Azospirillum melinis sp. nov., a group of diazotrophs isolated from tropical molasses grass

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Fifteen bacterial strains isolated from molasses grass (Melinis minutiflora Beav.) were identified as nitrogen-fixers by using the acetylene-reduction assay and PCR amplification of nifH gene fragments. These strains were classified as a unique group by insertion sequence-PCR fingerprinting, SDS-PAGE protein patterns, DNA–DNA hybridization, 16S rRNA gene sequencing and morphological characterization. Phylogenetic analysis of the 16S rRNA gene indicated that these diazotrophic strains belonged to the genus Azospirillum and were closely related to Azospirillum lipoferum (with 97.8% similarity). In all the analyses, including in addition phenotypic characterization using BiolLog MicroPlates and comparison of cellular fatty acids, this novel group was found to be different from the most closely related species, Azospirillum lipoferum. Based on these data, a novel species, Azospirillum melinis sp. nov., is proposed for these endophytic diazotrophs of M. minutiflora, with TMCY 0552T (= CCBAU 5106001T = LMG 23364T = CGMCC 1.5340T) as the type strain.

INTRODUCTION

Association of nitrogen-fixing bacteria and herbaceous plants is a common phenomenon in nature. From this association, wild grasses can obtain nitrogen fixed by the bacteria and grow in nitrogen-deficient soils. Diverse endophytic diazotrophs have been isolated from maize, rice, sorghum, sugar cane, cameroon grass and other gramineous plants (Baldani et al., 1986, 1997; Olivares et al., 1996; Reis et al., 2004). Some of these plants could associate with a wide range of bacteria, such as in the case of Kollar grass (Leptochloa fusca (L.) Kunth), a pioneer plant grown on salt-affected, often flooded, low-fertility soils in the Punjab of Pakistan, which has been found to be associated with five nitrogen-fixing endophytic bacterial species (Reinhold-Hurek et al., 1993; Reinhold-Hurek & Hurek, 2000; Tan & Reinhold-Hurek, 2003).

To date, diverse nitrogen-fixing bacteria, including Azospirillum lipoferum, Azospirillum brasilense, Azospirillum halopraeferens, Azoarcus indigens, Azoarcus communis, Azovibrio restrictus, Azo-spira oryzae and Burkholderia tropica, have been isolated from the roots of numerous wild and cultivated grasses grown in tropical, subtropical and temperate regions all over the world (Kirchhof et al., 1997; Reinhold et al., 1986, 1987; Reinhold & Hurek, 1988; Reinhold-Hurek et al., 1993; Reinhold-Hurek & Hurek, 2000; Reis et al., 2004; Tarrand et al., 1978). Among these bacteria, Azospirillum species have been isolated from roots of numerous wild and cultivated grasses, cereals, food crops and soils in various regions. Based on their microaerophilic and nitrogen-fixing characteristics, semi-solid nitrogen-free medium (Döbereiner, 1980) was the key to the successful isolation of these bacteria. At present, eight species have been described within this genus, including the two original species, Azospirillum lipoferum and Azospirillum brasilense (Tarrand et al., 1978), and the later-described species Azospirillum amazonense (Magalhães et al., 1983), Azospirillum halopraeferens (Reinhold et al., 1987), Azospirillum irakense (Khammas et al., 1989), Azospirillum lurgimobile (Sly & Stackebrandt, 1999), Azospirillum doebereinerae (Eckert et al., 2001) and Azospirillum oryzae (Xie & Yokota, 2005). It has been reported that Azospirillum strains can enhance the growth of plants by the production of phytohormones (Bashan & Holguin, 1997). They are also possible
suppliers of nitrogen to their host plants (Döbereiner, 1983; Okon, 1985).

