Enterococcus saccharolyticus subsp. taiwanensis subsp. nov., isolated from broccoli

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A coccal strain isolated from fresh broccoli was initially identified as Enterococcus saccharolyticus; however, molecular identification and phenotypic traits did not support this identification. DNA–DNA hybridization with the type strain of E. saccharolyticus (76.4% relatedness), DNA G+C content (35.7 mol%), phylogenetic analysis based on 16S rRNA, pheS and rpoA gene sequences, rep-PCR fingerprinting and profiles of cellular fatty acids, whole-cell proteins and enzyme activities, together with carbohydrate metabolism characteristics, indicated that this strain is distinct and represents a novel subspecies, for which the name Enterococcus saccharolyticus subsp. taiwanensis subsp. nov. is proposed. The type strain is 812T (=NBRC 109476T =BCRC 80575T). Furthermore, we present an emended description of Enterococcus saccharolyticus and proposal of Enterococcus saccharolyticus subsp. saccharolyticus subsp. nov. (type strain ATCC 43076T =CCUG 27643T=CCUG 33311T =CIP 103246T =DSM 20726T =JCM 8734T =LMG 11427T =NBRC 100493T =NCIMB 702594T).

Enterococci are Gram-positive, catalase-negative, facultatively anaerobic cocci typically associated with the gastrointestinal tract of humans and animals (Rahkila et al., 2011). Enterococcus saccharolyticus has been found previously in various animal and environmental samples (Watts, 1989; ben Omar & Ampe, 2000; Ampe et al., 2001; Layton et al., 2010). Strain 812T was previously isolated from fresh broccoli and initially identified as E. saccharolyticus based on 16S rRNA gene sequences (Chen et al., 2013). However, at least four nucleotide differences were observed between the 16S rRNA gene sequences of strain 812T and E. saccharolyticus BCRC 80584T. In order to identify strain 812T, sequence analysis of two housekeeping genes, pheS and rpoA (Naser et al., 2005; Švec et al., 2005a; Morandi et al., 2012), and phenotypic characterization were performed. The purpose of the present study was to establish the taxonomic position of this bacterial strain.

The type strain of E. saccharolyticus, BCRC 80584T, was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and was grown in trypti-case-soy yeast extract (TSY) medium at 37 °C. The Gram reaction and catalase activity of strain 812T were determined using the methods of Kozaki et al. (1992). Phenotypic characteristics, including isomers of lactic acid produced, lactic acid fermentation type, salt tolerance and growth temperature range, were analysed based on methods described by Kozaki et al. (1992). Carbohydrate metabolism tests were performed using the API 50 CHL fermentation kit (bioMérieux) according to the manufacturer’s instructions. Enzyme activities of strain 812T and E. saccharolyticus BCRC 80584T were assessed using the API ZYM system (bioMérieux). The tests were performed according to the manufacturer’s instructions. After incubating at 37 °C for 4 h, the reaction was terminated by the addition of one drop each of API ZYM reagents A and B, and the results were examined.

Amplification and sequencing of the 16S rRNA gene were carried out as described by Chen et al. (2010). Amplification and sequencing of the housekeeping genes pheS and rpoA were performed using primers pheS-21-F (5′-CAYCCNG-CHCGYGYATGC-3′), pheS-22-R (5′-CCWARVCCRAAR-GCAAARCC-3′), rpoA-21-F (5′-ATGATYGARTTTGAA-9), which were designed and reported by Naser et al.
hybridization experiments were performed at 37°C dilution wells as described previously (Ezaki, 2007). DNA–DNA relatedness values were determined previously (Tamaoka & Komagata, 1984; Wang et al., 2006). All sequences were aligned using CLUSTAL W software (Thompson et al., 1997). Distances were calculated according to Kimura’s two-parameter model (Kimura, 1980). Phylogenetic trees were then reconstructed using the neighbour-joining method (Saitou & Nei, 1987), with bootstrap analysis based on 1000 iterations. The MEGA 5.05 package (Tamura et al., 2011) was used for all analyses.

Genomic DNA was extracted from cells grown in TSY broth for 24 h at 30°C and purified using the Qiagen Blood & Cell Culture DNA kit. The DNA G+C content was determined using reversed-phase HPLC as described previously (Tamaoka & Komagata, 1984; Wang et al., 2007). DNA–DNA relatedness values were determined using the fluorometric hybridization method in micro-dilution wells as described previously (Ezaki et al., 1989; Goris et al., 1998; Wang et al., 2007). DNA–DNA hybridization experiments were performed at 37°C and were repeated five times.

Extraction of fatty acids and determination of the cellular fatty acid profile were performed by using the Sherlock Microbial Identification System (version 6.0), according to the instructions of the Microbial Identification System (MIDI).

