Saccharibacter floricola gen. nov., sp. nov., a novel osmophilic acetic acid bacterium isolated from pollen

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Three Gram-negative, aerobic, rod-shaped bacterial strains were isolated, from the pollen of Japanese flowers, as producers of xylitol; these strains were subjected to a polyphasic taxonomic study. Phylogenetic analyses of the 16S rRNA gene sequences demonstrated that these three isolates formed a new cluster within a group of acetic acid bacteria in the α -*Proteobacteria*. The characteristics of the three isolates were as follows: (i) their predominant quinone was Q-10; (ii) their cellular fatty acid profile contained major amounts of 2-hydroxy acids and an unsaturated straight-chain acid (C_{18:1}ω7c); and (iii) their DNA G + C contents were in the range 51.9–52.3 mol%, which is around the lower limit of the reported range for the genera of acetic acid bacteria. The negligible or very weak productivity of acetic acid from ethanol and the osmophilic growth properties distinguished these strains from other acetic acid bacteria. The unique phylogenetic and phenotypic characteristics suggest that the three isolates should be classified within a novel genus and species with the proposed name *Saccharibacter floricola* gen. nov., sp. nov. The type strain is strain S-877^T (=AJ 13480^T=JCM 12116^T=DSM 15669^T).

Acetic acid bacteria are generally characterized by their remarkable ability to oxidize alcohols and sugars, and especially by their ability to oxidize ethanol to acetic acid, and they have been widely used for the commercial production of vinegar, gluconate and sorbose. Acetic acid bacteria are currently assigned to six genera in the family *Acetobacteraceae*, namely *Acetobacter*, *Gluconobacter*, *Acidomonas*, *Gluconacetobacter*, *Asaia* and *Kozakia*, which can be differentiated by means of phenotypic and chemotaxonomic properties (Yamada *et al.*, 2000; Lisdiyanti *et al.*, 2002). On the basis of 16S rRNA gene sequence analysis, these bacteria are affiliated to a group of acidophilic bacteria in the α -subclass of the *Proteobacteria* (Sievers *et al.*, 1994). The acetic acid bacteria are known to be well adapted to sugary and alcoholized fluid, their main habitats being vinegar, fruit juice, sap water, alcoholic beverages and flowers.

During the course of a screening programme for microbial producers of xylitol, which is a promising low-calorie

sweetener with anti-dental caries properties, we isolated several potent producing strains from flower samples (our unpublished results). Although these strains were found to be in the family *Acetobacteraceae* (Lisdiyanti *et al.*, 2002), they had rather unusual characteristics for this group of bacteria, such as negligible or weak capacities to produce acetic acid from ethanol and an osmophilic growth profile. In this paper, we report a detailed description of the taxonomic characteristics of these isolates and propose a novel genus and species, *Saccharibacter floricola* gen. nov., sp. nov., based on physiological and phylogenetic studies.

Three bacterial strains (S-877^T, S-1009 and S-1019) were isolated from an enrichment culture with a high glucose concentration. The medium used (YUG medium) comprised 1.0% (w/v) yeast extract (Difco), 0.1% (w/v) urea and 20% (w/v) glucose. After incubation with shaking at 28 °C for 7 days, the enrichment cultures with flower samples were appropriately diluted and then plated on YUG agar medium [containing 1.5% (w/v) agar]. For phenotypic characterization and collection of biomass, the strains were cultivated aerobically at 28 °C using YPG medium [1% (w/v) yeast extract (Difco), 1% (w/v) peptone (Difco) and 7% (w/v) glucose, adjusted to pH 6.0 with HCl] unless otherwise specified. Solid media were prepared by adding 1.5% (w/v) agar (Difco).

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain S-877^T is AB110421.

Cells of all isolates were Gram-negative, strictly aerobic, non-motile rods measuring $0.8\text{--}1.0 \times 2.5\text{--}4.0\text{ }\mu\text{m}$. Colonies of the three isolates on YPG agar were circular, entire and pale in colour. Endospores were not produced. The bacterial colonies produced neither soluble pigments, mucous substances nor cellulosic pellicles.