Molasses grass (Melinis minutiflora Beauv.) is a well-known pasture and fodder grass in various tropical countries. The leaves are covered with hairs that exude a sticky secretion and contain a volatile oil that gives the grass a strong and distinctive odour. The odour of fresh grass is believed to repel insects, snakes and ticks. The entire plant has insecticidal properties and has been cultivated in Brazil and Central Africa for this purpose (Thompson et al., 1978). Khan et al. (1997) reported that molasses grass planted in corn fields significantly reduces crop damage by repelling destructive moths and summoning the pests’ insect enemies. Although molasses grass has a foul odour at certain stages, dried grass is free of this odour and serves as foliage. Molasses grass is also drought-resistant and tolerant of soils of fairly low fertility, and has been used as a pioneer species after clearing of poor soils. These features indicate that molasses grass may have obtained nitrogen as a nutrient from some kind of nitrogen-fixing bacteria associated with it, although no relevant information is available at present.

In the present study, we isolated and characterized some nitrogen-fixing bacteria from molasses grass grown as pioneer plants in poor soils in a tropical region of China. The aims were to verify the association of this grass with diazotrophs and to classify the nitrogen-fixing bacteria isolated from the plants.

METHODS

Bacterial isolation, quantification and growth media. Stems and roots of molasses grass were collected from Zhuhai city in Guangdong Province, China. Plant roots and stems were washed with sterilized distilled water and rinsed with 86 % ethanol for 30 s, then submerged in 0·1 % HgCl₂ for 5 min and washed six times with sterilized distilled water. The sterilized samples were then ground in 0·9 % NaCl solution (1: 5, v/w). Various carbon sources in semi-solid LGI medium (Cavalcante & Döbereiner, 1988; Martínez et al., 2003; Salles et al., 2000) and semi-solid NFB medium (Eckert et al., 2001) were used to screen for the maximum number of putatively endophytic diazotrophs. Small vessels (approx. 10 ml) containing 5 ml NFB or LGI semi-solid nitrogen-free medium were inoculated with serial dilutions of root extracts. With the aim of estimating the bacterial content in the plant tissues by using the most-probable number (MPN) method, each dilution was inoculated into three vessels. After incubation at 97 °C for 3 min, 30 cycles at 95 °C for 50 s, 57 °C for 35 s and 72 °C for 8 s, and final extension at 72 °C for 5 min. The products were separated by electrophoresis in 1·2 % agarose gels, which were stained with ethidium bromide (0·5 mg l⁻¹) for 30 min. Patterns were visualized and photographed using a digital camera (GIS-1000; Tanon) under UV light.

Insertion sequence-based PCR (IS-PCR) fingerprinting. This was performed to evaluate the genotypic diversity of the isolates. The IS-primer J3 (5'-GCTCAGGTTGAGTGCTTGG-3') was chosen according to previous studies (Adhikari et al., 1999; Weiner et al., 2004). All amplifications were carried out in a final volume of 50 µl and were performed with a programmable thermal cycler (model PTC-100; MJ Research). The reaction mixtures for IS-PCR contained (final concentrations): 50 pmol primer, 30 ng template DNA extracted from the isolate (Tan et al., 2001b), 200 µM of each dNTP (Sigma) and 3 U Taq polymerase. PCR began with a denaturation step at 97 °C for 5 min followed by 30 cycles of denaturation at 97 °C for 50 s, annealing at 60 °C for 50 s and extension at 72 °C for 2 min each. The final extension cycle was at 72 °C for 5 min. After completion of the PCR, samples were stored at 4 °C until gel electrophoresis. The PCR products (10 µl aliquots) were separated in 6 % non-denatured polyacrylamide/bisacrylamide gels (19: 1) in 0·5 × TBE buffer (89 mM Tris, 89 mM boric acid and 0·5 M EDTA, pH 8·0). The gels were stained with ethidium bromide and photographed as described for PCR of nifH. A dendrogram was created from a matrix of band matching by using the unweighted pair group method with arithmetic means (UPGMA) (Adhikari et al., 1999; Weiner et al., 2004).

SDS-PAGE of whole-cell protein patterns. Methods of cell preparation, protein extraction and data analysis described previously (Tan et al., 2001a) were used to estimate the diversity of the bacterial isolates.

