Lactobacillus apodemi sp. nov., a tannase-producing species isolated from wild mouse faeces

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A Gram-positive, rod-shaped, non-endospore-forming bacterium, strain ASB1^T, able to degrade tannin, was isolated from faeces of the Japanese large wood mouse, *Apodemus speciosus*. Comparative analysis of the 16S rRNA gene sequence revealed that the strain could be assigned as a member of the genus *Lactobacillus*. The nearest phylogenetic neighbours were determined as *Lactobacillus animalis* DSM 20602^T (98.9% 16S rRNA gene sequence similarity) and *Lactobacillus murinus* ASF 361 (98.9%). Subsequent polyphasic analysis, including automated ribotyping and DNA–DNA hybridization experiments, confirmed that the isolate represents a novel species, for which the name *Lactobacillus apodemi* sp. nov. is proposed. The DNA G + C content of the novel strain is 38.5 mol%. The cell-wall peptidoglycan is of type A4 α L-Lys–D-Asp. The type strain is ASB1^T (=DSM 16634^T = CIP 108913^T).

In recent years, several studies have reported the presence of bacterial species with tannase activity in the guts or faeces of different animals (Osawa *et al.*, 1995a, b). The Japanese large wood mouse (*Apodemus speciosus*) inhabits various types of forests in Japan. It feeds on acorns in autumn and winter which may contain considerable amounts of tannins (Sasaki *et al.*, 2005). Fresh faecal pellets were collected from wild wood mice from the forests of Kyotanabe, Japan.

Bacterial strains were isolated from spread plates (tannic acid-treated brain heart infusion agar, prepared as described by Osawa, 1990) of serial diluted faecal samples incubated under anaerobic conditions at 37 °C. For further testing, the strains were grown in MRS medium (Difco). Colony and cell morphology were determined after 2 days of incubation. The ability to grow at 15 or 45 °C was tested in MRS broth (Difco). Anaerobic growth was tested on MRS plates incubated using the Anaerocult A mini system (Merck). Catalase activity was determined by using reagent droppers (Becton Dickinson). Lactic acid configuration was determined using the D-lactic acid/L-lactic acid test kit (Boehringer Mannheim/R-Biopharm) based on the UV method, according to the manufacturer's instructions.

Phenotypic properties were determined using an API 50 CHL kit (bioMérieux).

Genomic DNA was extracted from bacterial cells and purified using a previously described protocol (Pukall *et al.*, 1998). The primer pair 27f (5'-GAGTTTGATCCTGGCTC-AG-3') and 1527r (5'-AGAAAGGAGGTGATCCAGCC-3') was used for amplification of the 16S rRNA gene (Lane, 1991). PCR amplification of the 16S rRNA-encoding gene was performed as described earlier (Pukall *et al.*, 1999). Amplicons were sequenced by using the Dye-labelled Dideoxy Terminator cycle sequencing quick start kit and the CEQ 8000 Genetic Analysis System (both from Beckman Coulter). Sequences were manually aligned and compared with previously published sequences. These were stored in the Deutsche Sammlung von Mikroorganismen und Zellkulturen internal database consisting of more than 6000 16S rRNA gene sequence entries, including those from the Ribosomal Database Project (Maidak *et al.*, 2001) and EMBL. Similarity values were transformed into genetic distance values that compensate for multiple substitutions at any given site in the sequence (Jukes & Cantor, 1969). The neighbour-joining method contained in the PHYLIP package (Felsenstein, 1993) was used for the construction of a phylogenetic dendrogram. In addition, the DNAm1 algorithm for maximum-likelihood analysis (Felsenstein 1993) was applied, but revealed almost the same clusters for closely related species as shown in the neighbour-joining

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Lactobacillus apodemi* sp. nov. DSM 16634^T is AJ871178.

dendrogram. All analyses were done on a SUN SparcII workstation.

Automated ribotyping was carried out for strains ASB1^T (=DSM 16634^T), ASB2 (=DSM 16635), ASB6 (=DSM 16648), ASB8 (=DSM 16649) and ASB7 (=DSM 16748), that had been previously isolated from the faeces of Japanese large wood mice and found to be able to produce tannase (Sasaki *et al.*, 2005). Fingerprints were generated by using the RiboPrinter microbial characterization system (Qualicon). The RiboPrinter system combines molecular processing steps for ribotyping in a stand-alone, automated instrument. Steps include cell lysis, digestion of chromosomal DNA with a restriction enzyme, separation of fragments by electrophoresis, transfer of DNA fragments to a nylon membrane, hybridization to an *Escherichia coli* *rrnB* probe, chemiluminescent detection of the probe to the fragments containing *rrn* operon sequences, image detection and computerized analysis and storage of RiboPrint patterns. Sample preparation and analysis were performed according the manufacturer's instructions and the *EcoRI* restriction enzyme was used to generate restriction fragments. The band patterns were compared using BioNumerics software (Applied Maths). Clustering was performed by the UPGMA method based on the Pearson correlation coefficient using an optimization coefficient of 1.2 %.

Analysis of the peptidoglycan structure was carried out as described by Schleifer (1985) and Schleifer & Kandler (1972) with the modification that TLC on cellulose was applied instead of paper chromatography. Strain ASB1^T possesses

cell-wall peptidoglycan of type A4 α , L-Lys-D-Asp (type A11.31 according to the DSMZ catalogue of strains; DSMZ, 2001).

