Lactobacillus hordei sp. nov., a bacteriocinogenic strain isolated from malted barley

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A high-throughput screening effort, designed to isolate bacteriocin-producing lactic acid bacteria (LAB) from malted cereals, resulted in the isolation of four bacteriocin-producing strains that could not be assigned conclusively to recognized species. The four isolates (UCC128^T, UCC125, UCC126 and UCC127) were found to share identical (100 %) 16S rRNA gene sequences and were therefore deemed to belong to the same species. The strains were Gram-positive, catalase-negative, non-motile homofermentative LAB. The closest recognized relative to strain UCC128^T identified based on comparative 16S rRNA gene sequence analysis was *Lactobacillus mali* DSM 20444^T (97 % similarity). The strains were characterized phenotypically to identify specific growth requirements. DNA–DNA hybridization between strain UCC128^T and *L. mali* DSM 20444^T revealed a level of relatedness of only 39.4 %. This indicates that strain UCC128^T does not belong to the species *L. mali*. The four bacteriocin-producing strains are therefore considered to represent a novel species of the genus *Lactobacillus*, for which the name *Lactobacillus hordei* sp. nov. is proposed. The type strain is UCC128^T (=DSM 19519^T=LMG 24241^T).

Barley is a natural habitat for lactic acid bacteria (LAB) (O'Mahony *et al.*, 2000; Hartnett *et al.*, 2002). The numbers of LAB on barley have been shown to increase as a result of the malting process during brewing (O'Sullivan *et al.*, 1999), possibly because the steeping conditions create a favourable environment for their growth. However, LAB counts start to decrease during barley germination and continue to decline throughout the kilning process (O'Sullivan *et al.*, 1999). A screening project was undertaken to isolate bacteriocin-producing LAB from malted barley by means of a high-throughput approach with an automated colony-picker (Genetix) (Rouse *et al.*, 2007). In total, the screening process yielded approximately 77 798 LAB colonies, all of which were assayed for bacteriocin production. Of these, 179 LAB were shown to possess bactericidal activity and were selected for further investigation. Strain UCC128^T, which was shown to possess bactericidal activity, could not be assigned to a definite species grouping on the basis of initial 16S rRNA

gene sequencing. Three other bacteriocin-producing strains were isolated in the same way, all of which had 16S rRNA gene sequences that were identical to that of isolate UCC128^T. This study describes the phenotypic and genotypic characterization of these isolates, which are shown to represent a novel species of the genus *Lactobacillus*.

Strains UCC128^T, UCC125, UCC126 and UCC127 were isolated from malted barley obtained from a brewing facility in Belgium. The four cultures were routinely grown in MRS broth (Oxoid) at 30 °C for 16 h and maintained in 40 % glycerol at –80 °C. The purity of the cultures was checked by streaking them on MRS agar (Oxoid), incubating them anaerobically at 30 °C for 16 h and observation under a microscope. Reference strain *Lactobacillus mali* DSM 20444^T was grown according to the instructions outlined by the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Cell morphology was examined by streaking on MRS agar and incubating the plates at 30 °C anaerobically for 3 days. Overnight cultures grown in MRS broth were viewed by using phase-contrast microscopy (Leica DMLS). Strains UCC128^T, UCC125, UCC126 and UCC127 were subjected to phenotypic characterization. The Gram reaction was determined by Gram staining as described by Hucker (1921). Carbohydrate fermentation profiles were determined by

Abbreviation: LAB, lactic acid bacteria.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain UCC128^T is EU074850.

Phylogenetic trees showing the relationship between strain UCC128^T and related species of the genus *Lactobacillus* based on 16S rRNA gene sequences, constructed with the maximum-parsimony and maximum-likelihood methods, are available with the online version of this paper.

using an API 50CH test kit (bioMérieux). Catalase activity was investigated by adding 5% H_2O_2 to fresh colonies from MRS agar and examining for the production of O_2 bubbles. Growth at temperatures between 15 and 45 °C was examined in addition to the ability to grow between pH 3 and 9. Gas production from glucose was determined by growing the isolates overnight in MRS broth sealed with 2% sterile molten agar. Tolerance to salt was tested by growing the isolates in MRS broth and on MRS agar containing 1–10% NaCl (w/v), followed by incubation at 30 °C for 3 days. Sensitivity of the isolates to antibiotics was tested by placing discs containing the different antibiotics (Oxoid) on the surface of MRS agar, onto which the cultures had been spread-plated. Ampicillin (25 µg), penicillin (2 U), metronidazole (5 µg), amoxicillin (30 µg), trimethoprim (5 µg), chloramphenicol (30 µg), cephadrine (30 µg), oxacillin (1 µg), novobiocin (5 µg), nalidixic acid (30 µg), ceftizoxime (30 µg), gentamicin (30 µg), tetracycline (30 µg) and ciprofloxacin (5 µg) were tested. The isomer type of lactic acid was determined by using a DL lactate test kit (Boehringer).

