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Lactobacillus apinorum belongs to the fructophilic lactic acid bacteria

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Potential fructophilic characteristics of *Lactobacillus apinorum*, originally isolated from the guts of honeybees (*Apis mellifera*), were studied in the present study. The species showed typical fructophilic growth characteristics, i.e., active growth on D-fructose, poor growth on D-glucose, and accelerated growth on D-glucose in the presence of electron acceptors. Biochemical characteristics strongly supported classification of the species into fructophilic lactic acid bacteria (FLAB). Furthermore, genetic analyses suggested that the species underwent extensive gene reduction, similar to that recorded for *Lactobacillus kunkeei* and other FLAB. These data clearly indicated that *L. apinorum* is the second fructophilic species within the genus *Lactobacillus.*

Key words: fructophilic lactic acid bacteria, *Lactobacillus apinorum*, honeybees, genomics, reductive evolution

Fructophilic lactic acid bacteria (FLAB) differ from other lactic acid bacteria (LAB) in that they prefer D-fructose over D-glucose as a growth substrate [1]. Growth on D-glucose is usually poor but is dramatically enhanced in the presence of external electron acceptors such as pyruvate, oxygen and D-fructose [1, 2]. It is thus not unusual to isolate FLAB from fructose-rich environments, including flowers, fruits and the guts of fructose-feeding insects [1, 3]. The unique characteristics in FLAB were suggested to be obtained by a reductive evolution that took place to adapt to specific niches [4, 5]. All five species of the genus *Fructobacillus* are representatives of this unique microbial group [2, 6]. Until now, *Lactobacillus kunkeei* has been the only obligate FLAB species reported in the genus *Lactobacillus* [1, 5].

Lactobacillus apinorum was originally isolated from the honey stomach of *Apis mellifera* [7]. The species is phylogenetically closely related to *L. kunkeei*, as evident from the 98.9% 16S rRNA gene sequence similarity they share. *L. apinorum* produces acids only from the fermentation of D-glucose and D-fructose [7]. In our previous study [5], we revealed that *L. apinorum* lacks the bifunctional alcohol/acetaldehyde dehydrogenase gene (*adhE*) essential in heterolactic fermentation. These characteristics implied that the species would be a member of FLAB. However, fructophilic characteristics of the species have not been studied until now. In the present study, biochemical and genomic characteristics of *L. apinorum* were studied, focusing on its possible fructophilic characteristics.

L. apinorum JCM 30765^T was included in the present study. The strain was routinely cultured in FYP broth at 30°C. FYP broth is composed of 10 g/l D-fructose, 10 g/l yeast extract, 5 g/l polypeptone, 2 g/l sodium acetate, 0.5 g/l Tween80, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 0.01 g/l $MnSO_4 \cdot 4H_2O$, 0.01 g/l $FeSO_4 \cdot 7H_2O$, 0.01 g/l NaCl at pH 6.8.

Growth characteristics of the strain were determined in FYP broth, GYP broth, GYP broth supplemented with 0.5% pyruvate and GYP broth under aerobic conditions on an orbital shaker (120 rpm). GYP broth differed from FYP broth in that it contained 10 g/l of D-glucose instead of D-fructose. *L. apinorum* JCM 30765^T grew poorly on D-glucose but grew well on D-fructose (Fig. 1). Supplementation of GYP with pyruvate or aerobic culturing dramatically enhanced the growth of strain JCM 30765^T. The strain produced large quantities of lactate and acetate (17.1 mM and 10.0 mM, respectively) and low levels of ethanol (less than 0.1 mM) from metabolism of D-glucose under static conditions. Metabolic properties of carbohydrates in the strain were recorded using API CHL galleries (BioMérieux, Marcy L'Etoile, France), according to the manufacturer's instructions. Readings were taken for 7 days at 30°C. Strain JCM 30765^T metabolized D-fructose within 24 hr but took 3–4 days for metabolism of D-glucose. These characteristics are identical to those recorded for *L. kunkeei* and *Fructobacillus* spp. [1]

Recent studies have suggested that enzyme activities of alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) are key factors for requirement of electron acceptors when D-glucose is metabolized by FLAB [8]. The activities of both enzymes were thus determined. NADH oxidase activity, which is essential for the oxidation

