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Isolation and identification of *Gluconacetobacter azotocaptans* from corn rhizosphere ☆

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Abstract

Six acetic acid producing, diazotrophic bacteria were isolated from soil adhering to corn roots. These isolates were shown to be *Gluconacetobacter azotocaptans* and they shared some features with *G. johannae* and *G. diazotrophicus* but differed on the basis of colony morphology on different media, use of carbon sources and use of L-amino acids as a nitrogen source. The species identity was confirmed using 16S rDNA sequence analysis, PCR amplification of 16S rRNA gene with species-specific primers and amplified rDNA restriction analysis. This is the first report of the presence of this bacteria on corn plants.

Scope of the paper: This is the first report of the occurrence and association of *Gluconacetobacter azotocaptans* with corn.

Keywords: *Gluconacetobacter azotocaptans*; *Gluconacetobacter diazotrophicus*; *Gluconacetobacter johannae*; 16S rDNA sequence analysis; Species-specific PCR; Corn rhizosphere

Introduction

Gluconacetobacter (synonym *Acetobacter*) *diazotrophicus* is an endophytic bacterial species that occurs predominantly in vegetatively propagated plants. It has been isolated from numerous types of plant tissues including: the internal tissues of sucrose accumulating plants such as sugarcane, washed roots and aerial parts of *Pennisetum purpureum*, sweet potato stems and roots, rhizosphere soil of coffee plants as well as the surface sterilized stems and roots, inner tissues of *Eleusine coracana* and pine apple plants [6]. Recently, it was reported from wetland rice varieties, cultivated in India [7]. *G. diazotrophicus* was the only known nitrogen fixing species of this genus until Jimenez-Salgado et al. [4] isolated two other acetic acid producing, diazotrophic bacteria from the rhizosphere of coffee plants. The newly discovered diazotrophs share features with the genus *Gluconacetobacter* but differ from *G. diazotrophicus* with respect to morphological and biochemical traits as well as genetic and molecular features. Result from intensive taxonomic analysis by Fuentes-Ramirez et al. [3] led to their recommendation that these new

☆ *Note*: Nucleotide sequence data reported for isolate DS1 are available in the DDBJ/EMBL/GenBank databases under the Accession no. AY958232 for 16S rRNA gene sequence and Accession no: DQ073427 for amplification of 16S rDNA with species-specific primer.

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isolates be assigned to novel species within the family of the *Acetobacteraceae*. These isolates were named as *G. azotocaptans* and *G. johannae*.

In studies aimed at identifying the bacterial rhizosphere residents of corn plants grown in Southern Ontario, we isolated several bacterial strains from soil adhering to corn roots. Some of them were found to fix nitrogen and to produce acetic acid. We compared these isolates to the three characterized nitrogen fixing, acetic acid producing species of the genus *Gluconacetobacter* using biochemical tests and molecular detection methods described by Fuentes-Ramirez et al. [3]. The results showed that our isolates were *G. azotocaptans* and to our knowledge, this is the first description of this bacteria from corn rhizosphere.

Material and methods

Isolation of acetic acid producing nitrogen fixing bacteria

Two-month-old, field grown corn plants were collected from London and Delhi Research Stations, Agriculture and Agri-Food Canada, Ontario. The rhizosphere soil samples were directly inoculated into LGI medium [1]. In addition, rhizosphere soil samples collected from corn fields at the Delhi Research Station in year 2000 and 2004, and stored at 4 °C, were also used for bacterial isolation. Root samples were surface sterilized by using the method described by Coombs and Franco [2], sectioned into 1 cm fragments and inoculated into semi-solid LGI medium vials. After 2 weeks incubation at 30 °C, nitrogenase activity was detected by acetylene reduction assay (ARA) [5].

Pellicles of the ARA positive vials were sub-cultured in fresh LGI medium before they were streaked on LGI agar plates and incubated at 30 °C for 2 weeks. Acid producing yellow and yellowish orange colonies were selected for further analysis. Fourteen *Gluconacetobacter* strains were isolated and six of these were identified as *G. azotocaptans*. The isolates of *G. azotocaptans* and their sources are presented in Table 1.

