Ann Microbiol (2016) 66:329–342
DOI 10.1007/s13213-015-1113-2

ORIGINAL ARTICLE

Identification and characterization of the part of the bacterial community associated with field-grown tall fescue (*Festuca arundinacea*) cv. SFRO Don Tomás in Uruguay

María Cecilia de los Santos[1] · **Cecilia Taulé**[1] · **Cintia Mareque**[1] · **Martin Beracochea**[1] · **Federico Battistoni**[1]

Received: 9 March 2015 / Accepted: 3 June 2015 / Published online: 26 June 2015

Abstract The aims of this study were to isolate, characterize and identify the native culturable putative endophytic bacterial community associated with tall fescue (*Festuca arundinacea*) cv. SFRO Don Tomás, cultivated in Uruguay, and to study the effects of inoculation on cv. SFRO Don Tomás and the commercial cv. Tacuabé. A total of 342 isolates were collected from surface-sterilized roots, stems and seeds of healthy cv. SFRO Don Tomás. The functional ability of the isolates to produce indole, to solubilize minerals (P, Fe, K) and to biologically fix molecular nitrogen (N_2) was determined. Several infection traits, such as the ability to produce proteases, peroxidases, cellulases and hemicellulases, were identified in the isolates. Selected bacterial isolates were identified by 16S rRNA sequencing and shown to belong to a broad spectrum of genera, including *Bacillus*, *Microbacterium*, *Curtobacterium*, *Streptomyces*, *Acidovorax*, *Variovorax*, *Acinetobacter*, *Pseudomonas*, *Pantoea*, *Rhanella* and *Xanthomonas*. Plant growth promotion assays shown that ten isolates were able to promote the growth of cv. SFRO Don Tomás under gnotobiotic conditions, thereby highlighting the potential of these isolates in biotechnological applications as inoculant for this cultivar which is highly adapted to dry and cold seasons.

Keywords Putative endophytic bacteria · Tall fescue · Plant growth-promoting bacteria · Endophyte–host interaction

Introduction

Tall fescue (*Festuca arundinacea*) is an important cool-season forage grass native to North Africa and Northern Europe which was introduced to several parts of the world, such as the USA, Argentina, Uruguay, Australia and New Zealand, for livestock feed (Hoveland 2010). In Uruguay, whose economy mainly depends on livestock and meat export, tall fescue is the most widely cultivated cool-season grass, grown on 70 % of the total cultivated pasture area (500,000 ha), due to its higher productivity and persistence compared to perennial ryegrass (*Lolium perenne*) (Milne 2010). Under optimal hydric growth conditions tall fescue has a high dry weight yield and is perennial during the winter. Moreover, in Uruguay, its high persistence and productivity is associated with excellent drought and cold resistance, as well as a high competitive capacity relative to one of the principal Uruguayan undergrowths, Bermuda grass (*Cynodon dactylon*) (Carambula 2000). Tall fescue propagation is by seed, and the plants display low vigor in the first winter, which makes the plant suitable for sowing in mixtures with other, less competitive forages, such as the legumes *Trifolium repens* and *Lotus corniculatus* (Milne

Electronic supplementary material The online version of this article (doi:10.1007/s13213-015-1113-2) contains supplementary material, which is available to authorized users.

✉ Federico Battistoni
fbattistoni@iibce.edu.uy

María Cecilia de los Santos
cdelossantos@iibce.edu.uy

Cecilia Taulé
ctaule@iibce.edu.uy

Cintia Mareque
cmareque@iibce.edu.uy

Martin Beracochea
mberacochea@iibce.edu.uy

[1] Microbial Biochemistry and Genomics Department, Clemente Estable Biological Research Institute, Avenida Italia 3318, Montevideo 11600, Uruguay

2010). Tall fescue is important in rotation systems due to its contribution to the productivity persistence of the prairie, in complementation with legume crops, leading to an improvement of soil physicochemical properties. This type of agricultural system allows a more sustainable use of the soil and helps control erosion as well as maintain organic carbon levels in the soil (García Préchac et al. 2002).

Bacterial endophytes are defined as those bacteria which can be detected at a particular moment within the tissues of apparently healthy plants without producing symptoms (Schulz et al. 2006). In contrast with the well-studied symbiotic or pathogenic systems, little is known about the molecular basis of endophyte–plant interactions. Nevertheless, there is an abundance of evidence which testifies their beneficial effects on plant growth promotion (PGP) in several host plants (Hardoim et al. 2008; Ryan et al. 2008; Compant et al. 2010; Mei and Flinn 2010). Endophytic PGP can be achieved directly by biofertilization [fixation of molecular nitrogen (N_2); solubilization of phosphorus (P), potassium (K) and iron (Fe)] by the production and regulation of phytohormones or indirectly by stress tolerance, pathogen biocontrol or the induction of systemic resistance in the plants (Mercado-Blanco and Lugtenberg 2014). Taking this into account, the biotechnological exploitation of the endophyte–host interaction might play a significant role in improving the sustainability of agricultural systems.

During a long dry season in 2008, technicians from the Rural Promotion Society Ortiz (SFRO) identified a green, healthy and vigorous naturalized population of tall fescue alongside Route 8 km 145 in Lavalleja State, Uruguay (34°17′26.60″ S, 54°59′14.77″ W; 123 m a.s.l.). This population was harvested and multiplied and its productive behavior tested under commercial conditions, i.e. a sheep farm, where it showed an excellent response. This led to its registration as a cultivar under the name SFRO Don Tomás, and it has become an excellent model for studying the native culturable bacterial endophytic community associated with tall fescue. The application of native plant growth-promoting bacteria (PGPB) might improve yields by direct PGP or by biocontrol and/or by increasing plant tolerance to stress (Mercado-Blanco and Lugtenberg 2014). In this context and with the general aim of contributing to the economic and environmental sustainability of the tall fescue cv. SFRO Don Tomás by using naturally associated PGPB, the aims of this study were: (1) to obtain a large collection of culturable putative endophytic bacteria associated with tall fescue cv. SFRO Don Tomás, (2) to characterize this collection based on PGP and infection traits, and thus to identify isolates of interest for agricultural use and (3) to study the inoculation effects of selected isolates on the growth of tall fescue cv. SFRO Don Tomás and the commercial cv. Tacuabé. The data obtained will contribute to future research toward the development of an inoculant based on native endophytic PGPB for tall fescue cv. SFRO Don Tomás.

Material and methods

Isolation of putative endophytic bacteria from tall fescue cv. SFRO Don Tomás

Putative endophytes associated with tall fescue cv. Don Tomás were isolated from the seeds, roots and aerial parts of adult plants. Healthy plants were collected from three sites (A–C) in the Lavalleja Department in the eastern region of Uruguay (A: 34°18′30.5″S, 55°24′1.4″W, 125 m a.s.l.; B: 34°17′26.60″S, 54°59′14.77″W, 123 m a.s.l.; C: 34°14′54.0″S, 55°23′33.5″W, 120 m a.s.l.), where no fertilization had been applied for the preceding 4 years but where good productivity had been still registered over that period. The soil profiles of sites A and B were as follows: A (pH 5.7, 13 % sand, 54 % silt, 33 % clay, 2.5 % organic matter, total N 0.28 %), B (pH 5.8, 30 % sand, 67 % silt, 13 % clay, 2.9 % organic matter, total N 0.55 %). The protocol employed for bacterial isolation was as reported by Mareque et al. (2015) with slight modifications in the incubation times and the culture media employed. Seeds and plant tissues (root and stems) were surface-disinfected by incubation in 5 % $HClO_4$ for 45 min and in 4 % $HClO_4$ for 15 min, respectively. Dilutions of the suspension obtained were inoculated onto agar plates containing Tryptic Soy Agar (TSA; Difco Laboratories, Detroit, MI) and nitrogen-free combined carbon (NFCC) medium (Mirza and Rodrigues 2012) respectively, and in vials containing NFCC semisolid medium. NFCC plates were incubated at 30 °C under microaerobic conditions using the Microbiology Anaerocult® A Mini kit (Merck KGaA, Darmstadt, Germany) for 20 days, while TSA plates and NFCC vials were incubated under aerobic conditions at the same temperature. The isolates obtained were replicated twice in NFCC medium followed by two replications in TSA medium. Individual colonies were classified in TSA medium according their morphological features. This procedure was carried out with the aim of isolating both diazotrophic and heterotrophic bacteria.

The bacterial abundance in the roots and the aerial parts of plants collected from sites A and B was determined by counting the colony forming units per gram of fresh tissue (CFU g^{-1}) in TY (tryptone yeast extract) plates.

Screening of the bacterial collection for biofertilization and plant interaction traits

The entire bacterial collection was screened for putatively diazotrophic isolates by *nifH*-targeted PCR amplification using the primers PolF and PolR (Poly et al. 2001). The final reaction mixture (25 μl) consisted of 2.5 μl 10X Taq reaction buffer (Fermentas, Thermo Fisher Scientific, Waltham, MA) 3.0 mM $MgCl_2$, 0.16 mM dNTPs, 0.8 μM of both set of primers, 0.5 U Taq polymerase (Fermentas), 4 % bovine serum albumin and 4.0 μl of a cell lysate template and was

amplified as described by Taulé et al. (2012) and Mareque et al. (2015). The ability to fix N_2 was also tested in those isolates, which harbored the *nifH* gene, in vials containing LGI and NFCC, N-free semisolid media (Reis et al. 1994; Mirza and Rodrigues 2012). The vials were incubated at 30ºC for up to 7 days, and those that showed a growth pellicle were replicated into a new fresh vial containing the same medium (Baldani et al. 2014). Those isolates that were available to grow as a pellicle in the semisolid N-free media and which harbored the *nifH* genes were considered to be diazotrophs.