The peptidoglycan structure of the cell wall of strain UCC128^T was determined by the DSMZ according to the method of Rhuland *et al.* (1955). The DNA G + C content of strain UCC128^T was determined by the DSMZ via a HPLC analytical method. DNA was purified on hydroxyapatite according to the method of Cashion *et al.* (1977) and was hydrolysed and dephosphorylated according to Mesbah *et al.* (1989). HPLC was performed in accordance with the protocol outlined by Tamaoka & Komagata (1984). The G + C content was determined as described by Mesbah *et al.* (1989).

Genomic DNA was isolated from all four of the novel isolates according to the method of Sambrook *et al.* (1989) and was used as template for the amplification of a large portion of the 16S rRNA gene by PCR as described by Corsetti *et al.* (2005). DNA sequencing reactions were performed at MWG Biotech AG. To determine the phylogenetic position of strain UCC128^T, the nearly-complete 16S rRNA gene sequence was aligned with the corresponding gene sequences of other lactobacilli by using the CLUSTAL W program (Thompson *et al.*, 1994). The 16S rRNA gene sequence of the type strain of *Bacillus subtilis* was added to root the trees. Phylogenetic analysis and tree construction were performed by using the neighbour-joining method under Kimura's two-parameter substitution model (Kimura, 1980) and with the maximum-parsimony method with MEGA4 software (Tamura *et al.*, 2007). Phylogenetic trees were also constructed via the maximum-likelihood method by using PHYLML software (Guindon & Gascuel, 2003), which allows a general time-reversible model to be used to accommodate rate variation among sites. Bootstrap values for each tree were calculated by resampling 100 times.

Levels of DNA–DNA relatedness were determined by the DSMZ between strain UCC128^T and *L. mali* DSM 20444^T.

DNA–DNA hybridization was performed as described by De Ley *et al.* (1970) with modifications according to the method described by Huß *et al.* (1983), by using a model Cary 100 Bio UV/Vis-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in situ* temperature probe (Varian).

Colonies of strains UCC128^T, UCC125, UCC126 and UCC127 grown on MRS agar for 3 days at 30 °C were approximately 0.75–1 mm in diameter and appeared as smooth, circular, white colonies on the surface of the plates. Cells were rod-shaped, 0.5 × 0.8–1 µm in size, non-motile and occurred singly, in pairs or in short chains. Cells were Gram-positive (data not shown). Carbohydrate fermentation profiles revealed the four novel isolates to be capable of producing acid from D-glucose, D-fructose, mannose, mannitol, methyl α-D-mannoside, methyl α-D-glucopyranoside, amygdalin, arbutin, salicin, cellobiose, maltose, sucrose and β-gentiobiose. The isolates were catalase-negative (Table 1). Aesculin hydrolysis was observed and no gas was produced from glucose. Growth was observed at 21 and 42 °C, with optimal growth at 30 °C; no growth occurred at 15 or 45 °C. The strains were unable to grow at pH 3, but could grow at pH 4; in MRS the novel strains were able to grow at pH 8 but not at pH 9. The isolates were able to grow in the presence of 4% NaCl, but were unable to grow in 5% NaCl. The strains were sensitive to ampicillin, penicillin G, amoxicillin, trimethoprim, chloramphenicol, oxacillin, novobiocin, gentamicin, tetracycline and ciprofloxacin, but resistant to metronidazole, cephadrine, nalidixic acid and ceftizoxime.

The peptidoglycan structure of strain UCC128^T, as determined by the DSMZ, was identified to be of type A1γ, due to the presence of *meso*-diaminopimelic acid. *L. mali* DSM 20444^T also possesses this type of peptidoglycan structure. The DNA G + C content of strain UCC128^T was 36.5 mol%, which falls within the range (32–53 mol%) reported for species of the genus *Lactobacillus* (Kandler & Weiss, 1986).

Strains UCC128^T, UCC125, UCC126 and UCC127 were found to share 100% 16S rRNA gene sequence similarity, indicating that they should all be placed in the same species grouping. The closest relative to strain UCC128^T as revealed by 16S rRNA gene sequence analysis was *Lactobacillus* sp. 88 (98% similarity), an undescribed strain identified in a study of wine-associated LAB (Rodas *et al.*, 2005). The closest recognized relative to UCC128^T was *L. mali* DSM 20444^T (Carr & Davies, 1970), with 97% 16S rRNA gene sequence similarity. According to Stackebrandt & Goebel (1994), a similarity value of 97% or less with the closest relative may indicate that the isolate represents a novel species, rendering the determination of DNA–DNA relatedness values unnecessary. However, a more recent report suggests that DNA–DNA relatedness values should be determined for strains showing 16S rRNA gene sequence

Table 1. Differential phenotypic characteristics between strain UCC128^T and the closest related species in the genus *Lactobacillus*

Strains: 1, UCC128^T (strains UCC125, UCC126 and UCC127 gave identical results); 2, *L. mali* DSM 20444^T (data from Carr & Davies, 1970); 3, *L. satsumensis* JCM 12392^T (Endo & Okada, 2005); 4, *L. nagelii* ATCC 700692^T (Edwards *et al.*, 2000); 5, *L. algidus* JCM 10491^T (Kato *et al.*, 2000); 6, *L. vini* DSM 20605^T (Rodas *et al.*, 2006); 7, *L. animalis* JCM 5670^T (Kandler & Weiss, 1986); 8, *L. murinus* JCM 1717^T (Kandler & Weiss, 1986); 9, *L. equi* JCM 10991^T (Morotomi *et al.*, 2002). Strains 1–6: –, negative reaction; +, positive reaction; v, variable; NA, no data available; mDAP, meso-diaminopimelic acid. Strains 7–9: +, >90 % of strains positive; –, >90 % of strains negative; d, 11–89 % of strains positive; NA, no data available. All strains were catalase-negative.

