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ORIGINAL ARTICLE

The Mo- and Fe-nitrogenases of the endophyte *Kosakonia* sp. UYSO10 are necessary for growth promotion of sugarcane

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Abstract

Aim Sugarcane is a multipurpose crop primarily used to produce sugar, energy and bioethanol. It requires high amounts of N-fertilization for optimal growth, which increases production costs and environmental degradation. The contribution of biological nitrogen fixation to Uruguayan commercial sugarcane cultivars was demonstrated previously, and diazotrophic bacteria that were isolated from the stems were characterized and identified. From this collection, the isolate UYSO10 related to the *Kosakonia* genus (formerly *Enterobacter*) was described as a plant growth-promoting endophyte of sugarcane plants.

Purpose To evaluate the effect of the inoculation of wild-type and nitrogenase-deficient strains of *Kosakonia* sp. UYSO10 on sugarcane growth promotion under non-sterile conditions.

Methods *Kosakonia* sp. UYSO10 was inoculated onto sugarcane setts for plant growth promotion greenhouse experiments. Single and double mutants resulting to the nitrogenase-encoding genes (*nifH*, *anfH*) were constructed, and the phenotypes were evaluated in vitro and in vivo.

Results *Kosakonia* sp. UYSO10 is able to promote sugarcane growth under non-sterile conditions, that strain UYSO10 harbors two functional nitrogenases and the inactivation of both nitrogenase-encoding genes diminish its capacity of promoting growth on sugarcane.

Conclusion All together, the results obtained showed that the biological nitrogen fixation ability of *Kosakonia* sp. UYSO10 is required for sugarcane growth promotion.

Keywords BNF · Nitrogenase mutants · *nifH* · *anfH*

Abbreviations

BNF	Biological nitrogen fixation
PGP	Plant growth promotion
PGPB	Plant growth-promoting bacteria
Pi	Post-inoculation

Introduction

Several plant nutrients, including N and P, are often scarce in the soil and thus can limit the optimal development of crops (Masclaux-Daubresse et al. 2010; Sinclair and Rufty 2012). Although this can be resolved by chemical fertilization, it is very costly and is often applied in excess, which results in harm to the environment via the leaching of nitrates into the ground water and the surface runoff of phosphorous and nitrates, all of which result in the contamination of drinking water and aquatic ecosystems (Adesemoye and Kloepper 2009). These problems obviate the need for the development of new agricultural

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technologies that seek to attain more sustainable production systems with less loss of nutrients and pollution. One alternative is the use of plant growth-promoting bacteria (PGPB). These can stimulate the growth and enhance the health of several crops in various ways that are classified as direct or indirect mechanisms. Direct plant growth promotion (PGP) mechanisms include the increase of nutrient uptake due to biological nitrogen fixation (BNF) and mineral solubilization (P, Fe) as well as the production of phytohormones such as auxins and cytokinins. Indirect PGP mechanisms include biocontrol against phytopathogens and the induced systemic resistance (ISR) response (Mei and Flinn 2010; Compant et al. 2010).

In nature, plants and bacteria are naturally closely associated in several ways, including non-beneficial and beneficial interactions. Endophytic bacteria are those that can be detected at a particular moment within the tissues of apparently healthy plants (Hallmann et al. 1997; Schulz and Boyle 2006). They do not normally cause any substantial morphological changes inside the plant, such as those that occur with root nodule symbionts such as rhizobia in legumes, and they do not cause any disease damage symptoms, unlike phytopathogens. In comparison with symbionts and phytopathogens, little is known about the molecular basis of endophyte-plant interactions, although strong evidence of the beneficial effects on their hosts has been thoroughly reported (Friesen 2013; Hardoim et al. 2015).

Sugarcane (*Saccharum officinarum*) is a multipurpose crop used in Uruguay to produce sugar, bioethanol, animal feed, and energy. This crop requires high levels of N-fertilization for its optimal growth in the Uruguayan cropping latitudes (Fogliata 1995). In a previous study, we demonstrated the contribution of BNF to Uruguayan sugarcane cultivars using the ^{15}N isotope dilution method (Taulé et al. 2012). Additionally, a collection of putative endophytes associated with the same sugarcane cultivars was constructed. These were characterized genetically and biochemically, and selected diazotrophic strains were identified and their phylogenetic relationships examined (Taulé et al. 2012). A subset of these isolates was inoculated onto micropropagated sugarcane plants (cv. LCP 85384), and this experiment demonstrated that a group of strains, including the diazotrophic strain *Kosakonia* sp. UYSO10 (formerly *Enterobacter* sp.), was able to promote the growth of sugarcane plants under *gnotobiotic* conditions (Taulé et al. 2016). In addition, microscopy studies of strain UYSO10 allowed us to define it as a sugarcane endophyte (Taulé et al. 2016).

