Enterococcus diestrammenae sp. nov., isolated from the gut of *Diestrammena coreana*

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A novel Gram-stain-positive, facultatively anaerobic, non-motile and lactic-acid-producing bacterium, designated strain ORL-24^T, was isolated from the gut of the camel cricket, *Diestrammena coreana*. Optimal growth occurred at 37 °C, pH 8 and with 0 % (w/v) NaCl. The ratio of L-lactate to D-lactate in strain ORL-24^T was 96 : 4. Lancefield antigen D was not detected. The strain was negative for oxidase activity and catalase activity. According to a phylogenetic analysis based on 16S rRNA gene sequences, strain ORL-24^T was most closely related to the type strain of *Enterococcus asini* (96.9 % similarity). Comparative *pheS* and *rpoA* sequence analyses of strain ORL-24^T indicated that the strain belonged to the genus *Enterococcus*. The major fatty acids were $C_{16:0}$ and $C_{18:1}\omega9c$. The DNA G+C content was 41.3 mol%. Based on phenotypic, genotypic and phylogenetic analyses, strain ORL-24^T represents a novel species of the genus *Enterococcus*, for which the name *Enterococcus diestrammenae* is proposed. The type strain is ORL-24^T (=KACC 16708^T=JCM 18359^T).

Insects are the most abundant class of animals on the Earth and their digestive tracts contain various micro-organisms (Dillon & Dillon, 2004; May & Beverton, 1990). Many gut micro-organisms have critical roles in the physiology of their host insects. In particular, insect health is affected by the lactic acid bacteria harboured in their guts (Vásquez *et al.*, 2012). Thus, identifying the bacteria present in the guts of insects is useful for studying the interactions between insects and their gut micro-organisms.

The genus *Enterococcus* was first described by Schleifer and Kilpper-Balz (1984). Species of the genus *Enterococcus* are Gram-stain-positive, non-spore-forming, facultatively anaerobic microbes that produce lactic acid. Their DNA G+C contents are 35.1–44.9 mol% (Švec & Devriese, 2009). The aim of this study was to determine the taxonomic position of a novel bacterial strain, ORL-24^T, which was isolated from an insect's gut.

Strain ORL-24^T was isolated from the gut of the camel cricket, *Diestrammena coreana*, in South Korea. Cells were isolated with the standard dilution-plating method using Lactobacilli MRS agar (MRSA; BBL) medium at 15 °C for 3 days. To obtain pure cultures, a single colony was transferred repeatedly to fresh MRSA. All tests were performed in triplicate. Gram staining was carried out using a Gram staining kit (bioMérieux) according to the manufacturer's instructions, and confirmed by the non-staining method (Buck, 1982). The cell morphology and Gram staining of strain ORL-24^T were examined using a light microscope (ECLIPSE 50*i*; Nikon). A cellular motility test was conducted in semi-solid agar (Tittsler & Sandholzer, 1936). Growth under anaerobic conditions was monitored by incubation on MRS plates in an anaerobic chamber (N_2 : H_2 : CO_2, 90 : 5 : 5) at 37 °C for 7 days. The formation of lactate isomers in the fermented broth was determined enzymically using the UV method (R-Biopharm). Growth of strain ORL-24^T on enterococci selective media Slanetz–Bartley agar (Oxoid) and kanamycin aesculin azide agar (Sigma) was tested. Production of Lancefield antigen D was tested using the Streptococcal grouping kit (Oxoid). Growth at different temperatures (4, 10, 15, 20, 25, 30, 37, 45, 55 and 65 °C), NaCl concentrations (0, 1, 2, 3, 4, 5, 8, 10, 12 and 15 %, w/v) and pH levels (pH 4.0–10.0, at intervals of 1.0 pH unit) were tested in MRS broth (MRS; BBL). The pH was adjusted using 10 mM MES ($C_6H_{13}NO_4S$ for pH 4, 5 and 6), 10 mM TAPS ($C_7H_{17}NO_6S$ for pH 7, 8 and 9) or 10 mM Na_2HPO_4 (for pH 10). Strain ORL-24^T was cultivated in MRS broth at 37 °C in all experiments, unless stated otherwise. After 24 h, 48 h and 7 days incubation, the turbidity of cultures was measured as the OD_{600} using a spectrophotometer (SYNERGY MX; BioTek). Cells of