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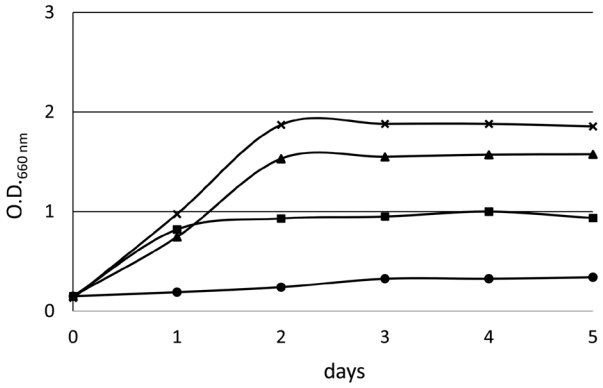

Fig. 1. Growth characteristics of *Lactobacillus apinorum* JCM 30765^T on glucose (circle), on fructose (square), on glucose in the presence of pyruvate (triangle) and on glucose under aerobic conditions (cross).

of NADH to NAD$^+$ when LAB are grown in the presence of oxygen, was also determined. Culturing, preparation of cell-free extracts and enzyme assays were performed as described previously [5], but with a slight modification. The cells were disrupted in a test tube containing 0.3 g sterile glass beads (0.1 mm in diameter). Beating of the cells was performed using a Mini Beadbeater-1 (model 3110BX, BioSpec Products, Bartlesville, OK, USA). The following method was used: Five cycles of beating for 30 sec each at 4,800 rpm, with 30 sec intervals on ice. Cells debris was harvested (9,000 X *g*, 1 min, 4°C), and the cell-free supernatant was collected for enzyme activity tests. Strong NADH oxidase activity (2,982 mU/mg protein) was recorded, but no ADH or ALDH activity was recorded. These results corresponded with those recorded for *Fructobacillus* species [5, 8]. This agreed well with our previous finding that *L. apinorum* JCM 30765^T lacks the *adhE* gene encoding AdhE protein with bifunctional alcohol/acetaldehyde dehydrogenase activities [5].

Genomic data of *L. apinorum* Fhon13^T was obtained from the newly published genome database for lactobacilli, DAGA (DFAST Archive of Genome Annotation, https://dfast.nig.ac.jp) [9]. The genes of the strain were used for Cluster of Orthologous Groups functional classification and for prediction of metabolic pathways by using the KEGG Automatic Annotation Server as described previously [5]. *L. kunkeei* YH-15^T, type strains of the five *Fructobacillus* spp. and a group of lactobacilli possessing small genomes (<2.06 Mbp, n=28) designed in a previous study [5] were included as controls. *L. apinorum* possessed 1.46 Mbp genomes containing 1,315 CDSs. These values are similar to those of previously studied FLAB [4, 5] but smaller than those of a group of lactobacilli possessing small genomes (average ± SD:1.74 ± 0.24 Mbp and 1,682 ± 230). The functional gene content profiles indicated that *L. apinorum* possesses genomic characteristics similar to those recorded for *L. kunkeei* and *Fructobacillus* spp., especially regarding the small ratio of genes involved in carbohydrate transport and metabolism (class G). The ratio of gene contents in the class G was 3.5% for *L. apinorum* (Fig. 2) and 6.2% for small genome