Estimation of the DNA G+C content of strain ASB1^T followed the procedure described by Sasaki *et al.* (2005). Levels of total DNA-DNA hybridization of genomic DNA extracted from the novel ASB strains and type strains of *Lactobacillus animalis* and *Lactobacillus murinus* were determined as described by Sasaki *et al.* (2005).

16S rRNA gene sequence analysis indicated that strain ASB1^T is a member of the genus *Lactobacillus*. *L. murinus* and *L. animalis* were determined to be the nearest phylogenetic neighbours to strain ASB1^T, showing sequence similarity values of 98.9 %. The position of strain ASB1^T relative to its phylogenetic neighbours is shown by neighbour-joining analysis (Fig. 1). In addition, a second algorithm, the maximum-likelihood method, included within the DNAmI program (Felsenstein, 1993), was applied to confirm the phylogenetic position of strain ASB1^T. The most closely related species grouped together in both dendrograms. Strain ASB1^T formed a stable cluster together with *L. animalis* and *L. murinus*. Both algorithms placed strain ASB1^T near to the *Lactobacillus mali* group and the *Lactobacillus salivarius* group (Hammes & Hertel, 2003). Partial sequences for all novel ASB strains tested revealed nearly identical stretches. Further, the close relationship of the novel ASB strains was confirmed by automated ribotyping (Fig. 2). The similarity of riboprint patterns obtained for the novel ASB strains, compared with strain ASB1^T, ranged between 87.5 and 93.9 %. When compared

Fig. 1. Neighbour-joining tree showing the nearest phylogenetic neighbours for strain ASB1^T. Bootstrap values (percentages of 1000 replications) with >70 % confidence are shown at branching points. Bar, 1 nucleotide substitution per 100 positions.

Fig. 2. Ribotype patterns obtained from ASB strains compared with those derived from *L. animalis* DSM 20602^T and *L. murinus* DSM 20452^T. Cluster analysis was performed by UPGMA based on the Pearson correlation coefficient using an optimization coefficient of 1.2. VCA indicates a standard *EcoRI* batch.

with strain ASB1^T, the similarity values of fingerprints derived from *L. animalis* DSM 20602^T and *L. murinus* DSM 20452^T were determined to be low (< 50 %).

Finally, DNA–DNA hybridization experiments performed previously (Sasaki *et al.*, 2005) showed that all the ASB strains tested could be representatives of the same species, with a relative DNA–DNA hybridization with strain ASB1^T of between 73.8 and 97.3 %. In contrast, relative binding of DNA from strain ASB1^T with the type strains of *L. animalis* and *L. murinus* gave values of < 15 % (Sasaki *et al.*, 2005).

Phenotypic properties differentiating strain ASB1^T from the type strains of *L. animalis* and *L. murinus* are summarized in Table 1.

The above evidence and the phenotypic characteristics described previously (Sasaki *et al.*, 2005) and summarized within the species description confirm that the ASB strains represent a novel species, for which the name *Lactobacillus apodemi* sp. nov. is proposed. The type strain is ASB1^T (= DSM 16634^T = CIP 108913^T).

Description of *Lactobacillus apodemi* sp. nov.

Lactobacillus apodemi (a'po.de.mi. N.L. gen. n. *apodemi* of *Apodemus*, the genus name of the field mouse, *Apodemus speciosus*, from which the organism was first isolated).

Gram-positive, non-motile, non-endospore-forming, catalase-negative rods, 0.5–1 × 5–6 µm in size and occurring

as single cells or in pairs. After anaerobic growth at 37 °C for 48 h, colonies on MRS agar are 2–3 mm in diameter. Colonies are white with an opaque border, smooth and convex. Growth on MRS agar also occurs under micro-aerophilic conditions and reduced growth is detected under aerobic conditions. Best growth occurs at temperatures between 25 and 37 °C. In MRS broth, growth occurs at 45 °C, but not at 15 °C. Produces gallic acid from tannic acid (tannase-positive), but does not convert gallic acid further to pyrogallol (Sasaki *et al.*, 2005). Only L-lactate is produced. Gas is not produced from glucose as tested in MRS broth with a Durham tube. Acid is produced from galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, arbutin, maltose, lactose, melibiose, sucrose, trehalose and D-raffinose. Acid is not produced from glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, methyl D-xyloside, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl D-mannoside, methyl D-glucoside, amygdalin, inulin, melezitose, starch, glycogen, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. Ribose, salicin and cellobiose are variable. Aesculin is hydrolysed. The DNA G+C content of the type strain is 38.5 mol% and the cell-wall peptidoglycan is of type A4α, L-Lys–D-Asp (type A11.31).

The type strain, ASB1^T (= DSM 16634^T = CIP 108913^T), was isolated from faeces of the Japanese large wood mouse, *Apodemus speciosus*.

Table 1. Comparison of phenotypic properties of *L. apodemi* sp. nov. ASB1^T and its nearest phylogenetic neighbours

Characteristic	<i>L. apodemi</i> ASB1 ^T	<i>L. animalis</i> JCM 5670 ^T	<i>L. murinus</i> JCM 1717 ^T
Tannase activity	+	–	–
Utilization of:			
Mannitol	–	–	+
Trehalose	+	–	–
Gentiobiose	–	–	+

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