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Abbreviation: FAME, fatty acid methyl ester.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *pheS* and *rpoA* gene sequences of strain OLR-24^T are JQ650245, JQ691735 and JQ691736, respectively.

A supplementary figure and a supplementary table are available with the online version of this paper.

strain ORL-24^T were found to be cocci or diplococci (1.0–1.3 μm in diameter), Gram-stain-positive, non-motile and facultatively anaerobic. The ratio of L-lactate to D-lactate was 96 : 4. The growth of strain ORL-24^T was observed on kanamycin aesculin azide agar, whereas the isolate did not grow on Slanetz–Bartley agar. Strain ORL-24^T was negative for Lancefield antigen D production. Strain ORL-24^T grew at 10–37 °C, pH 6.0–10.0 and in the presence of 0–5 % (w/v) NaCl, with optimal growth at 30–37 °C, pH 8.0–9.0 and in the presence of 0 % (w/v) NaCl. The isolate was distinguished from its closest phylogenetic relatives by the ability to grow on Slanetz–Bartley agar. Other phenotypic differences between strain ORL-24^T and the type strains of closely related species of the genus *Enterococcus* are shown in Table 1.

In the phylogenetic analysis, the 16S rRNA gene of strain ORL-24^T was amplified by colony PCR using a PCR pre-mix (iNtRon Biotechnology) and two universal

Table 1. Differential characteristics of strain ORL-24^T and its closest phylogenetic relatives

Strains: 1, ORL-24^T; 2, *Enterococcus asini* KCTC 13286^T; 3, *Enterococcus pallens* DSM 15690^T; 4, *Enterococcus canintestini* LMG 13590^T; 5, *Enterococcus dispar* KCTC 13288^T; 6, *Enterococcus hermanniensis* LMG 12317^T; 7, *Enterococcus faecalis* JCM 5308^T. All data were obtained in the current study. All strains assimilated L-lactic acid and adenosine (Biolog GP2). All strains produced acid from D-ribose, D-glucose, D-fructose, D-mannose, *N*-acetylglucosamine, arbutin, aesculin, salicin, cellobiose, maltose, trehalose and gentiobiose (API 50CHL). All strains were positive for esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities (API ZYM). Characteristics giving the same results for strain ORL-24^T and strains of more than three other species were omitted. +, Positive; −, negative; ±, positive or negative.

Characteristics	1	2	3	4	5	6	7
Growth at 45 °C	−	+	+	+	−	−	+
Growth on Slanetz-Bartley agar	−	+	+	+	+	+	+
Group D antigen	−	+	+	−	−	−	+
Assimilation of:							
D-Fructose	−	+	+	+	+	−	+
α-D-Glucose	−	+	+	+	+	−	+
D-Mannose	−	+	+	+	+	−	+
Pyruvic acid methyl ester	+	−	−	+	−	+	+
Pyruvic acid	+	−	−	+	−	+	+
N-Acetyl-β-D-mannosamine	−	−	+	+	−	−	+
D-Ribose	−	−	+	+	−	+	+
D-Psicose	−	−	+	+	+	−	+
Maltose	−	+	−	+	−	−	+
N-Acetyl-D-glucosamine	−	+	+	+	+	+	+
Acid production from:							
Melibiose	−	−	+	+(−†)	+	−	−
Raffinose	−	−	+	+(−†)	+	−	−
Glycerol	−	−	+	+(±†)	+	−	+
D-Tagatose	−	−	+	+	+	−	+
D-Mannitol	−	−	+	−	−	+	+
Gluconate	−	−	+	−	+	−	+
L-Rhamnose	−	+	−	−	−	+(±‡)	+
Starch	−	+(±*)	−	+(−†)	−	−	+
Turanose	−	+(−*)	+	+	+	−	−
Sucrose	−	+(−*)	+	+	+	−	+
D-Xylose	+	+	−	−	−	−	−
Methyl α-D-glucoside	+	+(±*)	−	+	+	−	−
5-Ketogluconate	+	+(−*)	+	−	−	+(−‡)	−
Enzyme activity							
Cystine arylamidase	−	−	−	+	+	−	+
α-Glucosidase	−	−	+	+	−	−	+
Valine arylamidase	−	+	−	+	+	−	+
β-Galactosidase	+	+	−	−	−	−	−