Phenotypic characterization

Colony morphology and pigmentation was examined on LGI, potato agar with 10% cane sugar and GYC media [4] agar plates. Biochemical tests for compounds used as sole carbon source and L-amino acids as sole nitrogen source were carried out as mentioned by Fuentes-Ramirez et al. [3]. *Gluconacetobacter diazotrophicus* PAL 5 was provided by Agribiotics Inc., (Cambridge, Ontario, Canada) and used as reference strain for comparison. Results of biochemical tests for *G. azotocaptans* and *G. johannae* were as by Fuentes-Ramirez et al. [3].

16S rDNA sequence analysis

For DNA isolation, a single bacterial colony of isolate DS1 was inoculated into 5 ml of a rich medium ($g l^{-1}$; glucose, 2.0; glutamic acid, 1.5; peptone, 1.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; yeast extract, 2.0; pH, 6). After overnight growth at 30 °C, QIAGEN blood and cell culture DNA Midi kit was used for DNA isolation. The DNA was used as a template for PCR amplification of 16S rDNA. The primers and PCR conditions were those previously described by Mehnaz et al. [5]. The purified PCR product was directly sequenced on Applied Biosystems 3730 Analyzer. Amplification primers, as well as internal primers [8], were used for sequencing both strands of PCR products. The sequence was deposited in the GenBank (*Gluconacetobacter azotocaptans* DS1, Accession No. AY958232).

PCR with species-specific primers

PCR amplification of 16S rDNA of isolates DS1, DS3, DS4, DS7, DS8, and LS1 was performed by using two sets of primers, i.e., (1) universal primer U475 and specific primer for identification of *G. azotocaptans* L923Ga; (2) universal primer U475 and specific primer for identification of *G. johannae* L927Gj, designed by Fuentes-Ramirez et al. [3]. PCR conditions were same as mentioned by Fuentes-Ramirez et al. [3] except that the annealing temperature used for primers set 1 was 60 °C.

Table 1. Source of diazotrophic acetic acid bacteria from corn

Strain	Soil pH	Source	Region
DS1	5.50	Rhizosphere soil (year 2000)	Delhi, Ontario
DS3	6.62	Rhizosphere soil (year 2004)	Delhi, Ontario
DS4	6.18	Rhizosphere soil (year 2005)	Delhi, Ontario
DS7	6.31	Rhizosphere soil (year 2005)	Delhi, Ontario
DS8	6.25	Rhizosphere soil (year 2005)	Delhi, Ontario
LS1	7.44	Rhizosphere soil	London, Ontario

The PCR product for isolate DS1, obtained with primers set 1 was sequenced by using U475 and L923Ga primers. This sequence was submitted to Genbank (Accession No. DQ073427).

Amplified rDNA restriction analysis

The 16S rRNA gene of isolates DS1, DS3, DS4 and LS1 was amplified with primers FGPS4-281 and FGPS1509-153, as mentioned earlier [5]. Approximately 1500 ng PCR-amplified 16S rRNA gene fragment was restricted with 20 U of endonuclease *Rsa*I (New England Biolabs, Canada), by overnight incubation at 37 °C. Xylene cyanole FF (0.05%, Sigma) was used as loading dye [9]. The length of the restriction fragments was determined from their electrophoretic separation in 1% agarose gel, at 70 V for 2 h.

Results

Phenotypic characterization

Six grams negative, acid producing, nitrogen fixing bacteria were isolated from corn rhizosphere soil. Isolates DS1 (from soil of year 2000), DS3 (from soil of year 2004), DS4, DS7, DS8 (from soil of year 2005), were from Delhi Research Station. Isolate LS1 was from rhizosphere soil from London Research Station. These strains formed orange, round, mucous, smooth and convex colonies with translucent margins, on LGI plates. On GYC medium, they turned the off-white medium into dark brown color. On potato agar medium with 10% sucrose, a brownish liquid pigment was produced. *G. azotocaptans* is reported to have similar colony morphology on LGI, GYC and potato agar with 10% sucrose [3], as for these isolates. *G. johannae* produced yellow-orange, irregular, flat, smooth colonies on LGI but colony morphology on GYC and potato agar with 10% sucrose was similar to *G. azotocaptans* [3]. *G. diazotrophicus* PAL 5 produced dark orange, round, flat colonies on LGI, turned GYC medium into light brown color and formed small dark brown colonies on potato agar with 10% sucrose.

