Natural association of *Gluconacetobacter diazotrophicus* and diazotrophic *Acetobacter peroxydans* with wetland rice

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**Abstract**

The family *Acetobacteraceae* currently includes three known nitrogen-fixing species, *Gluconacetobacter diazotrophicus*, *G. johannae* and *G. azotocaptans*. In the present study, acetic acid-producing nitrogen-fixing bacteria were isolated from four different wetland rice varieties cultivated in the state of Tamilnadu, India. Most of these isolates were identified as *G. diazotrophicus* on the basis of their phenotypic characteristics and PCR assays using specific primers for that species. Based on 16S rDNA partial sequence analysis and DNA:DNA reassociation experiments the remaining isolates were identified as *Acetobacter peroxydans*, another species of the Acetobacteraceae family, thus far never reported as diazotrophic. The presence of *nifH* genes in *A. peroxydans* was confirmed by PCR amplification with *nifH* specific primers.

**Scope for the findings:** This is the first report of the occurrence and association of N\textsubscript{2}-fixing *Gluconacetobacter diazotrophicus* and *Acetobacter peroxydans* with wetland rice varieties. This is the first report of diazotrophic nature of *A. peroxydans*.

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**Keywords:** Wetland rice; Isolation; N\textsubscript{2} fixation; *Gluconacetobacter diazotrophicus*; *Acetobacter peroxydans*; *nifH*; 16S rDNA; DNA–DNA hybridization

**Introduction**

Nitrogen is known as an important element in plant growth since it is often the primary nutrient limiting the plant growth in many ecosystems. In agriculture, crop productivity is mainly supported by the use of inorganic N fertilizers, which are expensive and damaging for the environment. From ecological and economical perspectives plant-associated biological nitrogen fixation is desirable especially for economically important crops such as rice, which is the staple diet for more than two-fifths of the world’s population.
There are several reports on the association of nitrogen-fixing bacteria such as *Azospirillum* sp. with wheat [2], *Herbaspirillum seropedicae* with maize [3], *Glucanacetobacter diazotrophicus* with sugarcane [6,13] and *G. johannae* and *G. azotocaptans* with coffee plants [11]. Also *H. seropedicae* [3], *Burkholderia vietnamiensis* [14], *Rhizobium leguminosarum* bv. *trifolii* [45], *Azorarcus* [9], *Serratia marcescens* [16] and innumerable species of *Pseudomonas* [39,41] have been found in association with rice plants. In the family Acetobacteraceae, three nitrogen-fixing species have been described: *G. diazotrophicus*, *G. johannae* and *G. azotocaptans* [13,11,21]. *G. diazotrophicus*, originally described as *Acetobacter diazotrophicus* [13] and later transferred to the genus *Gluconacetobacter* [43], which was subsequently corrected to *Gluconacetobacter* [44], was the first nitrogen-fixing Acetobacteraceae species described and it has been investigated thoroughly with regard to its nitrogen-fixing ability [19,33]. Yet it is only since recently that there is evidence that it is a nitrogen contributor for crops.

Many reports on acetic acid bacteria mention that these strains are mainly associated with sugar or ethanol-rich environments [36,40], but recent studies show that their distribution is wider [21]. *G. diazotrophicus* was originally isolated from sugarcane [13], but recent reports show that this species is also associated with other sugar-rich plants as sweet sorghum, sweet potato and pineapple plants [30,37] and with sugar-poor plants as coffee and ragi [21,24]. *G. johannae* and *G. azotocaptans* were isolated from coffee [11].

The present study describes the identification and characterization of nitrogen-fixing acetic acid bacteria *G. diazotrophicus* and unreported *A. peroxydans* recovered from wetland rice cultivated in India.