Siderophore production was assayed according to Schwyn and Neilands (1987) using plates containing chromeazurol (CAS) medium. Plates were incubated for 72 h at 30 °C. Siderophore production was indicated by the presence of a yellow halo around the colony (positive test result).

The method described by Sylvester-Bradley et al. (1982) was used to identify isolates able to solubilize phosphates. Isolates were grown on plates containing GL medium (10 g glucose, 2 g yeast extract, 5 g K_2HPO_4, 10 g $CaCl_2$, 15 g agar) and incubated for 72 h at 30 °C. Phosphate-solubilizing ability was indicated by the presence of a translucent halo around the colony (positive test result).

For the detection of K-solubilizing isolates, the isolated bacteria were grown on plates containing Aleksandrov medium (10 g sucrose, 1.5 g K_2HPO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 1 g $CaCO_3$, 15 g of agar; Avakyan et al. 1986) and incubated at 30 °C. K-solubilizing ability was indicated by the presence of a translucent halo around the colony (positive test result).

The colorimetric method described by Sarwar and Kremer (1995) was employed to test the entire collection of isolates with the aim of detecting the production of indole-3-acetic acid (IAA) as described previously (Taulé et al. 2012).

Endoglucanase and hemicellulase activities were screened by culturing the isolates on solid TSA culture media supplemented with 0.2 % carboxymethyl cellulose or 0.5 % Avicel, respectively (Kim et al. 2008). Positive strains were determined by degradation halos around each colony.

Protease activity was evaluated in plates containing TSA medium supplemented with 5 % skimmed milk. Strains were considered to possess protease activity when a translucent halo was observed around the colonies (Martinez-Rosales and Castro-Sowinsky 2011).

For the determination of peroxidase activity, we grew strains in plates containing TSA medium supplemented with 250 mg l^{-1} of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS; Sigma-Aldrich, St. Louis, MO); for the determination of manganese (Mn)-peroxidases, the strains were grown on TSA medium with 250 mg l^{-1} ABTS + 100 mg l^{-1} $MnCl_2{\cdot}4H_2O$ (Sack et al. 1997). Strains were considered to possess peroxidase activity when the color of the colonies turned a dark green or brown, respectively.

Biofilm formation was screened in 96-well plates using the crystal violet method (Peeters et al. 2008). Each well containing 200 μl TSB medium was inoculated with each isolate at a final concentration of $1–2\times10^8$ cells ml^{-1} isolate. After incubation for 48 h at 30 °C without agitation, the supernatant was removed and the wells were washed with phosphate buffered saline (PBS), followed by staining with a 0.1 % CV solution for 20 min. The excess CV was removed by washing the plates with PBS, and the bound CV was solubilized with 95 % EtOH. The absorbance of the suspension was measured at 570 nm.

All determinations were performed in triplicate.

Physiological features of the bacterial collection

With the aim of determining the capacity of the isolates to grow on different C and N sources, we grew the entire bacterial collection on plates containing LGI medium supplemented with the C or N sources to be tested; these were provided at the same final concentrations as reported in the original protocol (Cavalcante and Dobereiner 1988). The C sources analyzed were maltose, mannitol, glucose, sucrose, malate, fructose, lactose, glycerine, pyruvic acid, unrefined sugar, ethanol and vinasse (byproduct generated during the fermentation of cane molasses). The N sources tested were $(NH_4)_2SO_4$, KNO_3, NH_4Cl, L-tyrosine, L-asparagine, L-glutamic acid and urea. All determinations were done in triplicate.

16S rRNA amplification, sequencing and phylogenetic analysis

Selected isolates were subject to 16S rRNA amplification and sequencing as described by Taulé et al. (2012). The 16S rRNA sequences were deposited in GenBank under the following accession numbers: KP704407–KP704439.

In the phylogenetic studies, the quality of the sequences obtained was checked manually and assembled using the software DNA Baser Sequence Assembler v3.× (2010) (http://www.dnabaser.com). The consensus sequences were aligned using Sina Web Aligner (Pruesse et al. 2007), imported into the ARB software package v 6.0.2 (Ludwig et al. 2004) and added to the database. Phylogenetic trees were generated using the neighbor-joining algorithm with 1000 bootstrap replicates.

PGP of tall fescue cvs. SFRO Don Tómas and Tacuabé under gnotobiotic conditions

Seeds of tall fescue cvs. Don Tomás and Tacuabé were surface-sterilized as described above and placed onto 0.8 % water agar plates at room temperature for 5 days. Two seedlings were then placed into each plant growth tube containing 20 ml of liquid Jensen medium (Vincent 1970).

The plants were inoculated with a cell suspension of each isolate to be tested at a final concentration of 1.0×10^8 cells ml^{-1}; there were eight replicates for each treatment. Plants without inoculation or chemical fertilization were used as negative controls, and a reference control without inoculation but with the addition of 0.05 % KNO_3 was employed since all the isolates tested were putative diazotrophs. Plants were maintained at 26 °C under a photoperiod of 16/8 h light/darkness in a growth room. The plants were harvested at 1-month post inoculation and dried at 60 °C until a constant weight was reached, at which time the total dry weight was determined.

Statistical analysis

For the statistical analyses, we performed an analysis of variance on parametric data using the SPSS for Windows v20.0 (IBM Corp., Armonk, NY) software program. When a significant difference could be confirmed, the treatment means were compared against the negative control using Dunnett's test. For nonparametric data we performed the Krustal–Wallis test. When a significant difference could be confirmed, the treatment means were compared against the negative control using the Mann–Whitman test. All significant differences between treatments were expressed as *p*-values at the 0.1, 0.05 and 0.01 levels.

Results

Isolation and characterization of putative endophytic bacteria associated with tall fescue cv. SFRO Don Tomás

A collection of 342 bacterial isolates associated with the seeds (6 isolates), roots (192) and aerial parts (144) of healthy tall fescue cv. SFRO Don Tomás plants was obtained. Because they were isolated from surface-sterilized material, we considered these to be putative endophytes unless proven otherwise by microscopy. Tall healthy fescue plants were collected from a crop region which had not been treated with chemical fertilization and where no tall fescue toxicity symptoms had been reported. In all, 179, 74 and 89 bacterial isolates were obtained using TSA, NFCC solid and NFCC semisolid media, respectively. The bacterial population from the inner plant tissues was enumerated in samples collected from sites A and B. In both cases, bacteria were more abundant in the roots (1×10^6 and 1×10^5 CFU g^{-1} for sites A and B, respectively) than in the aerial parts (1×10^5 and 1×10^3 CFU g^{-1} for sites A and B, respectively). Additionally, for both plant parts, bacterial abundance was higher at site A (1×10^6 and 1×10^5 for roots and aerial part, respectively) than at site B (1×10^5 and 1×10^3 for roots and aerial part, respectively).

PGP features, such as the ability to fix N_2, solubilize inorganic P, K and produce siderophores and IAA, was studied in the entire collection. Results from selected isolates, including their in vitro plant growth promotion and plant infection traits as well as their colony morphology, are shown in Table 1.

Of the 342 isolates tested, 45 putative diazotrophic isolates were detected by PCR amplification of the *nifH* gene. All of these were grown in vials containing LGI or NFCC N-free semisolid medium, resulting in 42 isolates capable of producing a growth pellicle. The production of the growth pellicle allowed them to be considered as potential diazotrophs (12.3 % of the collection). From this group of 42 potential diazotrophic isolates 20, 14 and 8 were isolated from TSA, NFCC (microaerobic) and NFCC (semisolid) culture media, respectively. In addition, 27 and 33 isolates (of the total 342 isolates) were able to produce IAA and siderophores, respectively, 36 isolates were able to solubilize phosphate and one isolate was able to to solubilize K.

The bacterial collection was also screened for the presence of plant infection traits, including hemicellulase, cellulase, protease and peroxidase activities, as well as biofilm formation. No isolates with hemicellulase, cellulase or peroxidase activity were present in the collection, but 45 and 140 isolates demonstrated protease activity and biofilm-forming ability, respectively.

The entire bacterial collection was also evaluated for its ability to grow on different C and N sources (Table S1, electronic supplementary material). In terms of growing on a sole C source, 5 % of the isolates grew on sugarcane vinasse, 19 % on ethanol, 34 % on malic acid, 46 % on lactose and 60–81 % on fructose, pyruvic acid, sucrose, crystal sugar, glycerine, maltose, mannitol and glucose. In terms of growing on a sole N source, 50 % of the isolates grew on $(NH_4)_2SO_4$, 58 % on NH_4Cl and 83–88 % on KNO_3, urea, L-tyrosine, L-glutamic acid and L-asparagine.