The hypothesis of the present work is that BNF is one of the mechanisms used by *Kosakonia* sp. UYSO10 to promote sugarcane growth. In particular, the aims were to evaluate the effect of the inoculation of *Kosakonia* sp. UYSO10 and nitrogenase mutants on sugarcane growth under non-sterile conditions.

Materials and methods

Plant growth promotion experiments under greenhouse conditions

Stem seed pieces ("setts") from sugarcane commercial cultivars TUC 7742 and LCP 85384 were first clean with water to remove soil particles and secondly washed with 70% EtOH to diminish the bacterial adhered to the surface, cut in order to leave one bud per sett, and sown into pots containing 1.5 kg of sterile sand/soil (2:1) as substrate. The soil was collected from a northern region of the country in which no fertilization or cropping activity had been reported over the preceding 10 years. The soil profile was as follows: pH 6.6, total N 0.4 mg/g organic C 3.4 mg/g, 7 μgP/g, 0.05 meq K/100 g, and 22.0 μg Fe/g. Each stem piece was inoculated with a suspension containing *Kosakonia* sp. UYSO10 (1×10^7 cells $plant^{-1}$) in the base of the stem at 20 and 65 days after sowing. In the positive control treatment, 0.18 g of urea was added in each pot containing one sett, while in the negative control, plants without N-fertilization remained uninoculated. The experiment included 10 replicates per treatment and was a completely randomized design. The pots were located in a greenhouse with a photoperiod of 16/8 h light/darkness and were watered with tap water as required.

In all the sugarcane PGP experiments, the plant height (measured from the stem base to the newest leaf blade joint) and the diameter of the basal stem were determined 4 months after the first bacterial inoculation. Roots and aerial parts were dried at 65 °C for dry weight determination. Total N content of the aerial parts was determined with the Kjeldjhal method at the Animal Nutrition Laboratory of the Faculty of Agronomy-UdelaR.

Parametric data were analyzed by ANOVA and means compared by the least significant differences test (LSD) at $p < 0.05$ (InfoStat 2008). Non-parametric data were analyzed by Kruskal-Wallis test and the treatment means were compared using the same test at $p < 0.05$ (Infostat 2008).

Genomics analysis of nitrogenase encoding operons

Nitrogenase genes (nif and anf operons) were identified on the genome draft annotation of the strain *Kosakonia* sp. UYSO10 (Beracochea et al. in preparation). The mentioned operons were compared with their homologs in the strains *Gluconacetobacter diazotrophic*us Pal5 (GCA_000021325.1), *Azotobacter vinelandii* DJ (GCA_000021045.1), and *Kosakonia radicincitans* DSM16656 (GCA_000280495.2). The aforementioned comparison was carried out using BLASTP version 2.6.0+ with an *e*-value of 0.001 for each strain using UYSO10 as the reference. The BLASTP results were processed with Easyfig 2.2.2 (Sullivan et al. 2011). For the phylogenetic study of the *nifH* and *anfH* genes, the aminoacidic sequences were

aligned using Muscle 3.8.31 (Edgar 2004). The phylogenetic reconstructions were generated using maximum likelihood with the Jones-Taylor-Thornton substitution model with 1000 bootstrap replicates with the software MEGA7 (Kumar et al. 2016).

Construction of Δ*nifH*, Δ*anfH*, and Δ*nifH*Δ*anfH* mutants

Mutant strains Δ*nifH*, Δ*anfH*, and double mutant Δ*nifH*Δ*anfH* were constructed using an adaptation of the method described by Martínez-García and de Lorenzo (2011) (Supplementary material). The primers nifH-TSF1-EcoRI, nifH-TS1R, nifH-TS2F, nifH-TS2R-BamHI, anfH-TS1F-EcoRI, anfH-TS1R, anfH-TS2F, and anfH-TS2R-BamHI (Table 2) were employed to amplify 500 bp fragments of the flanking upstream (TS1) and downstream (TS2) regions of the *nifH* and *anfH* genes, respectively. TS1 and TS2 fragments were joined together by SOE-PCR (Horton et al. 1989) employing TS1F and TS2R primers. The resulting 1.0-kb fragments were digested with *Eco*RI and *Bam*HI (Thermo, USA), ligated into the pEMG plasmid (containing I-SceI nuclease recognition site) (Martínez-García and de Lorenzo 2011) treated with the same enzymes, and transformed into DH5-α λpir competent cells. The presence of the plasmid in selected Km-resistant colonies was checked by PCR, and the accuracy of the cloned fragments was confirmed by sequencing the entire TS1-TS2 fragment. The resulting pEMGΔ*nifH* and pEMGΔ*anfH* vectors were transferred to UYSO10 strain by triparental conjugation using *E. coli* DH5-α (pRK2013) as a helper strain (Figurski and Helinski 1979). Cointegration of the recombinogenic plasmids in the chromosome of the resulting Km^r UYSO10 colonies was corroborated by PCR using appropriate TS1F and TS2R primers. In order to induce the resolution of the cointegrates, the pSW plasmid bearing a 3-methyl-benzoate (3 MB) inducible *I-sce*I nuclease gene was introduced into the cointegrated Km^r clones by triparental maiting. Transformed clones were grown overnight in a 5-mL tube with LB media, 100 mg mL^{-1} ampicillin, 40 mg mL^{-1} gentamicin, and 2 mM 3 MB for the induction of I-SceI nuclease and plated in LB Ap agar plates. Individual colonies were restreaked in LB + Ap and LB + Ap + Km to verify the resolution of the cointegrated constructs. The Km-sensitive clones were analyzed by colony PCR to identify the deletions of *nifH* or *anfH*.

