# *Lactobacillus paracollinoides* sp. nov., isolated from brewery environments

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Three novel strains isolated from brewery environments are described. These strains were Gram-positive, facultatively anaerobic, heterofermentative rods that did not exhibit catalase activity. Phylogenetic analysis based on 16S rRNA gene sequence similarity showed that these strains belong to the genus *Lactobacillus* and are most closely related to *Lactobacillus collinoides* (approximately 99 % similarity). The novel strains could be differentiated from *L. collinoides* on the basis of DNA–DNA relatedness, differences in beer-spoilage ability and the inability to utilize D-fructose. These isolates represent a novel species, for which the name *Lactobacillus paracollinoides* sp. nov. is proposed. The type strain is LA2$^T$ (=DSM 15502$^T$=JCM 11969$^T$).

Although the majority of bacteria are incapable of growing in beer, a limited number of species of lactobacilli exhibits strong beer-spoilage ability (Back, 1981). *Lactobacillus brevis* is known to be the most prevalent beer-spoilage species (Back *et al.*, 1988; Back, 1994a). Three brewery isolates, LA2$^T$, LA3 and LA4, that possess strong beer-spoilage ability have been reported previously (Funahashi *et al.*, 1998). Since these three strains showed identical ribotypes and morphological features, the isolates were indistinguishable at the strain level. Coupled with the fact that these three strains were isolated from one brewery, they may well be considered to be identical. The representative strain, LA2$^T$, did not show sufficient DNA–DNA relatedness to be classified as any of the validly published *Lactobacillus* species, although it was most closely related to *Lactobacillus collinoides* JCM 1123$^T$ on the basis of 16S rRNA gene sequence comparisons (Funahashi *et al.*, 1998). *L. collinoides* strains are not generally considered to be beer-spoilage bacteria (Back, 1994b; Carr & Davies, 1972, 1974), but LA2$^T$ was able to grow in beer.

Recently, strains LA7 and LA8 have been isolated from different breweries in Japan. These strains also exhibited strong beer-spoilage ability. 16S rRNA gene sequence analysis indicated that these strains are potentially related to *L. collinoides* or *Lactobacillus* sp. LA2$^T$ at the species level. These findings led us to characterize these novel beer-spoilage strains and to investigate their taxonomic relationship with *L. collinoides* and *Lactobacillus* sp. LA2$^T$. Based on these results, a novel species, *Lactobacillus paracollinoides* sp. nov., is described.

*Lactobacillus* strains used in this study were grown in MRS broth (Merck) at 25 °C under anaerobic conditions. Carbohydrate fermentation profiles were determined using the API 50CH system (bioMérieux). API tests were performed in accordance with the manufacturer's instructions. Each strain was examined for morphological features, motility and Gram staining by microscopy. Hydrogen peroxide (3 %) was used to test for catalase activity. Gas production from glucose was examined using Durham tubes. An F-kit DL-lactic acid (Boehringer Mannheim) was used to determine production of D- and L-lactic acids. Beer-spoilage ability was determined by inoculating degassed commercial beers (pH 4·2) with each strain at $3 \times 10^3$ cells ml$^{-1}$. The inoculated beers were incubated anaerobically at 25 °C and examined regularly for visible growth for up to 90 days (Suzuki *et al.*, 2002).

The ribotype of each strain was obtained using a RiboPrinter (Qualicon) in accordance with the manufacturer's instructions, with *Eco*RI as a restriction enzyme (Bruce *et al.*, 1995; Hubner *et al.*, 1995; Olsen *et al.*, 1991). G+C contents were determined by HPLC as described by Mesbah *et al.* (1989). Experimental procedures for 16S rRNA gene analysis were described previously (Funahashi *et al.*, 1998). Sequences were edited with the DNASIS PRO software package (Hitachi Software Engineering). The CLUSTAL W algorithm (Thompson *et al.*, 1994) provided in DNASIS PRO was used to align sequences and to construct a neighbour-joining tree with 1000 bootstrap iterations. A DNA–DNA hybridization study was carried out as described by De Ley *et al.* (1970) with some modifications (Escara & Hutton, 1980; Huß *et al.*, 1983). A model 2600

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The DDBJ accession number for the 16S rRNA gene sequence of strain LA2$^T$ is E16651.

spectrophotometer equipped with a model 2527-R thermoprogrammer and plotter (Gilford Instrument Laboratories) was used for determining DNA–DNA relatedness. Renaturation rates were computed with the program TRANSFER.BAS (Jahnke, 1992).

Strains LA2$^T$, LA7 and LA8 were Gram-positive rods that exhibited no catalase activity. When grown on MRS agar, their colonies were small and non-pigmented. Growth was observed at 15 °C but not at 45 °C in MRS broth. All strains produced predominantly D-lactic acid from glucose with a smaller amount of L-lactic acid; the proportion of D-lactic acid ranged between 66·5 and 79·1 %, depending on the strain. Gas production was observed for each strain. Compared with *L. collinoides* JCM 1123$^T$, the major difference in carbohydrate utilization profiles was the inability of the brewery isolates to ferment D-fructose. The ability to utilize L-arabinose was variable. Except for these differences, carbohydrate utilization by the isolates was identical to that observed in *L. collinoides* JCM 1123$^T$. The DNA G+C content of strain LA2$^T$ was 44·8 mol%, which is within the range for the genus *Lactobacillus* (32–53 mol%) (Kandler & Weiss, 1986).