Identification and phylogenetic analysis of tall fescue-associated isolates based on their partial 16S rRNA sequences

In the identification and phylogenetic analyses, we performed 16S rRNA sequencing of selected isolates and analyzed the sequence data by BLAST searches against the NCBI database. Isolates were selected for 16S rRNA sequencing on the basis of their in vitro PGP and plant infection traits. The nearest match from the GenBank database revealed a broad spectrum of isolates, including bacteria belonging to the phyla Firmicutes (*Bacillus*) and Actinobacteria (*Microbacterium*, *Curtobacterium*, *Streptomyces*) and a high number of isolates in the Proteobacteria. Among the latter, we identified isolates from the Betaproteobacteria (*Acidovorax*, *Variovorax*) and

Table 1 Plant growth promotion features of selected putative endophytic bacterial isolates from tall fescue (*Festuca arundinacea*) cv. SFRO Don Tomás

Isolate	Isolation culture medium[a]	Identification[b]	Phytostimulation and biofertilization[c]						Infection[d]					
			K	IAA	*nifH*	GP	Ca_3PO_4	SID	Mn-PER	PER	CE	HC	PROT	Biofilm formation
UYFA01	TSA	*Acinetobacter*	−	−	+	+	+	−	−	−	−	−	−	+
UYFA02	TSA	*Acinetobacter*	−	−	+	+	+	−	−	−	−	−	−	+
UYFA06	TSA	*Acinetobacter*	−	−	+	+	+	−	−	−	−	−	−	+
UYFA09	TSA	*Microbacterium*	−	−	+	−	−	−	−	−	−	−	−	+
UYFA10	TSA	*Pantoea*	−	+	−	ND	−	−	−	−	−	−	−	+
UYFA15	TSA	*Pantoea*	−	−	+	+	−	+	−	−	−	−	−	+
UYFA22	TSA	*Variovorax*	−	−	+	−	−	−	−	−	−	−	−	+
UYFA23	TSA	*Acidovorax*	−	−	+	+	−	−	−	−	−	−	−	+
UYFA24	TSA	*Acinetobacter*	−	−	+	+	+	−	−	−	−	−	−	+
UYFA26	TSA	*Microbacterium*	−	+	−	ND	−	−	−	−	−	−	−	+
UYFA39	TSA	*Acinetobacter*	−	−	+	+	+	−	−	−	−	−	−	+
UYFA61	TSA	*Microbacterium*	−	+	−	ND	−	−	−	−	−	−	−	−
UYFA68	TSA	*Microbacterium*	−	+	−	ND	−	−	−	−	−	−	−	−
UYFA95	TSA	*Pseudomonas*	−	−	+	+	−	−	−	−	−	−	−	+
UYFA105	TSA	*Xanthomonas*	−	+	−	ND	−	−	−	−	−	−	−	−
UYFA110	TSA	*Pantoea*	−	+	+	+	−	−	−	−	−	−	−	−
UYFA113	TSA	*Pseudomonas*	−	−	+	+	−	−	−	−	−	−	−	−
UYFA152	TSA	*Bacillus*	−	−	−	ND	−	−	−	−	−	−	−	+
UYFA153	TSA	*Streptomyces*	−	−	−	ND	−	−	−	−	−	−	−	+
UYFA154	TSA	*Bacillus*	−	−	−	ND	−	+	−	−	−	−	−	−
UYFA155	TSA	*Curtobacterium*	−	−	+	+	−	−	−	−	−	−	−	−
UYFA156	TSA	*Streptomyces*	−	−	+	+	−	NG	−	−	−	−	−	−
UYFA157	TSA	*Curtobacterium*	−	−	+	+	−	−	−	−	−	−	−	−
UYFA190	NFCCss	*Pantoea*	−	+	−	ND	−	−	−	−	−	−	−	+
UYFA198	NFCCss	*Pantoea*	−	+	+	+	−	−	−	−	−	−	−	+
UYFA214	TY	*Pseudomonas*	+	−	+	+	−	+	−	−	−	−	+	+
UYFA215	TY	*Xanthomonas*	−	−	+	+	−	NG	−	−	−	−	−	+
UYFA249	NFCCss	*Pseudomonas*	−	+	−	ND	−	+	−	−	−	−	+	+
UYFA288	NFCC	*Rahnella*	−	−	+	+	−	−	−	−	−	−	−	+
UYFA289	NFCC	*Rahnella*	+	+	+	+	−	+	−	−	−	−	−	+
UYFA298	NFCC	*Lelliottia*	−	+	+	+	−	−	−	−	−	−	−	+
UYFA330	NFCCss	*Rahnella*	−	+	+	+	−	−	−	−	−	−	−	−
UYFA337	NFCCss	*Rahnella*	−	−	+	+	−	−	−	−	−	−	−	−

[a] TSA, Tryptic soy agar medium; NFCCss, nitrogen-free combined carbon semisolid medium; TY, tryptone yeast extract

[b] Taxonomic identification was based on 16S rRNA similarity

[c] K, Potassium solubilization in plates containing Alexsandrov medium; IAA, indole-3-acetic acid production; ACC, aminocyclopropane-1-arboxylate deaminase activity, *nifH*, gene encoding enzyme involved in the fixation of atmospheric nitrogen into a form of nitrogen available to plant which was detected by PCR approach; GP, growth pellicle; Ca_3PO_4, solubilization of inorganic calcium phosphate; SID, siderophore production; ND, not determined; NG, no growth

[d] Mn-PER, Manganese-peroxidase; PER, peroxidase; CE, exo-cellulase activity; HC, hemicellulase activity; PROT, protease activity

Gammaproteobacteria classes (*Acinetobacter*, *Pseudomonas*, *Pantoea*, *Rhanella*, *Lelliottia*, *Xhantomonas*) (Table 2).

A phylogenetic tree was constructed based on the 16S rRNA sequences of 33 isolates (Fig. 1). Within the Proteobacteria, 23 isolates belonged to the class Gammaproteobacteria (Fig. 1; Table 2), of which isolates UYFA10, UYFA15, UYFA110, UYFA190 and UYFA198 were placed in the genus *Pantoea* and grouped in a well-supported cluster (Fig. 1), although only isolate UYFA110 was closely related to the reference strain *P. ananatis* AY530795 (Fig. 1). In a separate and well-

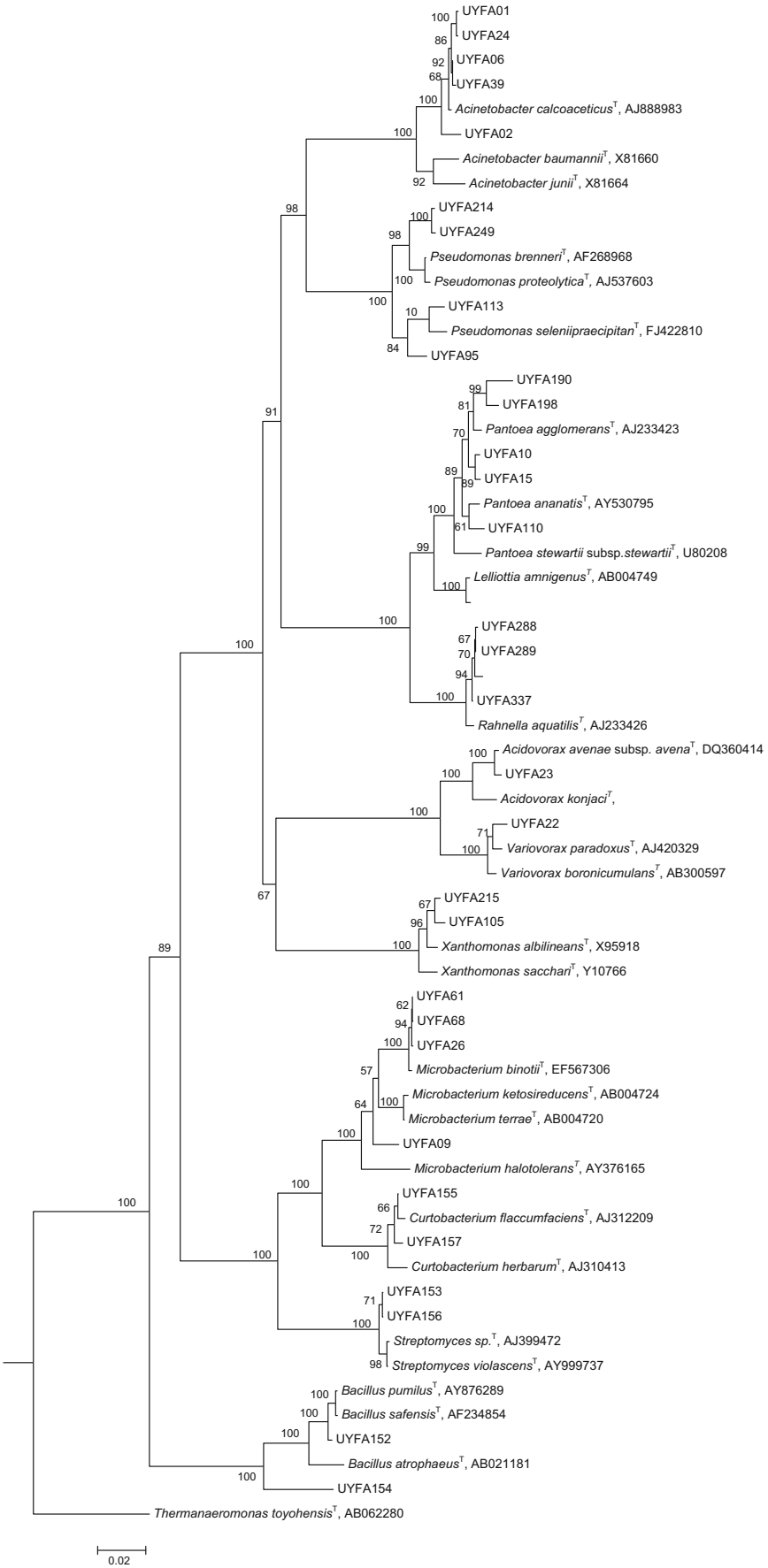
Table 2 16S rRNA nucleotide sequence similarities of selected putative endophytic bacterial isolates from the Uruguayan tall fescue cv. SFRO Don Tomás

