Enterobacter sacchari sp. nov., a nitrogen-fixing bacterium associated with sugar cane (Saccharum officinarum L.)

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Five nitrogen-fixing bacterial strains (SP1T, NN143, NN144, NN208 and HX148) were isolated from stem, root or rhizosphere soil of sugar cane (Saccharum officinarum L.) plants. Cells were Gram-negative, motile, rods with peritrichous flagella. DNA G+C content was 55.0 ± 0.5 mol%. Sequence determinations and phylogenetic analysis of 16S rRNA gene and rpoB indicated that the strains were affiliated with the genus Enterobacter and most closely related to E. radicincitans DSM 16656T and E. oryzae LMG 24251T. Fluorimetric determination of thermal denaturation temperatures after DNA–DNA hybridization, enterobacterial repetitive intergenic consensus PCR and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry differentiated the whole-genome, genotype and protein profiles from those of E. radicincitans and E. oryzae. The strains’ cell fatty acid composition differentiated them from E. radicincitans and E. oryzae by containing a higher level of summed feature 2 (C16:1ω7c and/or C16:1ω6c) and a lower level of C17:0 cyclo. Their physiological and biochemical profiles differentiated them from E. radicincitans by being positive for methyl red test, ornithine decarboxylase and utilization of putrescine, D-arabitol, L-fucose and methyl α-D-glucoside and being negative for arginine dihydrolase, and differentiated them from E. oryzae by being positive for aesculin hydrolysis and utilization of putrescine, D-arabitol and L-rhamnose and being negative for arginine dihydrolase, lysine decarboxylase and utilization of mucate. The five strains therefore represent a novel species, for which the name Enterobacter sacchari sp. nov. is proposed, with the type strain SP1T (=CGMCC 1.12102T=LMG 26783T).

Both pathogenic and beneficial bacteria to plants have been affiliated with several species of the genus Enterobacter in the family Enterobacteriaceae (Chung et al., 1993; Kämpfer et al., 2005; Madhaiyan et al., 2010; Peng et al., 2009; Zhu et al., 2011). Beneficial nitrogen-fixing bacteria belonging to several genera of the family Enterobacteriaceae have been found and have been shown to fix N2 in association with sugar cane (Saccharum officinarum L.) worldwide and to promote sugar cane growth (Govindarajan et al., 2007; Lin et al., 2012; Loiret et al., 2004; Magnani et al., 2010; Mehnaz et al., 2010; Mirza et al., 2001; Taulé et al., 2012). Strain SP1T was isolated from a stem of sugar cane cultivar GT11 grown in Nanning, Guangxi, China in 1994. Sugar cane internodes were surface-sterilized by immersing into

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains SP1T, HH148, NN143, NN144 and NN208 are J0001784, HQ204281, HQ204313, HQ204314 and HQ204315, for rpoB sequences are J0001786, J0001787, J0001788, J0001789 and J0001790 and for nifH sequences are J0001785, HQ204226, HQ204258, HQ204259 and HQ204260, respectively.

Five supplementary figures and two supplementary tables are available with the online version of this paper.
70 % ethanol for 2 min and 5 % (w/v) chloramine T solution for 5 min; the stem rind was removed and the inner tissues were cut into small pieces with a sterile scalpel; inner tissues were squashed in a sterile 5 % (w/v) cane sugar solution and 100 μl aliquots were incubated with a semisolid LGI medium containing 10 % (w/v) cane sugar (Cavalcante & Döbereiner, 1988) at 28 °C for 5 days. Bacterial pellicles on the media were streaked on LGI agar. Strain SP1T formed viscous colonies on LGI agar and reduced acetylene to ethylene at a rate of 140–150 nmol C4H4 h⁻¹ ml⁻¹ culture in liquid LGI supplemented with 0.002 % (w/v) yeast extract at an OD600 value of 1.0. Strain HX148 was isolated from rhizosphere soil of sugarcane cultivar ROC22 grown in Hengxian, Guangxi in 2008. Strains NN143, NN144 and NN208 were isolated from surface-sterilized roots of ROC22 plants grown in Nanning in 2008. Strains HX148, NN143, NN144 and NN208 were isolated on modified Ashby’s agar and showed nitrogenase activities (Lin et al., 2012). Partial 16S rRNA sequences encoding the iron protein of nitrogenase were obtained from the strains by PCR amplification as previously described (Lin et al., 2012).

Colonies of the five strains were light yellow, smooth, circular with entire margins and convex on nutrient agar (Difco) grown for 24 h. Colonies did not secret diffusible green fluorescent pigment on King’s medium B and were 0.3–0.9 mm wide and 0.9–1.8 mm long rods with peritrichous flagella (Fig. S1, available in IJSEM Online).