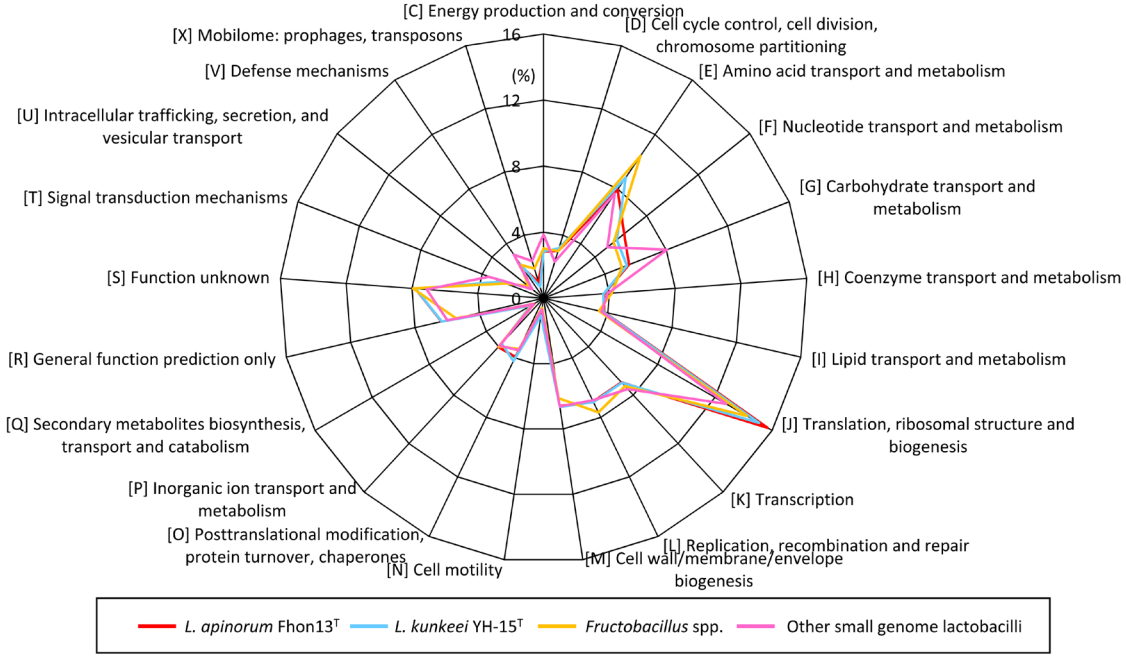

Fig. 2. Comparison of the ratio of gene content profiles obtained for *L. apinorum* Fhon13^T, *L. kunkeei* YH-15^T, *Fructobacillus* spp. and other small genome lactobacilli (<2.06 Mbp).

Data for the small genome lactobacilli (n=28) were obtained from Maeno *et al.* [5].

Table 1. Number of genes used for metabolic pathways in *L. apinorum*, *L. kunkeei, Fructobacillus* spp. and other small genome lactobacilli

Pathway definition	*L. apinorum* Fhon13^T	*L. kunkeei* YH-15^T	*Fructobacillus* spp.	Small genome lactobacilli[a]
Citrate cycle (TCA cycle) (map00020)	1	2	0 (0)	3.5 (2.0)
Fructose and mannose metabolism (map00051)	2	2	2.8 (0.8)	10.3 (6.0)
Galactose metabolism (map00052)	5	5	5.8 (0.8)	11.7 (4.2)
Fatty acid biosynthesis (map00061)	11	11	11.0 (0)	7.7 (4.6)
Ubiquinone and other terpenoid-quinone biosynthesis (map00130)	2	1	1.0 (0)	2.0 (1.9)
Oxidative phosphorylation (map00190)	10	11	9.2 (0.4)	11.7 (1.4)
Purine metabolism (map00230)	45	46	42.0 (5.5)	41.2 (5.2)
Tetracycline biosynthesis (map00253)	4	4	4.0 (0)	2.5 (2.0)
Lysine biosynthesis (map00300)	4	1	12.2 (0.4)	9.4 (4.2)
Amino sugar and nucleotide sugar metabolism (map00520)	13	13	11.2 (0.4)	19.6 (4.6)
Pyruvate metabolism (map00620)	14	16	12.0 (1.0)	13.6 (3.9)
Biotin metabolism (map00780)	5	5	4.0 (0)	3.4 (2.0)
2-Oxocarboxylic acid metabolism (map01210)	1	7	8.4 (4.6)	2.7 (2.5)
Fatty acid metabolism (map01212)	11	11	10.0 (0)	7.7 (4.6)
Biosynthesis of amino acids (map01230)	22	39	61.4 (14.2)	35.4 (10.7)
ABC transporters (map02010)	30	26	33.8 (3.1)	34.4 (6.9)
Two-component system (map02020)	13	12	19.4 (1.1)	20.4 (5.5)
Phosphotransferase system (PTS) (map02060)	0	0	0.4 (0.5)	11.3 (8.9)

[a]The values for *Fructobacillus* spp. and small genome lactobacilli indicate means and standard deviations (in parenthesis) of the number of genes used for the pathways.
Data for the small genome lactobacilli (n=28) were obtained from Maeno *et al.* [5].

lactobacilli [5]. Lack of the phosphotransferase system (PTS) and ubiquinone and other terpenoid-quinone biosynthesis in the strain (Table 1) was similar to that recorded for FLAB but different from that of other lactobacilli [5].

Based on the data provided, *L. apinorum* JCM 30765^T possesses biochemical and genomic characteristics similar to known FLAB and distinct from other lactobacilli. Fructophilic characteristics are well conserved in its closest relative, *L. kunkeei* [5, 10]. This would imply that fructophilic characteristics are shared in *L. apinorum,* although only the type strain was assessed in the present study. Indeed, *L. apinorum* Kvahm3N, which was used for the proposal of the species description as the type strain, possessed the same poor carbohydrate metabolic properties with the type strain [7]. *L. apinorum* would be thus the second obligate FLAB species in the genus *Lactobacillus*. The species shares the same habitats as *L. kunkeei* and *Fructobacillus*, and fructose-rich environments would thus lead fructophilic evolution to inhabitant LAB as suggested in a previous study [5].

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