The 16S rRNA gene was amplified by a PCR using crude cell lysates, as described by Iizuka *et al.* (1998). Amplified products were sequenced with a SequiTherm Long-Read cycle sequencing kit (Epicentre Technologies); this was followed by detection with a Pharmacia DNA sequencer according to the manufacturer's instructions. Almost complete 16S rRNA gene sequences of the three isolates were determined and the sequences were aligned with sequences of reference strains in the *Acetobacteraceae* obtained from the GenBank/EMBL/DDBJ databases. Gaps at the 5' and 3' ends of the alignment were omitted and the sequence at positions 46–1506, based on the *Escherichia coli* numbering system (Weisburg *et al.*, 1991), was used for the final analysis. Nucleotide substitution rates (K_{nuc} values) were calculated using Kimura's method (Kimura, 1980). The phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) with the CLUSTAL W program (Thompson *et al.*, 1994). The robustness of the topology of the neighbour-joining tree was estimated by means of a bootstrapped analysis with 1000 replicates (Felsenstein, 1985). The three isolates shared the same sequences, and a representative isolate (S-877^T) constituted a distinct cluster separate from any existing genera in the family *Acetobacteraceae* (Fig. 1). The tree topology was also supported by maximum-parsimony analysis (data not shown). The sequence similarity levels between isolate S-877^T and its closest relatives, *Gluconobacter cerinus* and *Gluconobacter oxidans*, were 94.0 and 93.9%, respectively. These relatively low similarities warrant the allocation of the novel isolates to a novel genus of acetic acid bacteria.

Fig. 1. Neighbour-joining phylogenetic tree showing the relationships of the novel isolates (represented by strain S-877^T) and related acetic acid bacteria, based on 16S rRNA gene sequences. Bootstrap values, shown at the nodes, were calculated from 1000 samplings. Bar, 1% estimated sequence divergence.

Isoprenoid quinones were extracted with acetone and analysed by TLC and HPLC according to the method of Hiraishi *et al.* (1984). The respiratory quinone type of the three isolates was Q-10. Chromosomal DNA was extracted from bacterial cells according to the method of Saito & Miura (1963) and further purified using a Qiagen Genomic-tip kit. The DNA G+C content was determined by HPLC using the method of Tamaoka & Komagata (1984). The G+C contents of isolates S-877^T, S-1009 and S-1019 were 52.3, 52.3 and 51.9 mol%, respectively. These values are around the lower limit of those of the genera in the family *Acetobacteraceae* (De Ley *et al.*, 1984; Lisdiyanti *et al.*, 2002). The cellular fatty acid compositions of the isolates were determined using the Microbial Identification System MIDI (Hewlett Packard).

The major fatty acids of the new isolates were a 2-hydroxy acid ($C_{16:0}$ 2-OH) and a straight-chain unsaturated acid ($C_{18:1\omega7c}$), which accounted for 31.1–41.0 and 22.0–29.8% of total fatty acids, respectively. The other fatty acids were $C_{14:0}$ (1.4–2.1%), $C_{16:0}$ (10.0–11.1%), $C_{19:0\omega8c}$ cyclo (2.7–3.4%), $C_{14:0}$ 2-OH (2.2–2.5%), $C_{18:1}$ 2-OH (0.8–1.0%), $C_{14:0}$ 3-OH (4.4–7.6%) and $C_{16:0}$ 3-OH (6.4–6.9%). Although these fatty acid profiles were roughly in agreement with reported data for the other genera of acetic acid bacteria (Yamada *et al.*, 1981; Franke *et al.*, 1999), the novel isolates contained unusually high levels of 2-hydroxy fatty acids.

DNA–DNA relatedness was measured according to the dot-blot hybridization method (Hiraishi *et al.*, 1991), using a Biotyne A membrane (Gibco-BRL). Labelling of DNA and detection of hybridized DNA were performed using the AlkPhos Direct system for chemifluorescence (Amersham Pharmacia Biotech) according to the manufacturer's directions. Quantification of dots was performed using a fluoroimage analyser (FLA3000; Fuji Film). DNA isolated from S-877^T hybridized strongly with that from the other two isolates, the similarity values being 71.8 and 99.6% for strains S-1009 and S-1019, respectively. These results indicate that these three isolates belong to a single species. On the other hand, DNA from *Gluconobacter oxydans* and *Acetobacter aceti*, which are representatives of the acetic acid bacteria, showed less than 5% relatedness with S877^T.

Phenotypic characteristics were examined principally according to Asai *et al.* (1964) and Yamada *et al.* (1976, 2000). The three isolates showed rather acidophilic growth, the pH range for growth of all three strains being 4.0–7.5 with an optimum between pH 5.0 and 7.0. The temperature range for growth was 20–33 °C with an optimum around 25–30 °C. The catalase test was positive and the oxidase and indole tests were negative for all three isolates. The isolates showed growth on mannitol agar and glutamate agar supplemented with 7% (w/v) glutamate, but they did not grow on normal glutamate agar supplemented with 1% (w/v) glutamate. Other phenotypic characteristics are summarized in Table 1.