The beer-spoilage ability of the strains was compared with that of three *L. collinoides* strains, JCM 1123$^T$, ATCC 27610 and ATCC 27611. Strains LA2$^T$, LA7 and LA8 exhibited strong beer-spoilage ability and formed visual turbidity in beer within 7 days. This degree of beer-spoilage ability is comparable with that of the most serious beer-spoilage bacterium, *Lactobacillus brevis* (Suzuki *et al.*, 2002). In contrast, none of the three *L. collinoides* strains tested in this study was able to grow in beer, even after 90 days of observation. Thus, strains LA2$^T$, LA7 and LA8 are distinguishable from *L. collinoides* in terms of beer-spoilage ability.

The 16S rRNA gene sequences of LA7 and LA8 showed approximately 99 % similarity to those of *L. collinoides* JCM 1123$^T$ (DDBJ accession number AB005893) and *Lactobacillus* sp. LA2$^T$ (E16651), suggesting that these four strains are closely related. DNA–DNA hybridization data showed that LA7 and LA8 showed 86·8 and 70·7 % relatedness, respectively, to LA2$^T$. In contrast, DNA similarity between the three brewery isolates and *L. collinoides* JCM 1123$^T$ was relatively low (46·8–57·6 %). These results, together with differences in beer-spoilage ability and the ability to utilize D-fructose, show that the three brewery isolates are most likely to be related at the species level and should be regarded as a species distinct from *L. collinoides*. Ribotyping of LA7 and LA8 yielded ribopatterns that were distinct from that of LA2$^T$, indicating these three brewery isolates are distinguishable at the strain level. A phylogenetic tree showing the relationship of LA2$^T$ with other *Lactobacillus* species is shown in Fig. 1.

Taken collectively, these results allowed us to assign the brewery isolates described in the present study to a novel species, for which the name *Lactobacillus paracollinoides* sp. nov. is proposed.

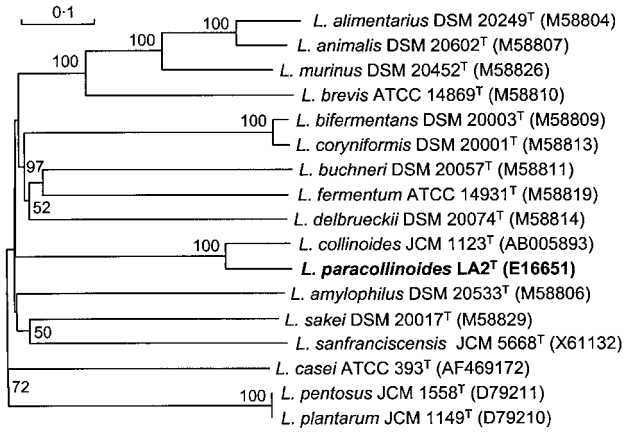


**Fig. 1.** Phylogenetic tree of *Lactobacillus paracollinoides* LA2$^T$ and other *Lactobacillus* strains derived from 16S rRNA gene sequence data (accession numbers given in parentheses), constructed using the neighbour-joining method. Percentages at nodes were obtained from 1000 bootstrap replications. Bar, 0·1 inferred substitutions per 100 nt.

## Description of *Lactobacillus paracollinoides* sp. nov.

*Lactobacillus paracollinoides* (pa.ra.col.li.noi′des. Gr. pref. *para* beside; N.L. masc. adj. *collinoides* hill-shaped, referring to the colony form of *Lactobacillus collinoides*; N.L. masc. adj. *paracollinoides* beside *collinoides*, referring to the close relationship to *L. collinoides*).

Cells are Gram-positive, non-motile, non-spore-forming rods, occurring singly or in short chains. Facultatively anaerobic, catalase-negative and heterofermentative. All strains so far isolated grow at 15 °C, but not at 45 °C. A predominant amount of D-lactic acid, with a smaller amount of L-lactic acid, is produced from glucose. Acid is produced from ribose, D-xylose, D-glucose, maltose and melibiose. Acid production from L-arabinose is variable. No acid is produced from glycerol, erythritol, D-arabinose, L-xylose, adonitol, methyl β-xyloside, D-fructose, D-mannose, D-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, *N*-acetyl-β-glucosamine, amygdalin, arbutin, salicin, cellobiose, lactose, sucrose, trehalose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol, gluconate or 2- or 5-ketogluconate.

The DNA G+C content of the type strain, strain LA2$^T$ (=DSM 15502$^T$=JCM 11969$^T$), is 44·8 mol%. Isolated from brewery environments. All the strains presently isolated exhibit strong beer-spoilage ability.

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