Isolate	Accession number	Best hit	Coverage	*e*-value	Maximum identity	Isolation site[a]	Plant organ origin
UYFA01	NR_117621.1	*Acinetobacter pittii* ATCC 19004	99 %	0.0	99 %	C	Aerial tissues
UYFA02	NR_117621.1	*Acinetobacter pittii* ATCC 19004	99 %	0.0	99 %	C	Aerial tissues
UYFA06	NR_117621.1	*Acinetobacter pittii* ATCC 19004	99 %	0.0	99 %	C	Aerial tissues
UYFA09	NR_042262.1	*Microbacterium oleivorans* BAS69	99 %	0.0	99 %	C	Aerial tissues
UYFA10	NR_074740.1	*Pantoea ananatis* AJ13355	99 %	0.0	98 %	C	Aerial tissues
UYFA15	NR_041978.1	*Pantoea agglomerans* DSM 3493	99 %	0.0	98 %	C	Aerial tissues
UYFA22	NR_074654.1	*Variovorax paradoxus* S110	100 %	0.0	98 %	C	Aerial tissues
UYFA23	NR_118396.1	*Acidovorax avenae* BC523	99 %	0.0	99 %	C	Aerial tissues
UYFA24	NR_117621.1	*Acinetobacter pittii* ATCC 19004	99 %	0.0	99 %	C	Aerial tissues
UYFA26	NR_118272.1	*Microbacterium neimengense* 7087	99 %	0.0	99 %	C	Aerial tissues
UYFA39	NR_117621.1	*Acinetobacter pittii* ATCC 19004	100 %	0.0	99 %	C	Aerial tissues
UYFA61	NR_118272.1	*Microbacterium neimengense* 7087	99 %	0.0	99 %	C	Root
UYFA68	NR_118272.1	*Microbacterium neimengense* 7087	99 %	0.0	99 %	C	Root
UYFA95	NR_025228.1	*Pseudomonas koreensis* Ps 9–14	100 %	0.0	99 %	A	Aerial tissues
UYFA105	NR_036968.1	*Xanthomonas translucens* XT 2	99 %	0.0	98 %	A	Aerial tissues
UYFA110	NR_115258.1	*Pantoea allii* BD 390	99 %	0.0	95 %	A	Aerial tissues
UYFA113	NR_109583.1	*Pseudomonas punonensis* LMT03	99 %	0.0	99 %	A	Aerial tissues
UYFA152	NR_118439.1	*Bacillus aerius* 24 K	99 %	0.0	99 %	–	Seed
UYFA153	NR_116508.1	*Streptomyces sampsonii* NRRL B12325	100 %	0.0	99 %	–	Seed
UYFA154	NR_112636.1	*Bacillus megaterium* NBRC 15308	99 %	0.0	99 %	–	Seed
UYFA155	NR_025467.1	*Curtobacterium flaccumfaciens* LMG 3645	100 %	0.0	99 %	–	Seed
UYFA156	NR_116508.1	*Streptomyces sampsonii* NRRL B12325	100 %	0.0	99 %	–	Seed
UYFA157	NR_025467.1	*Curtobacterium flaccumfaciens LMG 3645*	100 %	0.0	99 %	–	Seed
UYFA190	NR_114111.1	*Pantoea agglomerans* NBRC 102470	100 %	0.0	92 %	A	Aerial tissues
UYFA198	NR_114111.1	*Pantoea agglomerans* NBRC 102470	100 %	0.0	95 %	A	Aerial tissues
UYFA214	NR_024911.1	*Pseudomonas rhodesiae* CIP 104664	100 %	0.0	98 %	A	Root
UYFA215	NR_036968.1	*Xanthomonas translucens* XT 2	100 %	0.0	99 %	A	Root
UYFA249	NR_024911.1	*Pseudomonas rhodesiae* CIP 104664	98 %	0.0	99 %	A	Root
UYFA288	NR_074921.1	*Rahnella aquatilis* HX2	99 %	0.0	99 %	B	Root
UYFA289	NR_074921.1	*Rahnella aquatilis* HX2	100 %	0.0	99 %	B	Root
UYFA298	NR_024642.1	*Lelliottia amnigena* JCM1237	99 %	0.0	98 %	B	Root
UYFA330	NR_074921.1	*Rahnella aquatilis* HX2	99 %	0.0	98 %	B	Root
UYFA337	NR_074921.1	*Rahnella aquatilis* HX2	100 %	0.0	99 %	B	Root

[a] See section Isolation of putative endophytic bacteria from tall fescue cv. SFRO Don Tomás for a full description of the sampling sites

supported branch, isolate UYFA298, which has 99 % identity to *Lelliottia amnigena* JCM1237 (Table 2), was closely related to the reference strain *Enterobacter amnigenus* AB004749. The isolates within the genus *Rhanella* (UYFA288, UYFA289, UYFA330 and UYFA337) formed a well-supported cluster that was closely related to the reference strain *Rhanella aquatilis* AJ233426 (Fig. 1). *Acinetobacter* spp. were well represented in the collection and formed a cluster with two branches. In the first branch, isolates UYFA01, UYFA06, UYFA24 and UYFA39 grouped close to the reference strain *Acinetobacter calcoaceticus* AJ888983, while in the second one, isolate UYFA02 grouped alone with non-reference strains (Fig. 1). Analysis of the 16S rRNA sequences of isolates UYFA95, UYFA113, UYFA214 and UYFA249 showed that these isolates belonged to the genus *Pseudomonas* (Table 2; Fig. 1) and that they formed two well-supported branches. Among these isolates, only isolate

Fig. 1 Neighbor-joining phylogenetic tree based on bacterial 16S rRNA ▶ sequences of the representative isolates. The tree shows the phylogenetic affiliation of 30 partial 16S rRNA sequences of endophytic bacteria isolated from Tall fescue (*Festuca arundinacea*). *Numbers at branches* Bootstrap values of >50 % from 1000 replicates. *Thermanaeromonas toyohensis*^{T} AB062280 was used as an outgroup. *Scale bar* Number of nucleotide substitutions per site


UYFA01
UYFA24
UYFA06
UYFA39
Acinetobacter calcoaceticus^T, AJ888983
UYFA02
Acinetobacter baumannii^T, X81660
Acinetobacter junii^T, X81664
UYFA214
UYFA249
Pseudomonas brenneri^T, AF268968
Pseudomonas proteolytica^T, AJ537603
UYFA113
Pseudomonas seleniipraecipitan^T, FJ422810
UYFA95
UYFA190
UYFA198
Pantoea agglomerans^T, AJ233423
UYFA10
UYFA15
Pantoea ananatis^T, AY530795
UYFA110
Pantoea stewartii subsp.stewartii^T, U80208
Lelliottia amnigenus^T, AB004749
UYFA288
UYFA289
UYFA337
Rahnella aquatilis^T, AJ233426
Acidovorax avenae subsp. avena^T, DQ360414
UYFA23
Acidovorax konjaci^T,
UYFA22
Variovorax paradoxus^T, AJ420329
Variovorax boronicumulans^T, AB300597
UYFA215
UYFA105
Xanthomonas albilineans^T, X95918
Xanthomonas sacchari^T, Y10766
UYFA61
UYFA68
UYFA26
Microbacterium binotii^T, EF567306
Microbacterium ketosireducens^T, AB004724
Microbacterium terrae^T, AB004720
UYFA09
Microbacterium halotolerans^T, AY376165
UYFA155
Curtobacterium flaccumfaciens^T, AJ312209
UYFA157
Curtobacterium herbarum^T, AJ310413
UYFA153
UYFA156
Streptomyces sp.^T, AJ399472
Streptomyces violascens^T, AY999737
Bacillus pumilus^T, AY876289
Bacillus safensis^T, AF234854
UYFA152
Bacillus atrophaeus^T, AB021181
UYFA154
Thermanaeromonas toyohensis^T, AB062280
0.02

UYFA113 grouped with the reference strain *Pseudomonas seleniipraecipitans* FJ422810 (Fig. 1).

From the Gammaproteobacteria, two isolates in the genus *Xanthomonas*, UYFA105 and UYFA215, with 99 and 100 % identity with *X. translucens* XT2 (Table 2), clustered in a branch that was distantly related to the reference strains.