Corn isolates and *G. azotocaptans* utilized gluconate, sucrose, ethanol and showed slight growth on D-galactose, D-sorbitol [3]. Corn isolates also utilized glycerol and showed weak growth on glucose whereas *G. azotocaptans* grew well on glucose and did not utilize glycerol. The other carbon sources tested were not utilized (Table 2). *G. johannae* utilized D-glucose, D-galactose, D-xylose, D-raffinose, D-sorbitol, ethanol, sucrose, maltose, gluconate; showed slight growth on D-arabinose, D-mannitol, butanol, glycerol and no growth on methanol and mannose [3]. *G. diazotrophicus* PAL 5 did not use xylose, maltose, mannose, methanol, butanol and showed slight growth on ethanol. Rest of the carbon sources were utilized.

Corn isolates and *G. azotocaptans* [3] used L-alanine, L-aspartic acid, L-cystein, L-glutamic acid, L-leucine but not L-methionine, L-proline, L-tryptophan and L-threonine, as nitrogen source (Table 2). Corn isolates did not utilize L-lysine but *G. azotocaptans* showed slight growth on this nitrogen source. *G. johannae* is reported to have good growth on L-alanine, L-aspartic acid, L-tryptophan, slight growth on L-lysine, L-leucine and no growth on L-cystein, L-glutamic acid, L-methionine, L-proline and L-threonine [3]. *G. diazotrophicus* PAL 5 did not utilize L-threonine and showed weak growth on L-leucine and L-lysine but grew well on all the other L-amino acids tested as a nitrogen source. All taxa were positive for catalase and acetylene reduction and negative for oxidase and nitrate reduction.

16S rDNA sequence analysis

Approximately 1.7 kb fragment of the 16S rRNA gene of isolate DS1 was amplified and sequenced. On the basis of sequence analysis, it showed 99.7% homology (1441/1446 identities) with *G. azotocaptans* (Accession No. AF192761), 99.3% homology with *G. johannae* (1440/1450 identities; Accession No. AF111841) and 98.5% homology with *Acetobacter diazotrophicus* (synonym for *Gluconacetobacter diazotrophicus*; Accession No: X75618) with 1462 identities at 1485 positions.

PCR with species-specific primers

Amplification of the 16S rRNA gene of isolates DS1, DS3, DS4, DS7, DS8 and LS1 with universal primer U475 and a specific primer for *G. azotocaptans* L923Ga gave a ~470 bp product (Fig. 1). Amplification was not observed with primer L927Gj. PCR product of isolate DS1 was sequenced. A 469-bp sequence with U475 and L923Ga primers showed 100% homology (467/467 identities) with *G. azotocaptans* (Accession No. AF192761). Homology with *G. johannae* (Accession No. AF111841) was 99.1% for 457 identities over 461 positions. *G. diazotrophicus* (Accession No. AY230814) showed 448 identities over 449 positions, with 469 bp product of isolate DS1.

Amplified rDNA restriction analysis

After digestion with *Rsa*I, 16S rDNA of isolates DS1, DS3, DS4 and LS1 showed four bands on agarose gel (Fig. 2). Fragments ranged between 500 and 150 bp. *G. azotocaptans* [3] is reported to possess four *Rsa*I sites, generating fragments of 504, 404, 246, 159 and 134 bp in 3% gel [3]. *G. johannae* showed three *Rsa*I sites, giving

Table 2. Biochemical tests for identification of corn isolates and their comparison with *G. azotocaptans*, *G. johannae* and *G. diazotrophicus*

