

Pediococcus lolii sp. nov., isolated from ryegrass silage

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A Gram-positive, coccus-shaped, lactic acid bacterium, strain NGRI 0510Q^T, was isolated from ryegrass silage produced in Okinawa Prefecture, Japan. The cell is non-spore-forming, non-motile, and occurs in pairs or tetrads. The strain is homofermentative and produces D- and L-lactic acid from glucose. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain NGRI 0510Q^T belongs to the genus *Pediococcus* and clusters within the *Pediococcus acidilactici* and *Pediococcus pentosaceus* group, with 98.2 and 96.9% sequence identity, respectively. DNA–DNA relatedness between strain NGRI 0510Q^T and *P. acidilactici* JCM 8797^T and *P. pentosaceus* JCM 5890^T was 19.3 and 17.3%, respectively. Based on its phenotypic characteristics, phylogenetic relationship and DNA–DNA relatedness, NGRI 0510Q^T (=JCM 15055^T=DSM 19927^T) represents the type strain of a novel species, for which the name *Pediococcus lolii* sp. nov. is proposed.

The members of the genus *Pediococcus* are typical lactic acid bacteria (LAB) in that they are Gram-positive, catalase-negative and oxidase-negative. At present, *Pediococcus* includes ten species: *Pediococcus acidilactici* (Tanasupawat *et al.*, 1993), *P. claussenii* (Dobson *et al.*, 2002), *P. cellicola*, *P. damnosus*, *P. dextrinicus*, *P. ethanolidurans*, *P. inopinatus*, *P. parvulus*, *P. pentosaceus* and *P. stilesii* (Franz *et al.*, 2006). Two other species, the former *P. halophilus* and *P. urinaeequi*, have been reclassified as *Tetragenococcus halophilus* and *Aerococcus urinaeequi*. The members of the genus *Pediococcus* are homofermentative and produce lactic acid, but no CO₂, from glucose and are not able to reduce nitrate. The uniformly spherical cells are never ovoid or elongated, and they differ from all other LAB by forming tetrads via alternate division in two perpendicular directions. Most of the species have been isolated from a large variety of plants and fruits, fermenting vegetables, beer and silage.

Silage production is an important technique for preserving plant materials with high nutritive value for use during the winter in cold and temperate regions and during the dry

season in tropical areas. It is based on natural fermentation whereby LAB ferment sugars to mainly lactic acid under anaerobic conditions. In the present study, we isolated and characterized a pediococcal strain from grass silage produced in Okinawa Prefecture, Japan. The strain exhibits distinct phenotypic characteristics, divergent sequences of the 16S rRNA gene and 16S–23S rRNA intergenic spacer region, and low rates of DNA–DNA hybridization. All of these characteristics distinguish this strain from presently known species in the genus *Pediococcus*.

Strain NGRI 0510Q^T was isolated from ryegrass silage produced on Ishigaki Island, Okinawa Prefecture, Japan. Isolation and purification was carried out with GYP agar (Tanaka *et al.*, 1994) for 5 days at 37 °C under aerobic conditions. Analogous cultivation conditions were used for subsequent experiments, unless indicated otherwise. Cell morphology was determined using scanning electron microscopy and phase-contrast microscopy. Bacterial growth was monitored by measuring the optical density at 660 nm using a Mini Photo 518R (TAITEC). D/L-Lactic acid and ethanol production and the glucose remaining in the medium were measured using HPLC according to the method of Tanaka & Ohmomo (1994). Sugar fermentation patterns were determined using an API 50 CHL kit (bioMérieux) over a period of 72 h. Preparation of cell wall and determination of peptidoglycan was carried out by the method described by Komagata & Suzuki (1987),

Abbreviations: ERIC-PCR, enterobacterial repetitive intergenic consensus PCR; LAB, lactic acid bacteria; RAPD-PCR, random amplified polymorphic DNA PCR.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NGRI 0510Q^T is AB362985.

except that TLC was performed on cellulose sheets. All tests used for biochemical characterization were carried out at least in triplicate.

Genomic DNA was extracted and purified as described by Marmur (1961) with some modifications. To determine the genomic G + C content, the purified DNA was digested using nuclease P1 (Yamasa), after which the G + C content was determined by HPLC. DNA–DNA hybridization was carried out according to the method of Ezaki *et al.* (1989). The sequence of the 16S rRNA gene from the isolated strain was scanned using data registered in the DDBJ, GenBank and EMBL databases using DNASIS ver. 3.5 (Hitachi Software Engineering). GENETIX-MAC ver. 10 (GENETYX) was used to generate multiple alignments and to construct a phylogenetic tree. Distance matrices for the aligned sequences were calculated using the two-parameter method. The neighbour-joining method was used to construct the phylogenetic tree (Saitou & Nei, 1987), and the robustness of individual branches was estimated by bootstrapping with 1000 replicates.