The Betaproteobacteria were represented in the phylogenetic tree by isolates UYFA22 and UYFA23. Isolate UYFA22 belonged to genus *Variovorax* (Table 2) and clustered close to the reference strain *V. paradoxus* AJ420329, while isolate UYFA23, which belonged to genus *Acidovorax*, was closely related to the reference strain *Acidovorax avenae* subsp. *avenae*, DQ360414 (Fig. 1).

With respect to the Firmicutes, analysis of the 16S rRNA sequences of isolates UYFA152 and UYFA154 showed that these belonged to genus *Bacillus* and were grouped in a well-supported cluster. Whereas isolate UYFA152 grouped with the reference strains *Bacillus safensis* AF234854 and *B. pumilis* AY876289, isolate UYFA154 was distant from any reference strain (Fig. 1). In addition, two isolates, UYFA153 and UYFA156, which have an identity of 99 and 100 % with *Streptomyces sampsonii* NRRL and B12325, respectively (Table 2), were clustered in a separate branch distantly related to the reference strains (Fig. 1).

The Actinobacteria were also well represented in the collection (Table 2), and the phylogenetic tree based on 16S rRNA sequences showed that isolates from this phylum grouped in a single cluster (Fig. 1). The genus *Microbacterium* was represented by isolates UYFA26, UYFA61, UYFA68 and UYFA09. The latter isolate did not group with any reference strains, but the remaining isolates grouped together in a cluster closely related to the reference strain *M. binotii* EF567306 (Fig. 1). Additionally, two isolates of Actinobacteria with 100 % identity with *Curtobacterium flaccumfaciens* LMG 3645 were identified in the collection (Table 2). Of these, isolate UYFA155 clustered closely with the type strain *C. flaccumfaciens* AJ312209, while isolate UYFA157 grouped in another branch distant from any reference strains (Fig. 1).

PGP of tall fescue cv. Don Tomás and Tacuabé inoculated with selected bacterial isolates

Twenty-eight isolates were selected on the basis of their in vitro PGP and plant infection traits, as well as on their generic identity (Tables 1, 2). These selected isolates were tested as inoculants on tall fescue cv. SFRO Don Tomás and Tacuabé under gnotobiotic conditions. At 1 month post inoculation, plantlets were harvested and their stem heights and total dry weights determined (Table 3).

In the case of cv. SFRO Don Tomás, results from the statistical analysis showed that both parameters analyzed were significantly higher than the negative control for the isolates UYFA61, UYFA156, UYFA157 and UYFA215. Additionally, in isolates UYFA09, UYFA10, UYFA15, UYFA22, UYFA26 and UYFA249, the stem heights were significantly higher than those of the negative control, while plant dry weight was significantly higher for the isolate UYFA68 than for the negative control (Table 3).

In contrast to cv. SFRO Don Tomás, in the case of the commercial cv. Tacuabé, only isolates UYFA289 and UYFA337 were able to promote aerial dry weight and stem height, respectively (Table 4).

Discussion

A variety of bacteria with PGP traits were found to be present in the roots, stems and seeds of tall fescue cv. SFRO Don Tomás

A collection of 342 bacterial isolates was obtained from surface-desinfected roots, stems and seeds of tall fescue cv. SFRO Don Tomás. The bacterial population was more numerous in the roots than in the aerial parts, which is in agreement with reports that roots are the most common colonization sites for endophytic bacteria and that only a small proportion of endophytic bacteria are able to colonize the internal aerial tissues (Mercado-Blanco and Lugtenberg 2014).

Among all of the bacterial isolates collected, 45 harbored the *nifH* gene and were able to grow on NFCC N-free semisolid medium, while 42 were also able to grow on NFCC N-free semisolid medium under microaerophilic conditions; these isolates were therefore considered to be diazotrophic. In addition, numerous isolates from the collection had various PGP traits, including the ability to produce IAA and solubilize P, K and Fe; interestingly, however, all were negative for the plant infections traits evaluated, with the exception of biofilm formation. The roles of IAA production and the ability to solubilize P and Fe in beneficial bacteria–plant interactions and growth promotion have been well reported (Rodríguez and Fraga 1999; Shokri and Emtiazi 2010; Ahmed and Holmström 2014). Additionally, our bacterial collection was highly versatile in the use of different C and N sources. Taken together, these data highlight the biotechnological potential of the bacterial collection in the production of a bioinoculant for cv. SFRO Don Tomás.

Our phylogenetic analysis of selected isolates revealed a broad range of genera in the collection, including *Bacillus* from the Firmicutes, *Microbacterium*, *Curtobacterium* and *Streptomyces* from the Actinobacteria, *Acidovorax* and *Variovorax* from the Betaproteobacteria, as well as a large number of isolates belonging to the Gammaproteobacteria, such as *Acinetobacter*, *Pseudomonas*, *Pantoea*, *Rhanella*, *Lelliottia* and *Xanthomonas*. These results are in contrast with the composition of a collection of bacteria associated with tall fescue reported in New Zealand, where the identified isolates

Table 3 Effects of inoculation with putative bacterial endophytes on the growth of tall fescue cv. SFRO Don Tomás under gnotobiotic conditions

Experiment 1			Experiment 2			Experiment 3		
Treatment[a]	Stem height (cm)	Dry weight (g plant^{-1})	Treatment	Stem height (cm)	Dry weight (g plant^{-1})	Treatment	Stem height (cm)	Dry weight (g plant^{-1})
Acinetobacter sp. UYFA01	11.32	0.0115	*Microbacterium* sp. UYFA09	11.43**	0,0126	*Pantoea* sp. UYFA198	10.85	0.0133
Acinetobacter sp. UYFA02	11.67	0.0090	*Pantoea* sp. UYFA10	11.06*	0.0133	*Pantoea* sp. UYFA190	10.98	0.0107
Acinetobacter sp. UYFA06	8.88	0.0104	*Pantoea* sp. UYFA15	12.56***	0.0120	*Rahnella* sp. UYFA337	11.23	0.0117
Acinetobacter sp. UYFA24	10.72	0.0112	*Variovorax* sp. UYFA22	11.25**	0.0135	*Rahnella* sp. UYFA330	11.32	0.0119
Acinetobacter sp. UYFA39	10.38	0.0107	*Acidovorax* sp. UYFA23	10.28	0.0095	*Rahnella* sp. UYFA288	11.36	0.0106
Microbacterium sp. UYFA68	13.50	0.0167**	*Microbacterium* sp. UYFA26	13.19***	0.0119	*Rahnella* sp. UYFA289	10.54	0.0092
Xanthomonas sp. UYFA105	12.11	0.0132	*Microbacterium* sp. UYFA61	14.14***	0.0159***	*Lelliottia* sp. UYFA298	12.06	0.0131
Pantoea sp. UYFA110	11.58	0.0127	*Pseudomonas* sp. UYFA95	9.26	0.0109	*Pseudomonas* sp. UYFA249	13.23**	0.0118
Curtobacterium sp. UYFA155	11.03	0.0135	*Pseudomonas* sp. UYFA113	10.98	0.0079	*Xanthomonas* sp. UYFA215	13.77**	0.0178**
Streptomyces sp. UYFA156	19.14***	0.0237***	*Curtobacterium* sp. UYFA157	12.4***	0.0137***	Control (−)	10.58	0.0100
Control (−)[a]	11.62	0.0100	Control (−)	8.22	0.0094	Control (+)	13.54**	0.0171**
Control (+)[b]	19.01 ***	–	Control (+)	15.40***	0.0184***			

Results presented in the table are from three independent experiments (1, 2, 3). Within the columns, treatment means with asterisk are significantly different compared with the control (−) at *$p<0.1$, **$p<0.05$), ***$p<0.01$

[a] Control (−) are uninoculated plants or plants grown in a system with N fertilization

[b] Control (+) are plants grown in a system with N fertilization

Table 4 Effects of inoculation with putative bacterial endophytes on the growth of tall fescue cv. Tacuabé under gnotobiotic conditions

Experiment 1			Experiment 2		
Treatment[a]	Stem height (cm)	Dry weight (g plant^{-1})	Treatment	Stem height (cm)	Dry weight (g plant^{-1})
Acinetobacter sp. UYFA01	11.26	0.0186	*Pseudomonas* sp. UYFA95	11.78	0.0261
Acinetobacter sp. UYFA02	10.69	0.0219	*Xanthomonas* sp. UYFA105	12.51	0.0222
Acinetobacter sp. UYFA06	10.64	0.0187	*Pantoea* sp. UYFA110	12.9	0.0258
Microbacterium sp. UYFA09	11.16	0.0231	*Pseudomonas* sp. UYFA113	11.97	0.0324
Pantoea sp. UYFA10	12.86	0.0214	*Curtobacterium* sp. UYFA155	11.98	0.0284
Pantoea sp. UYFA15	11.29	0.0202	*Streptomyces* sp. UYFA156	11.36	0.0285
Variovorax sp. UYFA22	11.31	0.0219	*Curtobacterium* sp. UYFA157	13.49	0.0256
Acidovorax sp. UYFA23	11.05	0.0289	*Pantoea* sp. UYFA190	13.14*	0.0301
Acinetobacter sp. UYFA24	12.56	0.0222	*Pantoea* sp. UYFA198	12.93	0.0272
Microbacterium sp. UYFA26	12.83	0.0226	*Pseudomonas* sp. UYFA214	13.55	0.0274
Acinetobacter sp. UYFA39	11.75	0.0257	*Xanthomonas* sp. UYFA215	11.38	0.0243
Microbacterium sp. UYFA61	11.06	0.0214	*Pseudomonas* sp. UYFA249	12.27	0.0280
Microbacterium sp. UYFA68	11.51	0.0226	*Rahnella* sp. UYFA288	12.05	0.0273
Control (−)[a]	11.28	0.0210	*Rahnella* sp. UYFA289	12.01	0.0242*
Control (+)[b]	19.08***	0.0341**	*Lelliottia* sp. UYFA298	13.01	0.0258
			Rahnella sp. UYFA330	13.02	0.0230
			Rahnella sp. UYFA337	14.09**	0.0250
			Control (−)	11.85	0.0304
			Control (+)	29.2***	0.1045***

Results presented in the table are from two different experiments (1, 2). Within the columns, treatment means with asterisk are significantly different compared with the control (−) at *$p<0.1$, **$p<0.05$), ***$p<0.01$

[a] Control (−) are uninoculated plants or plants grown in a system with N fertilization

[b] Control (+) are plants grown plants grown in a system with N fertilization

were mostly members of the Gammaproteobacteria (Monk et al. 2009).

