

Lactobacillus sobrius sp. nov., abundant in the intestine of weaning piglets

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To obtain porcine isolates related to *Lactobacillus amylovorus*, we screened strains from piglet intestine grown on *Lactobacillus*-specific MRS agar for hybridization to a fluorescent 16S rRNA-targeted DNA probe. Six strains were isolated and further characterized by phenotypic and molecular taxonomic methods. The isolates were Gram-positive, catalase-negative, facultatively anaerobic rods. They had similar phenotypic characteristics and displayed genomic DNA–DNA relatedness values of > 78 % to each other, indicating that they belong to a single species. Comparative 16S rRNA gene sequence analysis demonstrated that the novel isolates were members of *Lactobacillus* rRNA group I, which includes *Lactobacillus delbrueckii*, the type species of the genus. Based on 16S rRNA gene sequence similarity, *Lactobacillus kitasatonis* (99 %), *Lactobacillus crispatus* (98 %) and *Lactobacillus amylovorus* (97 %) were the nearest relatives of the novel isolates, but their DNA–DNA relatedness was found to be lower than 49 %. One of the isolates, strain OTU171-001^T, was further characterized using physiological and biochemical tests. Together, the results enabled genotypic and phenotypic differentiation of strain OTU171-001^T from the other species that showed 16S rRNA gene sequence similarity values greater than 97 %. Strain OTU171-001^T merits species status and the name *Lactobacillus sobrius* sp. nov. is proposed. The type strain is OTU171-001^T (= DSM 16698^T = NCCB 100067^T).

Lactobacilli are characterized as Gram-positive, non-spore-forming rods and are catalase-negative, non-motile, do not usually reduce nitrate and utilize glucose fermentatively (Hammes *et al.*, 1991). Members of the genus *Lactobacillus* are not only found on plants and in plant-derived materials, such as silage, grains and foods, but also in the gastrointestinal tract of humans and animals (Vaughan *et al.*, 2002). Recent studies have shown a specific response of a novel and abundant *Lactobacillus amylovorus*-like phylotype to dietary oligosaccharides in the guts of weaning piglets (Konstantinov *et al.*, 2004). Therefore, culturing and further analysis were needed in order to gain insight into the systematic position of the *L. amylovorus*-like strains. Here we

report on the isolation of strains related to *Lactobacillus amylovorus* DSM 20531^T from pig intestinal sources and their characterization using a polyphasic approach. The results of this approach indicate that these strains represent a novel species for which the name *Lactobacillus sobrius* sp. nov. is proposed.

A DNA oligonucleotide probe L^{*}-OTU171-0088-a-A-18 (5'-CGCTTCCCAACGTCATT-3') (Konstantinov *et al.*, 2004) targeting the 16S rRNA gene of the *L. amylovorus*-like phylotype OTU171 was used to screen a range of *Lactobacillus* isolates from piglets (21 days of age) housed at different locations. In total, 192 isolates grown on MRS *Lactobacillus*-selective agar (Difco) were screened by fluorescence *in situ* hybridization (FISH) using the CY3-labelled phylotype-specific probe in combination with image analysis as described by Konstantinov *et al.* (2004). Two *L. amylovorus*-like strains were identified in the faeces of piglets housed on a farm near Wageningen, the Netherlands, and were isolated as strains OTU171-001^T and OTU171-002. Three strains (OTU171-003, OTU171-004 and OTU171-005) were isolated from the faeces of piglets kept on a farm near Bologna, Italy, and one isolate was found in an ileal lumen sample from a piglet reared on a farm near Bristol,

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Abbreviations: FISH, fluorescence *in situ* hybridization; FOS, fructo-oligosaccharides; SBP, sugar beet pulp.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Lactobacillus sobrius* sp. nov. OTU171-001^T is AY700063.

A figure showing the results of SDS-PAGE analysis and a table detailing the results of DNA–DNA hybridization studies are available as supplementary material in IJSEM Online.

UK (OTU171-006). These six isolates were selected for further characterization by phenotypic and molecular taxonomic methods. *Lactobacillus acidophilus* DSM 20079^T, *L. amylovorus* DSM 20531^T, *Lactobacillus crispatus* DSM 20584^T, *Lactobacillus gallinarum* DSM 10532^T, *Lactobacillus helveticus* DSM 20075^T and *Lactobacillus kitasatonis* JCM 1039^T were used as reference strains.