### Materials and methods

#### Isolation of diazotrophic acetic acid bacteria from rice

Samples were collected in triplicate from rhizosphere soil, roots and stems from four different rice varieties (*Oryza sativa* cv. Co 36, cv. Co 39, cv. Ponni and cv. IR 50) cultivated in flooded soils of Tamilnadu, a Southern state in India, during either flowering stage or just before flowering stage. The rhizosphere soil samples were serially diluted and 0.1 ml aliquots were inoculated into vials containing 5 ml of N-free semisolid LGI medium with an initial pH of 6.0 with 1, 10 or 30% cane sugar (w/v) and 0.005% yeast extract (w/v) [31] and incubated at 32 °C for 5 days. The roots were surface sterilized with 70% ethanol for 5 min and 0.2% mercuric chloride for 30 s and then washed several times with sterile water. Basal stem portions were cut into 5 cm pieces, surface sterilized, by dipping in 95% ethanol and then washed six times with sterile water. From each end of the stem pieces 1 cm portions were removed by cutting with a sterile blade [16]. The outer sterility of the root and stem samples was verified by rolling them on Tryptic Soy agar (Sigma). Afterwards the samples were homogenized with a mortar and pestle in sterile phosphate-buffered saline (PBS) and serially diluted. 0.1 ml aliquots were inoculated into vials containing the semisolid LGI medium described for the rhizosphere soil.

Vials with a white to yellow surface pellicle were assayed for acetylene reduction activity as described by Hardy et al. [17]. Pellicles of the nitrogenase positive vials were sub-cultured in fresh medium before they were streaked on LGI agar plates with 10% cane sugar (w/v) and incubated at 32 °C for 10 days. Acid-producing deep yellow and yellowish orange colonies with dark center were picked up for further analysis. The isolates and their sources are presented in Table 1. Strains TNCSF 42, TNCSF 47, TNCSF 36 and TNCSF 49 are deposited in the BCCM/LMG Bacteria Collection as LMG 22174, LMG 22175, LMG 21769 and LMG 21770, respectively.

### Reference strains

The reference strains used in this study were *G. diazotrophicus* LMG 7603T, *G. johannae* ATCC 700987T, *G. azotocaptans* ATCC 700988T and *A. peroxydans* LMG 1635T.

### Phenotypic characterization

Colonies morphology and pigmentation were observed on LGI agar with 10% (w/v) cane sugar and potato agar with 10% (w/v) cane sugar [6]. Pigmentation was also checked on GYC agar, pH 4.5 [26]. Oxidase and catalase tests were determined using commercially available discs (Hi media). The ability to utilize various carbon substrates was assayed in LGI medium with NH₄Cl (0.1%) and supplemented with 0.5% (w/v) of the appropriate filter sterilized carbon compound instead of cane sugar. The ability to utilize various amino acids was tested in LGI medium with sorbitol (0.5%) instead of cane sugar, and supplemented with 0.1% of an appropriate filter sterilized L-amino acid. LGI medium with both NH₄Cl (0.1%) and sorbitol (0.5%), and LGI medium lacking both, was used as positive and negative controls, respectively.
Species-specific PCR for *G. diazotrophicus*, *G. azotocaptans* and *G. johannae*

The isolates and the type strains of the nitrogen-fixing acetic acid bacteria *G. diazotrophicus* LMG 7603\(^T\), *G. johannae* ATCC 700987\(^T\) and *G. azotocaptans* ATCC 700988\(^T\) were grown in SYP [4] for 48 h at 32°C. PCR amplifications were performed with supernatant of a single colony that was suspended in 50 \(\mu\)l sterile water and boiled for 5 min at 95°C [22]. The PCR for the detection of *G. diazotrophicus* was performed by genetic method based on 16S rRNA gene sequence with the species-specific primers AC (5\(^{\prime}\)-CTG-TTTCCCGCAAGGGAC-3\(^{\prime}\)) and DI (5\(^{\prime}\)-GCG-CCCCATTGCTGGGTT-3\(^{\prime}\)) [35]. The species-specific PCR for *G. johannae* and *G. azotocaptans* were performed with the universal primer U475 (5\(^{\prime}\)-AAT-GACTGGGCGTAAAG-3\(^{\prime}\)) and with one specific primer: L927Gj (5\(^{\prime}\)-GAAATGAACATCTCTGCT-3\(^{\prime}\)) for *G. johannae*, and L923Ga (5\(^{\prime}\)-AATGCTCAATCTGA-3\(^{\prime}\)) for *G. azotocaptans* according with the conditions described previously [11].