Repetitive sequence-based (rep)-PCR fingerprint profile analysis was performed by using primers REP1R-I (5′-IIICGICGICATCGCC-3′) and REP2-I (5′-IIICGNCAGCN-CATCGNGGC-3′) described by Svec et al. (2005b). PCR amplification was performed under the conditions described by Versalovic et al. (1994).

Nucleotide sequences of the 16S rRNA (~1498 nt), pheS (~435 nt) and rpoA (~770 nt) genes were determined. Phylogenetic analysis of the 16S rRNA gene sequences obtained in this study and from GenBank indicated that strain 812T should be classified within E. saccharolyticus owing to its very low sequence divergence (Fig. 1). Strain 812T showed the highest sequence similarity (99.73 %) to E. saccharolyticus BCRC 80584T. However, at least four nucleotide differences were observed between the 16S rRNA gene sequences of strain 812T and E. saccharolyticus BCRC 80584T.

When comparing the pheS gene sequence with those held in the GenBank database, strain 812T also showed the highest sequence similarity (86.86 %) to E. saccharolyticus BCRC 80584T. However, similarities to members of other Enterococcus species were lower than 85 %. The results of phylogenetic analysis of the pheS gene are shown in Fig. 2. Comparison of the rpoA gene with that of other Enterococcus type strains was also performed. The results indicated that strain 812T again had the highest sequence similarity (97.83 %) to E. saccharolyticus BCRC 80584T. Similarities to members of other Enterococcus species were lower than 90 %. The results of phylogenetic analysis of the rpoA gene are shown in Fig. 3.

Phylogenetic analyses were also carried out using the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971, 1977) methods. Bootstrap analysis was performed using 1000 iterations. Similar results were obtained as for the neighbour-joining method (compare Figs 1–3 and Figs S1–S6, available in IJSEM Online).

The DNA G+C content of strain 812T was 35.7 mol%, differing from that of E. saccharolyticus BCRC 80584T (37.6 mol%) (Table 1). DNA–DNA hybridization experiments were performed using DNA derived from strain 812T and E. saccharolyticus BCRC 80584T. Mean DNA relatedness between strain 812T and E. saccharolyticus BCRC 80584T was 76.4 % (SD 5.5 %), indicating that strain 812T should be grouped with E. saccharolyticus. The rep-PCR fingerprint profiles obtained demonstrated the separation of strain 812T from other E. saccharolyticus strains (BCRC 80584T, NCIMB 702609 and NCIMB 702612) (Fig. S7).

In addition to phylogenetic analyses, a number of phenotypic tests were performed. Strain 812T showed homolactic acid fermentation, production of L-lactic acid and growth in medium containing 6 % (w/v) NaCl. Strain 812T could not grow at pH 4.0. These characteristics were similar to those of E. saccharolyticus BCRC 80584T (Table 1). Acid production from carbohydrates was assessed using the API 50 CHL fermentation kit after 48 h of incubation. When comparing the results obtained in this study with the description of Farrow et al. (1984), differences in fermentation ability were observed in five carbohydrates studied, dulcitol, D-tagatose, gluconate, D-arabitol and melezitose (Table 1). However, results for fermentation of ribose, starch, inulin and 2-ketogluconate by E. saccharolyticus BCRC 80584T were different from the results described by Farrow et al. (1984) (Table 1). Enzyme activities evaluated by using the API ZYM system are shown in Table S1. Characteristics that differed between strain 812T and E. saccharolyticus BCRC 80584T were activities of x-glucosidase, β-glucosidase and x-glucosaminidase.

The cellular fatty acid profile was determined using the Sherlock Microbial Identification System. The major fatty acids detected in strain 812T were C18:1ω7c (45.15 %), C16:0 (22.37 %), C14:0 (10.89 %), C12:0 (0.48 %), C18:1ω9c (0.35 %) and summed feature 3 (C16:0ω7c and/or iso-C15:0 2-OH; 20.76 %). The fatty acid profile of E. saccharolyticus BCRC 80584T, reported previously by Tyrrell et al. (2002), was not identical to the profile of strain 812T (Table 1).

