Sporolactobacillus putidus sp. nov., an endospore-forming lactic acid bacterium isolated from spoiled orange juice

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A Gram-positive, endospore-forming, lactic acid bacterium was isolated from spoiled orange juice. The organism, strain QC81-06^T, grew microaerobically or anaerobically at 30–45 °C (optimum 35 °C) and pH 3.5–5.5 (optimum pH 4.5), and produced acid from various sugars. D-Lactic acid was produced. It contained menaquinone-7 as the major isoprenoid quinone. The G+C content of the genomic DNA was 47.5 mol%. The predominant cellular fatty acids of the strain were iso-C_{16:0}, anteiso-C_{15:0} and anteiso-C_{17:0}. Phylogenetic analyses based on the 16S rRNA gene and *gyrB* gene (DNA gyrase B subunit gene) revealed that strain QC81-06^T clustered with *Sporolactobacillus* species but the strain was clearly distinct from other *Sporolactobacillus* species with significant bootstrap values. The levels of 16S rRNA gene and *gyrB* gene sequence similarities between strain QC81-06^T and the other strains of the cluster were 96.0–97.0 % and 75.1–77.2 %, respectively. On the basis of these results, strain QC81-06^T should be classified as a novel *Sporolactobacillus* species for which the name *Sporolactobacillus putidus* is proposed. The type strain is strain QC81-06^T (=DSM 21265^T=JCM 15325^T).

The genus *Sporolactobacillus* (Kitahara & Suzuki, 1963) consists of Gram-positive, catalase-negative, endospore-forming, homofermentative lactic acid bacteria. The type species is *Sporolactobacillus inulinus*, which was first isolated from chicken feed (Kitahara & Suzuki, 1963; Kitahara & Lai, 1967). Later Yanagida *et al.* (1987a, b, 1997) proposed four species and one subspecies, Chang *et al.* (2008) proposed one species, and *Bacillus laevolacticus* (Andersch *et al.*, 1994) was transferred to the genus *Sporolactobacillus* (Hatayama *et al.*, 2006), so the genus *Sporolactobacillus* now comprises seven species and one subspecies (*S. inulinus*, *S. lactosus*, *S. laevolacticus*, *S. nakayamae*, *S. nakayamae* subsp. *racemicus*, *S. kofuensis*, *S. terrae* and *S. vineae*). A living culture of *S. lactosus* has unfortunately been lost, so the strain can not be used as an experimental reference in a taxonomic study at present. Although *Sporolactobacillus* species are typically isolated from soil, they are occasionally isolated from fermented or spoiled foods (Yanagida *et al.*, 1997). During the course of a microbiological survey of spoiled orange juice, we ascertained that the spoilage was caused by an unusual

heat-tolerant lactic acid bacterium. In this paper, we describe a novel Gram-positive, endospore-forming species of the genus *Sporolactobacillus*.

Strain QC81-06^T was isolated from a spoiled orange juice sample with an organic acid odour by using the dilution plating technique on MRS agar (Merck). Based on partial 16S rRNA gene sequence analyses (Goto *et al.*, 2000, 2002), the strain was grouped into the *Sporolactobacillus* cluster. The strain, however, was found to be distinct from previously described species of the genus *Sporolactobacillus*.

Cell morphology was examined by phase-contrast microscopy with Plate Count Agar (PCA; Merck). Motility was observed using the prick test with GYP medium [2 % (w/v) glucose, 1 % (w/v) yeast extract (Difco), 1 % (w/v) peptone (Difco), 1 % (w/v) sodium acetate and 0.5 % (v/v) salt solution (4 % MgSO₄·7H₂O, 0.2 % MnSO₄·4H₂O, 0.2 % FeSO₄·7H₂O and 0.2 % NaCl); pH 6.8 with 1 M NaOH] containing 0.15 % agar (Difco) (Okada *et al.*, 1986). The catalase activity of the cells grown on GYP agar was determined by adding a 3 % hydrogen peroxide solution. The oxidase activity of the cells grown on GYP agar was determined by a cytochrome oxidase test strip (Nissui Pharmaceutical). The effect of NaCl concentration on growth was determined in GYP broth with different NaCl concentrations (3, 5, 7 and 10 %) added. The growth temperature range (15–50 °C) was examined in GYP broth by measuring the OD₆₆₀. The pH range for growth was examined in GYP broth with different pH values (pH 3.5–

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *gyrB* gene sequences of strain QC81-06^T are AB374522 and AB374529, respectively.