For the construction of the double mutant Δ*nifH*Δ*anfH*, the same protocol for the construction of the Δ*anfH* strain was used but starting with the Δ*nifH* strain. Finally, the pSW plasmid was eliminated after three consecutive passes in liquid LB without antibiotic pressure. Curing of the plasmid was verified in all cases by direct colony PCR amplification, using the diagnostic primer pair pSW-F and pSW-R (Table 2).

The complementation plasmids pnifHkosa and panfHkosa (Table 1) were constructed by cloning the *nifH* and *anfH* genes in the broad host range vectors pSEVA232 and pSEVA632, respectively (Silva-Rocha et al. 2013). Primers nifH-F-EcoRI/nifH-R-BamHI and anfH-F-KpnI/anfH-R-BamHI were used for the amplification of the entire *nifH* and *anfH* genes from UYSO10 strain. The obtained fragments were digested with indicated enzymes, ligated to the pSEVA232 (Km^r) and pSEVA632 (Gm^r) vectors digested with appropriated enzymes and transformed into DH5-α competent cells. Blue/white selection was carried out in LB plates with X-gal 40ugmL^{-1} and IPTG 0,1 mM and appropriated antibiotics. Individual white colonies were transferred to fresh LB plates and screened for the presence of the gene insert by PCR. Accuracy of the cloned genes was corroborated by sequencing the entire inserted fragments using the primers PS1 (Table 2) and M13R (universal primer). Plasmids were mobilized from *E. coli* to UYSO10 by triparental mating using *E. coli* DH5-α (pRK2013) as a helper strain (Figurski and Helinski 1979).

Characterization of UYSO10 Δ*nifH*, Δ*anfH*, and Δ*nifH*Δ*anfH* mutant strains

The growth of all the strains (wt and mutants) was investigated in LB broth with the aim to determine if the mutations affected the growth under non-BNF conditions. For that, the OD_{620nm} of the strains were measured by an automated spectrophotometer Varioskan Flash (ThermoScientific), every 2 h during 6 h.

The ability to fix N_2 was tested for every strain by the evaluation of the nitrogenase activity using the acetylene reduction assay (ARA) (Hardy et al. 1968). In order to do so, bacteria were inoculated into vials containing NFCC liquid media (Mirza and Rodrigues 2012), for 4 days at 30 °C, with and without of Mo 9.1 μM. After that, 10% of the gas volume was replaced by acetylene, incubated for 3 days at 30 °C and analyzed for ethylene production by gas chromatography (GC-2010 Plus+, Shimazu), with a column TG-Bond (30 m × 0.32 mm ID × 10 μm) (Thermo Scientific). All the treatments were tested in quintupled, while un-inoculated vials were used as negative control.

Effects of nitrogenase mutations on the sugarcane plant growth promotion

Micropropagated plantlets of cv. LCP 85384 were inoculated with 1×10^7 cells $plant^{-1}$ of each strain to be tested as previously described (Taulé et al. 2016). Strains tested as inoculants were the wild-type UYSO10, the simple mutants (Δ*nifH* and Δ*anf*); the double mutant (Δ*nifH*Δ*anfH)*; as well as the respective complementant strains (Δ*nifH*Δ*anfH* pnifHkosa and Δ*nifH*Δ*anfH* panfHkosa).