Table 1. Characteristics that differentiate *Saccharibacter* gen. nov. from other genera of acetic acid bacteria

+, Positive; –, negative; W, weakly positive; ND, not determined. Data for reference taxa were taken from Urakami *et al.* (1989) and Lisdiyanti *et al.* (2002).

Characteristic	<i>Sacchari- bacter</i>	<i>Kozakia</i>	<i>Asaia</i>	<i>Gluconaceto- bacter</i>	<i>Glucono- bacter</i>	<i>Acido- monas</i>	<i>Acetobacter</i>
Flagellation	Non- motile	Non- motile	Peritrichous or non-motile	Peritrichous or non-motile	Polar or non-motile	Non- motile	Peritrichous or non-motile
Pigmentation	–	–	+ / –	–	+	–	–
Production of water-soluble brown pigment(s)	–	–	–	+ / –	– / +	–	–
Production of cellulose	–	–	–	+ / –	–	–	–
Production of mucous substance(s) from sucrose	–	+	–	–	–	–	– / +
Oxidation of:							
Acetate	–	W	W	+	–	+	+
Lactate	W	W	W	+	–	–	+
Production of acetic acid from ethanol	W / –	+	–	+	+	+	+
Growth in the presence of 0.35 % (v/v) acetic acid in CaCO ₃ -free AG broth at pH 3.5*	–	+	–	+	+	+	+
Growth in the presence of 30 % (w/v) D-glucose	+	–	+	+ / –	–	ND	–
Growth on YPM agar:†							
Without D-mannitol	–	W	W	W	W	ND	W
With D-mannitol	+	+	+	+	+	ND	W
Growth on 1 % (w/v) glutamate agar	–‡	–	+	+	–	ND	+
Assimilation of ammoniacal nitrogen on Hoyer–Frateur medium with:							
D-Glucose	–	–	+	+ / –	–	ND	–
D-Mannitol	–	–	+	+ / –	–	ND	–
Ethanol	–	–	–	–	–	ND	+ / –
Assimilation of ammoniacal nitrogen on Frateur-modified Hoyer medium with:							
D-Glucose	+ / –	+ / –	+	+ / –	+	ND	–
D-Mannitol	+ / –	+	+	+ / –	+	ND	–
Ethanol	–	–	–	–	–	ND	+ / –
Utilization of methanol	–	–	–	–	–	+	–
Ketogenesis from glycerol	–	+	+ / –	+ / –	+	–	+ / –
Production of keto-D-gluconates from D-glucose:							
2-Keto-D-gluconate	+	+	+	+	+	ND	+ / –
5-Keto-D-gluconate	+	+	+	+ / –	+	ND	+ / –
Acid production from:							
L-Arabinose	+	+	+	+ / –	+	–	+ / –
D-Arabinose	–	+ / –	+	–	+	ND	–
D-Xylose	+	+	+	+ / –	+	–	+ / –
L-Rhamnose	–	–	+ / –	–	–	ND	–
D-Glucose	+	+	+	+	+	+	+ / –
D-Galactose	+	+	+	+	+	–	+ / –
D-Mannose	+	+	+	+ / –	+	–	+ / –
D-Fructose	+ / –	–	+	+	+	–	–
L-Sorbose	–	–	+	+ / –	+	ND	–
Melibiose	+	+	+	–	+	ND	–
Sucrose	+	+ / –	+	–	+	–	–
Raffinose	–	+	–	–	–	ND	–
D-Mannitol	+	–	+ / –	+ / –	+	–	–

Table 1. cont.

Characteristic	<i>Sacchari- bacter</i>	<i>Kozakia</i>	<i>Asaia</i>	<i>Gluconaceto- bacter</i>	<i>Glucono- bacter</i>	<i>Acido- monas</i>	<i>Acetobacter</i>
D-Sorbitol	—	—	+/-	—	+	—	—
Dulcitol	—	—	+	—	—	ND	—
Glycerol	—	+	+	+	+	—	—
Ethanol	—	+	—	+	+	+	+
Major ubiquinone	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-9
DNA G + C content (mol%)	52–53	56–57	59–61	55–66	54–63	63–66	52–60

*For AG broth, see Yamada *et al.* (2000).

†For YPM agar, see Asai *et al.* (1964).

‡Growth occurred at 7% (w/v) glutamate, but not at 1% (w/v) glutamate.

