Fructobacillus tropaeoli sp. nov., a fructophilic lactic acid bacterium isolated from a flower

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A fructophilic lactic acid bacterium, designated strain F214-1^T, was isolated from a flower of *Tropaeolum majus* in South Africa. Based on phylogenetic analysis of 16S rRNA gene sequences, the strain formed a subcluster with *Fructobacillus ficulneus* and *Fructobacillus pseudoficulneus* and, based on *recA* gene sequences, the strain formed a subcluster with *F. ficulneus*. DNA–DNA hybridization studies showed that strain F214-1^T was phylogenetically distinct from its closest relatives. Acid was produced from the fermentation of D-glucose, D-fructose and D-mannitol only. D-Fructose was the preferred sole carbon and energy source and was fermented more rapidly than D-glucose. Growth of the strain on D-glucose under anaerobic conditions was very weak but external electron acceptors such as oxygen and pyruvate enhanced growth on D-glucose. Lactic acid and acetic acid were produced from D-glucose in equimolar amounts. Ethanol was produced at very low levels, despite the strain's obligately heterofermentative metabolism. Based on these data, strain F214-1^T represents a novel species of fructophilic bacteria in the genus *Fructobacillus*, for which the name *Fructobacillus tropaeoli* sp. nov. is proposed. The type strain is F214-1^T (=JCM 16675^T =DSM 23246^T).

At the time of writing, the genus *Fructobacillus* comprised four species: *Fructobacillus fructosus*, *Fructobacillus durionis*, *Fructobacillus ficulneus* and *Fructobacillus pseudoficulneus* (Endo & Okada, 2008). These four species were formerly classified as species of the genus *Leuconostoc* (Antunes *et al.*, 2002; Chambel *et al.*, 2006; Leisner *et al.*, 2005) but were reclassified by Endo & Okada (2008) as members of a novel genus, *Fructobacillus*, based on their phylogenetic positions and their morphological and biochemical characteristics. Species of the genus *Fructobacillus* typically show a preference for D-fructose over D-glucose as a growth substrate and, as such, are classified as fructophilic lactic acid bacteria (Endo & Okada, 2008). Two distinct groups of fructophilic lactic acid bacteria have been reported; obligate fructophiles and facultative fructophiles (Endo *et al.*, 2009). The four species of the genus *Fructobacillus*,

as well as *Lactobacillus kunkeei*, are regarded as obligately fructophilic and the novel species *Lactobacillus florum* is regarded as facultatively fructophilic (Endo *et al.*, 2009, 2010). The two groups differ in their ability to grow on D-glucose without electron acceptors and to produce ethanol from D-glucose (Endo *et al.*, 2009, 2010).

During the course of our studies on fructophilic lactic acid bacteria, one strain, designated F214-1^T, was isolated from a flower in South Africa. The strain showed biochemical characteristics typical of obligately fructophilic lactic acid bacteria and, based on 16S rRNA gene sequence analyses, formed a phylogenetic subcluster with *F. ficulneus* and *F. pseudoficulneus* in the *Fructobacillus* cluster. Based on DNA–DNA relatedness values, strain F214-1^T was genetically distinct from these close relatives. Here, we describe the taxonomic characterization of strain F214-1^T and propose that it represents a novel species of the genus *Fructobacillus*.

Strain F214-1^T was isolated from a nasturtium flower (*Tropaeolum majus*) in Stellenbosch, Western Cape, South Africa, in June 2009. Fresh flowers were collected and

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *recA* gene sequences of strain F214-1^T are AB542054 and AB542055, respectively.