16S rRNA gene sequencing and phylogenetic analysis. Fragments of the 16S rRNA gene were amplified from genomic DNA of the isolates by using the forward primer 25F (5'-AACCTCAAGAGGTGTGATTCGTC-3') and reverse primer 1492R (5'-TCGTCAGCAGTTGTGATCT-3'), as described by Hurek et al. (1997). The purified PCR products were sequenced directly as reported by Hurek et al. (1997), by using the sequencing primers 35F (5'-CTAAAAAGGTTTAGTGGCTGCTAGTTG-3'), 342F (5'-TCCTACGGGAGAGCAGG-3') and 930F (5'-GTTTAAATTCTTAAACAGTGTGAC-3'). The sequences determined, together with some related sequences selected from GenBank with the BLAST program, were aligned by using the RDP program (Maidak et al., 1999). Alignment gaps and ambiguous bases were excluded from the calculation of similarity. The tree topology was inferred by using the neighbour-joining method (Saitou &
Nei, 1987) and the phylogenetic tree was visualized and bootstrapped by using the TREECON software package (Van de Peer & De Wachter, 1994).

DNA base composition and DNA–DNA hybridization. DNA was isolated and purified as described by Marmur (1961) and the DNA base composition was determined spectrophotometrically (De Ley et al., 1970). DNA from Escherichia coli K-12 was used as a standard for estimation of G + C content. DNA–DNA relatedness was determined by using the initial renaturation rate method (De Ley et al., 1970) in 2 × SSC, as modified by Tan et al. (2001c).

Cellular fatty acid analysis. The bacteria were incubated for 36 h in NFB medium as mentioned above. Methods described by Sasser (1990) were used to harvest the cells, extract the fatty acids and to transform the fatty acids into methyl esters. Fatty acid analysis was performed using an SP-2100 (Tanon) gas chromatograph equipped with a fused silica capillary column, SE-34 (20 m × 0.22 mm i.d.). Cellular fatty acid profiles were analysed by comparing the quantity of each compound determined as a percentage of the total fatty acids and the different kinds of fatty acids.

Physiological characterization using the Biolog system. A representative strain, TMCY 0552T, and Azospirillum lipoferum DSM 1691T were comparatively characterized by using Biolog GN2 MicroPlates (Hayward). Overnight cultures were used to inoculate the GN2 MicroPlates, according to the manufacturer’s instructions. The GN2 MicroPlates were incubated in the dark at 28 °C for 24 h and the development of colour in each test well was measured using an automated plate reader (Benchmark; Bio-Rad). Increases in absorbance were recorded, relative to a negative control. Similarity between the two strains was calculated using the formula $S_{ax}=2ad/(2a+b+c)$, where $S_{ax}$ is the simple matching coefficient, $a$ is the number of positive characteristics shared by the two strains, and $b$ and $c$ the number of positive characteristics unique to each strain.

Perchlorate reduction. As a phenotypic characteristic, perchlorate reduction was determined for all the isolates and related reference strains by using anaerobic culturing techniques as described by Bruce et al. (1999), Coates et al. (1999) and Tan & Reinhold-Hurek (2003).

RESULTS

Bacterial isolation and ARA

A total of 15 endophytic bacteria were isolated from the root and stem samples of molasses grass in this study and numbered TMCY 41, TMCY 055, TMCY 243, TMCY 244, TMCY 255, TMCY 0256, TMCY 023, TMCY 0551, TMCY 0832, TMCY 066, TMCY 225, TMCY 0552T, TMCY 0831, TMCY 24 and TMCY 025. These isolates were obtained from both NFB and LGI media with dl-malic acid and sucrose as the sole carbon source, respectively. The MPN varied from $6 \times 10^5$ to $2 \times 10^7$ per gram fresh plant tissue. The bacteria were all Gram-negative, facultatively anaerobic, non-motile, straight or curved rods. Acetylene reduction was detected in all isolates 4 h after the acetylene injection. Strain TMCY 0552T was able to reduce acetylene to ethylene with a mean value of 90 nmol ethylene h$^{-1}$ (10$^8$ cells)$^{-1}$ at 28 °C, without addition of yeast extract. This amount is comparable with values obtained for other Azospirillum species (Eckert et al., 2001; Reinhold et al., 1987). In the PCR of the nifH gene, the expected fragment of about 360 bp was obtained from all isolates and the positive control strain Azospirillum brasilense Sp 7T (Fig. 1), further confirming the nitrogen-fixing capacity of these isolates.

