

Reclassification of *Lactobacillus amylophilus* LMG 11400 and NRRL B-4435 as *Lactobacillus amylotrophicus* sp. nov.

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The taxonomic position of six *Lactobacillus amylophilus* strains isolated from swine waste-corn fermentations was reinvestigated. All strains were included in a multilocus sequence analysis (MLSA) study for species identification of *Lactobacillus* using the genes encoding the phenylalanyl-tRNA synthase alpha subunit (*pheS*) and RNA polymerase alpha subunit (*rpoA*). Partial *pheS* and *rpoA* gene sequences showed that strains LMG 11400 and NRRL B-4435 represent a separate lineage that is distantly related to the type strain of *L. amylophilus*, LMG 6900^T, and to three other strains of the species. The MLSA data showed that the two strains LMG 11400 and NRRL B-4435 constituted a distinct cluster, sharing 100% *pheS* and *rpoA* gene sequence similarity. The other reference strains clustered together with the type strain of *L. amylophilus*, LMG 6900^T, and were clearly differentiated from strains LMG 11400 and NRRL B-4435 (80 and 89% *pheS* and *rpoA* gene sequence similarity, respectively). The 16S rRNA gene sequences of the latter two strains are 100% identical, with the nearest phylogenetic neighbour *L. amylophilus* LMG 6900^T showing only 97.2% 16S rRNA gene sequence similarity. Further polyphasic taxonomic study based on whole-cell protein fingerprinting, DNA–DNA hybridization and biochemical features demonstrated that the two strains represent a single, novel *Lactobacillus* species, for which the name *Lactobacillus amylotrophicus* sp. nov. is proposed. The type strain is LMG 11400^T (=NRRL B-4436^T=DSM 20534^T).

Lactobacillus amylophilus was first described by Nakamura & Crowell (1979) based only on the phenotypic characterization of six strains (NRRL B-4435, LMG 11400, LMG 6900^T, NRRL B-4438, NRRL B-4439 and NRRL B-4440) isolated from swine waste-corn fermentations. Few species of

Lactobacillus are able to utilize amylose as a carbon and energy source. *L. amylophilus* is recognized as a starch-utilizing species and displays extracellular amyolytic enzyme activity. *L. amylophilus* is a homofermentative lactic acid bacterium, which ferments starch to L(+)-lactic acid (Nakamura & Crowell, 1979). The strains of *L. amylophilus* also fermented fructose, galactose, glucose, mannose and maltose. Two strains (NRRL B-4435 and LMG 11400) fermented amygdalin, cellobiose and salicin weakly after 3 days or more (Nakamura & Crowell, 1979). By further genotypic investigation, Nakamura (1982) showed high DNA–DNA reassociation values (90–98%) among all these strains of the species. In an ongoing study of lactic acid bacteria diversity, these six strains of *L. amylophilus* were included in a multilocus sequence analysis (MLSA) study applied for the identification of *Lactobacillus* species. The results demonstrated that two strains, LMG 11400 and NRRL B-4435, occupied a distinct position. Further genomic and phenotypic research revealed that they represent a single novel species.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of LMG 11400^T and NRRL B-4435 are AM236149 and AM236150, respectively. Other accession numbers reported in this paper are: partial *pheS* gene sequences for strains LMG 11400^T, NRRL B-4435, NRRL B-4438, NRRL B-4439 and NRRL B-4440, AM236139–AM236143; partial *rpoA* gene sequences for strains LMG 11400^T, NRRL B-4440, NRRL B-4435, NRRL B-4438 and NRRL B-4439, AM236144–AM236148.

A distance-matrix tree based on 16S rRNA gene sequences is available as supplementary material in IJSEM Online.

The following culture collection strains were included in this study: *L. amylophilus* LMG 6900^T (=NRRL B-4437^T), LMG 11400 (=NRRL B-4436), NRRL B-4435, NRRL B-4438, NRRL B-4439 and NRRL B-4440. All strains were cultivated and maintained on de Man, Rogosa and Sharpe (MRS) medium (Oxoid) and incubated at 37 °C for 24 h, unless otherwise indicated.