*Data from de Vaux *et al.* (1998).
†Data from Naser *et al.* (2005b).
‡Data from Koort *et al.* (2004).

bacterial-specific primers: forward primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-GGYTACCTTGTTACGACTT-3′) (Lane, 1991). The PCR product was purified with a PCR purification kit (Qiagen) and sequencing was performed as described previously (Kim *et al.*, 2013). The 16S rRNA gene sequence fragments were assembled using SeqMan (DNASTAR), which produced an almost complete 16S rRNA gene sequence (1439 bp). The 16S rRNA gene sequence comparison was performed using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). Strain ORL-24^T had the highest levels of 16S rRNA gene sequence similarity to *Enterococcus asini* AS2^T (Y11621) (96.9 %), *Enterococcus pallens* ATCC BBA-351^T (DQ411812) (96.7 %), *Enterococcus canintestini* LMG 13590^T (AJ888906) (96.5 %), *Enterococcus dispar* ATCC 51266^T (AF061007) (96.5 %) and *Enterococcus hermanniensis* LMG 12317^T (AY396047) (96.2 %). The 16S rRNA gene sequence was aligned with those of the most closely related species using the multiple alignment program CLUSTAL W (Thompson *et al.*, 1994). The phylogenetic relationships between strain ORL-24^T and the closely related type strains were determined using MEGA5 (Tamura *et al.*, 2011). Phylogenetic correlations were determined using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Kluge & Farris, 1969) and maximum-likelihood (Felsenstein, 1981) methods with 1000 bootstrap replicates. Strain ORL-24^T was also investigated using *pheS* and *rpoA* gene sequence analysis to produce a more detailed phylogenetic description (Naser *et al.*, 2005a). Phylogenetic analyses based on the *pheS* and *rpoA* genes using the neighbour-joining method (Saitou & Nei, 1987) were performed in the same way as the 16S rRNA gene sequence analysis. The phylogenetic analyses based on the 16S rRNA, *pheS* and *rpoA* gene sequences indicated that strain ORL-24^T clustered with species in the genus *Enterococcus* (Fig. 1 and Fig. S1 available in IJSEM Online). The type strains of *Enterococcus asini* (KCTC 13286^T), *Enterococcus dispar* (KCTC 13288^T), *Enterococcus pallens* (DSM 15690^T), *Enterococcus canintestini* (LMG 13590^T), *Enterococcus hermanniensis* (LMG 12317^T) and *Enterococcus faecalis* (JCM 5803^T) were obtained from the Korean Collection of type Cultures (KCTC), German Culture Collection (DSMZ), the Belgian Coordinated Collections of Microorganisms/Laboratorium voor Microbiologie, Ghent University (BCCM/LMG) and Japan Collection of Microorganisms (JCM) and used for comparative experiments.