Four supplementary figures and one supplementary table are available with the online version of this paper.

crushed in a sterile plastic bag. A 1 ml aliquot of FYP broth, composed of (g l⁻¹) D-fructose (10), yeast extract (10), polypeptone (5), sodium acetate (2), Tween 80 (0.5), MgSO₄·7H₂O (0.2), MnSO₄·4H₂O (0.01), FeSO₄·7H₂O (0.01), NaCl (0.01), cycloheximide (0.05) and sodium azide (0.05; pH 6.8), was added to the crushed samples and the bags were incubated at 30 °C for 24 h. After incubation, each sample was inoculated into FYP broth by using a loop and incubated at 30 °C under aerobic conditions on an orbital shaker (120 r.p.m.) for 2 days. The culture was then streaked onto FYP agar plates containing (l⁻¹) 5 g CaCO₃ and 12 g agar. The plates were incubated at 30 °C under aerobic conditions until visible colonies were observed. Colonies were selected based on differences in morphology, such as colony size and shape, and the clearance zone formed from hydrolysis of CaCO₃ by lactic acid, inoculated into FYP broth and GYP broth and incubated statically for 24 h at 30 °C. GYP broth differed from FYP broth by containing 10 g l⁻¹ D-glucose instead of D-fructose.

Strain F214-1^T grew well in FYP broth but poorly in GYP broth and was, therefore, regarded as a fructophilic lactic acid bacterium. The strain was stored at -80 °C in nutrient broth containing 20 % (v/v) glycerol. Strains *F. durionis* LMG 22556^T, *F. ficulneus* JCM 12225^T, *F. fructosus* NRIC 1058^T and *F. pseudoficulneus* DSM 14568^T were cultured in FYP broth and used as reference strains.

The 16S rRNA gene sequence of strain F214-1^T was determined as described previously (Endo & Okada, 2005). The closest recognized relatives of the isolate were determined by performing database searches and sequences of closely related species were retrieved from DDBJ. Multiple sequence alignments were carried out by using CLUSTAL_X, version 1.18 (Thompson *et al.*, 1997). Distance matrices for the aligned sequences were calculated by using the two-parameter method of Kimura (1980) and phylogenetic trees were reconstructed by using the neighbour-joining, maximum-likelihood and maximum-parsimony methods in PHYLIP version 3.65 software as described previously (Endo & Okada, 2006). 16S rRNA gene sequences (~1450 bp) of the isolate and related species of lactic acid bacteria were used to reconstruct phylogenetic trees. The highest sequence similarities to strain F214-1^T were 99.2 and 98.0 % to *F. pseudoficulneus* and *F. ficulneus*, respectively. In the tree reconstructed using the neighbour-joining method, the strain formed a subcluster with *F. pseudoficulneus* and *F. ficulneus* within the *Fructobacillus* phylogenetic cluster (Fig. 1). An identical tree topology was obtained when using the maximum-likelihood and maximum-parsimony methods (see Supplementary Figs S1 and S2, available in IJSEM Online).

As an additional genetic marker, the *recA* gene of strain F214-1^T was sequenced and used for phylogenetic analysis. The *recA* gene encodes a recombinase A protein and using this sequence in phylogenetic analyses gives greater resolution than the 16S rRNA gene sequence when classifying members of the genera *Leuconostoc* and *Fructobacillus*

(Ehrmann *et al.*, 2009; Endo & Okada, 2008). Amplification, purification and partial sequencing of the *recA* gene were performed as described previously (Endo & Okada, 2008). Phylogenetic analysis was conducted as described above and a phylogenetic tree was reconstructed by the neighbour-joining method using ~500 bp *recA* gene sequences of the isolate and related species of lactic acid bacteria. *Lactococcus lactis* IL1403 was used as an outgroup. In the phylogenetic tree, strain F214-1^T formed a subcluster with *F. ficulneus* within the *Fructobacillus* cluster (Fig. 2). Sequence similarities based on the *recA* gene of strain F214-1^T and related species of the genus *Fructobacillus* ranged from 83.0 to 85.6 %. Other studies have shown that *recA* gene sequence similarities were >99 % between different strains of *Lactobacillus plantarum* but <87 % between different species of the genus *Lactobacillus* (Torriani *et al.*, 2001), supporting the assumption that strain F214-1^T represents a distinct species and not a separate strain.