The experiment setup was randomized with 10 replicates per treatment. Plants were maintained at a temperature of 30 °C with a photoperiod of 16/8 h light/dark. At 25 dpi, plants were transferred to pots containing 1.5 kg of soil/

Table 1 Strains and plasmids used in this study

Name	Feature	Reference
Kosakonia sp. UYSO10	Wild-type strain	(Taulé et al. 2012)
E. coli DH5α	supE44 ΔlacU169(ϕ80lacZΔM15) hsdR17 recA1 gyrA96 thi-1 relA1	(Hanahan 1983)
E. coli DH5α λpir	λpir lysogen of DH5 α	(Martínez-García and de Lorenzo 2011)
pEMG	Ori6K, Km^r, mob, LacZα	(Martínez-García and de Lorenzo 2011)
pEMGΔnifH	Derivates from pEMG. Non-replicable in *Kosakonia*. Recombinogenic. Contains 500 bp region of homologies flanking *nifH* gene	This work
pEMGΔanfH	Derivates from pEMG. Non-replicable in *Kosakonia*. Recombinogenic. Contains 500 bp region of homologies flanking *anfH* gene	This work
pSEVA232	pBBR1, Km^r, mob. pUC19/LacZ	(Silva-Rocha et al. 2013)
pnifHkosa	pSEVA232 with *nifH* gene cloned in EcoRI/BamHI	This work
pSW-2	Gm^R, oriRK2, xylS, Pm → I-sceI (transcriptional fusion of I-sceI to Pm)	(Martínez-García and de Lorenzo 2011)
pSEVA632	pBBR1, Gm^r, mob. pUC19/LacZ	(Silva-Rocha et al. 2013)
panfHkosa	pSEVA632 with *anfH* cloned in KpnI/BamHI	This work

sterile sand (2:1) as substrate and maintained in the growth chamber. The soil was collected from the same region as previously mentioned. The height and diameter of the stems, as the plant dry weight, were determined after 4 months pi.

Results

Plant growth promotion of commercial sugarcane cultivars by *Kosakonia* sp. UYSO10

Greenhouse experiments were conducted to determine if the commercial sugarcane cultivars LCP 85384 and TUC 7742 respond equally to inoculation with the diazotrophic strain *Kosakonia* sp. UYSO10. The results showed that only cultivar LCP 85384 responds to the bacterial inoculation. In this case, the inoculated treatment resulted in a significantly higher stem (height and diameter) and root dry weight in comparison with the un-inoculated control (negative control). When the shoot dry weight was measured, the bacterial inoculation treatment was not significantly different from the negative control but showed significantly higher N accumulation than the negative control (Table 3).

Nitrogenase mutation effects on the growth and the N_2-fixation ability of *Kosakonia* sp. UYSO10

The bioinformatic analysis showed that genome of the strain *Kosakonia* sp. UYSO10 contains two types of nitrogenases, the classical FeMo-nitrogenase (encoded by the *nif* regulon) and the alternative Fe-nitrogenase (encoded by the *anf* regulon) (Fig. 1). The gene composition and organization of the *Kosakonia* sp. UYSO10 *nif* regulon were compared to (1) the closely related strain *K. radicincitans* DSM16656 and (2) the model sugarcane endophyte *G. diazotrophicus* PA15 (Fig. 1, Table S1 Supplementary material). The comparison to strain DSM16656 revealed that the *nif* regulon gene composition is almost identical to that of strain UYSO10. In particular, the *nifH* gene shared 100% amino acid (aa) identity between the strains, while the aa identity ranged from 99.2 to 99.8% for the other genes of the regulon (Fig. 1; Table S1 Supplementary material). In the second case, the *nif* structural genes *nifHDK* are arranged in a cluster on both strains with a low percentage of aa identity. In addition, the *nifL* gene is missing in the *G. diazotrophicus* PA15 genome (Bertalan et al. 2009).

Alternatively, the gene composition and organization of the *Kosakonia* sp. UYSO10 *anf* operon were compared to that of strain DSM16656 and the model organism *A. vinelandii* DJ. In the first case, the strain UYSO10 *anf* regulon has 100% aa identity with that of strain DSM16656 (Fig. 1; Table S1). In the second case, the strain UYSO10 *anf* regulon shares the cluster *anfHDGK*, responsible for the nitrogenase synthesis on *A. vinelandii* DJ, with a low percentage of aa identity (Fig. 1; Table S1).