Figures showing cells of strain QC81-06^T and phylogenetic trees based on 16S rRNA gene and *gyrB* gene sequences showing the position of strain QC81-06^T in the *Sporolactobacillus* cluster are available with the online version of this paper.

8.0) adjusted by the addition of 1 M or 2 M HCl and 1 M NaOH. Acidification was examined with API 50 CH test strips in API 50 CHL Medium (bioMérieux). Acidification was observed for 14 days. The colour change of the medium from blue to yellow was regarded as acidification by metabolism of the carbon source. Lactic acid production was tested using an F-kit (D-lactic acid/L-lactic acid; Roche Diagnostics).

Cells for cell wall amino acids and sugar, quinone and cellular fatty acid analysis were grown on GYP medium at 35 °C for 3 days. Cell walls were prepared from approximately 100 mg (dry weight) bacterial cells. Amino acids in an acid hydrolysate of the cell walls were identified by using two-dimensional descending chromatography on cellulose TLC plates (Tokyo Kasei), using the method of Harper & Davis (1979), and by HPLC, as phenylthiocarbamoyl derivatives, with a model LC-10AD HPLC apparatus (Shimadzu) equipped with a Wakopak WS-PTC column (Wako Pure Chemical Industries). Cell walls were hydrolysed using 2 M HCl at 100 °C for 3 h, and sugars were analysed by TLC (Hasegawa *et al.*, 1983) or by GLC with an Agilent Ultra 2 capillary column (30 m × 0.25 mm i.d.) at a column temperature of 170–250 °C after converting into alditol acetate derivatives (Sawadecker *et al.*, 1965). Quinones were

extracted from freeze-dried cells as described previously (Sano *et al.*, 1996) and analysed using an Alliance HPLC system equipped with a Waters 2996 Photodiode Array detector. A Mightysil RP-18GP column (ϕ 4.6 × 150 mm; Kanto Chemical) was used for separation. Methanol/methanol:2-propanol (1:1, v/v) were used for the mobile phase. Quinones were identified by their UV spectra. Cells for cellular fatty acid analysis were saponified, methylated to create fatty acid methyl esters and identified with the Sherlock Microbial Identification System, version 4.02 (MIDI).

Preparation of genomic DNA and 16S rRNA gene sequencing were carried out as described previously (Goto *et al.*, 2002). The DNA G+C content was determined by the method of Tamaoka & Komagata (1984). Almost complete 16S rRNA gene sequences were determined using a 16S rRNA Gene kit (Applied Biosystems). A partial *gyrB* gene covering positions 274–1525 in the *Escherichia coli gyrB* gene was amplified using the deoxyinosine nucleotide containing primers UP1Gi (5'-GAA GTC ATC ACC GTT CTG CAY GSI GGI GGI AAR TTY RG-3') and GB-Igc-R (5'-GTG AGT AAC AAT GTA CGD ATR TGI GCI CCR TCI AC-3'). PCR was performed using TaKaRa LA *Taq* polymerase (Takara) under the following conditions: one cycle of 95 °C for

Table 1. Differential phenotypic characteristics of strain QC81-06^T and related *Sporolactobacillus* strains

Strains: 1, QC81-06^T; 2, *S. inulinus* IFO 13595^T; 3, *S. kofuensis* JCM 3419^T; 4, *S. laevolacticus* IAM 12321^T; 5, *S. nakayamae* subsp. *nakayamae* JCM 3514^T; 6, *S. nakayamae* subsp. *racemicus* JCM 3417^T; 7, *S. terrae* JCM 3516^T; 8, *S. vineae* SL153^T. +, Positive result; –, negative result; ND, no data. Acidification was examined with API 50 CH test strips in API 50 CHL Medium (bioMérieux).