The 16S rRNA gene and rpoB sequences of the five strains were amplified using the primers 27F/1492R and CM7/CM31b (Lane, 1991; Mollet et al., 1997). The PCR products (approximately 1500 bp and 900 bp, respectively) were cloned, sequenced (Invitrogen) and BLASTed (Altschul et al., 1997). The sequences showed highest sequence similarity with those from bacteria belonging to the genus Enterobacter (data not shown).

The DNA G+C contents of the five strains were determined using HPLC (Mesbah et al., 1989) and were 55.0±0.5 mol% (means of three independent analyses of the same DNA sample from each strain). The values are consistent with the DNA G+C contents of members in the genus Enterobacter (Hoffmann et al., 2005; Madhaiyan et al., 2010; Peng et al., 2009; Stephan et al., 2008).

The obtained 16S rRNA gene and rpoB sequences of the five strains were phylogenetically analysed with those of the type strains of species of the genus Enterobacter and the type strains of type species of other genera in the family Enterobacteriaceae. Bootstrap values (≥50 %) based on 1000 replications are shown at branch nodes. Bar, 0.01 substitutions per nucleotide position.

Fig. 1. Phylogenetic tree constructed using the neighbour-joining algorithm based on 16S rRNA gene sequences of strains SP1T, HX148, NN143, NN144 and NN208, the type strains of species of the genus Enterobacter and the type strains of type species of other genera in the family Enterobacteriaceae. Bootstrap values (≥50 %) based on 1000 replications are shown at branch nodes. Bar, 0.01 substitutions per nucleotide position.

The 16S rRNA gene sequences of the five strains showed 98.90–99.43 % similarity to each other and formed a monophyletic group with E. radicicinits DSM 16656T, E. oryzae LMG 24251T, E. arachidis NCIMB 14469T, E. cowanii CIP 107300T and the type species E. cloacae ATCC 13047T (Fig. 1). The five strains grouped most closely with a cluster containing E. radicicinits and E. oryzae (Fig. 1). The sequence similarity of strain SP1T to E. radicicinits DSM 16656T and E. oryzae LMG 24251T was 98.26 % and 98.15 % and to the type species E. cloacae ATCC 13047T was 98.16 %. Stackebrandt & Ebers (2006) showed that <98.7–99 % 16S rRNA gene sequence similarity indicates genomic uniqueness. Therefore, the five strains may belong to a novel species of the genus Enterobacter.

The rpoB sequences of the five strains showed 98.49–99.01 % similarity to each other and formed a monophyletic group with E. radicicinits DSM 16656T, E. oryzae LMG 24251T, E. cowanii CIP 107300T, E. helveticus LMG 23732T, E. turicensis LMG 23730T and E. arachidis NCIMB 14469T but not with the type species E. cloacae ATCC 13047T (Fig. S2). Previous phylogenetic analyses of rpoB sequences have shown that E. radicicinits, E. oryzae, E. arachidis, E. cowanii, E. helveticus and E. turicensis but not with E. cloacae belong to a monophyletic group (Kämpfer et al., 2005; Madhaiyan et al., 2010; Peng et al., 2009). The five strains also grouped most closely with a cluster containing E. radicicinits DSM 16656T and E. oryzae LMG 24251T (Fig. S2). The sequence similarities of strain SP1T to E. radicicinits DSM 16656T and E. oryzae LMG 24251T were 94.13 and 92.46 % respectively, which are lower than the intraspecies similarity range of 98–100 % found for rpoB in the family Enterobacteriaceae (Mollet et al., 1997). Therefore, the rpoB sequence similarities and phylogenetic analysis give support to the five strains belonging to a novel species of the genus Enterobacter.

The degree of genomic DNA–DNA relatedness between strain SP1T and the other four isolates or the type strains of their closest neighbours was determined using the fluorimetric method described by Gonzalez & Saiz-Jimenez (2005) based on thermal denaturation (De Ley et al., 1970). Double-stranded DNA was selectively bound to fluorescent
SYBR Green I dye (Invitrogen) and its thermal denaturation was followed by quantifying a decrease in fluorescence at increasing temperatures with an ABI Prism 7500 Realtime PCR System (Applied Biosystems) (Zhu et al., 2011). Melting curves were generated with the accompanying software of the PCR system and are presented in Fig. S3. The melting temperature or the thermal denaturation midpoint ($T_m$) and $\Delta T_m$ (the difference between the $T_m$ of the genomic DNA of the reference strain and the $T_m$ of the hybridized DNA) (Gonzalez & Saiz-Jimenez, 2005) are presented in Table 1. The $\Delta T_m$ between strain SPI$^T$ and the other four strains ranged from 0.9 to 2.1 °C, and between strain SPI$^T$ and E. radicincitans DSM 16656$^T$ and E. oryzae LMG 24251$^T$ were 7.1 and 18.2 °C, respectively. Because the definition of a species generally includes strains with $\Delta T_m \leq 5$ °C (Wayne et al., 1987), the five strains belong to a novel species of the genus Enterobacter.