To elucidate the role of both nitrogenases on the plant growth promotion effects of the sugarcane plants previously described, mutants for the structural *nifH* and *anfH* genes were constructed (Supplementary material). The phenotypes of the single (Δ*nifH*, Δ*anfH*) and double (Δ*nifH*Δ*anfH*) mutants

Table 2 Primers used in this work

	Primer sequence (5′-3′)	Reference
nifH-TSF1-EcoRI	Cgaattcctccattaaacgcaccggctg	This work
nifH-TS1R	cttcctgttggcgggcgtgcatcggtgtttctcctgttggttgtgtg	This work
nifH-TS2F	Gatgcacgcccgccaacaggaag	This work
nifH-TS2R-BamHI	cgggatccgatccctttggtcagcgggaacag	This work
anfH-TS1F-EcoRI	Gcgaattcgtttgccatcccgcaagggg	This work
anfH-TS1R	gactgtaaaataaccggacgaagatatttcctcttgatgaacaac	This work
anfH-TS2F	Ttcgtccggttattttacagtc	This work
anfH-TS2R-BamHI	Cgggatcccctgcgcaatggcggcaatatc	This work
pnifH-F-EcoRI	Gcgaattccctgtgccagctcatttatcc	This work
pnifH-R-BamHI	cgggatccgcagagcggcgaccaggttttg	This work
nifH-R-BamHI	Cgggatcctcaaaccgcgttttcttctgcg	This work
panfH-F-KpnI	Ggggtaccctgaatttcacttccgcgaacg	This work
panfH-R-BamHI	cgggatcccggatcgcagccatgaataaacac	This work
anfH-R-BamHI	cgggatccttaatcgctcagaccatatttaacg	This work
pSW-F	ggacgcttcgctgaaaacta	(Martínez-García and de Lorenzo 2011)
pSW-R	Aacgtcgtgactgggaaaac	(Martínez-García and de Lorenzo 2011)
PS1	Agggcggcggatttgtcc	(Silva-Rocha et al. 2012)

and the complements (Δ*nifH* pnifHkosa, Δan*fH* panfHkosa, Δ*nifH*Δa*nfH* pnifHkosa, and Δ*nifH*Δa*nfH* panfHkosa) were studied in vitro and in vivo.

For the in vitro studies, wild-type and nitrogenase mutant strains were tested for their ability to grow in LB medium (non BNF conditions) (Fig. 2). The results showed that under non-BNF conditions, all the strains have the same growth behavior; thus, the fitness is not affected by the mutation.

In addition, and with the aim of testing the nitrogenase activity, every strain was inoculated into vials containing liquid NFCC media without N, and an ARA assay was conducted. Under these conditions, only the double mutant (Δ*nifH*Δ*anfH*) was unable to fix nitrogen (Table 4). In addition, results showed that the absence of BNF in the double mutant was overcome when this mutant was complemented *in trans* with a plasmid encoding the *nifH* or *anfH* genes with their respective promoters (Table 4).

Nitrogenase mutations effects on the plant growth promotion of sugarcane plants

The in vivo characterization of the nitrogenase mutants was evaluated by studying the inoculation effects of the wild-type and mutant strains on micropropagated sugarcane plants cv. LCP 85384 under growth chamber conditions (Fig. 3). In this case, the substrate used was a mixture of sterile sand and soil from the Bella Union region (Uruguay) with a sugarcane crop history without the addition of N-chemical fertilization.

A statistical analysis of the biometric parameters evaluated showed that the inoculation with the simple mutants (Δ*nifH* or

Table 3 Effects of inoculation with *Kosakonia* sp. UYSO10 on sugarcane setts from cv. LCP 85384, in pots at greenhouse condition (4 months pi)

Treatment	Stem height (cm)	Stem diameter (mm)	Dry weight (g plant^{-1})		N concentration (mg N g^{-1} dry weight)	N accumulation (mg plant^{-1})
			Roots	Shoot		
Negative control	13.7 a[b]	6.97 a	0.83 a	1.79 a	5.90 a	10.57 a
Kosakonia sp. UYSO10	16.00 b	7.72 b	1.78 b	2.36 a	6.06 a	14.30 b
Positive control	22.20 c	9.06 c	1.95 b	4.56 b	7.06 b	32.17 c

[a] Negative controls: plants uninoculated and without N-fertilization, while positive controls are plant supplemented with urea (equivalent to 100 kg N ha^{-1})

[b] Means within two treatments that have the same letter are not significantly different at $p < 0.05$

