Lactobacillus heilongjiangensis sp. nov., isolated from Chinese pickle

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A Gram-stain-positive bacterial strain, S4-3^T, was isolated from traditional pickle in Heilongjiang Province, China. The bacterium was characterized by a polyphasic approach, including 16S rRNA gene sequence analysis, *pheS* gene sequence analysis, *rpoA* gene sequence analysis, *dnaK* gene sequence analysis, fatty acid methyl ester (FAME) analysis, determination of DNA G+C content, DNA–DNA hybridization and an analysis of phenotypic features. Strain S4-3^T showed 97.9–98.7 % 16S rRNA gene sequence similarities, 84.4–94.1 % *pheS* gene sequence similarities and 94.4–96.9 % *rpoA* gene sequence similarities to the type strains of *Lactobacillus nantensis*, *Lactobacillus mindensis*, *Lactobacillus crustorum*, *Lactobacillus futsaii*, *Lactobacillus farciminis* and *Lactobacillus kimchiensis*. *dnaK* gene sequence similarities between S4-3^T and *Lactobacillus nantensis* LMG 23510^T, *Lactobacillus mindensis* LMG 21932^T, *Lactobacillus crustorum* LMG 23699^T, *Lactobacillus futsaii* JCM 17355^T and *Lactobacillus farciminis* LMG 9200^T were 95.4, 91.5, 90.4, 91.7 and 93.1 %, respectively. Based upon the data obtained in the present study, a novel species, *Lactobacillus heilongjiangensis* sp. nov., is proposed and the type strain is S4-3^T (=LMG 26166^T=NCIMB 14701^T).

Pickle is rich in lactic acid bacteria (LAB). In recent years, many novel LAB species and subspecies have been isolated from pickle, such as *Lactobacillus senmaizukei* (Hiraga *et al.*, 2008), *Lactobacillus nodensis* (Kashiwagi *et al.*, 2009), *Lactobacillus kisonensis* (Watanabe *et al.*, 2009), *Lactobacillus otakiensis* (Watanabe *et al.*, 2009), *Lactobacillus rapi* (Watanabe *et al.*, 2009), *Lactobacillus sunkii* (Watanabe *et al.*, 2009), *Lactobacillus kimchicus* (Liang *et al.*, 2011), *Lactobacillus koreensis* (Bui *et al.*, 2011), *Lactobacillus delbrueckii* subsp. *sunkii* (Kudo *et al.*, 2012), *Lactobacillus xiangfangensis* (Gu *et al.*, 2012), *Lactobacillus futsaii* (Chao *et al.*, 2012), *Lactobacillus kimchiensis* (Kim *et al.*, 2013) and *Leuconostoc miyukkimchii* (Lee *et al.*, 2012). In the present study, a Gram-stain-positive bacterial strain, S4-3^T, was isolated from traditional pickle in Heilongjiang Province, China. The bacterium was characterized by a polyphasic approach. The strains used in this study are listed in Table 1.

The GenBank/EMBL/DDBJ accession numbers obtained in the present study are: 16S rRNA gene sequence of *Lactobacillus heilongjiangensis* S4-3^T, JF411966; *rpoA* gene sequences of *Lactobacillus kimchiensis* JCM 17702^T, *Lactobacillus nantensis* LMG 23510^T, *Lactobacillus crustorum* LMG 23699^T and *Lactobacillus heilongjiangensis* S4-3^T, HF679049 and JF411980–JF411982, respectively; *dnaK* gene sequences of *Lactobacillus futsaii* JCM 17355^T, *Lactobacillus kimchii* LMG 19822^T, *Lactobacillus alimentarius* LMG 9187^T, *Lactobacillus farciminis* LMG 9200^T, *Lactobacillus mindensis* LMG 21932^T, *Lactobacillus nantensis* LMG 23510^T, *Lactobacillus crustorum* LMG 23699^T and *Lactobacillus heilongjiangensis* S4-3^T, HF679040–HF679041 and JF411983–JF411988, respectively; *pheS* gene sequence of *Lactobacillus heilongjiangensis* S4-3^T, JF411971.