The biochemical analyses of strain ORL-24^T and the reference species were performed using cells cultivated at 37 °C for 72 h on MRS medium. The catalase and oxidase activities were determined using 3 % (v/v) hydrogen peroxide solution and 1 % (w/v) tetramethyl-*p*-phenylenediamine (bioMérieux), respectively. Sole carbon source assimilation and acid production from carbohydrates were

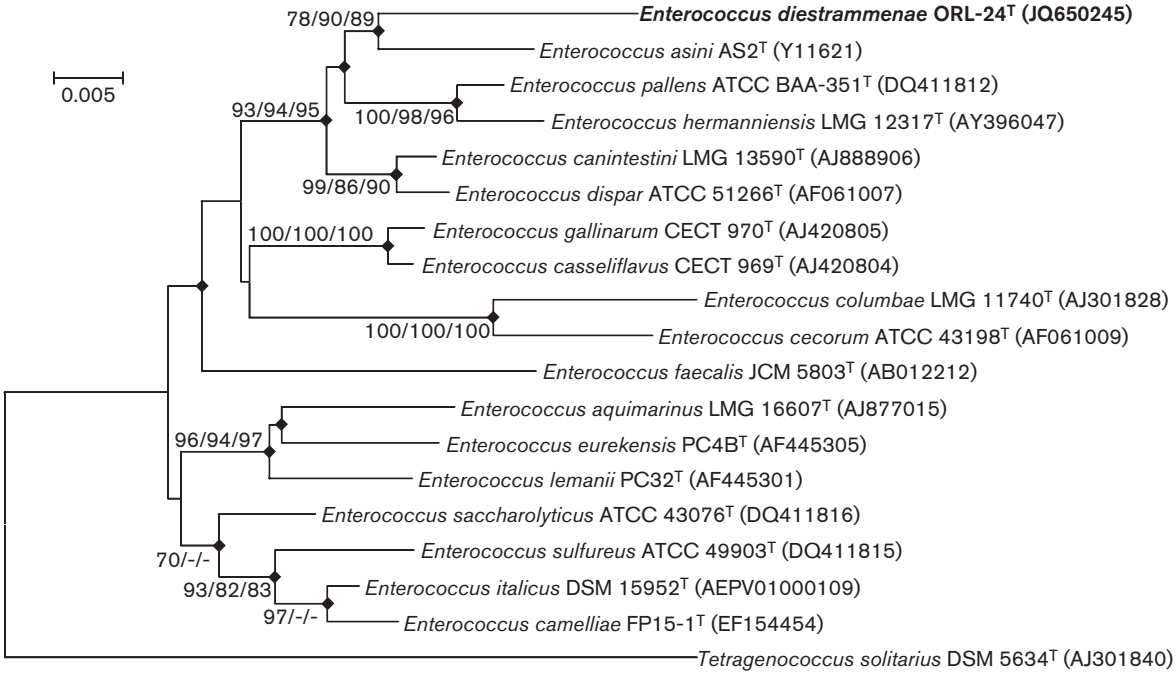

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the taxonomic position of strain ORL-24^T. Filled diamonds indicate that identical branches are present in the phylogenetic trees generated using the neighbour-joining, maximum-parsimony and maximum-likelihood methods. The numbers at nodes represent bootstrap values (>70 %, dashes indicate values below 70 %) based on 1000 replicates. *Tetragenococcus solitarius* DSM 5634^T was used as an outgroup. Bar, 0.005 accumulated changes per nucleotide.

tested using GP2 MicroPlates (Biolog) with GN/GP inoculating fluid (Biolog) and API 50 CH test strips (bioMérieux) with 50 CHL medium (bioMérieux), respectively, according to the manufacturer's instructions. The enzyme activities were determined using API ZYM test strips (bioMérieux). Table 1 shows that strain ORL-24^T and the type strains of reference species were biochemically distinct. The complete results of biochemical test are shown in Table S1.