Most of the genera identified in our study have been previously reported as being associated with several agronomical crops, including phytopathogens such as *Acidovorax oryzae*, *Xanthomonas albilineans*, *X. sacchari*. However, the initial plant material which we collected for bacterial isolation had no disease symptoms, which is an essential prerequisite for endophytic bacteria (Hallmann et al. 1997; Rosenblueth and Martínez-Romero 2006). Within the Gammaproteobacteria, isolates of the genera *Pantoea*, *Acinetobacter*, *Rhanella* and *Pseudomonas* have been previously reported as being associated with and/or endophytic within several poaceous plants, including sweet sorghum (*Sorghum bicolor*), sugarcane (*Saccharum officinarum*), maize (*Zea mays*), rice (*Oryza sativa*), grapevine (*Vitis vinifera*), canola (*Brassica napus*) and others (Bell et al. 1995; Misko and Germida 2002; Hallmann and Berg 2006; Rosenblueth and Martínez-Romero 2006; Taulé et al. 2012; Mareque et al. 2015). Of particular significance is that *Pseudomonas* and *Rhanella* isolates have been previously reported as being associated with tall fescue (Monk et al. 2009).

With respect to the Betaproteobacteria, the only two isolates identified in this study belonged to the genera *Variovorax* (UYFA22) and *Acidovorax* (UYFA23). Isolates from these genera have been reported to be endophytic and plant-associated bacteria with canola (Germida et al. 1998; Graner et al. 2003).

The Actinobacteria in our collection were represented by isolates belonging to the genera *Microbacterium*, *Curtobacterium* and *Streptomyces*. Species of the genus *Microbacterium* have been reported to be endophytes of marigold (*Calendula officinalis*), rice, common bean (*Phaseolus vulgaris*), potato (*Solanum tuberosum*) and maize (Zinniel et al. 2002; Sturz and Kimpinski 2004). In addition, the *Streptomyces* strain EN27 has been reported to be an endophyte of wheat (*Triticum aestivum*) seeds (Coombs and Franco 2003). Interestingly, both *Streptomyces* isolates identified in our study (UYFA153 and UYFA156) were obtained from surface-desinfected tall fescue seeds. On the other hand, *Curtobacterium* spp. have been reported to be endophytes of red clover (*Trifolium pratense*), potato, strawberry (*Fragaria* spp.) and *Eucalyptus* spp. (Sturz et al. 1997; Zinniel et al. 2002; Procópio et al. 2009; de Melo Pereira et al. 2012).

Finally, for Firmicutes, isolates were identified as *Bacillus*, and they also came from surface-desinfected seeds of tall fescue. *Bacillus* isolates have been reported to be associated with and/or endophytic within several important crops, including

citrus species, maize, cotton (*Gossypium* spp.) and carrots (*Daucus carota*) (McInroy and Kloepper 1994; Araújo et al. 2001; Surette et al. 2003). In particular, the partner *B. pumilus* JP12 strain–tall fescue has been demonstrated to be very successful in the phytoremediation of soils contaminated with heavy metals (Lu et al. 2013; Lu and Zhang 2014).

To our knowledge this study reveals several novel isolates associated with tall fescue, belonging to the genera *Bacillus*, *Streptomyces*, *Microbacterium*, *Xanthomonas*, *Acidovorax*, *Variovorax*, *Pantoea* and *Acinetobacter*. These isolates possess a number of PGP traits and consequently are excellent candidates as PGP inoculants in biotechnological application.

Isolates associated with tall fescue can promote its growth

Isolates selected according to their in vitro PGP traits were evaluated as inoculants under gnotobiotic conditions in two tall fescue cultivars, cv. SFRO Don Tomás and the commercial cv. Tacuabé. Three sets of PGP isolates were identified in cv. SFRO Don Tomás under the conditions studied. The first set (UYFA09, UYFA15, UYFA22, UYFA157, UYFA249) was able to promote the height of stems, the second set (UYFA68) promoted the dry weight of the plants and the third set (UYFA61, UYFA156, UYFA215) was able to promote both the aforementioned parameters. It is interesting to note that all of the isolates identified as belonging to the genus *Microbacterium* were able to promote plant growth. *Microbacterium* strains have been reported as growth promoters of apple trees (*Malus domestica*) and potato (Sessitsch et al. 2004; Karlidag et al. 2007), and the authors of both of these studies speculated that the ability to fix N_2 and to produce IAA were most probably the mechanisms involved in the reported PGP effects. In the present study, *Microbacterium* isolates UYFA26, UYFA61 and UYFA68 were defined as IAA producers, but they were negative for all of the other PGP traits tested, while isolate UYFA09 was *nifH* positive and negative for all others PGP traits. Therefore, it is reasonable to speculate that these PGP traits are also involved in the PGP effects observed with these isolates.

The seed-borne *Streptomyces* isolate UYFA156 was able to increase both plant dry weight and stem height. *Streptomyces* strains are well known for their ability to produce a wide range of secondary metabolites (Castillo et al. 2002; Nassar et al. 2003). In addition, several *Streptomyces* isolates have been described as growth promoters of many crops, such as chickpea (*Cicer arietinum*), wheat, soybean (*Glycine max*), sorghum and rice, through both indirect (biocontrol) and/or direct mechanisms (phytostimulation) (Nassar et al. 2003; Gopalakrishnan et al. 2011; Sadeghi et al. 2012). In these studies, the isolates possessed several PGP traits, such as IAA production, Fe and P solubilization and cellulase, protease, lipase and chitinase activites. Moreover, the *Streptomyces* isolate, *S. griseoluteus*, was found to be capable of producing relatively high levels of polyamines, which were involved in the observed PGP effect (Nassar et al. 2003). Our data show that isolate *Streptomyces* sp. UYFA156 is a diazotroph which tested negative for the other PGP traits examined. Therefore, it can be speculated that this mechanism may be involved in the PGP effect observed in this strain in cv. SFRO Don Tomás.

Curtobacterium sp. UYFA157 was also able to promote both growth parameters evaluated. *Curtobcterium* spp. have been reported as plant growth promoters of cucumber and other crop plants through the biological control of plant diseases (Raupach and Kloepper 1998; Ramamoorthy et al. 2001). Nevertheless, we classified isolate UYFA157 as a diazotroph; therefore, it is possible that this mechanism is involved in the observed PGP effect.

Regarding the PGP isolates *Pantoea* sp. UYFA15 and *Pseudomonas* sp. UYFA249, the former was described as a diazotroph, while the latter was able to produce IAA and siderophores. *Pantoea* spp. have been reported to be PGPB of several important agronomical crops, such as maize, rice, sugarcane, sweet sorghum, canola, lentil (*Lens culinaris*) and pea (*Pisum sativum*) (Verma et al. 2001; Sergeeva et al. 2007; Montañez et al. 2012; Taulé et al. 2012; Quecine et al. 2012; Mareque et al. 2015). *Pseudomonas* spp. are well-known PGPB of several plants, such as olive tree (*Olea europaea*), spruce (*Picea*), *Eucalyptus*, cactus (*Pachycereus pringlei*), tomato (*Solanum lycopersicum*) and wheat (Chanway et al. 2000; Zhang et al. 2000; Ran et al. 2005; Shaharoona et al. 2007; Puente et al. 2009; Richardson et al. 2009; Mercado-Blanco and Lugtenberg 2014). In some of these studies the mechanism involved in the PGP effects included P and N uptake, growth stimulation and IAA production (Richardson et al. 2009). Therefore, it is possible that the production of IAA and siderophores by isolate UYFA249 is involved in the PGP effect observed in tall fescue.

Finally, the *nifH*-positive isolate UYFA22, which has 100 % identity with *Variovorax paradoxus* strain S110, was able to promote the stem height of tall fescue cv. SFRO Don Tomás. *Variovorax* spp. have been described as PGPB of pea and maize in which the probable PGP mechanism involved is ACC-deaminase activity (Dodd et al. 2009).