Characteristic	1	2	3*	4*	5*	6*	7	8*
Hydrolysis of starch	–	–	–	+	–	ND	+	ND
Optimum pH for growth	4.5	7.0–7.5	ND	ND	ND	ND	5.5	ND
pH range for growth	4.0–4.5	5.0–8.0	ND	4.5–7.7	ND	ND	5.0–5.5	6.0–7.0
Optimum growth temperature (°C)	35	35–40	ND	40	ND	ND	35	37
Growth temperature range (°C)	30–45	20–45	25–40	15–40	15–40	15–40	25–45	25–40
Growth in 3 % NaCl	–	+	+	+	+	+	+	+
Acid production from:								
Glycerol	–	+	ND	ND	ND	ND	–	ND
Galactose	+	–	+	ND	+	+	–	–
L-Sorbose	–	–	ND	ND	ND	ND	+	+
Sorbitol	–	+	–	ND	+	+	–	+
Methyl α -D-glucoside	–	+	+	ND	–	–	–	+
Aesculin	–	–	ND	ND	ND	ND	+	–
Salicin	–	–	–	ND	–	+	–	–
Cellobiose	–	–	–	ND	–	+	–	–
Maltose	+	+	+	ND	+	+	+	+
Melibiose	–	–	–	ND	–	+	–	–
Trehalose	+	+	+	ND	–	+	+	+
Inulin	–	+	+	ND	–	+	–	–
Raffinose	–	+	+	ND	+	+	–	–
Starch	–	–	+	+	–	+	–	–
Turanose	+	+	ND	ND	ND	ND	–	+
D-Tagatose	+	+	ND	ND	ND	ND	+	–

*Data were obtained from Chang *et al.* (2008), Hatayama *et al.* (2006) and Yanagida *et al.* (1997).

5 min, followed by 35 cycles of 95 °C for 30 s and 50 °C for 1 min, followed by 72 °C for 90 s, then a final 5 min incubation at 72 °C. Sequencing was performed using primers UP1s (5'-GAAGTCATCACCGTTCTGCA-3') and UP2rs (5'-AGCAGGGTACGGATGTGCGAGCC-3'), as described by Yamamoto & Harayama (1995). The products were analysed with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Sequence analysis was performed using Gene Works (version 2.0; IntelliGenetics) and the GenBank/EMBL/DBJ databases. Multiple sequence alignment was performed using CLUSTAL W version 1.8 (Thompson *et al.*, 1994). The *gyrB* gene sequences and their deduced amino acid sequences were aligned by CLUSTAL W version 1.8 and the alignments were manually corrected. Alignment gaps and unidentified base positions were not taken into account for these calculations. A phylogenetic reconstruction was produced using MEGA3 (Kumar *et al.*, 2001) with the following settings: neighbour-joining (Saitou & Nei, 1987) based on Kimura's two-parameter model (Kimura, 1980). The robustness of individual branches was estimated by bootstrapping with 1000 replicates (Felsenstein, 1985).

Cells of strain QC81-06^T were Gram-positive, motile rods (0.5–0.8 × 3.0–8.0 µm) and formed oval spores subterminally in swollen sporangia (see Supplementary Fig. S1 available in IJSEM Online). Colonies were flat, smooth and creamy-white after 72 h culture on PCA. Growth of strain QC81-06^T occurred anaerobically at 30 and 45 °C, but not at 25 or 50 °C. The optimal growth temperature was 35 °C. Strain QC81-06^T grew well at pH 4.5, with growth inhibited at pH 3.0 and 6.0. The strain failed to grow on GYP containing more than approximately 3 % (w/v) NaCl. Strain QC81-06^T did not exhibit any catalase, oxidase or nitrate reductase activity. Acid was produced from galactose, D-glucose, D-fructose, D-mannose, mannitol, N-acetylglucosamine, maltose, sucrose, trehalose, turanose and D-tagatose. D-Lactic acid was produced from glucose. The differential phenotypic characteristics of strain QC81-06^T and *Sporolactobacillus* strains are shown in Table 1.