Cell shape, size and arrangement, Gram-stain and appearance of colonies were determined by using cells grown on MRS agar plates for 2 days at 37 °C. Production of gas from glucose was also examined. The *L. amylovorus*-like isolates hybridizing to the OTU171 probe were Gram-positive, non-spore-forming and non-motile rods and colonies of these strains were white with circular to irregular shapes. Strains were tested for carbohydrate fermentation abilities using the API 50 CHL system (bioMérieux). In addition, the degradation of fructooligosaccharides (FOS) and sugar beet pulp (SBP) by the strains was tested using MRS as basal medium (without carbohydrates) supplied with 1% FOS or SBP as carbon sources. All strains were found to display similar fermentation characteristics. The phenotypic characteristics that differentiate the *L. amylovorus*-like strains from the reference strains are summarized in Table 1. In contrast to *L. kitasatonis*, all of the novel isolates were able to ferment D-raffinose. The strains grew also in MRS-FOS and MRS-SBP media to a final cell density (OD₆₀₀) of 1.81 and 2.7, respectively. The strains produced DL-lactic acid, did not grow at 15 °C, did grow at 45 °C, were catalase-negative and did not produce gas from glucose. DNA G+C content was calculated based on the determination of genomic DNA T_m

(Marmur & Doty, 1962) and the values obtained were estimated to range from 35 to 36 mol% (Table 1).

16S rRNA gene sequences (approximately 1.5 kb) were determined for the six representative strains as described by Konstantinov *et al.* (2004). Phylogenetic analysis was performed using the ARB software package (Ludwig *et al.*, 2004) (Fig. 1). Sequence similarity calculations performed after a neighbour-joining analysis indicated that the closest relatives of strain OTU171-001^T were *L. kitasatonis* (99%), *L. crispatus* (98%), *L. amylovorus* (97%) and *L. gallinarum* (97%).

Extraction of whole-cell proteins and their separation by SDS-PAGE were performed using standard protocols (Sambrook *et al.*, 1989). SDS-PAGE protein fingerprints were compared using the BIONUMERICS software package version 3.0 (Applied Maths). When analysed using SDS-PAGE, the whole-cell proteins of the six OTU171 strains displayed marked differences from those of the reference strains (see Supplementary Fig. S1 in IJSEM Online). After cluster analysis of the SDS-PAGE protein profiles, the fingerprints of the novel isolates formed a coherent cluster with a similarity greater than 85%; they were related to the type strains examined with a similarity below 70%.

DNA-DNA hybridization experiments were performed with strains OTU171-001^T, OTU171-002 and OTU171-003 and type strains of the nearest *Lactobacillus* species by filter hybridization according to Klijn *et al.* (1994) except that [α -³²P]dCTP was used for the nick translation and the hybridization temperature was 59 °C (~25 °C below T_m).

Table 1. Phenotypic characteristics and DNA G+C content of *L. amylovorus*-like OTU171 strains and closely related lactobacilli

Taxa: 1, *L. amylovorus*-like OTU171 strains (*L. sobrius* sp. nov.); 2, *L. kitasatonis*; 3, *L. amylovorus*; 4, *L. crispatus*; 5, *L. gallinarum*; 6, *L. gasei*; 7, *L. acidophilus*; 8, *L. johnsonii*; 9, *L. helveticus*. Data for taxa 2–9, belonging the *L. acidophilus* group, are from Mukai *et al.* (2003). +, Positive; D, strain-dependent; D–, usually negative; –, negative; w, weak reaction. All strains produce acid from D-glucose, galactose, D-fructose, D-mannose and maltose and produce DL-lactic acid. No strains produce acid from arabinose, xylose, rhamnose, melezitose or sorbitol.

Characteristic	1	2	3	4	5	6	7	8	9
DNA G+C content (mol%)	35–36	37–40	36–38	33–36	36–37	33–34	32–40	33–35	38–40
Growth at 15 °C	–	–	–	–	+	–	–	+	–
Fermentation of:									
Amygdalin	D, w	–	D	+	+	+	+	+	–
Cellobiose	D	D	D	+	+	+	+	+	–
Lactose	+	D, w	+	+	D	+	+	+	+
Mannitol	D, w	D, w	–	D	–	–	–	–	–
Melibiose	D	–	D	+	+	w	–	D	–
D-Raffinose	+	–	D	+	+	–	+	D	–
Ribose	D, w	–	–	D	–	–	–	–	–
Salicin	D, w	D	+	+	+	+	+	+	–
Sucrose	+	+	+	+	+	+	+	+	–
Trehalose	D	D–	–	D	–	+	+	D	D