Random amplified polymorphic DNA (RAPD)-PCR and enterobacterial repetitive intergenic consensus (ERIC)-PCR were performed as we described previously (Fujino *et al.*, 2008). RAPD-PCR was carried out using the Arbitrary Primer Set and Gene Taq FP polymerase (Nippon Gene). The primers AP-A-02 (5'-TGGATTGGTC-3'), AP-A-22 (5'-GATCGCATTG-3'), AP-A-24 (5'-GATCATGGTC-3') and AP-A-25 (5'-GATCATAGCG-3') were used in this study. The primers used for ERIC-PCR were ERIC1R (5'-ATGTAAGCTCCTGGGGATTAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') (Dalla-Costa *et al.*, 1998).

Cells of strain NGRI 0510Q^T were cocci with a diameter of 0.8–1.0 µm. They occurred singly and in pairs or tetrads, the last being typical for pediococci (Simpson & Taguchi, 1995). Strain NGRI 0510Q^T could not grow at 48 °C but did grow at 15 °C. The optimum pH for growth was 6.0–6.8. Lactic acid [about 50 % (–)-D-/50 % (+)-L-] was produced from glucose by homofermentation, and the strain grew as a facultative anaerobe. No catalase activity was present. Phenotypic test results are summarized in Table 1. Growth at 48 °C and maltose fermentation are key for presumptive identification of *P. pentosaceus* and *P. acidilactici*, respectively (Holzapfel *et al.*, 2006); thus, NGRI 0510Q^T is distinguished from *P. pentosaceus* by its inability to produce acid from maltose, and from *P. acidilactici* by its inability to grow at 48 °C. Cells of strain NGRI 0510Q^T contain diaminopimelic acid in their peptidoglycan. The mean DNA G + C content of strain NGRI 0510Q^T was 41.0 mol%, which is within the range for genus *Pediococcus* (34–42 mol%).

The complete 16S rRNA gene sequence of strain NGRI 0510Q^T was compared with those of the most closely related species retrieved from the aforementioned databases. Construction of a phylogenetic tree rooted at *Bifidobacterium bifidum* DSM 20456^T revealed that strain

Table 1. Characteristics that differentiate strain NGRI 0510Q^T from related pediococci species

Taxa: 1, *P. lolii* sp. nov.; 2, *P. acidilactici*; 3, *P. pentosaceus*; 4, *P. stilesii*; 5, *P. claussenii*. Data for *Pediococcus* species were obtained from Tanasupawat *et al.* (1993), Dobson *et al.* (2002) and Franz *et al.* (2006). +, Positive; –, negative; w, weak reaction.

| Characteristic | 1 | 2 | 3 | 4 | 5 |
|--------------------------|------|------|------|------|--------|
| Acid from: | | | | | |
| L-Arabinose | + | + | + | – | – |
| Lactose | – | – | – | – | – |
| Galactose | + | + | + | + | – |
| Sucrose | – | – | + | – | – |
| D-Xylose | + | + | w | – | – |
| Rhamnose | + | – | – | – | – |
| Trehalose | – | + | + | – | + |
| Raffinose | – | – | w | – | – |
| Maltose | – | – | + | + | + |
| Melibiose | – | – | + | – | – |
| Growth at 48 °C | – | + | – | – | – |
| Lactate formed | DL | DL | DL | DL | L(+) |
| DNA G + C content (mol%) | 41.0 | 43.5 | 37.8 | 38.0 | 40.5 |

NGRI 0510Q^T clusters within the *P. acidilactici*–*P. pentosaceus*–*P. stilesii* group (Fig. 1). The similarities of the 16S rRNA gene sequence of strain NGRI 0510Q^T to those of *P. acidilactici* DSM 20284^T, *P. pentosaceus* DSM

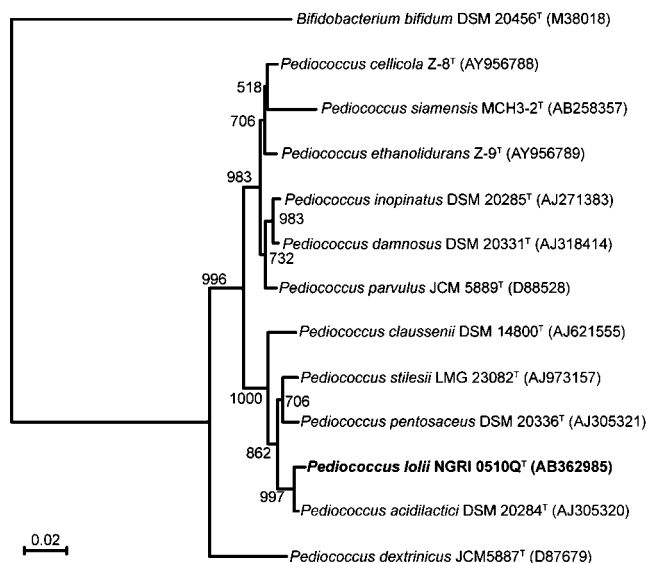


Fig. 1. Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences. The relationships between strain NGRI 0510Q^T and related species are shown. *Bifidobacterium bifidum* DSM 20456^T was used as an outgroup. Bootstrap values based upon 1000 replicates are included at the major branch points. Bar, 0.02 nucleotide substitutions per site.