The three isolates differed from *Acetobacter*, *Gluconobacter*, *Acidomonas*, *Gluconacetobacter* and *Kozakia* in the lack of production of acetate from ethanol and in the absence of growth in 0.35% (v/v) acetic acid at pH 3.5. The isolates could be differentiated from *Asaia* by the absence of oxidation of acetate and by the absence of assimilation of ammoniacal nitrogen on Hoyer–Frature medium (De Ley & Frature, 1974) with glucose or mannitol.

Another unique property of the three isolates is their osmophilic growth characteristics. To investigate the effect of osmophilic pressure on the growth of the isolates, growth rates at various glucose levels were determined by monitoring optical density at 600 nm using an automatic growth analyser (Bioscreen C; Labsystems), as shown in Fig. 2. The novel isolate, strain S-877^T, showed not only high osmotolerance but also a requirement for very high glucose levels, growing in the range 5–40% (w/v), with an optimum at 10% (w/v) glucose. In contrast, growth of the

known ‘osmotolerant’ species such as *Gluconacetobacter diazotrophicus* and *Asaia bogorensis* was inhibited by increased glucose concentrations, although both species were able to grow in the presence of 30% (w/v) glucose (Gillis *et al.*, 1989; Yamada *et al.*, 2000). The other isolates (S-1009 and S-1019) showed growth profiles similar to that of strain S-877^T (data not shown).

In conclusion, the three isolates constitute a distinct phylogenetic lineage among the acetic acid bacteria and have unique phenotypic characteristics, as mentioned above. We propose placement of the isolates in a novel species of a novel genus, namely *Saccharibacter floricola* gen. nov., sp. nov.

Description of *Saccharibacter* gen. nov.

Saccharibacter (Sac.cha.ri.bac'ter. L. neut. n. *saccharum* or *saccharon* sugar; N.L. masc. n. *bacter* from Gr. n. *baktron* rod; N.L. masc. n. *Saccharibacter* a rod that grows well in a sugar-rich environment).

Cells are Gram-negative, non-motile, straight rods measuring 0.8–1.0 × 2.5–4.0 µm. Chemo-organotrophic and strictly aerobic. Catalase-positive. Oxidase-negative. Non-pigmented. Produces neither cellulosic pellicles nor water-soluble mucous substances. The optimum pH for growth is around 5.0–7.0; there is no growth above pH 8.0 or below pH 4.0. Growth occurs in the glucose range 2–40% (w/v), with an optimum around 10% (w/v). Grows on mannitol and 7% (w/v) glutamate agar but not on 1% (w/v) glutamate agar. Forms negligible or very little acetic acid from ethanol. Produces gluconic acid, 2-keto-D-gluconic acid and 5-keto-D-gluconic acid from glucose. Prefers high glucose concentration for growth [e.g. 10% (w/v) glucose]. Does not oxidize acetate to CO₂ and water. Oxidation of lactate is weak. Ammonia is not assimilated on Hoyer–Frature medium with glucose, mannitol or ethanol. Does not utilize methanol. Does not produce dihydroxyacetone from glycerol. Acid is produced from L-arabinose, D-xylose, D-glucose, D-galactose, D-mannose, melibiose, sucrose and mannitol, but not from D-arabinose,

Fig. 2. Effect of glucose concentration on growth of isolate S-877^T (●), *Gluconacetobacter diazotrophicus* DSM 5601^T (◇), *Asaia bogorensis* JCM 10569^T (□) and *Acetobacter aceti* NCIB 8621^T (○). One hundred per-cent growth corresponds to the maximum specific growth rate (h⁻¹) under optimum conditions.

L-rhamnose, L-sorbose, raffinose, D-sorbitol, dulcitol, glycerol or ethanol. Production of acid from L-sorbose is variable. The DNA G+C content is about 52–53 mol%. The major quinone type is Q-10. The major cellular fatty acids are a 2-hydroxy acid ($C_{16:0}$ 2-OH; 31.1–41.0 %) and a straight-chain unsaturated acid ($C_{18:1\omega7}$; 22.0–29.8 %). The type species is *Saccharibacter floricola*.

Description of *Saccharibacter floricola* sp. nov.

Saccharibacter floricola (flo.ri.co'la. L. n. *flos* -oris a flower; L. suff. -cola derived from L. n. *incola* a dweller; N.L. n. *floricola* flower-dweller).

Has all the characteristics that define the genus. Strains have been isolated from Japanese flowers.

The type strain is strain S-877^T (=AJ 13480^T=JCM 12116^T=DSM 15669^T), which was isolated from pollen collected in Kanagawa Prefecture, Japan. The DNA G+C content of strain S-877^T is 52.3 mol%.

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