All strains were incubated aerobically at 30 °C on mMRS medium (pH 6.2–6.4) consisting of 0.5 % peptone, 0.5 % meat extract, 1 % tryptone, 0.5 % yeast extract, 1 % glucose, 0.1 % Tween 80, 0.2 % $K_2HPO_4 . 3H_2O$, 0.5 % sodium acetate anhydrous, 0.2 % diammonium hydrogen citrate, 0.025 % $MnSO_4 . H_2O$ and 0.01 % $MgSO_4 . 7H_2O$.

The DNA for gene amplification was prepared using genomic DNA extraction kits (TIANGEN). Amplification of the 16S rRNA gene was performed using the primers developed by An *et al.* (2006). The *pheS* and *rpoA* genes were amplified using the primers of De Bruyne *et al.* (2007) and the protocols of Naser *et al.* (2005). The *dnaK* gene was amplified using the primers and protocol of Huang *et al.* (2010). Purification and sequencing of PCR products were carried out by the Shenggong Company in Shanghai, China. The resulting sequences, together with those of related strains obtained from the GenBank database were aligned using CLUSTAL W. A phylogenetic tree was reconstructed using the neighbour-joining method with the maximum composite likelihood model. The bootstrap analysis was performed based on 1000 replicates. The MEGA5 package (Tamura *et al.*, 2011) was used for all the analyses. 16S rRNA gene sequence analysis indicated that strain S4-3^T was phylogenetically related to *Lactobacillus nantensis*, *Lactobacillus mindensis*, *Lactobacillus crustorum*, *Lactobacillus futsaii*, *Lactobacillus farciminis* and *Lactobacillus kimchiensis* (Fig. 1). 16S rRNA gene sequence similarities between S4-3^T and *Lactobacillus nantensis* LMG 23510^T, *Lactobacillus mindensis* LMG 21932^T, *Lactobacillus crustorum*

Table 1. Bacterial strains used in this study

Strain	Isolation source
Lactobacillus heilongjiangensis sp. nov. S4-3^T (=LMG 26166^T=NCIMB 14701^T)	Pickle
Lactobacillus nantensis LMG 23510^T	Sourdough
Lactobacillus mindensis LMG 21932^T	Sourdough
Lactobacillus crustorum LMG 23699^T	Sourdough
Lactobacillus futsaii JCM 17355^T	Fermented mustard
Lactobacillus farciminis LMG 9200^T	Sausage
Lactobacillus kimchiensis JCM 17702^T	Pickle (Kimchi)
Lactobacillus alimentarius LMG 9187^T	Marinated fish product

LMG 23699^T, *Lactobacillus futsaii* YM 0097^T, *Lactobacillus farciminis* LMG 9200^T and *Lactobacillus kimchiensis* L133^T were 98.7, 98.3, 98.6, 97.9, 98.4 and 98.4 %, respectively. Strain S4-3^T showed 84.4–94.1 % *pheS* gene sequence similarities (Fig. 2) and 94.4–96.9 % *rpoA* gene sequence similarities (Fig. 3) to the type strains of *Lactobacillus nantensis*, *Lactobacillus mindensis*, *Lactobacillus crustorum*, *Lactobacillus futsaii*, *Lactobacillus farciminis* and *Lactobacillus kimchiensis*, respectively. *dnaK* gene sequence similarities (Fig. 4) between S4-3^T and *Lactobacillus nantensis* LMG 23510^T, *Lactobacillus mindensis* LMG 21932^T, *Lactobacillus crustorum* LMG 23699^T, *Lactobacillus futsaii* JCM 17355^T and *Lactobacillus farciminis* LMG 9200^T were 95.4, 91.5, 90.4, 91.7 and 93.1 %, respectively.

The API 50 CH system (bioMérieux) was used to determine the carbohydrate fermentation profile. Test preparations were incubated at 30 °C, and readings were made after 48 h. The results are given in the species description below. Characteristics that differentiate strain S4-3^T from its closest relatives are summarized in Table 2.

Whole-cell fatty acids were analysed as fatty acid methyl esters (FAMEs) with the Sherlock Microbial Identification system (MIDI). The cultures were incubated for 24 h at 30 °C on mMRS solid medium. FAMEs were extracted and prepared according to the protocol of Sasser (1990). Comparative fatty acid compositions of strain S4-3^T and phylogenetically related reference strains are given in Table 3.