IS-PCR fingerprinting

All 15 diazotrophic isolates showed very similar patterns, but were different from that for the reference strain Azospirillum lipoferum DSM 1691T (see Supplementary Fig. S1 in IJSEM Online). Most of the amplified fragments were between 240 and 1700 bp. All the isolates and the reference strain showed identical patterns between 339 and 1700 bp. The novel isolates had three or four fragments between 240 and 339 bp, whereas the reference strain Azospirillum lipoferum DSM 1691T showed only one fragment of 339 bp. The presence of these small fragments resulted in the novel isolates clustering together and they could be clearly differentiated from Azospirillum lipoferum in the clustering analysis (Fig. 2). The novel diazotrophic isolates were grouped together at a similarity level of 78% and were separated from Azospirillum lipoferum DSM 1691T at a level of 67% similarity.

SDS-PAGE of whole-cell proteins

All 15 diazotrophic isolates had almost identical protein patterns (data not shown). Combined with the results of IS-PCR fingerprinting, five diazotrophic isolates were selected as representatives for further comparison with Azospirillum lipoferum DSM 1691T by SDS-PAGE (Fig. 3). The five diazotrophic isolates had similar protein patterns, and with

Fig. 1. Electrophoretic patterns of PCR products of the nifH gene showing that all of the 15 strains isolated from molasses grass harbour the nitrogen-fixing gene. Lanes: 1, genomic DNA from Azospirillum brasilense Sp 7T (positive control); 2–16, TMCY 0256, TMCY 41, TMCY 0552T, TMCY 0551, TMCY 023, TMCY 055, TMCY 025, TMCY 225, TMCY 24, TMCY 0831, TMCY 025, TMCY 24 and TMCY 0832, respectively; M, lambda DNA/PstI marker.
at least eight protein bands that were different from those of *Azospirillum lipoferum* DSM 1691$^\mathrm{T}$ (Fig. 3).

**Sequencing of the 16S rRNA gene and phylogenetic analysis**

Four strains, TMCY 41, TMCY 0552$^\mathrm{T}$, TMCY 243 and TMCY 244, which were randomly selected to represent the diazotrophs isolated from molasses grass, were sequenced. They had almost identical 16S rRNA gene sequences. In the reconstructed phylogenetic tree (Fig. 4), these four strains formed a group within the genus *Azospirillum*, indicating that they were a monophyletic group. These four strains were more closely related to *Azospirillum lipoferum* DSM 1691$^\mathrm{T}$ (97.5 ± 0.6 % similarity) than to *Azospirillum largimobile* ACM 2041$^\mathrm{T}$ and other species (similarity of 97.4 ± 0.6 % or less).

**DNA base composition and DNA–DNA hybridization**

The G+C content of the genomic DNA of strain TMCY 0552$^\mathrm{T}$ was 68.7 ± 0.6 mol%, which is in the range for the genus *Azospirillum* (64–71 mol%). Results of the DNA–DNA hybridization are presented in Table 1. DNA–DNA relatedness among the strains isolated from molasses grass varied from 81 to 95 %, with a mean of 88.7 %, indicating that they represented the same genomic species. In contrast, the DNA–DNA relatedness was 54–57 %, with a mean of 55.6 %, among *Azospirillum lipoferum* DSM 1691$^\mathrm{T}$ and three strains isolated from molasses grass. The corresponding data were 30–34 % (mean 32 %) among *Azospirillum brasilense* Sp 7$^\mathrm{T}$ and three strains isolated from molasses grass.