The cell wall peptidoglycan of strain QC81-06^T contained mesodiaminopimelic acid, alanine and glutamic acid. The cell wall sugar of strain QC81-06^T contained glucose and galactose. These characteristics of the isolate agreed with those of *S. inulinus*, *S. kofuensis* and *S. terrae*. The major respiratory quinone of strain QC81-06^T was menaquinone-7 (MK-7), which accounted for 85 % of the total, with MK-3 as the vestigial quinone (15 %). The major fatty acids of strain QC81-06^T were iso-C_{16:0}, anteiso-C_{15:0} and anteiso-C_{17:0}. This pattern is similar to that of *S. kofuensis* and *S. laevolacticus* (Table 2). However, the content of anteiso-C_{15:0} and anteiso-C_{17:0} is more similar to that of *S. vineae*.

The G+C content of strain QC81-06^T was 47.5 mol%. This was about the same as that of *S. inulinus* (47 %), somewhat higher than that of *S. laevolacticus* (43–45 %), *S.*

Table 2. Cellular fatty acid composition (%) of strain QC81-06^T and related *Sporolactobacillus* strains

Strains: 1, QC81-06^T; 2, *S. inulinus* IFO 13595^T; 3, *S. kofuensis* JCM 3419^T; 4, *S. laevolacticus* IAM 12321^T; 5, *S. nakayamae* subsp. *nakayamae* JCM 3514^T; 6, *S. nakayamae* subsp. *racemicus* JCM 3417^T; 7, *S. terrae* JCM 3516^T; 8, *S. vineae* SL153^T. tr, Trace. All strains were cultivated for 3 days on GYP agar at 35 °C.

Fatty acid	1	2	3*	4*	5*	6*	7	8*
C _{12:0}	—	—	—	—	—	tr	—	—
C _{14:0}	tr	tr	tr	tr	tr	tr	tr	tr
C _{16:0}	4.19	2.38	3.88	2.56	7.65	9.43	1.16	5.37
C _{18:0}	tr	—	—	tr	tr	tr	7.34	—
C _{20:0}	—	—	—	—	—	—	20.39	—
iso-C _{14:0}	tr	2.76	tr	tr	tr	2.11	tr	—
iso-C _{15:0}	3.16	5.78	5.91	6.63	2.99	9.10	3.00	13.28
iso-C _{16:0}	13.99	12.38	10.84	10.52	7.37	11.93	tr	4.68
iso-C _{17:0}	2.61	tr	2.60	5.05	1.82	6.00	1.32	6.09
iso-C _{18:0}	tr	—	—	tr	—	tr	tr	—
iso-C _{19:0}	—	—	—	—	—	—	2.43	—
iso-C _{20:0}	—	—	—	—	—	—	tr	—
anteiso-C _{11:0}	—	tr	—	—	—	—	—	—
anteiso-C _{13:0}	—	tr	—	—	—	—	—	—
anteiso-C _{15:0}	17.52	38.64	26.24	25.96	29.74	27.21	42.43	15.17
anteiso-C _{17:0}	55.54	34.04	48.22	46.58	44.89	29.29	7.50	53.79
anteiso-C _{19:0}	tr	—	tr	tr	tr	—	10.78	—
C _{18:1} ω9c	tr	1.37	tr	tr	2.30	1.94	tr	—
C _{16:1} ω7c	—	—	—	—	tr	tr	—	—
iso-C ₁₅ 2OH	—	—	—	—	—	—	—	—

*Data were obtained from Chang *et al.* (2008), Hatayama *et al.* (2006) and Yanagida *et al.* (1997).

kofuensis (43 %) and *S. terrae* (43–46 %), and somewhat lower than that of *S. vineae* (50.6 %).