20336^T and *P. stilesii* LMG 23082^T were 98.2, 96.9 and 96.3 %, respectively. Given the high degree of similarity between the 16S rRNA gene sequence of strain NGRI 0510Q^T and those of species of pediococci, the levels of DNA–DNA relatedness between the isolate and *P. acidilactici* DSM 20284^T and *P. pentosaceus* DSM 20336^T were determined and found to be 19.3 and 17.3 %, respectively, which is lower than the DNA–DNA relatedness threshold for a species (70 %) as recommended by Wayne *et al.* (1987).

All RAPD-PCR and ERIC-PCR fingerprints for strains NGRI 0510Q^T, *P. acidilactici* DSM 20284^T and *P. pentosaceus* DSM 20336^T were distinctly different (Fig. 2). In ERIC-PCR fingerprints, a few similarly sized fragments were observed between strain NGRI 0510Q^T and *P. acidilactici* DSM 20284^T, but obvious differentiation was seen in the RAPD-PCR pattern. Thus strain NGRI 0510Q^T could be separated from *P. acidilactici* DSM 20284^T and *P. pentosaceus* DSM 20336^T by genotyping.

Based on its phenotypic characteristics, phylogenetic relationship and DNA–DNA relatedness, a novel species of the genus *Pediococcus*, *Pediococcus lolii* sp. nov., is proposed to accommodate strain NGRI 0510Q^T.

Description of *Pediococcus lolii* sp. nov.

Pediococcus lolii (lo'li.i. L. gen. n. *lolii* of ryegrass).

Cells are Gram-positive, catalase-negative, non-motile and non-spore-forming. The spherical cells occur in pairs or tetrads, and are 0.8–1.0 µm in diameter after 24 h growth in GYP broth under aerobic conditions at 37 °C. Colonies are off-white, low-convex, circular, entirely opaque, and about 1–2 mm in diameter after 24 h of cultivation on GYP agar at 37 °C. The cells grow well in liquid or solid

GYP under facultatively anaerobic conditions. Growth occurs at 15–43 °C, with optimum growth occurring at 37 °C. Lactic acid [about 50 % (–)-D-/50 % (+)-L-] is produced from glucose by homofermentation. Lactic acid, but not gas, is produced from glucose fermentation. The optimum pH for growth is 6.0–6.8 (range, pH 4.0–8.0). Acid is produced from L-arabinose, ribose, D-xylose, galactose, glucose, fructose, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose and gentiobiose. The peptidoglycan structure is of the diaminopimelic acid type, and the DNA G+C content of the type strain is 41.0 mol%.

The type strain NGRI 0510Q^T (=JCM 15055^T=DSM 19927^T) was isolated from ryegrass silage produced on Ishigaki Island in Okinawa Prefecture, Japan.

Acknowledgements

We thank Dr J. P. Euzéby, Ecole Nationale Vétérinaire, Toulouse, France for his suggestion regarding the nomenclature for the novel species. This research was supported in part by Grants-in-Aid for Scientific Research (A) 07556094 of MEXT, Japan.

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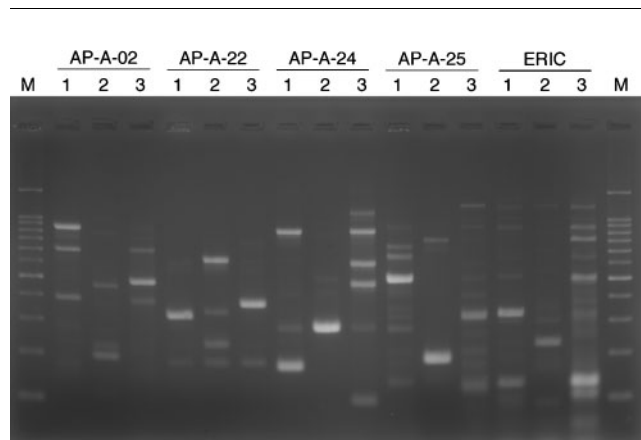


Fig. 2. RAPD-PCR and ERIC-PCR fingerprinting of strain NGRI 0510Q^T and type strains of related species. Primers AP-A-02, AP-A-22, AP-A-24 and AP-A-25 were used. Lanes: M, size markers (200 bp DNA ladder; TOYOBO); 1, NGRI 0510Q^T; 2, *P. pentosaceus* DSM 20336^T; 3, *P. acidilactici* DSM 20284^T.

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