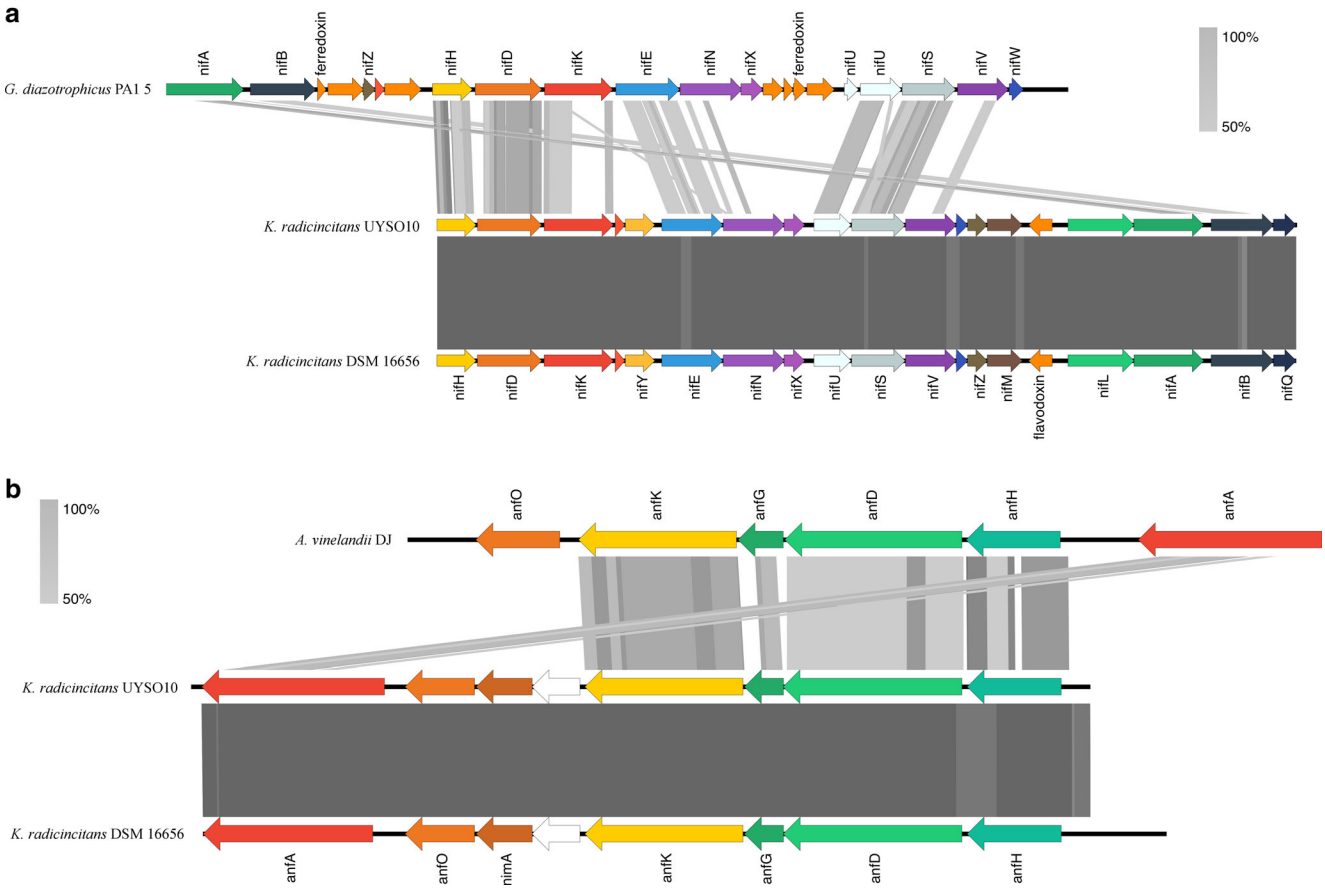

Fig. 1 A schematic comparison of nitrogenase-encoding *nif* and *anf* operons in different plant-associated bacteria. **a** *nif* operon comparison between the strains *Kosakonia* sp. UYSO10, *Kosakonia radicincitans* DSM16656^T, and *Gluconacetobacter diazotrophicus* PAl 5; **b** *anf* operon comparison between the strains *Kosakonia* sp. UYSO10, *Kosakonia radicincitans* DSM16656^T, and *Acetobacter vinelandii* DJ

Δ*anfH*) was not significantly different than that of the wild type. Nevertheless, when the double mutant (Δ*nifH*Δ*anfH*) was inoculated, the height, stem diameter, and the aerial dry weight were significantly lower (9, 13, and 32%, respectively) than the wild-type treatment. In addition, the inoculation with the double mutant strain complemented with a plasmid encoding each mutated gene controlled by its own promoters (strains Δ*nifH*Δa*nfH* pnifHkosa and Δ*nifH*Δa*nfH* panfHkosa) recovered the wild-type plant growth promotion effects (Fig. 3).