Protein analysis using SDS-PAGE and 2D-DIGE methods was applied to compare the protein profiles of strain 812T.
and *E. saccharolyticus* BCRC 80584<sup>T</sup>. Cell pellets were harvested, washed and resuspended in 20 ml PBS (pH 7.5). Cell disruption was performed using a TS-0.75 kW continuous high pressure cell disrupter (Constant Systems) at 20 000 p.s.i. and cell debris was removed by centrifugation at 15 000 g for 20 min at 4 °C. SDS-PAGE

**Fig. 1.** Neighbour-joining tree of strain 812<sup>T</sup> and related enterococci based on 16S rRNA gene sequences. Bootstrap values based on 1000 iterations are indicated as percentages at branch points. GenBank accession numbers are given in parentheses. *Bacillus subtilis* NCDO 1769<sup>T</sup> was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

**Fig. 2.** Neighbour-joining tree of strain 812<sup>T</sup> and related enterococci based on pheS sequences. Bootstrap values based on 1000 iterations are indicated as percentages at branch points. *Streptococcus gordonii* LMG 14516<sup>T</sup> was used as an outgroup. Bar, 0.05 substitutions per nucleotide position.
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Fig. 3. Neighbour-joining tree of strain 812 T and related enterococci based on rpoA sequences. Bootstrap values based on 1000 iterations are indicated as percentages at branch points. Streptococcus gordonii LMG 14516 T was used as an outgroup. Bar, 0.05 substitutions per nucleotide position.

Analysis of supernatants showed different protein expression patterns between strain 812 T and E. saccharolyticus BCRC 80584 T (Fig. S8). This result was further confirmed by 2D-DIGE analysis (Fig. S9). Protein samples were labelled with Cy3 or Cy5 (CyDye DIGE Fluor minimal dyes; GE Healthcare). Samples were then mixed and loaded on IPG strips (13 cm, pH 4–7 linear; GE Healthcare) using an IPGphor IEF system (GE Healthcare) according to the manufacturer’s recommendations. The second dimension was performed in an SEM 600 electrophoresis unit (GE Healthcare). After electrophoresis, the gel was scanned on an FLA-5100 scanner (Fujifilm) and the image was analysed using Multi Gauge software (Fujifilm). The resulting image revealed that a significant number of the protein spots did not overlap, indicating that the protein profiles of the two strains were different.

16S rRNA gene sequencing, DNA–DNA hybridization and SDS-PAGE of whole-cell proteins are currently among the techniques most commonly used for identification of species of the genus Enterococcus. However, the 16S rRNA gene sequence has limited discriminating power for several closely related enterococcal species (Naser et al., 2005). Protein-encoding gene sequences are more variable and have proved useful in the differentiation of closely related species with almost identical 16S rRNA gene sequences (Dellaglio et al., 2005; Naser et al., 2005). Based on the data reported by Sistek et al. (2012), the threshold similarity value for interspecies differentiation is less than 99 % for rpoA gene sequences and less than or equal to 90 % for pheS gene sequences. Strain 812 T showed 86.86 % pheS gene sequence similarity to E. saccharolyticus BCRC 80584 T, suggesting that strain 812 T cannot be assigned to the same taxon.

The data reported here indicate the independent status of the isolated strain in the genus Enterococcus. DNA–DNA hybridization and 16S rRNA gene sequence analysis confirm that strain 812 T belongs to a distinct group within E. saccharolyticus. However, data obtained in the current study, such as pheS and rpoA gene sequences, ability to ferment carbohydrates and cellular fatty acid profile, suggest that strain 812 T should not be designated as a strain of E. saccharolyticus as currently described.

Based on the results obtained in this study, the isolated strain represents a subspecies that is distinct from that represented by E. saccharolyticus BCRC 80584 T, and a number of phenotypic characteristics clearly distinguish strain 812 T from E. saccharolyticus BCRC 80584 T (Table 1). In conclusion, a novel subspecies of E. saccharolyticus is proposed for the isolated strain from broccoli, represented by 812 T as the type strain, for which the name Enterococcus saccharolyticus subsp. taiwanensis subsp. nov. is proposed. The description of Enterococcus saccharolyticus subsp. taiwanensis leads to the automatic creation of Enterococcus saccharolyticus subsp. saccharolyticus. An emended description of Enterococcus saccharolyticus is also provided.

Emended description of Enterococcus saccharolyticus (Farrow et al. 1985) Rodrigues and Collins 1991

In addition to the characteristics described by Farrow et al. (1984), the following features are found. In the API ZYM strip, positive for esterase (C4), esterase lipase (C8), valine aminopeptidase, cystine aminopeptidase, chymotrypsin and acid phosphatase and negative for α-glucosidase, β-glucosidase, α-glucosaminidase, α-mannosidase and α-fucosidase.
Description of *Enterococcus saccharolyticus* subsp. *saccharolyticus* subsp. nov.

The subspecies is created automatically with the same authors as those of *Enterococcus saccharolyticus* subsp. *taiwanensis* according to the rules of nomenclature (Garrity et al., 2011; Poli et al., 2012). The description is the same as given previously for *Streptococcus saccharolyticus* by Farrow et al. (1984) and emended in this study for *Enterococcus saccharolyticus*. The type strain is ATCC 43076T = CCUG 27643T = CIP 103246T = DSM 20726T = JCM 8734T = LMG 11427T = NBRC 100493T = NCIMB 702594T.