Strain ORL-24^T and the strains of the reference species were harvested from MRSA plates after incubation at 37 °C for 48 h and used in the chemotaxonomic analysis. The cellular fatty acid methyl esters (FAMEs) of strain ORL-24^T and the reference species were obtained by saponification, methylation and extraction, as described by the Sherlock Microbial Identification System (MIDI, 1999). The FAMEs were analysed by gas chromatography (Agilent 7890 gas chromatograph; Agilent Technologies) and identified using the microbial identification program (Sherlock software 6.0B) (Sasser, 1990) based on the TSBA6 library. The major fatty acids (>10 % of total fatty acids) of strain ORL-24^T in the exponential phase were $C_{18:1}\omega9c$ (67.9 %) and $C_{16:0}$ (22.7 %). The majority of the cellular fatty acid profile of the isolate was generally similar to those of the reference species; however, strain ORL-24^T was distinguishable by differences in the components present in minor proportions. The complete cellular fatty acid compositions of strain ORL-24^T and the reference species are given in Table 2.

Genomic DNA was extracted from strain ORL-24^T according to the method described by Rochelle *et al.* (1992). The G+C content of the genomic DNA was estimated using a fluorimetric method with SYBR Gold I and a real-time PCR thermocycler (Biorad) (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 the calibration references. The G+C contents of the genomic DNA of strain ORL-24^T was 41.3 mol%. The genomic DNA G+C content of species with validly published names that belong to the genus *Enterococcus* are in the range of 35.1–44.9 mol% (Švec & Devriese, 2009).

Based on its phenotypic, phylogenetic and genotypic characteristics, it is suggested that strain ORL-24^T represents a novel species of the genus *Enterococcus*, for which the name *Enterococcus diestrammenae* sp. nov. is proposed.

Table 2. Cellular fatty acid composition (%) of strain ORL-24^T and the reference strains

Strains: 1, ORL-24^T; 2, *Enterococcus asini* KCTC 13286^T; 3, *Enterococcus pallens* DSM 15690^T; 4, *Enterococcus canintestini* LMG 13590^T; 5, *Enterococcus dispar* KCTC 13288^T; 6, *Enterococcus hermanniensis* LMG 12317^T; 7, *Enterococcus faecalis* JCM 5308^T. All data were obtained in the current study. Values are expressed as percentages of the total fatty acids. Fatty acids that represented <0.1 % in all species were omitted. TR, Trace amount (<0.5 %); −, not detected.

Fatty acid	1	2	3	4	5	6	7
Saturated acids							
$C_{12:0}$	TR	1.5	TR	1.3	TR	0.9	TR
$C_{14:0}$	1.6	6.0	2.8	6.4	1.1	5.1	5.1
$C_{16:0}$	22.7	22.4	23.7	19.6	22.1	25.0	21.8
$C_{17:0}$	−	−	−	−	TR	−	TR
$C_{18:0}$	1.0	0.8	1.3	0.7	1.7	1.3	1.9
Unsaturated acids							
anteiso-$C_{17:1}\omega9c$	−	−	−	TR	−	−	−
$C_{17:1}\omega8c$	TR	−	TR	TR	TR	TR	−
$C_{18:1}\omega9c$	67.9	54.9	38.5	45.2	66.0	32.8	8.3
$C_{18:1}\omega7c$ 11-methyl	−	−	−	−	−	−	TR
cyclo-$C_{19:0}\omega8c$	−	−	−	−	−	−	21.0
Branched acids							
iso-$C_{15:0}$	−	−	−	−	−	−	TR
cyclo-$C_{17:0}$	−	−	−	−	−	−	0.8
iso-$C_{19:0}$I	−	TR	−	−	−	−	−
iso-$C_{19:0}$	0.5	0.7	2.7	1.5	1.7	0.5	0.6
Summed features*							
2	−	TR	−	−	−	−	−
3	1.7	6.9	3.6	14.1	1.6	3.1	12.0
7	−	−	22.2	−	−	28.0	16.1
8	3.9	5.7	4.4	8.7	5.0	2.9	11.7

*Summed features represent two or three fatty acids that could not be separated by the Microbial Identification System. Summed feature 2 comprises $C_{14:0}$ 3-OH/iso-$C_{16:1}$I; summed feature 3 comprises $C_{16:1}\omega7c$/iso-C_{15} 2-OH; summed feature 7 comprises an unknown constituent with equivalent chain-length of 18.846/$C_{19:1}\omega6c$/cyclo-$C_{19:0}\omega10c$; summed feature 8 comprises $C_{18:1}\omega6c$/$C_{18:1}\omega7c$.