Characteristic	1	2	3	4	5	6	7	8	9
Acid production from:									
Arabinose	–	+	–	–	+	+	d	+	–
Cellobiose	+	+	–	+	v	+	+	+	–
Galactose	–	+	v	+	+	NA	+	+	+
Mannitol	+	+	+	+	–	–	–	d	+
Melezitose	–	–	v	–	–	–	–	–	–
Methyl α -D-glucopyranoside	+	+	+	+	–	–	+	NA	–
Rhamnose	–	+	+	+	–	–	–	–	d
Salicin	+	+	+	+	+	+	+	d	d
Sorbitol	–	–	+	+	–	–	+	–	d
Trehalose	–	+	+	+	+	+	–	d	–
Gas from glucose	–	–	–	–	–	–	–	+	–
Lactate isomer	L	L	L	DL	L	DL	L	L	DL
Growth at 15 °C	–	+	+	+	+	–	–	–	–
Peptidoglycan type	mDAP	mDAP	mDAP	mDAP	mDAP	L-Lys-D-Asp	L-Lys-D-Asp	L-Lys-D-Asp	NA
DNA G + C content (mol%)	36.5	32–34	40	NA	36.5	39.4	41–44	43–44	38–40

similarity of 98.7–99 % (Stackebrandt & Ebers, 2006), to allow for more precise species classification. Levels of 16S rRNA gene similarity were also calculated between strain UCC128^T and the type strains of other recognized species of the genus *Lactobacillus*, including *Lactobacillus satsumensis* (95 %), '*Lactobacillus mobilis*' (95 %), *Lactobacillus nagelii* (95 %), *Lactobacillus murinus* (94 %), *Lactobacillus algidus* (94 %) and *Lactobacillus animalis* (94 %). Fig. 1 depicts the phylogenetic relationship between strain UCC128^T and its closest related species, resulting from alignments of their 16S rRNA gene sequences according to

the neighbour-joining method. Similar tree topologies were obtained by using the maximum-parsimony and maximum-likelihood methods (see Supplementary Figs S1 and S2, respectively, in IJSEM Online).

DNA–DNA hybridization between UCC128^T and *L. mali* DSM 20444^T revealed a level of relatedness of 39.4 %, well below the threshold value of 70 % recommended by Wayne *et al.* (1987) for defining a bacterial species. Therefore, strain UCC128^T does not belong to the species *L. mali*. On the basis of the data presented here, we propose that strains

Fig. 1. Phylogenetic tree depicting the relationship between strain UCC128^T and its closest relatives as deduced from 16S rRNA sequence analysis. The tree was constructed by using the neighbour-joining method and approximately 1400 nt of the 16S rRNA gene was used for sequence alignment. Bar, 0.01 nucleotide substitutions per site.

UCC128^T, UCC125, UCC126 and UCC127 represent a novel species of the genus *Lactobacillus*, for which the name *Lactobacillus hordei* sp. nov. is proposed.

Description of *Lactobacillus hordei* sp. nov.

Lactobacillus hordei (hor'de.i. L. gen. neut. n. *hordei* of from barley).

Cells are Gram-positive rods, approximately 0.5×0.8 – $1 \mu\text{m}$ in length, non-motile, occurring singly, in pairs or in chains. Colonies are white, smooth and circular on MRS agar after 2 days incubation at 30°C and measure 0.75 – 1 mm in diameter. Facultatively anaerobic, catalase-negative and homofermentative. Optimum growth occurs at 30°C ; no growth occurs at 15 or 45°C . Aesculin is hydrolysed. Growth is observed at pH 4 and 8, but not at pH 3 or 9. Able to ferment D-glucose, D-fructose, mannose, mannitol, methyl α -D-mannoside, methyl α -D-glucopyranoside, amygdalin, arbutin, salicin, cellobiose, maltose, sucrose and β -gentiobiose, but not glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β -xyloside, galactose, sorbose, rhamnose, dulcitol, inositol, sorbitol, N-acetylglucosamine, lactose, melibiose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, turanose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, or 2- or 5-ketogluconate. The peptidoglycan structure is of A1 γ type. The DNA G+C content is 36.5 mol%.

The type strain, UCC128^T (=DSM 19519^T=LMG 24241^T), was isolated from malted barley. UCC125, UCC126 and UCC127, isolated from similar sources, are other strains of the species.

Acknowledgements

This work was supported by the Irish Government under National Development Plan 2000–2006. We would also like to thank Professor Hans Trüper for his assistance with the Latin translation of the species name.

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