Tests	Corn isolates ($n = 6$)	*G. azotocaptans* ($n = 3$)[a]	*G. johannae* ($n = 4$)[a]	*G. diazotrophicus* ($n = 1$)
Growth on:				
D-Glucose	±	+	+	+
D-Galactose	±	±	+	+
D-Xylose	−	−	+	−
D-Raffinose	−	−	+/±	+
D-Arabinose	−	−	±/−	+
D-Sorbitol	+/±	+/±	+/±	+
D-Mannitol	−	−	±/−	+
Maltose	−	±/−	+/±	−
Glycerol	+/±	−	±/−	+
Ethanol	+/±	+/±	+	±
Butanol	−	−	±	−
Growth on L-amino acids in the presence of sorbitol as carbon source:				
L-Cystein	+	+	−	+
L-Glutamic acid	+	+	−	+
L-Leucine	+/±	±	±	±
L-Lysine	−	±	±	±
L-Methionine	−	−	−	+
L-Proline	−	−	−	+
L-Tryptophan	−	−	+	+

For growth on different carbon and nitrogen sources, pure cultures of corn isolates and *G. diazotrophicus* PAL 5 were streaked in duplicate. Growth results were recorded after 10 days incubation at 30 °C. These tests were repeated three times. All strains showed good growth on media containing gluconate, sucrose, mannose, L-alanine, L-aspartic acid and no growth on methanol and L-threonine. All strains were positive for acetylene reduction, catalase enzyme and negative for nitrate reduction and oxidase enzyme.

+, good growth;−, no growth; ±, slight growth.

[a]Data for reference strains *G. azotocaptans* and *G. johannae* were taken from Fuentes-Ramirez et al. [3]. Results like +/± or ±/− describe range.

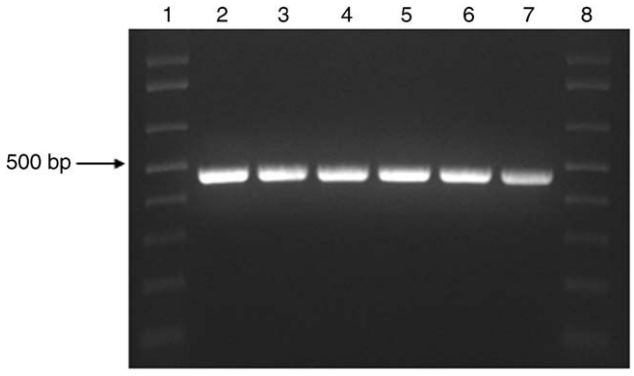

Fig. 1. Amplification of 16S rRNA gene of corn isolates with a universal primer U475 and a specific primer for *G. azotocaptans* L923Ga [3]. Lanes: 1 and 8, 1 kb plus DNA ladder; 2, Isolate DS1; 3, Isolate DS3; 4, Isolate DS4; 5, Isolate DS7; 6, Isolate DS8; 7: Isolate LS1.

fragments of 504, 403, 405 and 135 bp [3]. According to Fuentes-Ramirez et al. [3] the 159, 134 bp fragments in *G. azotocaptans* and 403, 405 bp fragments in *G. johannae* migrated together in agarose gel electrophoresis. Therefore, four bands for *G. azotocaptans* and three bands for *G. johannae* were observed.

Discussion

It is considered that the isolation of acetic acid forming bacteria and their assignment to either the genus *Acetobacter* or *Gluconobacter* generally pose few problems [11]. *Coffea arabica* was reported as a new host for *G. diazotrophicus* and some other unidentified nitrogen fixing, acetic acid producing bacteria which shared similarities and differences with *G. diazotrophicus* [4]. Two of these were named as novel species of the family *Acetobacteraceae* [3]. Strain CFN-Cf55^T was named as *G. johannae* and strain CFN-Ca54^T as *G. azotocaptans*. The isolation of additional strains of these two species was not achieved despite several attempts by Fuentes-Ramirez et al. (personal communication with Caballero-Melado J.). We isolated a group