Discussion

The plant growth promotion effect depends on plant-bacterial specificity

Many diazotrophic bacteria have been isolated from different sugarcane cultivars grown in several regions of the world (James 2000; Thaweenut et al. 2011; Fischer et al. 2012; Beneduzi et al. 2013). In addition, the PGP effects on sugarcane from associated or endophytic bacteria, such as *G. diazotrophicus*, *Herbaspirillum seropedicae*, *H. rubrisubalbicans*, *Azospirillum amazonense*, and

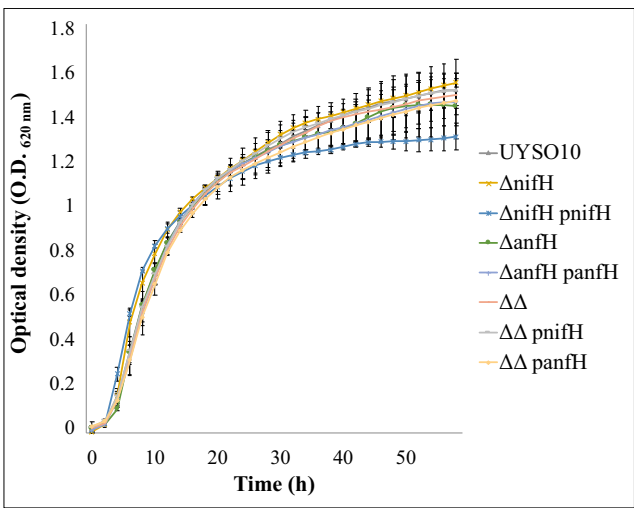

Fig. 2 Growth capacity of *Kosakonia* sp. UYSO10 wild-type and nitrogenase mutant strains, in 96-well microtiter plate containing LB growth media (non-BNF conditions)

Table 4 Acetylene reduction activity (ARA) of strains *Kosakonia* sp. UYSO10 and nitrogenase mutants

	Culture media	
	NFCC + Mo 9.1 μM	NFCC-Mo
Strain	Ethylene production	
UYSO10	+	+
Δ*nifH*	+	+
Δ*nifH* pnifHkosa	+	+
Δ*anfH*	+	+
Δ*anfH* panfHkosa	+	+
Δ*nifH*Δ*anfH*	–	–
Δ*nifH*Δ*anfH* pnifHkosa	+	+
Δ*nifH*Δ*anfH* panfHkosa	+	+

NFCC nitrogen-free combined carbon medium (Mirza and Rodrigues 2012)

Burkholderia spp., have been thoroughly studied (Sevilla et al. 2001; Oliveira et al. 2002, 2006; da Silva et al. 2012).

In a previous study, a collection of putatively endophytic diazotrophic bacteria associated with commercial Uruguayan sugarcane cultivars was constructed, and this was phenotypically and genotypically characterized (Taulé et al. 2012). In addition, a set of these isolates were tested as inoculants in PGP assays on micropropagated sugarcane cv. LCP 85384 under gnotobiotic conditions, and as a result, the strain *Kosakonia* sp. UYSO10 was determined to be one of the best plant promoters (Taulé et al. 2016). In this study, the inoculation of strain UYSO10 into setts of the two principal commercial sugarcane cultivars planted in Uruguay (LCP 85384 and TUC 7742), under greenhouse conditions, showed plant growth promotion ability only for the cv. LCP 85384. These results clearly showed a specificity of the plant response to the inoculation conforming to several reports that note that the PGP effects are dependent on the biotic and abiotic conditions as well as the specificity and compatibility of the plant and bacterial genotypes (Reis Junior et al. 2000; Oliveira et al. 2006; Govindarajan et al. 2008; Carvalho et al. 2011). In addition, the inoculation of sugarcane setts with the strain UYSO10 increases the N accumulation by 35% compared to the negative control in the cv. LCP 85384, as previously observed in micropropagated sugarcane plants (Taulé et al. 2016). As described, this cultivar can obtain a significant amount of its N needs via the BNF (Taulé et al. 2012). Several strains from the genus *Kosakonia* have been described to be plant-associated PGPB (Kämpfer et al. 2005; Witzel et al. 2012; Brock et al. 2013; Bergottini et al. 2015), and the PGP mechanism involved was the BNF in some cases (Zhu et al. 2012; Madhaiyan et al. 2013). The combination of these results led us to evaluate the role of the BNF in the plant-growth promotion observed.