The DNA for DNA G+C content determinations and DNA–DNA relatedness analyses was prepared using Power-Microbial Maxi DNA isolation kits (Mo Bio Laboratories). DNA G+C content was measured using the thermal melting protocol of De Ley (1970) and using

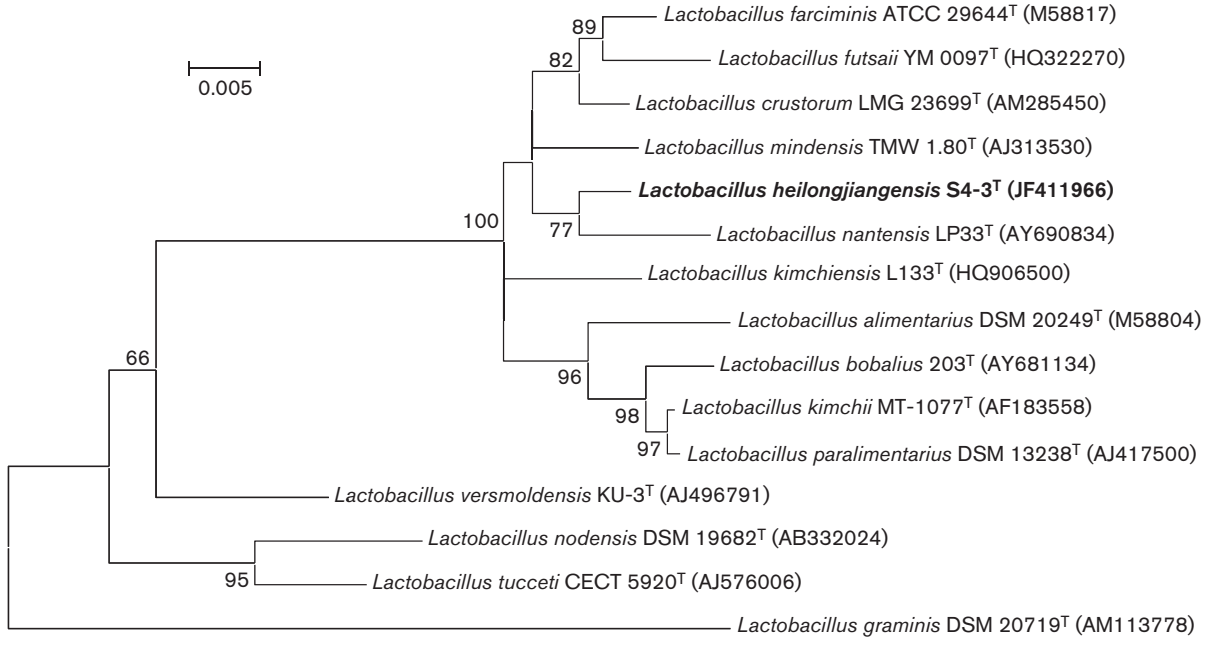

Fig. 1. Neighbour-joining tree showing the 16S rRNA gene phylogenetic relationships of *Lactobacillus heilongjiangensis* sp. nov. S4-3^T and phylogenetically related reference strains. Bar, 0.5 % substitution per site. *Lactobacillus graminis* DSM 20719^T (AM113778) was used as the outgroup.