DNA–DNA relatedness between strain F214-1^T and reference strains *F. durionis* LMG 22556^T, *F. fructosus* NRIC 1058^T, *F. ficulneus* JCM 12225^T and *F. pseudoficulneus* DSM 15468^T and the DNA G + C content of the isolate were determined as described by Endo & Okada (2006). Extraction and purification of bacterial DNA were performed according to the methods of Marmur (1961) with modifications (Ezaki *et al.*, 1983). Strain F214-1^T showed DNA–DNA relatedness values of 11–19 % with the reference strains (Supplementary Table S1) and had a G + C content of 44 mol%.

Morphological, physiological and biochemical characteristics of strain F214-1^T were determined as described previously (Endo *et al.*, 2009). FYP broth was used as basal medium. Acid production from carbohydrates and enzyme activities were determined for strain F214-1^T and the four reference strains using API 50 CH and API ZYM strips (bioMérieux). Detailed characteristics of strain F214-1^T and reference strains of the genus *Fructobacillus* are given in the species description and in Table 1. Of the 49 carbohydrates tested, strain F214-1^T only produced acid from D-fructose, D-glucose and D-mannitol and metabolized D-fructose faster than D-glucose. These results and those from enzymic assays were very similar between strain F214-1^T and other members of the genus *Fructobacillus* (Table 1). These shared physiological and biochemical properties make classification based on these characteristics difficult. On the other hand, sequence analysis of housekeeping genes, such as *recA*, can be a powerful tool for classifying species of the genus *Fructobacillus*.

Growth characteristics of strain F214-1^T were determined as described previously (Endo *et al.*, 2009). Broth containing D-glucose, D-fructose or D-glucose with 1 % pyruvate was used in this study. To determine growth enhancement in the presence of O₂, cells of strain F214-1^T were grown in broth containing D-glucose under aerobic (120 r.p.m. by orbital shaker) and static conditions. Growth on GYP agar under aerobic and anaerobic (Anaerobic system BR0038B; Oxoid) conditions was also determined. Presence of end products from the fermentation of D-glucose was determined as

Fig. 1. Phylogenetic relationship of strain F214-1^T to related species of lactic acid bacteria based on 16S rRNA gene sequences. The tree was reconstructed by using the neighbour-joining method. *Bacillus subtilis* NCDO 1769^T was used as an outgroup. Bootstrap percentages >70% are given at branching points. Bar, 0.01 substitutions per nucleotide position.

described previously (Endo *et al.*, 2009). Strain F214-1^T grew very poorly on D-glucose but grew well on D-fructose (Supplementary Fig. S3). However, the novel strain grew very well on D-glucose in the presence of 1% pyruvate and under aerobic (shaking) conditions (Supplementary Fig. S3), which suggests that it requires external electron acceptor(s) for the metabolism of D-glucose. Culturing under aerobic conditions enhanced the growth of the strain on agar medium (Supplementary Fig. S4). Colonies grown on GYP agar for 2 days under aerobic conditions were ~1–2 mm in diameter but under anaerobic conditions were only ~0.1–0.2 mm in diameter (Anaerobic system BR0038B; Oxoid). Strain F214-1^T produced gas from D-glucose but produced almost equimolar amounts of lactic acid and acetic acid and very little ethanol (molar ratio of 1:1:0.005, respectively). These characteristics were unique to strain F214-1^T when compared

with the characteristics of species of the obligately heterofermentative group of lactic acid bacteria but correlated well with those of the obligately fructophilic group of lactic acid bacteria, which includes species of the genus *Fructobacillus* (Endo & Okada, 2008; Endo *et al.*, 2009).

Based on these data, strain F214-1^T represents a novel species of obligately fructophilic lactic acid bacteria in the genus *Fructobacillus*, for which the name *Fructobacillus tropaeoli* sp. nov. is proposed. The type strain is F214-1^T (=JCM 16675^T =DSM 23246^T).

Description of *Fructobacillus tropaeoli* sp. nov.

Fructobacillus tropaeoli (tro.pae.o'li. N.L. n. *Tropaeolum* a botanical generic name; N.L. gen. n. *tropaeoli* of *Tropaeolum*, isolated from *Tropaeolum majus*).