The use of housekeeping genes in bacterial taxonomy offers higher resolution than 16S rRNA gene sequence data at the species level as it integrates information from different molecular markers from throughout the bacterial chromosome (Stackebrandt *et al.*, 2002; Zeigler, 2003). Sequencing of the housekeeping genes encoding the phenylalanyl-tRNA synthase alpha subunit (*pheS*) and RNA polymerase alpha subunit (*rpoA*) provided robust identification of all enterococcal species (Naser *et al.*, 2005a) and proved to be an efficient screening tool for the delineation of novel taxa (Naser *et al.*, 2005b; Švec *et al.*, 2005). In this study, we initially focused on partial *pheS* (304 bp) and *rpoA* (355 bp) gene sequences of *L. amylophilus* strains as a screening test to investigate their relatedness. Bacterial genomic DNA was extracted following the methodology described by Gevers *et al.* (2001). Amplification and sequencing reactions were performed as described by Naser *et al.* (2005a). Neighbour-joining trees of the investigated housekeeping genes showed that strains LMG 11400 and NRRL B-4435 constituted a distinct cluster, yielded 100 % *pheS* and *rpoA* gene sequence similarity, and represented a separate lineage distantly related to *L. amylophilus* LMG 6900^T (80 and 89 % *pheS* and *rpoA* gene sequence similarity, respectively) and to other lactobacilli (Fig. 1).

The phylogenetic position of strains LMG 11400 and NRRL B-4435 was also studied by complete 16S rRNA gene sequence analysis. Genomic DNA was prepared according to the protocol of Niemann *et al.* (1997). 16S rRNA gene amplification, purification and sequencing were performed as described by Vancanneyt *et al.* (2004) with the following modifications. PCR-amplified 16S rRNA genes were purified by using a NucleoFast 96 PCR Clean-up kit (Macherey-Nagel). Sequencing reactions were performed by using a BigDye Terminator cycle sequencing kit (Applied Biosystems) and purified by using a Montage SEQ₉₆ sequencing reaction cleanup kit (Millipore). Sequencing was performed by using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Sequence assembly was done by using the program Genebuilder (Applied Maths). The 16S rRNA gene sequences of strains LMG 11400 (1488 bp) and NRRL B-4435 (1525 bp) and gene sequences retrieved from EMBL were aligned and a phylogenetic tree was constructed from the global alignment by using the neighbour-joining method using the BioNumerics software package, version 4.50 (Applied Maths). Bootstrapping analysis was undertaken to test the statistical reliability of the topology of the neighbour-joining tree using 1000 bootstrap resamplings of the data (see Supplementary Fig. S1 available in IJSEM Online). The tree topology obtained with the neighbour-joining method was

evaluated and confirmed by maximum-parsimony analysis using BioNumerics (data not shown). Strains LMG 11400 and NRRL B-4435 were classified in the genus *Lactobacillus*, with *L. amylophilus* LMG 6900^T as their nearest neighbour, having 97.2 % 16S rRNA gene sequence similarity, a value that is generally accepted as borderline for species delineation (Stackebrandt & Goebel, 1994). Other *Lactobacillus* species showed less than 92 % 16S rRNA gene sequence similarity.

Protein fingerprinting is considered as a valuable method for species identification of most lactic acid bacteria (Vandamme *et al.*, 1996). Strains LMG 11400 and NRRL B-4435 and strains LMG 6900^T, NRRL B-4438, NRRL B-4439 and NRRL B-4440 of *L. amylophilus* were investigated using PAGE of whole-cell proteins. Whole-cell protein extracts were prepared and SDS-PAGE was performed as described by Pot *et al.* (1994). Densitometric analysis, normalization and interpolation of protein profiles, and numerical analysis were performed by using the GelCompar software package, versions 3.1 and 4.0, respectively (Applied Maths). Strains LMG 6900^T, NRRL B-4438, NRRL B-4439 and NRRL B-4440 showed identical protein patterns. Strains LMG 11400 and NRRL B-4435 could be differentiated from each other by the varying position of a dominant protein band in the molecular mass range of 40 to 50 kDa. After omitting this variable region from the cluster analysis, strains LMG 11400 and NRRL B-4435 constituted a highly homogeneous cluster showing a more distant relationship to the type strain of *L. amylophilus*, LMG 6900^T, and the other three reference strains of the species (Fig. 2). Strain-specific variable dense bands may indicate the presence of S-layer proteins, which are highly expressed and form a paracrystalline coat outside the bacterial cell wall (Sleytr & Sara, 1997). As these proteins are surface-exposed and play a role in survival and adaptation to the environment, differences between members of the same species can be seen in protein SDS-PAGE (Boot *et al.*, 1996).