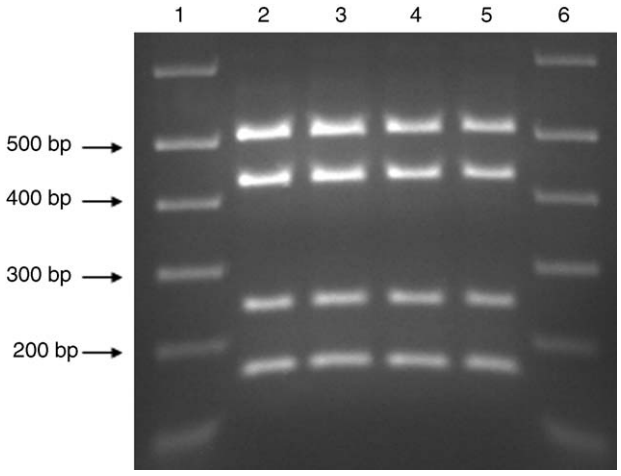

Fig. 2. Amplified rDNA restriction analysis profile of corn isolates digested with endonuclease *Rsa*I. Lanes: 1 and 6, 1 kb plus DNA ladder; 2, Isolate DS1; 3, Isolate DS3; 4, Isolate DS4; 5, Isolate LS1.

of nitrogen fixing, acetic acid producing bacteria from corn rhizosphere soil collected from two locations in Onatrio, Canada. Yellow pellicle formation in semisolid LGI medium and colony morphology suggested that these strains belong to the family *Acetobacteracea*. Initially, we used an API 20NE bacterial identification kit (data not shown) to characterize these isolates but this kit did not help, as the data bank provided with the kit does not have this genus listed. Fatty acid analysis identified the bacteria as *Acetobacter pasteurianus* (unpublished results), but this is a non-nitrogen fixing species. Colony morphology on different media and carbon and nitrogen source utilization pattern identified the isolates as *G. azotocaptans* and differentiated them from *G. diazotrophicus* PAL 5 and *G. johannae*.

Phenotypic identification was confirmed by 16S rDNA sequence analysis.16S rRNA gene amplification of these isolates with primer L927Gj was not observed even when we lowered the annealing temperature to 60 and 50 °C. Fuentes-Ramirez et al. [3] used 62°C annealing temperature for *G. johannae* and 67 °C for *G. azotocaptans*. Melting temperature (T_m) for these primers (L927Gj = 52.1 °C, L923Ga = 53.6 °C, U475 = 56 °C) is lower than annealing temperatures used by Fuentes-Ramirez et al. [3]. Therefore, we used a lower temperature in addition to these specific ones. Amplification with L923Ga primer (specific for *G. azotocaptans*) at 67 °C annealing temperature showed a very weak band in our experiments (data not shown) and therefore we used 60 °C as annealing temperature. At a low annealing temperature, this primer amplified an approximately 470 bp fragment. Fuentes-Ramirez et al. [3] reported a fragment of approximately 400 bp (result details not published). Although the product size of our isolates was bigger than that reported earlier, the sequence analysis of this product confirmed 100% homology with the sequence of *G. azotocaptans* CFN-Ca54^T (Accession No. AF192761). *Rsa*I digestion of 16S rDNA sequence of isolates DS1, DS3, DS4 and LS1 further confirmed the identification. Use of xylene cyanole dye (migrates slower than most DNA) helped to visualize the smallest band (~150 bp) found on the gel which we were unable to see with bromo-phenol blue (migrates faster than DNA, [10]). The number and sizes of fragments differentiated them from *G. johannae*.

On the basis of our results we are convinced that isolates DS1, DS3, DS4, DS7, DS8 and LS1 are new strains of *G. azotocaptans*. Environmental distribution of *G. azotocaptans* (considering that this species was described by analyzing a very limited number of isolates, i.e., 3) and its ecology is not understood. We were unable to locate any further reports on this species and to our best knowledge this is the first reported case of this bacterium from corn rhizosphere. We were unable to isolate *G. azotocaptans* from corn roots from both locations. Isolation of this bacterium from three batches of rhizosphere soil collected in three different years from Delhi suggests that this bacterium is persistent in this soil. This long-term survival in soil was not found by Caballero Mellado et al. (personal communication).

In a separate study, isolate DS1 has been shown to produce IAA (106 ng/ml), fix nitrogen (40 n.mol/h/mg bacterial protein), solubilize phosphate, and provide biological control against several fungal pathogens and promote the growth of three corn varieties in sterilized sand and unsterilized soil, under green house conditions (manuscript submitted). Experiments on beneficial effects of the association of *G. azotocaptans* with vegetables (cucumber, radish, pepper, tomato) are in progress. It is conceivable that *G. azotocaptans* can be used as a biofertilizers for corn production in Ontario and Canada.

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