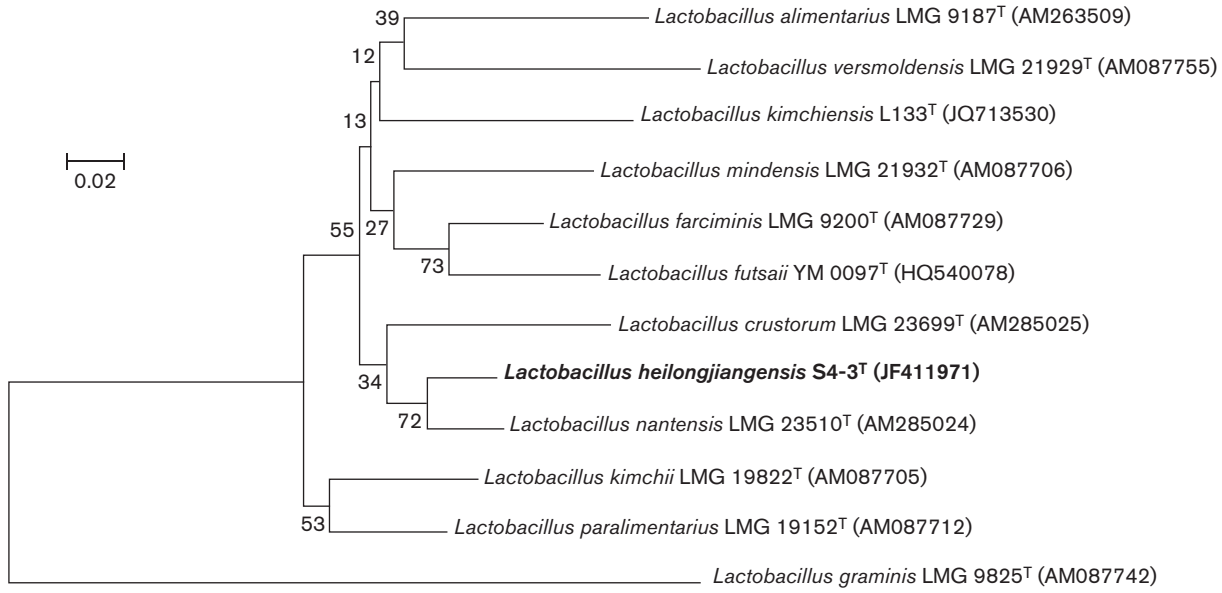

Fig. 2. Neighbour-joining tree showing the phylogenetic relationships of *Lactobacillus heilongjiangensis* sp. nov. S4-3^T and phylogenetically related reference strains based on *pheS* gene sequences. Bar, 2 % substitution per site. *Lactobacillus graminis* LMG 9825^T (AM087742) was used as the outgroup.

Escherichia coli K-12 as the standard. DNA–DNA hybridizations were performed by the initial renaturation rate method (De Ley *et al.*, 1970). The DNA G+C content of strain S4-3^T was 38.9 mol%. Low to medium DNA–DNA relatedness (0–49 %) was obtained between strain S4-3^T and the seven type strains of *Lactobacillus nantensis*, *Lactobacillus mindensis*, *Lactobacillus crustorum*, *Lactobacillus futsaii*, *Lactobacillus farciminis*, *Lactobacillus kimchiensis* and *Lactobacillus alimentarius*, confirming that strain S4-3^T represents a novel species within the genus *Lactobacillus*.

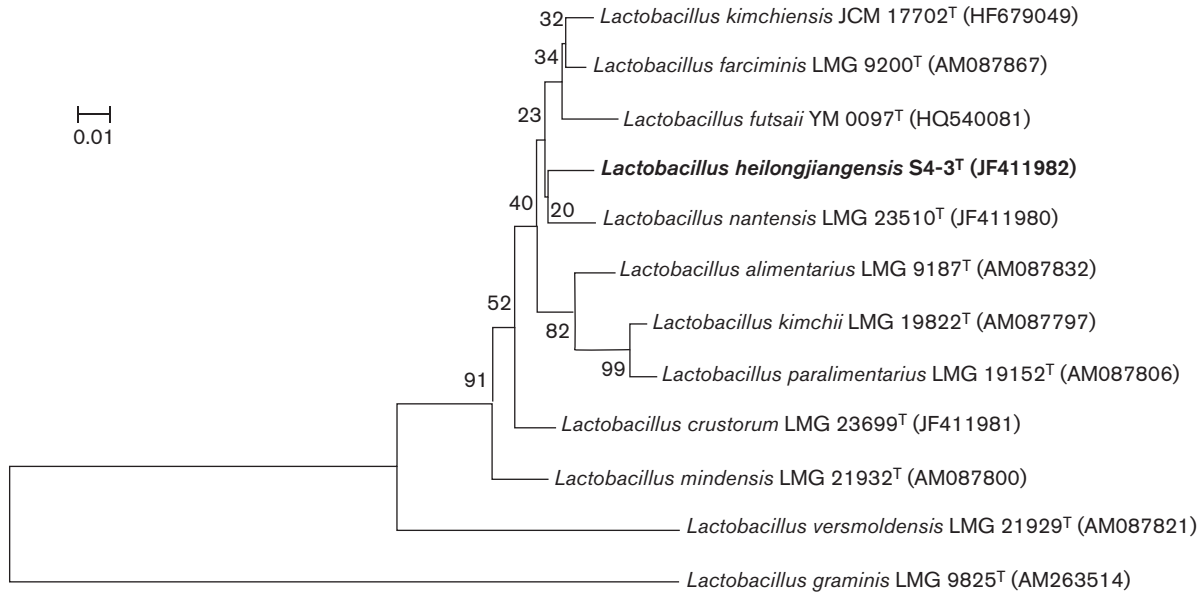

Fig. 3. Neighbour-joining tree showing the phylogenetic relationships of *Lactobacillus heilongjiangensis* sp. nov. S4-3^T and phylogenetically related reference strains based on *rpoA* gene sequences. Bar, 1 % substitution per site. *Lactobacillus graminis* LMG 9825^T (AM263514) was used as the outgroup.