The DNA G+C content was determined for strain LMG 11400. Cells were cultivated in MRS broth at 37 °C for 24 h. DNA was extracted from 0.5–0.75 g (wet weight) by using the protocol described by Marmur (1961) with the following modifications: (i) cells were suspended overnight in Tris/HCl buffer containing lysozyme (8 mg ml⁻¹) before addition of SDS and (ii) lysed cells were treated with proteinase K (360 mg l⁻¹; Merck) at 37 °C for 2 h. For determination of the DNA G+C content, DNA was degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture was then separated by HPLC using a Waters SymmetryShield C8 column maintained at a temperature of 45 °C. The solvent was 0.3 M NH₄H₂PO₄ (pH 4.4) with 1.5 % acetonitrile. Non-methylated λ-phage DNA (Sigma) was used as the calibration reference. The DNA G+C content of strain LMG 11400 was 43.5 mol%. In comparison, Nakamura & Crowell (1979) reported comparable DNA G+C contents for strains LMG 11400 and NRRL B-4435 (44 mol%) and the other four reference strains of *L. amylophilus* (46 mol%).

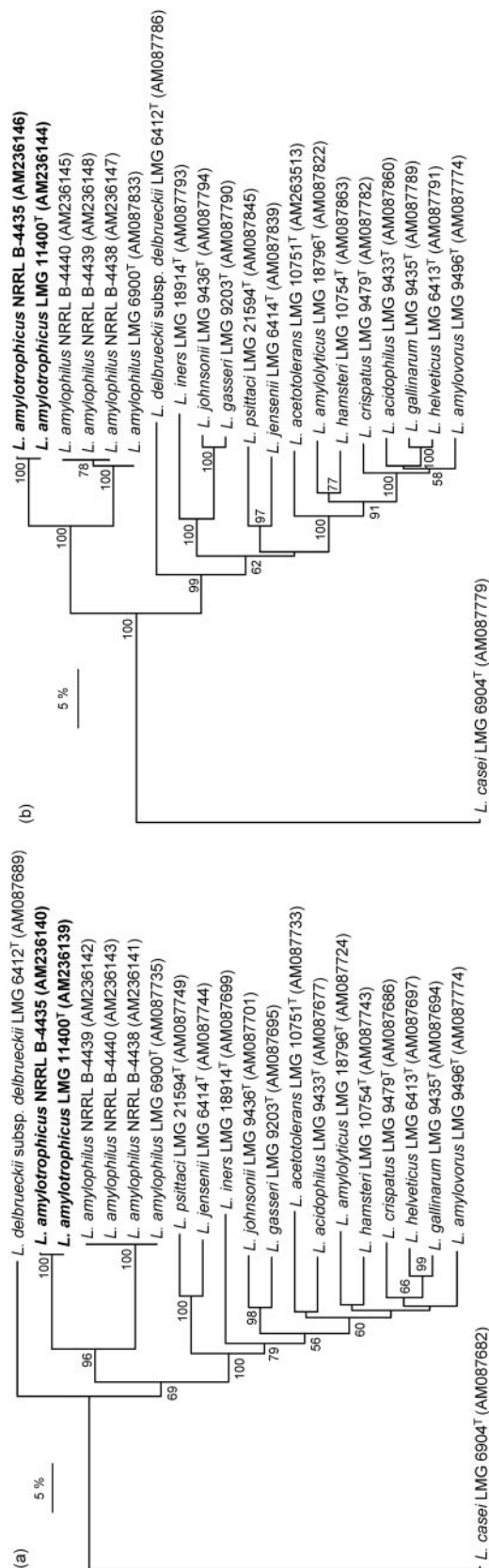


Fig. 1. Neighbour-joining trees showing relationships between strains LMG 11400 and NRRL B-4435 (*L. amylophilus* sp. nov.), *L. amylophilus* strains and other *Lactobacillus* reference species based on comparisons of *pheS* (a) and *rpoA* (b) gene sequences. *Lactobacillus casei* LMG 6904^T was used as an outgroup. Bootstrap percentages ($\geq 50\%$) after 1000 simulations are shown. Bars, 5% sequence divergence. Accession numbers are given in parentheses.

The heterogeneity observed in this study among strains of *L. amylophilus* is clearly in contrast to the reported data of Nakamura (1982) and indicates that it is necessary to reinvestigate the DNA–DNA reassociation among these strains. DNA–DNA hybridizations were performed between strains LMG 11400 and NRRL B-4435 and strains LMG 6900^T and NRRL B-4439 of *L. amylophilus*. Genomic DNA was prepared as described by Naser *et al.* (2006). The microplate method was used as described by Ezaki *et al.* (1989) and Goris *et al.