Almost complete 16S rRNA gene sequences of strain QC81-06^T and six type strains of *Sporolactobacillus* species were determined (1501–1503 bp). Phylogenetic analysis based on the 16S rRNA gene sequences comprising 1468 nucleotides showed that strain QC81-06^T was located in the phylogenetic cluster composed of species of the genus *Sporolactobacillus* (Fig. 1). Similar topologies were obtained when other treeing methods (minimum evolution and maximum parsimony) were used (see Supplementary Figs S2 and S3 in IJSEM Online). The sequence similarity values between strain QC81-06^T and the other strains of *Sporolactobacillus* were 96.0–97.0 %. The closest relative to strain QC81-06^T was *S. terrae* JCM 3516^T (97.0 %), followed by *S. vineae* (96.6 %), *S. laevolacticus* (96.6 %) and *S. kofuensis* (96.6 %).

Partial *gyrB* gene sequences for strain QC81-06^T and the six *Sporolactobacillus* strains were determined (1131–1167 bp). Phylogenetic analysis based on the alignment of the *gyrB* gene sequences (1128 nucleotides) showed that strain QC81-06^T clustered with species of the genus *Sporolactobacillus*

Fig. 1. Phylogenetic tree (constructed using the neighbour-joining method) showing the position of strain QC81-06^T in the *Sporolactobacillus* cluster based on 16S rRNA gene sequences. *Bacillus subtilis* IAM 12118^T (AB042061) served as an outgroup. The final dataset included 1468 unambiguously aligned sites. The numbers indicate the percentage from 1000 replicate bootstrap samplings (frequencies of less than 50 % are not shown). Bar, 0.01 substitutions per nucleotide position.

(Fig. 2). Similar topologies were obtained when other treeing methods (minimum evolution and maximum parsimony) were used (see Supplementary Figs S4 and S5 in IJSEM Online). The sequence similarity values between strain QC81-06^T and the other strains of *Sporolactobacillus* were 75.1–77.2 %.

On the basis of all of the data obtained, strain QC81-06^T is considered to represent a novel species within the genus *Sporolactobacillus*, for which the name *Sporolactobacillus putidus* sp. nov. is proposed.

Description of *Sporolactobacillus putidus* sp. nov.

Sporolactobacillus putidus (pu'ti.dus. L. masc. adj. *putidus* stinking).

Gram-positive, anaerobic, motile, endospore-forming straight rods with rounded ends (0.5–0.8 × 3.0–8.0 µm). The endospores are oval and subterminal with swollen

sporangia. Colonies on plate count agar are circular and 1–2 mm in diameter after 72 h, and are not pigmented. The temperature range is 30–45 °C (optimum growth temperature: 35 °C). The optimum pH is 4.5–5.0; growth does not occur at pH 3.0 or 6.0. Oxidase and catalase activities are negative. Starch is hydrolysed. Nitrate reduction is negative. Acid is produced from galactose, D-glucose, D-fructose, D-mannose, mannitol, N-acetylglucosamine, maltose, sucrose, trehalose, turanose and D-tagatose. D-Lactic acid is produced. The major fatty acids are iso-C_{16:0}, anteiso-C_{15:0} and anteiso-C_{17:0}. The main quinone is menaquinone 7. The G + C content is 47.5 mol%.

The type strain is strain QC81-06^T (=DSM 21265^T=JCM 15325^T), isolated from spoiled orange juice.

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Fig. 2. Phylogenetic tree (constructed using the neighbour-joining method) showing the position of strain QC81-06^T in the *Sporolactobacillus* cluster based on the *gyrB* gene sequences. *Bacillus subtilis* IAM 12118^T (Z99104) served as an outgroup. The final dataset included 1128 unambiguously aligned sites. The numbers indicate the percentage from 1000 replicate bootstrap samplings (frequencies of less than 50 % are not shown). Bar, 0.05 substitutions per nucleotide position.

acid, cell wall amino acids and sugar analysis. We also thank Ms. Wasada (Mitsui Norin Co. Ltd) for her technical assistance.

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