**Cellular fatty acid analysis**

The cellular fatty acids of strain TMCY 0552$^\mathrm{T}$, representing the diazotrophs isolated from molasses grass, and *Azospirillum lipoferum* DSM 1691$^\mathrm{T}$ had four common main components at retention times of 9-0, 9-8, 12-6 and 20-3 min (see Supplementary Fig. S2 in IJSEM Online). These main common components made up 94 and 84.6 %, respectively, of the detected components of TMCY 0552$^\mathrm{T}$ and *Azospirillum lipoferum* DSM 1691$^\mathrm{T}$ (Table 2). Strain TMCY 0552$^\mathrm{T}$ had two components at retention times of 8 and 10-8 min that were absent from *Azospirillum lipoferum* DSM 1691$^\mathrm{T}$. In addition, minor components at retention times of 13-1, 18-0, 20-5, 21-4 and 22-4 min were clear in *Azospirillum lipoferum* DSM 1691$^\mathrm{T}$, but appeared as trace peaks (less than 2 %) in TMCY 0552$^\mathrm{T}$. The relative abundance of each compound was also different in the two strains.

**Physiological characterization**

Using the Biolog MicroPlates, we found that strain TMCY 0552$^\mathrm{T}$ and *Azospirillum lipoferum* DSM 1691$^\mathrm{T}$ both could utilize 42 compounds, but both could not use 17 compounds. TMCY 0552$^\mathrm{T}$ could utilize 31 compounds, but not five other compounds that could be used by *Azospirillum lipoferum* (see Supplementary Table S1 in IJSEM Online). The $S_{\text{SM}}$ between strain TMCY 0552$^\mathrm{T}$ and *Azospirillum lipoferum* DSM 1691$^\mathrm{T}$ was 70 %.

**Perchlorate reduction**

As reported previously (Tan & Reinhold-Hurek, 2003), some endophytic diazotrophs have the ability of dissimilatory (per)chloride reduction. In this study, none of the diazotrophic strains isolated from molasses grass could grow...
with perchlorate as the terminal electron acceptor, whereas *Azospirillum lipoferum* DSM 1691<sup>T</sup> could.

**DISCUSSION**

Endophytic diazotrophic bacteria have been isolated from a wide range of plants grown in poor soils and have been shown to be able to supply fixed nitrogen to their host plants. The vigorous growth of molasses grass in non-fertilized soils may also be related to this association. In the present study, we isolated 15 bacterial strains from surface-sterilized roots and stems by using nitrogen-free semi-solid media. All of the strains were identified as diazotrophic bacteria based upon the results of ARA and PCR of the *nifH* gene. In an inoculation test, these strains could promote the growth of molasses grass and increase tolerance to acid soil

<table>
<thead>
<tr>
<th>Strain</th>
<th>TM CY 243</th>
<th>TM CY 244</th>
<th>TM CY 41</th>
<th>TM CY 066</th>
<th>TM CY 0831</th>
<th>TM CY 24</th>
<th>TM CY 0832</th>
<th>TM CY 0552&lt;sup&gt;T&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Azospirillum lipoferum</em> DSM 1691&lt;sup&gt;T&lt;/sup&gt;</td>
<td>90</td>
<td>86</td>
<td>95</td>
<td>84</td>
<td>92</td>
<td>93</td>
<td>81</td>
<td>100</td>
</tr>
<tr>
<td><em>Azospirillum brasilense</em> Sp 7&lt;sup&gt;T&lt;/sup&gt;</td>
<td>57</td>
<td>54</td>
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<tr>
<td><em>Alcaligenes faecalis</em> subsp. <em>parafaecalis</em> G&lt;sup&gt;+&lt;/sup&gt; (AJ242966)</td>
<td>32</td>
<td>30</td>
<td>34</td>
<td></td>
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*E. coli* K-12 was used as a negative control.
Table 2. Comparison of the cellular fatty acid composition of Azospirillum melinis sp. nov. TMCY 0552T and Azospirillum lipoforum DSM 1691T, determined as methyl esters by gas chromatography