Fig. 2. Phylogenetic relationship of strain F214-1^T to related species of lactic acid bacteria based on *recA* gene sequences. The tree was reconstructed by using the neighbour-joining method. *Lactococcus lactis* IL1403 was used as an outgroup. Bootstrap percentages >70% are given at branching points. Bar, 0.1 substitutions per nucleotide position.

Table 1. Differential characteristics of strain F214-1^T and related members of the genus *Fructobacillus*

Strains: 1, strain F214-1^T; 2, *F. durionis* LMG 22556^T; 3, *F. ficulneus* JCM 12225^T; 4, *F. fructosus* NRIC 1058^T; 5, *F. pseudoficulneus* DSM 15468^T. All results from this study. All strains were positive for acid phosphatase and phosphoamidase activities and negative for caprylate esterase (C8), leucine, valine and cysteine aminopeptidase, β -galactosidase, β -glucuronidase, α - and β -glucosidase, β -glucosaminidase, α -mannosidase and α -fucosidase activities. +, Positive; –, negative; w, weakly positive. For acid production, the number of days required for fermentation are indicated in parentheses.

Characteristic	1	2	3	4	5
Acid production from:					
D-Fructose	+ (1)	+ (1)	+ (1)	+ (1)	+ (1)
D-Glucose	+ (2)	+ (3)	+ (4)	+ (4)	+ (3)
D-Mannitol	+ (5)	+ (5)	+ (6)	+ (6)	+ (6)
Sucrose	–	+ (3)	w	–	–
Turanose	–	+ (4)	w	–	–
D-Ribose	–	w	–	–	–
Gluconate	–	w	w	–	–
Methyl α -D-glucopyranoside	–	+ (6)	w	–	–
Maltose	–	+ (7)	w	–	–
Trehalose	–	+ (6)	+ (5)	–	–
Enzyme activities (API ZYM)					
Alkaline phosphatase	+	w	+	w	w
Butyrate esterase (C4)	–	–	w	–	w
Myristate lipase (C14)	–	–	–	–	w
Trypsin	–	w	w	–	w
Chymotrypsin	+	+	+	w	w
α -Galactosidase	–	w	w	–	w

Cells are Gram-reaction-positive, non-motile rods, 0.8 $\times$ 1.5–6 μ m. Cells occur singly, in pairs or in chains. Facultatively anaerobic and catalase-negative. Obligately fructophilic lactic acid bacteria. Growth on D-glucose is enhanced with external electron acceptors, such as pyruvate and oxygen. Growth on D-glucose is suppressed under anaerobic conditions. After incubation for 2 days on GYP agar, colonies are white, smooth and ~1–2 mm in diameter under aerobic conditions but only ~0.1–0.2 mm in diameter under anaerobic conditions. Heterofermentative and produces lactic and acetic acid from D-glucose at a ratio of 1:1 along with very small amounts of ethanol. Gas is produced from D-glucose, D- and L-lactate are produced at a ratio of 9:1 and mannitol is produced from D-fructose. Nitrate is not reduced. Acid is only produced from D-fructose, D-glucose and mannitol. D-Fructose is fermented faster than D-glucose. Acid is not produced from D- or L-arabinose, D- or L-arabitol, N-acetylglucosamine, maltose, ribose, adonitol, amygdalin, arbutin, cellobiose, dulcitol, aesculin, erythritol, D- or L-fucose, β -gentiobiose, 2- or 5-ketogluconate, potassium gluconate, methyl α -D-glucoside, glycerol, glycogen, myo-inositol, inulin, D-lyxose, D-mannose, methyl α -D-mannoside, melezitose,

raffinose, rhamnose, sucrose, salicin, starch, sorbitol, L-sorbose, D-tagatose, trehalose, turanose, xylitol, L-xylose, methyl β -xyloside, D-galactose, lactose, melibiose or D-xylose. Dextran is not produced from sucrose. Grows at 15 °C and weakly at 10 °C but not at 45 °C. Grows at pH 4–8 and in the presence of 2.5 % (w/v) NaCl. Does not grow in the presence of 300 g l^{–1} D-fructose. Cells do not contain meso-diaminopimelic acid in their peptidoglycan.