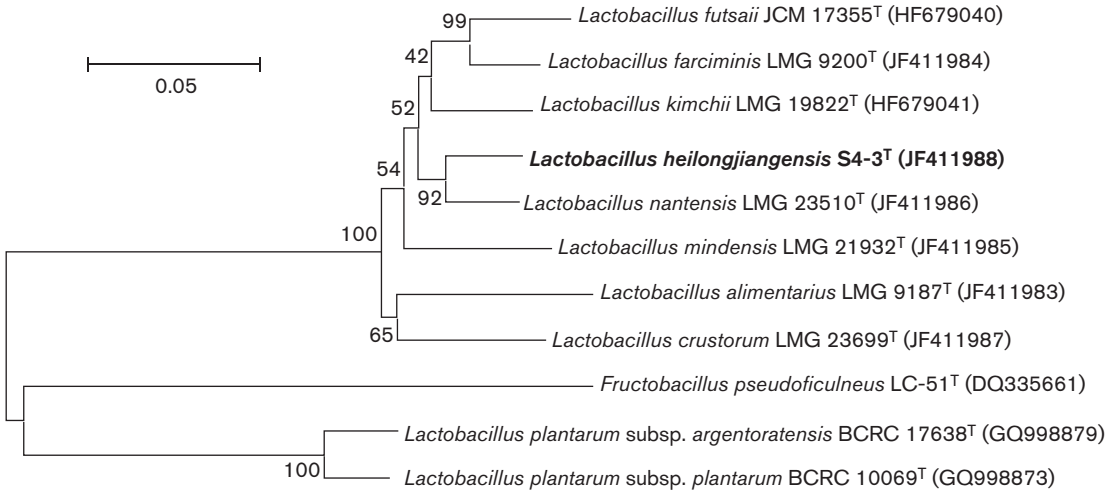

Fig. 4. Neighbour-joining tree showing the phylogenetic relationships of *Lactobacillus heilongjiangensis* sp. nov. S4-3^T and phylogenetically related reference strains based on *dnaK* gene sequences. Bar, 5 % substitution per site. *Fructobacillus pseudoficulneus* LC-51^T (DQ335661) was used as the outgroup.

Table 2. Features distinguishing between *Lactobacillus heilongjiangensis* sp. nov. and phylogenetically related species using API 50 CH

1, *Lactobacillus heilongjiangensis* sp. nov. S4-3^T (data from this study); 2, *Lactobacillus nantensis* (Valcheva *et al.*, 2006); 3, *Lactobacillus mindensis* (Ehrmann *et al.*, 2003); 4, *Lactobacillus crustorum* (Scheirlinck *et al.*, 2007); 5, *Lactobacillus futsaii* (Chao *et al.*, 2012); 6, *Lactobacillus farciminis* (Kandler & Weiss, 1986); 7, *Lactobacillus kimchiensis* (Kim *et al.*, 2013); 8, *Lactobacillus alimentarius* (Kandler & Weiss, 1986). +, 90 % or more strains positive; −, 90 % or more strains negative; ±, 11–89 % of strains positive; w, positive to weak reaction.