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Strain</th>
<th>Retention time (min)</th>
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<tbody>
<tr>
<td></td>
<td>TMCY 0552T</td>
<td>DSM 1691T</td>
</tr>
<tr>
<td>Main common</td>
<td>71-4</td>
<td>43-6</td>
</tr>
<tr>
<td></td>
<td>4-8</td>
<td>7-7</td>
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<tr>
<td></td>
<td>5-8</td>
<td>16-7</td>
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<tr>
<td></td>
<td>12-0</td>
<td>16-6</td>
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<tr>
<td>Total</td>
<td>94</td>
<td>84-6</td>
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<tr>
<td>Different</td>
<td>2-7</td>
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<td>4-1</td>
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<td>tr</td>
<td>2-4</td>
</tr>
<tr>
<td>Total</td>
<td>6-0</td>
<td>15-4</td>
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</table>

and they were reisolated from inoculated plants (data not shown). According to the concept of Hallmann et al. (1997), the diazotrophic bacteria isolated from molasses grass could be defined as endophytes, because they were isolated from surface-disinfected tissues and did not visibly harm the plant. The density of these bacteria was also in the range for endophytic bacteria [$10^7$–$10^8$ (g fresh tissue)$^{-1}$] (Hallmann et al., 1997; McInroy & Kloeper, 1995). These results demonstrated that the endophytic diazotrophic strains are potential biofertilizers for molasses grass, similar to some Azospirillum lipoforum strains (Vessey, 2003).

At present, the taxonomy of Azospirillum species is mainly based on a polyphasic approach including 16S rRNA gene sequencing, DNA–DNA hybridization, fatty acid composition, DNA G+C content and phenotypic characterization (Eckert et al., 2001; Sly & Stackebrandt, 1999; Xie & Yokota, 2005). This polyphasic approach is necessary because the consensus among different analyses can offer stable classification results, whereas a single analysis may be not able to differentiate the species correctly. For example, Schenk & Werner (1988) reported that Azospirillum lipoforum and Azospirillum brasilense had similar fatty acid profiles, and therefore it was difficult to distinguish these two long-established species by using fatty acid profiles. In the present study, high similarity was observed among the 15 diazotrophic strains isolated from molasses grass in SDS-PAGE of proteins, IS-PCR fingerprinting patterns, 16S rRNA gene sequencing and DNA–DNA hybridization, as well as in cellular and colony morphology. These data clearly indicated that the strains represented the same species within the genus Azospirillum. The small differences among them in IS-PCR fingerprinting and SDS-PAGE of whole-cell proteins showed that they were from a diverse population. The 97-5 % and lower similarities of the 16S rRNA gene sequence to other defined Azospirillum species, the low-to-medium DNA–DNA relatedness with Azospirillum lipoforum and Azospirillum brasilense, and the differences in IS-PCR fingerprinting, SDS-PAGE of proteins, lipid acids and Biolog MicroPlate tests from the most closely related species, Azospirillum lipoforum, indicated that the new strains were distinct from this recognized species. In addition, the group of new strains could also be distinguished from other recognized Azospirillum species (Table 3). Taking together all the results and the current definition of bacterial species, we propose that the novel group of endophytic diazotrophic bacteria isolated from molasses grass represents a novel species, Azospirillum melinis sp. nov.

