY33^T to the reference strains. The DDH values for strain ORY33^T were 13±0 % (reciprocal 23±0 %) with *W. koreensis* S-5623^T and 23±1 % (reciprocal 19±1 %) with *W. kandleri* NRIC 1628^T. The DDH value was below the novel genotypic species threshold of 70 % (Wayne *et al.*, 1987).

On the basis of the phenotypic, genotypic and phylogenetic analyses described herein we propose that strain ORY33^T represents a novel species belonging to the genus *Weissella*, for which the name *Weissella diestrammenae* sp. nov. is proposed.

Description of *Weissella diestrammenae* sp. nov.

Weissella diestrammenae (di.es.tram.me′nae. N.L. gen. n. *diestrammenae* of Diestrammena, referring to *Diestrammena coreana*, a camel cricket from the gut of which this bacterium was isolated).

Cells are facultatively anaerobic, Gram-stain-positive, coccoid- or rod-shaped, 1.1 μm long and 0.6 μm wide, non-motile and oxidase- and catalase-negative. Colonies incubated on MRSA medium at 30 °C for 48 h are approximately 1 mm in diameter, circular, moist, smooth and convex with a creamy colour. Growth occurs at 4–37 °C in

Table 2. Cellular fatty acid composition (%) of strain ORY33^T and the most closely related species

Strains: 1, ORY33^T; 2, *W. koreensis* S-5623^T; 3, *W. kandleri* NRIC 1628^T. All data were obtained in the current study. Values represent the percentage of total fatty acids. TR, Trace amount (<0.5 %); −, not detected.

Fatty acid	1	2	3
Saturated acids			
$C_{10:0}$	TR	−	TR
$C_{12:0}$	2.1	TR	TR
$C_{14:0}$	9.8	0.8	0.9
$C_{16:0}$	19.7	20.3	20.1
$C_{17:0}$	−	TR	TR
$C_{18:0}$	1.2	1.3	1.1
Unsaturated acids			
$C_{16:1}\omega9c$	−	−	TR
$C_{17:1}\omega8c$	TR	TR	TR
$C_{18:1}\omega9c$	58.5	71.6	70.4
$C_{20:4}\omega6,9,12,15c$	−	−	TR
Branched acids			
iso-$C_{19:1}$ I	TR	TR	TR
Summed features*			
3	2.6	1.5	2
5	−	−	TR
8	5.5	3.9	3.9

*Summed features are denoted when two or three fatty acids could not be separated using the Microbial Identification System. Summed features 3 comprises $C_{16:1}\omega7c/C_{16:1}\omega6c$. Summed features 5 comprises $C_{18:2}\omega6,9c$/anteiso-$C_{18:0}$. Summed features 8 comprises $C_{18:1}\omega7c/C_{18:1}\omega6c$.

0–4 % (w/v) NaCl and at pH 5–8. Optimal growth conditions are 30 °C, in the presence of 0 % (w/v) NaCl and pH 7. Able to hydrolyse arginine and produce gas from glucose. The cell wall contains Lys–Ala–Ser. Acid is produced from L-arabinose, D-ribose, D-glucose, D-mannose, *N*-acetylglucosamine, potassium gluconate, L-xylose and maltose (API 50CHL), and the type of acid produced is D-lactic acid. The assimilated carbon substrates are dextrin, *N*-acetyl-D-glucosamine, D-fructose, D-gluconic acid, α-D-glucose, D-mannose, D-ribose, adenosine, inosine, uridine, L-lactic acid and maltose (Biolog GP2). Enzyme activity is positive for alkaline phosphatase, acid phosphatase and naphthol-AS-BI-phosphohydrolase (API ZYM). The predominant cellular fatty acids are $C_{18:1}\omega9c$, $C_{16:0}$ and $C_{14:0}$. The genomic DNA G+C content of the type strain is 45 mol%.

The type strain is ORY33^T (=KACC 16890^T=JCM 18559^T), isolated from the gut of a camel cricket (*Diestrammena coreana*) in South Korea.

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