The genotypes of the five strains, E. radicincitans DSM 16656$^T$ and E. oryzae LMG 24251$^T$ were analysed using enterobacterial repetitive intergenic consensus (ERIC)-PCR as described by Fernández-Baca et al. (2001). The ERIC-PCR patterns of the five strains were closely similar to each other and indicated a distinct genotype different from E. radicincitans and E. oryzae (Fig. S4).

The protein profiles of the five strains, E. radicincitans DSM 16656$^T$ and E. oryzae LMG 24251$^T$ were analysed by being positive for aesculin hydrolysis and utilization of L-fucose, D-arabitol and L-rhamnose and negative for arginine dihydrolase, and from E. oryzae with an approximate similarity of 75% or greater to each other, constituted a distinct branch of the dendrogram and were distinguished from E. radicincitans and E. oryzae with an approximate similarity of 55 and 40% (Fig. S5). Therefore, the MALDI-TOF MS analysis differentiated the protein profiles of the five strains from those of E. radicincitans and E. oryzae and supports the finding that the five strains belong to a novel species of the genus Enterobacter.

Cell fatty acid compositions of the five strains, E. radicincitans DSM 16656$^T$ and E. oryzae LMG 24251$^T$ were analysed as previously described (Zhu et al., 2011). The total cellular fatty acids were extracted at stationary phase from bacterial cells grown at 28 °C on tryptic soy agar (Difco) for 48 h. The major fatty acids detected in the five strains were C$\_16:0$ (33.88–37.08%), C$\_18:1\omega7c$ (12.33–16.2%), C$\_17:0$ cyclo (10.2–12.27%) and summed feature 2 (C$\_16:1\omega7c$ and/or C$\_16:1\omega6c$; 16.49–19.46%) (Table S2). This fatty acid composition is typical of members of the genus Enterobacter (Hoffmann, et al., 2005; Kämpfer, et al., 2005; Peng, et al., 2009; Stephan et al., 2008; Zhu, et al., 2011). The five strains contained a higher level of summed feature 2 and a lower level of C$\_17:0$ cyclo compared with their closest neighbours E. radicincitans DSM 16656$^T$ and E. oryzae LMG 24251$^T$ (Table S2).

The physiological, biochemical and carbon substrate utilization profiles of the five strains were determined using the API 20E kit (bioMérieux) and GEN III MicroPlates (Biolog). The five strains showed characteristics in accordance with the genus Enterobacter, such as being positive for motility test and Voges–Proskauer test and negative for H$_2$S production from thiosulfate and utilization of L-alanine, cellobiose, citrate, d-fructose, D-galactose, d-glucose, glycerol, maltose, d-mannitol and D-mannose (Grimont & Grimont, 2006; Hoffmann, et al., 2005). They were differentiated from E. cloacae by being positive in methyl red test and utilization of d-arabitol and L-fucose, from E. radicincitans by being positive for methyl red test, ornithine decarboxylase and utilization of putrescine, D-arabitol, L-fucose and $\alpha$-methyl-D-glucoside and negative for arginine dihydrolase, and from E. oryzae by being positive for aesculin hydrolysis and utilization of putrescine, D-arabitol and L-rhamnose and negative for

Table 1. DNA–DNA hybridization melting temperature ($T_m$) and differences in $T_m$ ($\Delta T_m$) between strain SPI$^T$ and its closest relatives

<table>
<thead>
<tr>
<th>Strain</th>
<th>$T_m$ (°C)</th>
<th>$\Delta T_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPI$^T$</td>
<td>76.7 ± 0.2</td>
<td>0</td>
</tr>
<tr>
<td>HX148</td>
<td>75.8 ± 0.3</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td>NN143</td>
<td>75.4 ± 0.2</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>NN208</td>
<td>74.2 ± 0.3</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>NN144</td>
<td>75.5 ± 0.4</td>
<td>1.2 ± 0.6</td>
</tr>
<tr>
<td>E. oryzae LMG 24251$^T$</td>
<td>58.5 ± 0.7</td>
<td>18.2 ± 0.9</td>
</tr>
<tr>
<td>E. radicincitans DSM 16656$^T$</td>
<td>69.6 ± 0.3</td>
<td>7.1 ± 0.5</td>
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</tbody>
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were taken on an AUTOFLEX Analyser (Bruker Daltonics) using the linear positive ion extraction mode at a laser frequency of 50 Hz and within a mass range from 2000 to 20 000 Da (Decristophoris et al., 2011). Escherichia coli DH5$x$ was used as an external protein calibration mixture followed by the Bruker Test Standard (Figueras et al., 2011). Raw mass spectrum smooth, baseline correction and peak detection were performed using the corresponding programs installed in the MS system. Resulting protein mass spectra were exported to FLEX ANALYSIS (Bruker Daltonics) and analysed. The characteristic mass peaks (predominantly for ribosomal proteins) range of 2000 to 20 000 Da from all the strains are displayed in Table S1. The percentage similarity of identical mass peaks was calculated and used to generate dendrograms by the BIOTYPER system (Bruker Daltonics). The five strains clustered together with a similarity of 75% or greater to each other, constituted a distinct branch of the dendrogram and were distinguished from E. radicincitans and E. oryzae with an approximate similarity of 55 and 40% (Fig. S5). Therefore, the MALDI-TOF MS analysis differentiated the protein profiles of the five strains from those of E. radicincitans and E. oryzae and supports the finding that the five strains belong to a novel species of the genus Enterobacter.