In contrast to cv. SFRO Don Tomás, our PGP assays showed that only the two isolates related to *Rhanella*, UYFA289 and UYFA337, were able to promote the growth of the commercial tall fescue cv. Tacuabé. Both isolates are described here as diazotrophs, while UYFA288 was also able to produce IAA. Both isolates were closely related to the type strain of *Rahnella aquatilis* AJ233426. *R. aquatilis* strains have been reported to be PGPB of several important agronomical crops, such as apple, tomato, barley (*Hordeum vulgare*), chickpea, pea and maize (Calvo et al. 2007; Vyas et al. 2010).

It is interesting to note that the ability of different isolates to promote plant growth was dependent on the cultivar. Moreover, a larger number of PGPB isolates were observed for cv. Don

Tomás, highlighting the fact that plant-associated bacteria are often plant genotype dependent since the bacterial collection was constructed from cv. Don Tomás (Long et al. 2008).

In all of the PGPB strains mentioned here, additional studies are required to determine which mechanism is involved in their PGP abilities. However, regardless of the mechanism put into play in the growth promotion test under gnotobiotic conditions, the significance of this work lies in the diversity of bacteria with proven PGP capacity which were identified under in vitro and in vivo conditions. To our knowledge, this is the first report in which isolates belonging to the genera *Microbacterium*, *Pantoea*, *Variovorax*, *Streptomyces and Pseudomonas* are reported as PGPB in tall fescue.

From the biotechnology perspective, PGP experiments will be performed with this set of isolates as inoculants under greenhouse conditions. Special attention will be paid to isolates UYFA61, UYFA156 and UYFA215, which we found promoted both stem height and plant dry weight.

Concluding remarks

The results of our study reveal that a wide variety of putative endophytes are associated with the seeds, roots and stems of tall fescue cv. SFRO Don Tomás and that these also harbor several PGP and plant infection features. Ten of the isolates were able to promote the growth of cv. SFRO Don Tomás under gnotobiotic conditions, of which four were able to promote both growth parameters evaluated (stem height and plant dry weight). In addition to reporting known phenotypes, we describe novel strains for the first time as putative endophytes associated with tall fescue as well as PGPB for this crop. These results stress the biotechnology potential of the bacterial collection constructed in this study, and the PGPB strains identified will be tested in a more complex system with the aim of producing a PGP inoculant for tall fescue cv. SFRO Don Tomás.

Acknowledgments This work was supported by grants from the Alianza para el desarrollo fund (Project ALI-1-2012-1-3269) of the Uruguayan National Agency for Innovation and Research (ANII), and the Uruguayan Program for the Development of the Basic Science (PEDECIBA). The authors are very grateful to the members of the Rural Promotion Society Ortiz (SFRO) Ing. Agr. Santiago Larghero and José Mesa.

References

Ahmed E, Holmström SJM (2014) Siderophores in environmental research: roles and applications. Microb Biotechnol 7:196–208. doi:10.1111/1751-7915.12117

Araújo WL, Maccheroni W Jr, Aguilar-Vildoso CI, Barroso PAV, Saridakis HO, Azevedo JL (2001) Variability and interactions between endophytic bacteria and fungi isolated from leaf tissues of citrus rootstocks. Can J Microbiol 47:229–236. doi:10.1139/cjm-47-3-229

Avakyan Z, Pivovarova T, Shinner F (1986) Properties of a new species, *Bacillus mucilaginosus*. Mikrobiologiya 55:477–482

Baldani JI, Reis VM, Videira SS, Boddey LH, Baldani VLD (2014) The art of isolating nitrogen-fixing bacteria from non-leguminous plants using N-free semi-solid media: a practical guide for microbiologists. Plant Soil 384:413–431. doi:10.1007/s11104-014-2186-6

Bell CR, Dickie GA, Harvey WLG, Chan JWYF (1995) Endophytic bacteria in grapevine. Can J Microbiol 41:46–53. doi:10.1139/m95-006

Calvo J, Calvente V, de Orellano ME, Benuzzi D, de Tosetti MI (2007) Biological control of postharvest spoilage caused by *Penicillium* expansum and *Botrytis cinerea* in apple by using the bacterium *Rahnella aquatilis*. Int J Food Microbiol 113:251–257

Carambula M (2000) Producción y manejo de pasturas. Editorial Hemisferio Sur, Montevideo

Castillo UF, Strobel GA, Ford EJ, Hess WM, Porter H, Jensen JB, Albert H, Robison R, Condron MA, Teplow DB, Stevens D, Yaver D (2002) Munumbicins, wide-spectrum antibiotics produced by NRRL 30562, endophytic on *Kennedia nigriscans*. Microbiology 148:2675–2685

Cavalcante V, Dobereiner J (1988) A new acid-tolerant nitrogen fixing bacterium associated with sugarcane. Plant Soil 108:23–31

Chanway CP, Shishido M, Nairn J, Jungwirth S, Markham J, Xiao G, Holl F (2000) Endophytic colonization and field responses of hybrid spruce seedlings after inoculation with plant growth-promoting rhizobacteria. For Ecol Manag 133:81–88

Compant S, Clément C, Sessitsch A (2010) Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biol Biochem 42:669–678. doi:10.1016/j.soilbio.2009.11.024

Coombs JT, Franco CM (2003) Visualization of an endophytic *Streptomyces* species in wheat seed. Appl Environ Microbiol 69:4260–4262. doi:10.1128/AEM.69.7.4260-4262.2003

De Melo Pereira GV, Teixeira Magalhães K, Rainildes Lorenzetii E, Pereira Souza T, Freitas Schwan R (2012) A multiphasic approach for the identification of endophytic bacterial in strawberry fruit and their potential for plant growth promotion. Microb Ecol 63:405–417

Dodd IC, Jiang F, Teijeiro RG, Belimov AA, Hartung W (2009) The rhizosphere bacterium *Variovorax paradoxus* 5C-2 containing ACC deaminase does not increase systemic ABA signaling in maize (*Zea mays L.*). Plant Signal Behav 4:519–521. doi:10.1111/j.1469-8137.2008.02657.x.plant

García Préchac F, Ernst O, Siri G, Terra JA (2002) Integrating no-till into livestock pastures and crops rotations in Uruguay. In: 25th Annu Souther Conserv Tillage Conf Sustain Agric, pp 74–80

Germida JJ, Siciliano SD, Renato de Freitas J, Seib AM (1998) Diversity of root-associated bacteria associated with field-grown canola (*Brassica napus* L.) and wheat (*Triticum aestivum* L.). FEMS Microbiol Ecol 26:43–50. doi:10.1111/j.1574-6941.1998.tb01560.x

Gopalakrishnan S, Pande S, Sharma M, Humayun P, Kiran BK, Sandeep D, Vidya MS, Deepthi K, Rupela O (2011) Evaluation of actinomycete isolates obtained from herbal vermicompost for the biological control of *Fusarium* wilt of chickpea. Crop Prot 30:1070–1078. doi:10.1016/j.cropro.2011.03.006

Graner G, Persson P, Meijer J, Alstrom S (2003) A study on microbial diversity in different cultivars of *Brassica napus* in relation to its wilt pathogen, *Verticillium longisporum*. FEMS Microbiol Lett 29:269–276

Hallmann J, Berg G (2006) Spectrum and population dynamics of bacterial root endophytes. In: Schulz B, Boyle C, Sieber T (eds) Microbe root endophytes. Springer, Berlin Heidelberg, pp 15–31

Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper J (1997) Bacterial endophytes in agricultural crops. Can J Micorbiol 43:895–914

Hardoim PR, van Overbeek LS, Van Elsas JD (2008) Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol 16:463–471. doi:10.1016/j.tim.2008.07.008

Hoveland CS (2010) Origin and history. In: Hannaway DB, West CP (eds) Tall fescue for the twenty-first century. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, pp 3–10

Karlidag H, Esitken A, Turan M, Sahin F (2007) Effects of root inoculation of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrient element contents of leaves of apple. Sci Hortic (Amsterdam) 114:16–20. doi:10.1016/j.scienta.2007.04.013

Kim S-J, Lee C-M, Han B-R, Kim M-Y, Yeo Y-S, Yoon S-H, Koo B-S, Jun H-K (2008) Characterization of a gene encoding cellulase from uncultured soil bacteria. FEMS Microbiol Lett 282:44–51

Long HH, Schmidt DD, Baldwin IT (2008) Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses. PLoS ONE 3:e2702. doi:10.1371/journal.pone.0002702

Lu M, Zhang Z-Z (2014) Phytoremediation of soil co-contaminated with heavy metals and deca-BDE by co-planting of *Sedum alfredii* with tall fescue associated with *Bacillus cereus* JP12. Plant Soil 382:89–102. doi:10.1007/s11104-014-2147-0

Lu M, Zhang Z-Z, Wu X-J, Xu Y-X, Su X-L, Zhang M, Wang J-X (2013) Biodegradation of decabromodiphenyl ether (BDE-209) by a metal resistant strain, *Bacillus cereus* JP12. Bioresour Technol 149:8–15. doi:10.1016/j.biortech.2013.09.040

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Förster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, König A, Liss T, Lüssmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH (2004) ARB: a software environment for sequence data. Nucleic Acids Res 32:1363–1371