16S rDNA sequencing and phylogenetic analysis

DNA for 16S rDNA sequencing was prepared by alkalic extraction from cells grown on medium 13 from the Catalogue of cultures of the BCCM/LMG Bacteria Collection [20] at 28°C. A fragment of the 16S rRNA gene of strains TNCSF 36 and TNCSF 49 was amplified using oligonucleotide primers complementary to highly conserved regions of the 16S bacterial rRNA genes. The forward primer was 5\(^{\prime}\)-AGAGTTTGATCCTGGCT-CAG-3\(^{\prime}\) (positions 8-27, according to the *Escherichia coli* numbering system), the reverse primer 5\(^{\prime}\)-AAG-GAGGTGATCCAGCCGCA-3\(^{\prime}\) (positions 1541-1522). PCR products were purified using a QIA quickPCR Purification Kit (Qiagen), according to the manufacturer’s instructions. Purified PCR products were partially sequenced by using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit and an Applied Biosystems 377 DNA sequencer, using the protocols of the manufacturer (Applied Biosystems). The sequencing primers used were *Gamma, Gamma and PD* [8]. Sequence assembly was performed using the program Auto Assembler (Applied Biosystems). The partial 16S rRNA gene sequences determined and sequences of strains belonging to the same phylogenetic group, retrieved from the EMBL library, were aligned and a phylogenetic tree was constructed by the neighbor-joining method using the Bio Numerics 1.0 software package (Applied Maths). The comparison of the sequence data was based on 900 bases of the 16S rRNA genes. Unknown bases were discarded from the calculations. Bootstrapping analysis was undertaken to test the statistical reliability of the topology of the tree using 1000 bootstrap resamplings of the data. The strain numbers, species names and accession numbers of the 16S rDNA sequences retrieved from EMBL for use in the phylogenetic analysis are presented in Fig. 1.
DNA preparation for G+C content determination and for DNA–DNA hybridizations

High-molecular-weight DNA for determination of the G+C content and for DNA–DNA hybridizations was prepared from cells grown aerobically on Z1 medium (2.0% yeast extract, 2.0% calcium lactate and 1.5% agar) at 28°C by the method of Wilson [42] with minor modifications [7].

Determination of the DNA G+C content

The G+C content of the DNA was determined by HPLC according to the method of Mesbah et al. [25]. Non-methylated phage lambda DNA (Sigma) was used as the calibration reference.

DNA–DNA hybridizations

DNA–DNA hybridizations were performed using a modification of the microplate method described by Ezaki et al. [10] and Goris et al. [15]. Hybridizations were performed under stringent conditions at 47°C in a hybridization solution containing 50% formamide (2 × SSC, 5 × Denhardt’s solution, 50% formamide, 2.5% dextran sulfate, low-molecular-mass denatured salmon sperm DNA to a final concentration of 100 μg ml⁻¹, 1.25 μg biotinylated probe DNA per milli-
The DNA relatedness percentages presented are means based on at least two hybridization experiments. Reciprocal reactions (e.g. \( A \times B \) and \( B \times A \)) were performed and the variation between them was within the limit of this method [15].

**nifH PCR**

The *nifH* gene was amplified by PCR using the primer set PoIF/PoIR and the conditions described previously [31].

**Nucleotide sequence accession numbers**

The partial 16S rDNA sequences of strains TNCSF 36 and TNCSF 49 determined in this study were deposited in EMBL under the accession numbers AJ517177 and AJ517178, respectively.