* (1998), using an HTS7000 BioAssay Reader (Perkin Elmer) for the fluorescence measurements. Biotinylated DNA was hybridized with unlabelled single-stranded DNA, which was bound non-covalently to microplate wells. Hybridizations were performed at 36 °C in hybridization mixture (2 × SSC, 5 × Denhardt’s solution, 2.5% dextran sulfate, 50% formamide, 100 µg denatured salmon sperm DNA ml⁻¹, 1250 ng biotinylated probe DNA ml⁻¹). Reciprocal reactions were performed. The DNA–DNA binding values reported are the mean values of a minimum of four hybridization experiments, including the reciprocal reactions. High DNA–DNA hybridization levels (94%) were found between strains LMG 11400 and NRRL B-4435, indicating clearly that the two strains belong to the same species, whereas low DNA–DNA reassociation values, in the range 36–40%, were observed between the latter two strains and the type strain of *L. amylophilus* and strain NRRL B-4439. These results contradict the DNA–DNA reassociation values reported by Nakamura (1982), who showed high DNA–DNA reassociation (90–98%) among strains of *L. amylophilus*.

Growth characteristics and colony morphology were investigated on MRS agar (pH 5.4) after 24 h of incubation at 28 °C under aerobic conditions. Carbohydrate fermentation tests were carried out using API50 CHL galleries following the instructions of the manufacturer (bioMérieux). The results are given below in the species description.

Due to the contradictory results between the present study and those performed by Nakamura & Crowell (1979) and Nakamura (1982), we reinvestigated the fermentation patterns of three sugars, amygdalin, salicin and cellobiose, using a conventional method, as in the original description by Nakamura & Crowell (1979). This is because the carbohydrate fermentation tests in the API50 CHL gallery are reliable for 48 h but not after 3–7 days. The same set of strains analysed by SDS-PAGE was screened for growth on amygdalin, salicin and cellobiose. Cultures were first

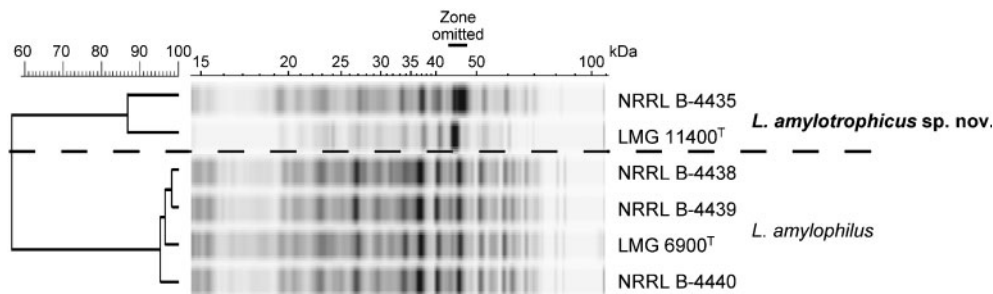


Fig. 2. Protein profiles and corresponding dendrogram derived from UPGMA linkage of correlation coefficients (r , expressed as a percentage for convenience) of strains LMG 11400 and NRRL B-4435 (*L. amylotrophicus* sp. nov.) and *L. amylophilus* strains.