In previous studies, nitrogen-fixing strains belonging to the genera Azospirillum, Azotobacter, Bacillus, Derxia, Enterobacter, Burkholderia, Herbaspirillum and Erwienia were isolated from sugar cane (Boddye et al., 2003). Kallar grass as a wild plant (Reinhold-Hurek et al., 1993; Reinhold-Hurek & Hurek, 2000; Tan & Reinhold-Hurek, 2003) and rice as a cultivated plant (Engelhard et al., 2005) also harboured various species of the genus Azotobacter. Compared with previously reported results, molasses grass showed a specific association with Azospirillum melinis sp. nov. Although various culture media and aerobic and anaerobic incubation conditions were used, only strains representing Azospirillum melinis were isolated from molasses grass in this study. As there is no related information available, we could only hypothesize that this limited diversity might be linked to the specificity of the association between molasses grass and its endophytes, or to the soil characteristics. More studies are needed to verify these hypotheses.

Description of Azospirillum melinis sp. nov.

Azospirillum melinis (me.lin’is. N.L. fem. n. melinis genus name of stinkgrass, Melinis minutiflora Beauv.; N.L. gen. n. melinis from stinkgrass, referring to its frequent occurrence in association with molasses grass).

Cells are straight or slightly curved rods, measuring 0.7–0.8 × 1.0–1.5 μm. Gram-negative and non-motile. Facultatively anaerobic and chemo-organotrophic. Colonies on NFB medium are circular, convex and translucent, with a diameter of 3 mm within 3 days at 28 °C. Growth occurs at 5–37 °C (optimum 20–33 °C). pH range for growth is between 4 and 8. Growth is inhibited by > 5 % NaCl, but at concentrations <3 % shows ARA activity. Arabinose, D-fructose, gluconate, glycerol, malate, mannitol, maltose and sorbitol can be used as sole carbon sources. Does not grow with perchlorate as the terminal electron acceptor under anaerobic conditions. Isolated at a high frequency as endophytes from molasses grass. The G+C content of genomic DNA of the type strain is 68.7 mol%. The closest phylogenetic related species, according to 16S rRNA gene sequence data, are

| Fatty acid Strain Retention time (min) |
|----------------|----------------|----------------|
|                | TMCY 0552T | DSM 1691T |
| Main common    | 71-4     | 43-6     | 9-0    |
|                | 4-8      | 7-7      | 9-8    |
|                | 5-8      | 16-7     | 12-6   |
|                | 12-0     | 16-6     | 20-3   |
| Total          | 94       | 84-6     |
| Different      | 2-7      | —        | 8      |
|                | 3-3      | —        | 10-8   |
|                | tr       | 3-8      | 13-1   |
|                | tr       | 2-4      | 18-0   |
|                | tr       | 2-7      | 20-5   |
|                | tr       | 4-1      | 21-4   |
|                | tr       | 2-4      | 22-4   |
| Total          | 6-0      | 15-4     |
Table 3. Characteristics that differentiate *Azospirillum melinis* sp. nov. from other *Azospirillum* species

<table>
<thead>
<tr>
<th>Characteristic</th>
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<tr>
<td>Biotin requirement</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<td>Growth in 3 % NaCl</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>V</td>
<td>–</td>
<td>+</td>
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<td>Carbon source:</td>
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<tr>
<td>N-Acetylglucosamine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>–</td>
<td>V</td>
<td>ND</td>
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<td>L-Arabinoose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>+</td>
<td>V</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>D-Cellobiose</td>
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<td>–</td>
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<td>+</td>
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<td>DNA G + C content (mol%)</td>
<td>68-7</td>
<td>69–70</td>
<td>70</td>
<td>66-8</td>
<td>69–71</td>
<td>66–68</td>
<td>68–70</td>
<td>64–67</td>
<td>70-7</td>
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</table>

*Data for the type strains from Ben Dekhil et al. (1997) and Sly & Stackebrandt (1999).

Azospirillum lipoforum, Azospirillum oryzae and Azospirillum largimobile. The type strain is TMCY 0552T (=CCBAU 5106001T = LMG 23364T = CGMCC 1.5340T), which was isolated from subtropical molasses grass grown in China.

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(Beijerinck) comb. nov. and Azospirillum brasilense sp. nov. Can J Microbiol 24, 967–980.