Cells are Gram-stain-positive, catalase-negative, coccoid and facultatively anaerobic and grow well anaerobically on TSY and blood agars at 30 °C. Colonies on TSY and blood agars are circular, smooth and non-pigmented. Utilizes D-glucose homofermentatively and does not produce gas from glucose. Produces L-lactic acid from glucose. Grows at 20–37 °C, but not at 10 or 45 °C. Grows in 6 % NaCl, but not at pH 4.0. Acid is produced from D-glucose, D-fructose, dulcitol, mannitol, sorbitol, methyl α-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, raffinose, gentiobiose, turanose, D-tagatose and gluconate. Acid is produced weakly from galactose, D-mannose and L-sorbos. Acid is not produced from glycerol, erythritol, D- or L-arabinose, ribose, D- or L-xylene, adonitol, methyl β-xylulose, rhamnose, inositol, methyl α-D-mannoside, inulin, starch, glycerogen, xylitol, D-lyxose, D- or L-fucose, D- or L-arabitol or 2- or 5-ketogluconate. Aesculin is hydrolysed. In API ZYM tests, positive for alkaline enzymes, catalase-negative, oxidase-negative, urease-negative, arginine dihydrolase-negative, ornithine decarboxylase-negative, citrase-positive, arginine-positive, hippurate-positive, gelatinase-negative, caseinase-negative, and lipase-negative.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>812T</th>
<th>E. saccharolyticus BCRC 80584T</th>
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<tr>
<td></td>
<td>This study</td>
<td>Data from references</td>
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<tr>
<td>DNA G+C content (mol%)</td>
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<td>37.6</td>
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<tr>
<td>Growth at:</td>
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<tr>
<td>10 °C</td>
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<tr>
<td>45 °C</td>
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<td>–</td>
</tr>
<tr>
<td>pH 4.0</td>
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<tr>
<td>Acid production from:</td>
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</tr>
<tr>
<td>Dulcitol</td>
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<tr>
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<tr>
<td>Gluconate</td>
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<tr>
<td>Mellezitose</td>
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<td>+</td>
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<tr>
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<td>Starch</td>
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<tr>
<td>2-Ketogluconate</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Cellular fatty acid content (%)</td>
<td></td>
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<tr>
<td>C12 : 0</td>
<td>0.48</td>
<td>ND 0.5</td>
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<tr>
<td>C14 : 0</td>
<td>10.89</td>
<td>ND 9.2</td>
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<tr>
<td>C15 : 0</td>
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<tr>
<td>C16 : 0</td>
<td>22.37</td>
<td>ND 17.1</td>
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<tr>
<td>C17 : 0</td>
<td>–</td>
<td>ND –</td>
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<tr>
<td>C18 : 0</td>
<td>–</td>
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<tr>
<td>C18 : 1ω9c</td>
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<tr>
<td>C18 : 1ω7c</td>
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<td>Summed feature 3*</td>
<td>20.76</td>
<td>ND 23.0</td>
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<tr>
<td>Summed feature 5*</td>
<td>–</td>
<td>ND 2.3</td>
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</table>

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contains C16 : 1ω7c and/or iso-C15 : 0 2-OH; summed feature 5 contains C18 : 2ω6,9c and/or anteiso-C17 : 0.

Table 1. Characteristics of strain 812T and *E. saccharolyticus* BCRC 80584T

Reference data for *E. saccharolyticus* were taken from Farrow et al. (1984) for strain NCDO 2594T with the exception of the cellular fatty acid profile, which were taken from Tyrrell et al. (2002) for strain BCRC 80584T. Both strains grow in 4 and 6.5 % (w/v) NaCl (confirmed in this study for *E. saccharolyticus* BCRC 80584T). +, Positive; –, negative; NA, no data available; ND, not done.

Description of *Enterococcus saccharolyticus* subsp. *saccharolyticus* subsp. nov.

*Enterococcus saccharolyticus* subsp. *taiwanensis* (tai.wa.n.en’sis. N.L. masc. adj. *taiwanensis* of or belonging to Taiwan, referring to the origin of the type strain).
phosphatase, esterase (C4), esterase lipase (C8), leucine aminopeptidase, valine aminopeptidase, cysteine aminopeptidase, chymotrypsin, acid phosphatase, phosphohydrolase, α-glucosidase, β-glucosidase and α-glucosaminidase and negative for lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase. The major cellular fatty acids are C₁₈:₁ω₇c, C₁₆:₀, C₁₄:₀ and summed feature 3 (C₁₆:₁ω7c and/or iso-C₁₅:₀ 2-OH).

The type strain is 812ᵀ (=NBRC 109476ᵀ = BCRC 80575ᵀ), with a DNA G+C content of 35.7 mol%.

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**References**