**Results**

**Isolation of diazotrophic acetic acid bacteria from rice**

Fifteen Gram-negative, acid-producing, nitrogen-fixing isolates were recovered from rhizosphere soil, roots and stems of rice cultivated in India (Table 1). The phenotypic characteristics of the isolates were determined and compared with those of the known nitrogen-fixing acetic acid bacteria *G. diazotrophicus*, *G. azotocaptans* and *G. johannae* (Table 2). One group of 9 strains showed phenotypic characteristics typical of *G. diazotrophicus*. They formed after 10 days of incubation yellow colonies (1.5–3.0 mm) on LGI agar with 10% cane sugar (w/v) and chocolate brown colonies on potato agar with 10% cane sugar (w/v). On GYC solid medium they produced water-soluble brown pigments. They also utilized the same carbon and nitrogen substrates as *G. diazotrophicus* (Table 3). The other group with the remaining 6 strains differed from the known nitrogen-fixing acetic acid bacteria. They formed after 10 days of incubation small (0.8–1.5 mm) yellowish-orange colonies with a dark center on LGI agar with 10% cane sugar (w/v) and pale brown colonies with a dark center on potato agar with 10% cane sugar (w/v). On GYC solid medium they did not produce the water-soluble brown pigments. They also differed in their ability to utilize some carbon and nitrogen substrates (Table 3). The isolation of these strains required successive sub-culturing of thepellicles up to 50–60 times in fresh medium. Gas production was noticed during the isolation process. These strains were only isolated from roots and stems but not from rhizosphere soil.

**PCR specific for *G. diazotrophicus*, *G. johannae and G. azotocaptans***

Recently, clear and fast PCR methods were developed for the identification of *G. diazotrophicus* [35], *G. johannae* and *G. azotocaptans* [11]. To examine whether the rice isolates could be classified as any of the known nitrogen-fixing acetic acid bacteria, PCR reactions with

<table>
<thead>
<tr>
<th>Determinative characters (^a)</th>
<th><em>G. d</em> (LMG 7603(^T))</th>
<th><em>G. j</em> (ATCC 700987)</th>
<th><em>G. a</em> (ATCC 700988)</th>
<th><em>G. d</em> (This study)</th>
<th><em>A. p</em> (This study)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size and shape of the colony on LGI (10% sugar, after 10 days)</td>
<td>1.5–3 mm, convex, round, smooth</td>
<td>1.5–3 mm, convex, round, smooth</td>
<td>1.5–3 mm, convex, round, smooth</td>
<td>1.5–3 mm, convex, round, smooth</td>
<td>0.8–1.5 mm, flat, dry</td>
</tr>
<tr>
<td>Pigmentation on LGI (10% sugar, after 10 days)</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Yellowish orange, dark centered</td>
</tr>
<tr>
<td>Pigmentation on potato agar (10% sugar, 10 days)</td>
<td>Chocolate brown</td>
<td>pale brown</td>
<td>brown (water soluble), agar diffusing</td>
<td>chocolate brown</td>
<td>pale brown, dark centered</td>
</tr>
<tr>
<td>Gas production during isolation process</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Successive sub-culturing is essential (~60 times)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Growth on methanol</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Organic acids</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>150 mM of nitrate</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\)All the isolates including the type stains were Gram negative, oxidase positive, catalase negative. Oxidized ethanol and glucose and the growth was positive in the presence of 30% sucrose and possessed acetylene reduction activity.

\(^b\)Growth positive with malate.
The described species-specific primers were performed. The 9 strains forming the yellow colonies on LGI medium reacted positive in the PCR assay specific for *G. diazotrophicus* (based on the amplification of a specific 16S rRNA gene fragment) giving a PCR product of 445 bp, the size expected for this species. The results showed that these isolates can be assigned to the species *G. diazotrophicus*. The 6 strains forming the yellowish-orange colonies on LGI medium did not yield any PCR product in any of the species-specific PCR tests. These results together with the phenotypic properties of these strains indicate that these isolates belong within the Acetobacteraceae family but not to any of the known nitrogen-fixing acetic acid bacterial species thus far described in this family. To identify these isolates further tests were performed.