Mareque C, Taulé C, Beracochea M, Battistoni F (2015) Isolation, characterization and plant growth promotion effects of putative bacterial endophytes associated with sweet sorghum (*Sorghum bicolor* (L) Moench). Ann Microbiol 65:1057–1067. doi:10.1007/s13213-014-0951-7

Martinez-Rosales C, Castro-Sowinsky S (2011) Antartic bacterial isolates that produce cold-active extracellular proteases at low temperature but are active and stable at high temperature. Polar Res 30:1–8

McInroy JA, Kloepper J (1994) Novel bacterial taxa inhabiting internal tissues of sweet corn and cotton. In: Ryder MH, Stephens PM, Bowen GD (eds) Improving plant productivity with rhizosphere bacteria. CSIRO, Melbourne

Mei C, Flinn BS (2010) The use of beneficial microbial endophytes for plant biomass and stress tolerance improvement. Recent Pat Biotechnol 4:81–95

Mercado-Blanco J, Lugtenberg B (2014) Biotechnological applications of bacterial endophytes. Curr Biotechnol 3:60–75. doi:10.2174/2211550113026660038

Milne G (2010) Management in New Zealand, Australia and South America. In: Hannaway DB, West CP (eds) Tall fescue in the twenty-first century. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, pp 101–117

Mirza BS, Rodrigues JLM (2012) Development of a direct isolation procedure for free living diazotrophs under controlled hypoxic conditions. Appl Environ Microbiol 78:5542–5549. doi:10.1128/AEM.00714-12

Misko AL, Germida JJ (2002) Taxonomic and functional diversity of pseudomonads isolated from the roots of field-grown canola. FEMS Microbiol Ecol 42:399–407

Monk J, Gerard E, Young S, Widdup K, Callaghan MO (2009) Isolation and identification of plant growth-promoting bacteria associated with tall fescue. Proc N Z Grassl Assoc 71:211–216

Montañez A, Blanco AR, Barlocco C, Beracochea M, Sicardi M (2012) Characterization of cultivable putative endophytic plant growth promoting bacteria associated with maize cultivars (*Zea mays* L.) and their inoculation effects in vitro. Appl Soil Ecol 58:21–28. doi:10.1016/j.apsoil.2012.02.009

Nassar AH, El-tarabily KA, Sivasithamparam K (2003) Growth promotion of bean (*Phaseolus vulgaris L* .) by a polyamine-producing isolate of *Streptomyces griseoluteus*. Plant Growth Reg 40:97–106

Peeters E, Nelis HJ, Coenye T (2008) Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. J Microbiol Methods 72:157–165

Poly F, Ranjard L, Nazaret S, Gourbiére F, Monrozier LJ (2001) Comparison of *nifH* gene pools in soils and soil microenvironments with contrasting properties. Appl Environ Microbiol 67:2255–2262

Procópio REL, Araújo WL, Maccheroni W Jr, Azevedo JL (2009) Characterization of an endophytic bacterial community associated with *Eucalyptus* spp. Genet Mol Res 8:1408–1422

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Jorg P, Glockner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 35:7188–7196

Puente M, Li C, Bashan Y (2009) Endophytic bacteria in cacti seeds can improve the development of cactus seedlings. Environ Exp Bot 66:402–408

Quecine MC, Araújo WL, Rossetto PB, Ferreira A, Tsui S, Lacava PT, Mondin M, Azevedo JL, Pizzirani-Kleiner AA (2012) Sugarcane growth promotion by the endophytic bacterium *Pantoea agglomerans* 33.1. Appl Environ Microbiol 78:7511–7518. doi:10.1128/AEM.00836-12

Ramamoorthy V, Viswanathan R, Raguchander T, Prakasam V, Samiyappan R (2001) Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. Crop Prot 20:1–11. doi:10.1016/S0261-2194(00)00056-9

Ran LX, Li ZN, Wu GJ, van Loon LC, Bakker PHM (2005) Induction of systemic resistance against bacterial wilt in *Eucalyptus urophylla* by fluorescent *Pseudomonas* spp. Eur J Plant Pathol 113:59–70. doi:10.1007/s10658-005-0623-3

Raupach GS, Kloepper JW (1998) Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology 88:1158–1164

Reis VMM, Olivares FLL, Dobereiner J (1994) Improved methodology for isolation of *Acetobacter diazotrophicus* and confirmation of its endophytic habitat. World J Microbiol Biotechnol 10:401–405

Richardson AE, Barea J-M, McNeill AM, Prigent-Combaret C (2009) Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil 321:305–339. doi:10.1007/s11104-009-9895-2

Rodríguez H, Fraga R (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol Adv 17:319–339

Rosenblueth M, Martínez-Romero E (2006) Bacterial endophytes and their interactions with hosts. Mol Plant Microbe Interact 19:827–837

Ryan RP, Germaine K, Franks A, Ryan DJ, Dowling DN (2008) Bacterial endophytes: recent developments and applications. FEMS Microbiol Lett 278:1–9. doi:10.1111/j.1574-6968.2007.00918.x

Sack U, Hofrichter M, Fritsche W (1997) Degradation of polycyclic aromatic hydrocarbons by manganese peroxidase of Nematoloma frowardi. FEMS Microbiol Lett 152:227–234

Sadeghi A, Karimi E, Dahaji PA, Javid MG, Dalvand Y, Askari H (2012) Plant growth promoting activity of an auxin and siderophore producing isolate of *Streptomyces* under saline soil conditions. World J Microbiol Biotechnol 28:1503–1509. doi:10.1007/s11274-011-0952-7

Sarwar M, Kremer RJ (1995) Determination of bacterially derived auxins using a microplate method. Lett Appl Microbiol 20:282–285

Schulz B, Boyle C, Schulz BJE, Boyle CJC, Sieber TN (2006) What are endophytes? Soil Biol 9:1–13. doi:10.1007/3-540-33526-9

Schwyn B, Neilands JB (1987) Universal chemical assay for detection and determination of siderophores. Anal Biochem 160:47–56

Sergeeva E, Hirkala DLM, Nelson LM (2007) Production of indole-3-acetic acid, aromatic amino acid aminotransferase activities and plant growth promotion by *Pantoea agglomerans* rhizosphere isolates. Plant Soil 297:1–13. doi:10.1007/s11104-007-9314-5

Sessitsch A, Reiter B, Berg G (2004) Endophytic bacterial communities of field-grown potato plants and their plant-growth-promoting and antagonistic abilities. Can J Microbiol 50:239–249. doi:10.1139/w03-118

Shaharoona B, Jamro G, Zahir Z, Arshad M, Memon K (2007) Effectiveness of various *Pseudomonas* spp. and *Burkholderia caryophylli* containing ACC-deaminase for improving growth and yield of wheat (*Triticum aestivum L*). J Microbiol Biotechnol 1300: 1300–1307

Shokri D, Emtiazi G (2010) Indole-3-acetic acid (IAA) production in symbiotic and non-symbiotic nitrogen-fixing bacteria and its optimization by Taguchi design. Curr Microbiol 61:217–225. doi:10.1007/s00284-010-9600-y

Sturz A, Kimpinski J (2004) Endoroot bacteria derived from marigolds (*Tagetes* spp.) can decrease soil population densities of root-lesion nematodes in the potato root zone. Plant Soil 262:241–249

Sturz A, Christie B, Matheson B, Nowak J (1997) Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. Biol Fertil Soils 25:13–19

Surette MA, Sturz AV, Lada RR, Nowak J (2003) Bacterial endophytes in processing carrots (Daucus carota L. var. sativus): their localization, population density, biodiversity and their effects on plant growth. Plant Soil 253:381–390

Sylvester-Bradley R, Askawa N, Latorraca S, Magalhães F, Oliveira L, Pereira R (1982) Levantamento quantitativo de microrganismos solubilizadores de fosfatos na rizosfera de gramíneas e leguminosas forrageiras na Amazônia. Acta Amaz 12:15–22

Taulé C, Mareque C, Barlocco C, Hackembruch F, Reis VM, Sicardi M, Battistoni F (2012) The contribution of nitrogen fixation to sugarcane (*Saccharum officinarum* L.), and the identification and characterization of part of the associated diazotrophic bacterial community. Plant Soil 356:35–49

Verma SC, Ladha JK, Tripathi K (2001) Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice. J Biotechnol 91:127–141

Vincent JM (1970) A manual for the practical study of root-nodule bacteria. Blackwell Scientific, Oxford

Vyas P, Joshi R, Sharma KC, Rahi P, Gulati A, Gulati A (2010) Cold-adapted and rhizosphere-competent strain of *Rahnella* sp. with broad-spectrum plant growth-promotion potential. J Microbiol Biotechnol 20:1724–1734

Zhang H, Hanada S, Shigematsu T, Shibuya K, Kamagata Y, Kanagawa T, Kurane R (2000) *Burkholderia kururiensis* sp. nov., a trichloroethylene (TCE) -degrading bacterium isolated from an aquifer polluted with TCE. Int J Syst Evol Microbiol 50:743–749

Zinniel DK, Lambrecht P, Harris NB, Feng Z, Kuczmarski D, Higley P, Ishimaru CA, Arunakumari A, Barletta RG, Vidaver AK (2002) Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. Appl Environ Microbiol 68:2198–2208. doi:10.1128/AEM.68.5.2198