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arginine dihydrolase, lysine decarboxylase and utilization of mucate (Table 2). Seventeen selected characteristics of the five strains are compared with the type strains of related species in the genus Enterobacter in Table 2.

The five strains represent a novel Enterobacter species, for which the name Enterobacter sacchari (type strain SP1$^{T}=$ CGMCC 1.12102$^{T}=$LMG 26783$^{T}$) is proposed.

**Description of Enterobacter sacchari sp. nov**

*Enterobacter sacchari* [sac. cha‘ri. N.L. gen. n. sacchari of Saccharum, isolated from Saccharum officinarum L. (sugar cane)].

The species description is based on five strains. Cells are motile rods (0.3–0.9 μm in width and 0.9–1.8 μm in length) with peritrichous flagella. Gram stain is negative. Colonies are cream on YDC agar and dark pink with translucent margins on TZC agar. Cells grow at pH 4–10 or in the presence of 0–4 % NaCl. Optimum growth occurs at 28–32 °C and pH 7. The major fatty acids are C$_{16:0}$, C$_{18:1}ω7c$, C$_{17:0}$ cyclo and summed feature 2 (C$_{16:1}ω7c$ and/or C$_{16:1}ω6c$). The DNA G+C content is 55.0 ± 0.5 mol%. With API 20E, positive for Voges–Proskauer and methyl red tests, ornithine decarboxylase, asacculin hydrolysis and 1-O-methyl-α-galactopyranoside but negative for indole production, H$_2$S production, oxidase, arginine dihydrolyase, lysine decarboxylase and utilization of mucate. With GEN III MicroPlate, positive for 1 % sodium lactate, fusidic acid, troleandomycin, rifampicin, lincomycin, guanidine HCl, niaproof 4, vancomycin, tetrazolium violet, tetrazolium blue, lithium chloride, aztreonam and sodium butyrate but negative for D-serine, minocycline, nalidixic acid, potassium tellurite and sodium bromate. The following compounds are utilized as a sole carbon source: citrate, sucrose, D-sorbitol, putrescine, α-methyl-D-glucoside, melibiose, D-arabitol, L-rhamnose, raffinose, α-D-glucose, gelatin, pectin, dextrin, α-lactose, D-mannose, D-mannitol, glycy1-L-proline, α-galacturonic acid, methyl pyruvate, maltose, D-fructose, L-alanine, L-galactonic acid lactone, β-methyl-D-glucoside, D-galactose, myo-inositol, D-glucic acid, L-lactic acid, cellubiose, D-salicin, 3-methyl glucose, glycy1-L-proline, D-aspartic acid, D-glucuronic acid, citric acid, gentiobiose, N-acetyl-D-glucosamine, D-fucose, D-glucose-6-PO$_4$, L-glutamic acid, N-acetyl-β-D-mannosamine, L-fucose, D-fructose-6-PO$_4$, mucic acid, turanose, N-acetyl-D-galactosamine, L-malic acid, acetic acid, inosine, L-serine, D-saccaric acid and bromosuccinic acid. The following compounds are not utilized as a sole carbon source: p-hydroxy-phenylacetic acid, Tween 40, γ-aminobutyric acid, D-lactic acid methyl ester, L-arginine, β-hydroxy-DL-butryic acid, α-ketobutyric acid, glycuronamide, α-ketogluutaric acid, acetoacetic acid, L-histidine, D-malic acid, propionic acid, D-aspartic acid, L-pyroglutamic acid, quinic acid, L-malic acid, stachyose, N-acetyl-neuraminic acid, D-serine and formic acid.

The type strain is SP1$^{T}$ (=CGMCC 1.12102$^{T}=$LMG 26783$^{T}$), isolated from a stem of sugar cane (*Saccharum officinarum* L.) cultivar GT11 being collected from Nanning, Guangxi Province, China.

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**References**