**Phylogenetic positions of TNCSF 36 and TNCSF 49**

The 16S rRNA gene of two representative strains of the group forming the yellowish orange colonies on LGI medium, TNCSF 36 and TNCSF 49, was partially sequenced (729 and 948 nucleotides, respectively, were determined) in order to determine the phylogenetic positions of the strains. Comparison of the partial 16S rDNA sequences of both strains revealed 99.7% sequence similarity and comparison of the partial 16S rDNA sequences determined with sequences of strains belonging to the same phylogenetic group, retrieved from the EMBL library, indicated that both strains belonged to the genus *Acetobacter* (Fig. 1). The 16S rDNA sequence of TNCSF 49 (948 nucleotides) showed significant sequence similarity for possible relatedness at the species level (>97%) with *A. peroxydans* IFO 13755T (100%), *A. pomorum* LTH 2458T (97.9%), *A. pasteurianus* LMG 1262T (97.5%), *A. lovaniensis* IFO 13753T (97.4%) and *A. syzygii* NRIC0483T (97.4%). To the other species of *Acetobacter* the similarity was below 97%.

**DNA relatedness and DNA base composition**

TNCSF 36 and TNCSF 49 were hybridized against each other and against the type strain of *A. peroxydans* LMG 1635T their closest relative (100% 16S rDNA sequence similarity). Both strains had a high value of DNA binding to one another (99%) and to LMG 1635T (87–89%). The DNA G+C content of TNCSF 36 and TNCSF 49 for both strains was 60.5 mol% which is comparable to the G+C content of *A. peroxydans* LMG 1635T (59.7 mol%). These data show that both strains belong to the species *A. peroxydans*.

**Presence of nif genes**

*A. peroxydans* belongs to the Acetobacteraceae family as *G. diazotrophicus*. Hitherto, *A. peroxydans* has never been described as a diazotrophic species. Because *nif*
genes are necessary for nitrogen fixation, a PCR with nifH specific primers was performed with the *A. peroxydans* isolates TNCSF 36 and TNCSF 49 and with the type strain of this species LMG 1635^T^. The strains yielded a PCR product of about 360 bp confirming the presence of *nif* genes (Fig. 2).

### Acetylene reduction of *A. peroxydans* LMG 1635^T^

The acetylene reduction (nitrogenase) activity of strain LMG 1635^T^ was examined by the acetylene reduction assay and compared to that of the novel *A. peroxydans* isolates TNCSF 36 and TNCSF 49 and with the type strain of this species LMG 1635^T^. The strains yielded a PCR product of about 360 bp confirming the presence of *nif* genes (Fig. 2).

### Discussion

Several studies have focused on nitrogen-fixing bacteria associated with rice [9,14,16,38,41,45] but the association of nitrogen-fixing acetic acid bacteria with rice cultivated under field conditions has never been reported. The present study shows that nitrogen-fixing acetic acid bacteria are found in natural association with rice plants.

The acetic acid bacteria isolates recovered from rice and capable of fixing nitrogen were assigned to the species *G. diazotrophicus* and *A. peroxydans*. The identification of the latter as a nitrogen-fixing species is surprising as this species was described already in 1925 [36,40] and was thus far never reported as diazotrophic. Although type strain and novel isolates of *A. peroxydans* showed low and inconsistent acetylene reduction activities, compared to *G. diazotrophicus* [3], their diazotrophic ability was confirmed by the presence of *nifH* genes. On this basis, the present study shows that nitrogen-fixing acetic acid bacteria are not restricted to the genus *Gluconacetobacter*.