inoculated in Difco MRS broth for 24 h and they were then transferred twice more to MRS broth, after which 1 ml culture was centrifuged at 7000 r.p.m. for 15 min and resuspended in 1 ml sterile physiological solution (0.85% w/v). The resulting suspension in physiological solution was used as the inoculum for the test media. All strains were incubated in 10 ml test tubes at 30 °C. The inoculum size was 1% (v/v) throughout the experiment. All experiments were performed twice. Growth on all three substrates, amygdalin, salicin and cellobiose, was observed for LMG 11400, which confirms data reported previously (Nakamura & Crowell, 1979). However, strain NRRL B-4435, which was expected to grow on amygdalin, salicin and cellobiose, showed growth on cellobiose and salicin after 7 days of incubation but it did not show any growth on amygdalin. Consequently, the phenotypic properties that can be used to differentiate the novel taxon from *L. amylophilus* are the production of acid from D-cellobiose and salicin. The production of acid from amygdalin is strain-dependent.

The same set of strains analysed by SDS-PAGE was tested for starch fermentation and the production of D- and L-lactic acid. Starch was used by all strains, as expected. Inoculating the strains on solid MRS medium with starch as a carbon source showed that all strains produced colonies with clearing zones around the colonies caused by degradation of the starch. This result confirmed that both *L. amylophilus* and the novel taxon are starch-utilizing species. The amounts of D- and L-lactic acid were determined with a Boehringer Mannheim D-lactic acid/L-lactic acid UV-method enzymic kit. Determinations of D- and L-lactic acid were performed on cultures grown on glucose as a carbon source after 24 h of incubation. Determination of the percentages of D- and L-lactic acid showed that all strains produced exclusively L(+)-lactic acid, which is in agreement with previous findings (Nakamura & Crowell, 1979).

The overall results of the present study allowed the assignment of two strains, LMG 11400 and NRRL B-4435, to a novel species, for which we propose the name *Lactobacillus amylotrophicus* sp. nov.

Description of *Lactobacillus amylotrophicus* sp. nov.

Lactobacillus amylotrophicus (a.my.lo.tro'phi.cus. Gr. n. *amulon* starch; N.L. adj. *trophicus* from Gr. adj. *trophikos* nursing, tending; N.L. masc. adj. *amylotrophicus* growing on starch, pertaining to the ability to ferment starch).

Cells are rod-shaped, occurring singly or in pairs and chains, 1.2–2.0 µm long and 0.9 µm wide, Gram-positive, catalase-negative, oxidase-negative, non-spore-forming and non-motile. After 24 h, colonies are white, smooth, circular and approximately 1 mm in diameter. Growth occurs at 15 °C but not at 45 °C. Facultatively anaerobic and produces L(+)-lactic acid homofermentatively from glucose and starch. No gas produced from glucose. Acid is produced in the API 50 CH kits in tests with D-galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, aesculin, D-cellobiose, D-maltose, starch, glycogen and gentiobiose. No acid is produced from glycerol, erythritol, D- or L-arabinose, D-ribose, D- or L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, arbutin, D-lactose, D-melibiose, sucrose, D-trehalose, inulin, D-melezitose, D-raffinose, xylitol, D-turanose, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol, gluconate or 2- or 5-ketogluconate. Production of acid from amygdalin is strain-dependent. The DNA G+C content of the type strain is 43.5 mol%.

The type strain is LMG 11400^T (=NRRL B-4436^T = DSM 20534^T), which was isolated from swine waste-corn fermentation.

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