Description of *Enterococcus diestrammenae* sp. nov.

Enterococcus diestrammenae [di.est.ram.me′nae. N.L. gen. n. *diestrammenae* of Diestrammena, isolated from the gut of *Diestrammena coreana* (order *Orthoptera*)].

Cells are non-motile, Gram-stain-positive, facultatively anaerobic and cocci or diplococci (1.0–1.3 μm in diameter). After cultivation at 37 °C for 3 days on MRS medium, the colonies are circular, opaque, ivory, smooth, raised and 1.0–2.0 mm in diameter. Optimal growth occurs at 30–37 °C, pH 8.0–9.0 and in the presence of 0 % (w/v) NaCl. The ratio of L-lactate to D-lactate is 96 : 4. The strain grows on kanamycin aesculin azide agar but not on Slanetz-Bartley agar. Lancefield antigen D is not detected. Oxidase- and catalase-negative. Assimilates α-ketovaleric acid, L-lactic acid, pyruvic acid methyl ester, pyruvic acid, adenosine, 2′-deoxyadenosine, thymidine and uridine, but not α-cyclodextrin, β-cyclodextrin, dextrin, glycogen, inulin, mannan, Tween 40, Tween 80, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, amygdalin, L-arabinose, D-arabitol, arbutin, cellobiose, D-fructose, L-fucose, D-galactose, D-galacturonic acid, gentiobiose, D-gluconic acid, α-D-glucose, *myo*-inositol, α-lactose, lactulose, maltose, maltotriose, D-mannitol, D-mannose,

melezitose, melibiose, methyl α-D-galactoside, methyl β-D-galactoside, 3-methyl glucose, methyl α-D-glucoside, methyl β-D-glucoside, methyl α-D-mannoside, palatinose, D-psicose, raffinose, L-rhamnose, D-ribose, salicin, sedoheptulosan, D-sorbitol, stachyose, sucrose, D-tagatose, trehalose, turanose, xylitol, D-xylose, acetic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketoglutaric acid, lactamide, D-lactic acid methyl ester, D-malic acid, L-malic acid, succinic acid monomethyl ester, propionic acid, succinamic acid, succinic acid, *N*-acetyl-L-glutamic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl L-glutamic acid, L-pyroglutamic acid, L-serine, putrescine, 2,3-butanediol, glycerol, inosine, adenosine-5′-monophosphate, thymidine-5′-monophosphate, uridine-5′-monophosphate, D-fructose-6-phosphate, α-D-glucose 1-phosphate, D-glucose 6-phosphate or DL-α-glycerol phosphate, according to Biolog GP2. Acid is produced from L-arabinose, D-ribose, D-xylose, methyl β-D-xyloside, D-galactose, D-glucose, D-fructose, D-mannose, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, trehalose, gentiobiose, 2-ketogluconate and 5-ketogluconate, but not from glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannoside, melibiose, sucrose, inulin, melezitose, raffinose, starch, glycogen, xylitol, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol or gluconate. The following enzymes are positive in the API ZYM test: esterase (C4), esterase lipase (C8), leucine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-galactosidase. Alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are negative. The major cellular fatty acids are $C_{18:1}\omega 9c$ and $C_{16:0}$.

The type strain is ORL-24^T (=KACC 16708^T=JCM 18359^T), which was isolated from the gut of the camel cricket, *Diestrammena coreana*, in South Korea. The DNA G+C content of the type strain is 41.3 mol%.

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