Acid production from:	1 (*n*=1)*	2 (*n*=5)	3 (*n*=4)	4 (*n*=4)	5 (*n*=3)	6	7 (*n*=1)	8
D-Arabinose	−	−	−	−	−	−	−	±
Ribose	−	w	−	−	−	−	−	+
Rhamnose	−	−	−	±	−	−	−	−
Mannitol	−	+	−	−	−	−	−	−
Sorbitol	−	+	−	−	−	−	−	−
Methyl α-D-Mannopyranoside	−	+	−	−	−	−†	−	−‡
Methyl α-D-Glucopyranoside	+	−	−	−	−	+†	−	−‡
N-Acetylglucosamine	+	+	+	+	+	+†	+	+‡
Amygdalin	+	+	±	±	+	−†	+	+‡
Arbutin	−	+	−	±	+	+†	+	+‡
Cellobiose	+	+	+	±	+	+	+	+
Maltose	+	+	w	±	±	+	+	+
Lactose	+	+	−	±	+	+	+	−
Melibiose	−	+	−	−	−	−	−	−
Sucrose	+	+	−	−	+	+	+	+
Trehalose	+	+	−	±	+	+	+	+
Raffinose	−	+	−	−	−	−	−	−
Gentiobiose	+	+	−	±	+	−†	+	+‡
Turanose	−	−	−	−	−	−†	+	−‡
Tagatose	+	+	−	±	±	+†	+	−‡
Gluconate	−	−	−	−	−	−	−	+

*n is the number of strains tested.
†Data from Kim *et al.* (2013) and Pang *et al.* (2012).
‡Data from Mañes-Lázaro *et al.* (2008) and Pang *et al.* (2012).

Table 3. Comparative fatty acid content (percentages) of *Lactobacillus heilongjiangensis* sp. nov. S4-3^T and phylogenetically related reference strains

Strains: 1, *Lactobacillus heilongjiangensis* sp. nov. S4-3^T; 2, *Lactobacillus nantensis* LMG 23510^T; 3, *Lactobacillus mindensis* LMG 21932^T; 4, *Lactobacillus crustorum* LMG 23699^T; 5, *Lactobacillus futsaii* JCM 17355^T; 6, *Lactobacillus farciminis* LMG 9200^T; 7, *Lactobacillus kimchiensis* JCM 17702^T; 8, *Lactobacillus alimentarius* LMG 9187^T. −, Not detected.

Fatty acid	1	2	3	4	5	6	7	8
12 : 0	−	0.61	−	0.60	−	−	0.67	−
14 : 0	3.34	3.31	4.58	3.15	2.48	2.82	3.67	2.64
16 : 0	46.79	42.12	46.31	43.66	33.80	35.65	39.30	37.20
17 : 0	0.42	−	−	−	−	−	−	−
18 : 0	3.37	2.73	2.61	3.35	4.54	3.71	3.07	2.75
18 : 1 ω9*c*	21.63	23.01	32.71	16.13	34.38	26.42	22.98	30.42
19 : 0 iso	−	7.02	2.62	6.33	7.51	7.91	−	5.84
19 : 1 iso I	1.23	1.42	−	1.38	1.87	1.65	−	1.30
Sum of 16 : 1ω6*c* and 16 : 1ω7*c*	2.47	2.72	1.75	2.35	2.47	2.81	2.27	2.48
Sum of 18 : 2 ω6, 9*c* and 18 : 0 anteiso	1.03	1.24	1.00	1.14	1.29	0.96	0.98	1.17
Sum of 19 : 1 ω6*c* and 19 : 0 cyclo ω10*c*	13.78	10.11	3.77	16.46	5.14	11.93	21.52	11.12
Sum of 18 : 1 ω6*c* and 18 : 1 ω7*c*	5.93	5.71	4.64	5.44	6.51	6.15	5.54	5.08

On the basis of the results presented here, strain S4-3^T is considered to represent a novel species within the genus *Lactobacillus*, for which the name *Lactobacillus heilongjiangensis* sp. nov. is proposed.

Description of *Lactobacillus heilongjiangensis* sp. nov.

Lactobacillus heilongjiangensis (hei.long.ji.ang.en′sis. N.L. masc. adj. *heilongjiangensis* pertaining to the Heilongjiang River, a river flowing through Heilongjiang Province of China, where the bacterium was first isolated).

Gram-stain-positive. Non-spore-forming rods. Catalase is not produced. Acid is produced from galactose, glucose, fructose, mannose, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, aesculin, salicin, cellobiose, maltose, lactose, sucrose, trehalose, gentiobiose and tagatose. Acid is not produced from glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β-D-xylopyranoside, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannopyranoside, arbutin, melibiose, inulin, melezitose, raffinose, starch, glycogen, xylitol, turanose, lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate.

The type strain is S4-3^T (=LMG 26166^T=NCIMB 14701^T). The DNA G+C content of strain S4-3^T is 38.9 mol%.

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