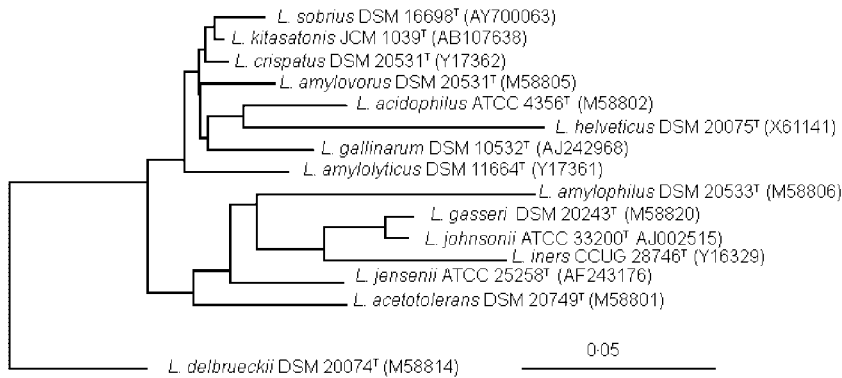


Fig. 1. Phylogenetic analysis of *Lactobacillus sobrius* OTU171-001^T and selected members of the *L. delbrueckii* 16S rRNA gene cluster of the genus *Lactobacillus*. The tree was calculated using the neighbour-joining method with partial 16S rRNA gene sequences (*Escherichia coli* positions 107–1433) using the ARB software package (Ludwig *et al.*, 2004). Bar, 5% sequence divergence.

Labelled DNA of strains OTU171-001^T, OTU171-002 and OTU171-003 reassociated at a high level (78–100%) with unlabelled DNA from the six OTU171 strains, while only low levels of reassociation (2–49%) were observed with the closely related *Lactobacillus* species examined (see Supplementary Table S1 in IJSEM Online).

Based on DNA–DNA hybridization values, the ability of the OTU171 strains to ferment D-raffinose and FOS and to grow at 45 °C, it is suggested that the six isolates are members of a single species that is distinct from the closest *Lactobacillus* species.

To assess the genomic diversity of the six isolates further, PFGE profiles of *ApaI*-digested chromosomal DNA were generated as described previously (McCartney *et al.*, 1996). The genome size of the isolates was calculated according to the λ PFGE standard ladder using the QUANTITY ONE program (Bio-Rad). All isolates displayed distinct PFGE profiles (data not shown) and a mean genome size of 1.2 Mb was calculated.

Based on the reported data, we propose the novel species *Lactobacillus sobrius* with the type strain OTU171-001^T.

Description of *Lactobacillus sobrius* sp. nov.

Lactobacillus sobrius (so'bri.us. L. masc. adj. *sobrius* sober, moderate; in Calvinism, referring to a hard working, invisible person).

Cells are Gram-positive, non-motile, non-spore-forming rods that are 0.6–1.0 μm in width and 2.0–20.0 μm in length and occur singly, in pairs or often in long chains. Colonies are 1.4–2 mm in diameter, circular to slightly irregular to rough in form and white when the organism is grown on MRS agar at 37 °C for 2 days. There is no growth at 15 °C, but the bacterium grows at 45 °C. The organism is facultatively anaerobic and produces D- and L-lactic acid homofermentatively. Catalase is not produced. Acid is produced without gas formation from D-glucose, D-mannose, maltose, galactose, D-fructose, lactose, aesculin, sucrose, starch, mannitol (two of six strains), cellobiose (three of six strains), salicin (two of six strains), trehalose (two of six strains), amygdalin (two of six strains, weak reaction),

N-acetylglucosamine (two of six strains), ribose (two of six strains, weak reaction), glycogen (three of six strains) and 5-ketogluconate (two of six strains, weak reaction). FOS and unidentified compounds of SBP are also fermented by all strains. There is no acid formation from glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, melezitose, rhamnose, adonitol, methyl β -xyloside, sorbitol, L-sorbose, dulcitol, inositol, methyl α -D-mannoside, methyl α -D-glucoside, arbutin, inulin, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate or 2-ketogluconate. DNA G+C content is 35–36 mol%.

The type strain, OTU171-001^T (=DSM 16698^T=NCCB 100067^T), was isolated from pig intestine.

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