The type strain, F214-1^T (=JCM 16675^T =DSM 23246^T), was isolated from a nasturtium flower collected at Stellenbosch, Western Cape, South Africa, in 2009. The DNA G+C content of the type strain is 44 mol%.

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References

- Antunes, A., Rainey, F. A., Nobre, M. F., Schumann, P., Ferreira, A. M., Ramos, A., Santos, H. & da, C. M. (2002). *Leuconostoc ficulneum* sp. nov., a novel lactic acid bacterium isolated from a ripe fig, and reclassification of *Lactobacillus fructosus* as *Leuconostoc fructosum* comb. nov. *Int J Syst Evol Microbiol* **52**, 647–655.
- Chambel, L., Chelo, I. M., Zé-Zé, L., Pedro, L. G., Santos, M. A. & Tenreiro, R. (2006). *Leuconostoc pseudoficulneum* sp. nov., isolated from a ripe fig. *Int J Syst Evol Microbiol* **56**, 1375–1381.
- Ehrmann, M. A., Freiding, S. & Vogel, R. F. (2009). *Leuconostoc palmae* sp. nov., a novel lactic acid bacterium isolated from palm wine. *Int J Syst Evol Microbiol* **59**, 943–947.
- Endo, A. & Okada, S. (2005). *Lactobacillus satsumensis* sp. nov., isolated from mashes of shochu, a traditional Japanese distilled spirit made from fermented rice and other starchy materials. *Int J Syst Evol Microbiol* **55**, 83–85.
- Endo, A. & Okada, S. (2006). *Oenococcus kitaharae* sp. nov., a non-acidophilic and non-malolactic-fermenting oenococcus isolated from a composting distilled shochu residue. *Int J Syst Evol Microbiol* **56**, 2345–2348.
- Endo, A. & Okada, S. (2008). Reclassification of the genus *Leuconostoc* and proposals of *Fructobacillus fructosus* gen. nov., comb. nov., *Fructobacillus durionis* comb. nov., *Fructobacillus ficulneus* comb. nov. and *Fructobacillus pseudoficulneus* comb. nov. *Int J Syst Evol Microbiol* **58**, 2195–2205.
- Endo, A., Futagawa-Endo, Y. & Dicks, L. M. T. (2009). Isolation and characterization of fructophilic lactic acid bacteria from fructose-rich niches. *Syst Appl Microbiol* **32**, 593–600.
- Endo, A., Futagawa-Endo, Y., Sakamoto, M., Kitahara, M. & Dicks, L. M. (2010). *Lactobacillus florum* sp. nov., a fructophilic species isolated from flowers. *Int J Syst Evol Microbiol* **60**, 2478–2482.
- Ezaki, T., Yamamoto, N., Ninomiya, K., Suzuki, S. & Yabuuchi, E. (1983). Transfer of *Peptococcus indolicus*, *Peptococcus asaccharolyticus*, *Peptococcus prevotii*, and *Peptococcus magnus* to the genus *Peptostreptococcus* and proposal of *Peptostreptococcus tetradius* sp. nov. *Int J Syst Bacteriol* **33**, 683–698.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Leisner, J. J., Vancanneyt, M., Van der Meulen, R., Lefebvre, K., Engelbeen, K., Hoste, B., Laursen, B. G., Bay, L., Rusul, G. & other authors (2005). *Leuconostoc durionis* sp. nov., a heterofermenter with no detectable gas production from glucose. *Int J Syst Evol Microbiol* **55**, 1267–1270.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Torriani, S., Felis, G. E. & Dellaglio, F. (2001). Differentiation of *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum* by *recA* gene sequence analysis and multiplex PCR assay with *recA* gene-derived primers. *Appl Environ Microbiol* **67**, 3450–3454.