The isolation of *G. diazotrophicus* from wetland rice is not so surprising. It is conceivable that the occurrence of this species, reported as a frequent colonizer of sugar-rich plants [6,30,37], in the rhizosphere as well as inside rice plants, originates from sugarcane residues left over during prior cropping. Some rice varieties sampled in Madhuranthagam and Kanchipuram (Table 1) were cultivated as a rotation crop in sugarcane fields. However, *G. diazotrophicus* was also recovered from the inside of rice plants cultivated in some other fields where there was no report of earlier sugarcane cultivation (e.g., strain TNCSF 42–LMG 21767 isolated from the root tissue of variety IR 50 cultivated at Thirumangalam, Madurai; Table 1). The mechanism of dispersion of *G. diazotrophicus* among non-vegetatively propagated plants such as rice remains to be revealed. The presence of VAM fungi spores, which can carry *G. diazotrophicus* [30], in soils and the presence of mealy bugs, known as host of *G. diazotrophicus* [1,5] and commonly occurring pests in sugarcane as well as in rice crops, could play a role.

In the present study the number of colonies of both *G. diazotrophicus* and *A. peroxydans*, isolated from the surface sterilized roots and stems of rice varieties cultivated in flooded fields in South India, was in the range of 10^2–10^3 CFU g\(^{-1}\) fresh weight. Previously, it has been reported that the number of *G. diazotrophicus* found in adult sugarcane plants were in the range of 10^5–10^7 CFU g\(^{-1}\) fresh tissues [6,32], but in mature sugarcane cultivated in Brazil, the number of *G. diazotrophicus* found, was only 10 to 10^2 CFU g\(^{-1}\) fresh weight [32]. It has been suggested that nitrogen fertilization in high levels promotes the growth of non-nitrogen-fixing bacteria and simultaneously inhibits the multiplication rate of diazotrophic bacteria [12,22,29]. So the isolation of *A. peroxydans* from high N fertilized samples might have been necessitated a specific enrichment of this bacterium with successive sub-culturing in the medium with a low pH and high sugar as suggested earlier [23,12,28,29]. Although, no exact data are

![Fig. 2. Agarose gel electrophoresis of nifH PCR amplification products of *A. peroxydans* LMG 1635^T^ (lane 1), TNCSF 36 (lane 3) and TNCSF 49 (lane 4). Lane 3: molecular weight standard (100-bp ladder).](image-url)
available about the levels of nitrogen fertilization applied in the fields where the samples were collected, generally 100–125 kg nitrogen ha⁻¹ is recommended in those areas. The low prevalence of acetic acid bacteria in association with rice can also be due to the low amount of their preferential sugar substrates present in the environment of this plant. Recently, it has been demonstrated that prevalence and persistence of *G. diazotrophicus* populations also differ according to the sugarcane varieties they harbor [27]. Because this study is the first report about the natural association of two nitrogen-fixing acetic acid bacterial species with rice plants, it would be of great interest to determine the occurrence and the persistence of these species in other rice varieties cultivated in different regions of India and worldwide. A similar study in South Korea (manuscript in preparation) indicates that *G. diazotrophicus* is naturally associated with South Korean rice variety Hwsanbyeo in low numbers (10⁴ g⁻¹ fresh tissue).

The association of nitrogen-fixing *G. diazotrophicus* and *A. peroxidans* with rice may be important for agriculture as both species may be supplying a part of the nitrogen that rice requires. In the case of sugarcane it has already been proven that *G. diazotrophicus* is a nitrogen contributor [34] and also could even more beneficial for sugarcane plant growth by mechanisms other than nitrogen fixation [27]. But in the case of rice plants though there was a report on the BNF activity [18], there is no evidence that any one of the associated bacterium/ or a group of bacteria reported so far be responsible for this observed BNF. Experiment on the beneficial effects of the association of *G. diazotrophicus* and *A. peroxidans* with rice plants are under progress which may throw some light on the role of these two bacteria with the rice plants.

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