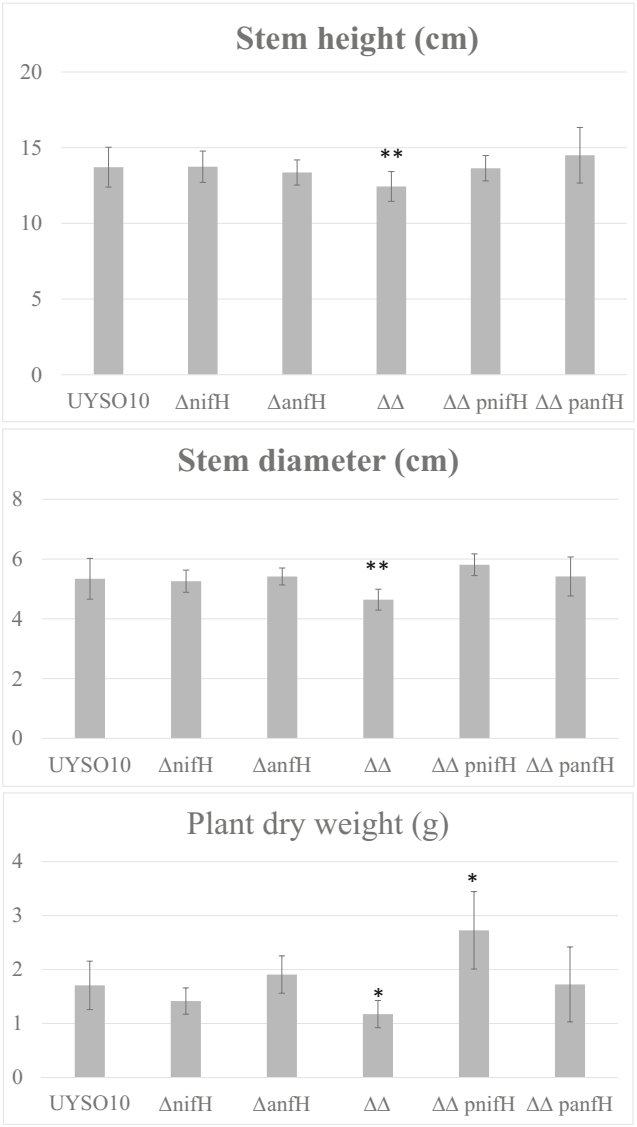

Fig. 3 Effects of inoculation with *Kosakonia* sp. UYSO10 and nitrogenase mutants on the growth of micropropagated sugarcane cv. LCP 85384 under growth chamber conditions. Treatments with an asterisk are significantly different compared with the wild-type strain at $^{**}p < 0.05$, $^{***}p < 0.01$

FeMo-nitrogenase and Fe-nitrogenase are present and active in *Kosakonia* sp. UYSO10

Analyses of the *Kosakonia* sp. UYSO10 genome revealed that this strain has two nitrogenase gene clusters (*nif* and *anf*) that encode FeMo and Fe-nitrogenases. To evaluate its role in the observer sugarcane growth promotion, simple and double mutants were constructed and characterized in vitro and in vivo. ARA assays detected nitrogenase activity in every strain evaluated, but not in the double mutant Δ*nifH*-Δ*anfH*, for every experimental condition. These results clearly indicate that only when both nitrogenase structural genes are deleted, then strain UYSO10 is unable to carry out the BNF process. This result was also reported for the strain *Rhodopseudomonas*

palustrus, a diazotrophic bacterium that contains three types of nitrogenases (Oda et al. 2005).

Recently, it was reported that the nitrogenase mutants Δ*nifH* and Δ*anfH*Δ*nifH* in the plant-associated strain *K. radicincitans* DSM16656^T were impaired in their ability to fix nitrogen (Ekandjo et al. 2018). The strain DSM16656^T has 98.91% genome similarity to the UYSO10 strain, and both strains have exactly the same nitrogenase *nif* and *anf* operon arrangement with almost 100% aa identity sequences. However, our results showed that both nitrogenases were active under the conditions analyzed, and these differences observed are likely to be related to the growth conditions and the methodology used to determine the BNF, or to the differences observed in the *anfH* gene sequence. In this sense, the phylogenetic tree based on the amino acid sequence of the AnfH protein showed that strain UYSO10 form a cluster with several *Kosakonia* strains, while the strain DSM 16656^T is located in a separated branch (Table S1 and Fig. S2). Nevertheless, the nitrogenase activity of strain UYSO10 was not significantly affected by the presence or absence of Mo, as was reported for strain DSM16656^T (Ekandjo et al. 2018) and other diazotrophic strains such as *R. palustris* and *Rhodospirillum rubrum* (Lehman and Roberts 1991; Oda et al. 2005). This is not the case for the strains *A. vinelandii* and *R. capsulatus*, where a Mo concentration of 1 μM or 10 nM inhibits the expression of the alternative nitrogenases (Joerger et al. 1990; Schneider et al. 1991), suggesting a diverse regulation mechanism process. More experiments are required to elucidate the factors involved in the regulation of the expression and activity of both nitrogenases in strain UYSO10.

The BNF process is involved in the sugarcane growth promotion by *Kosakonia* sp. UYSO10

Kosakonia sp. UYSO10 inoculation into micropropagated sugarcane clearly showed that the BNF is partially involved in the plant growth promotion of cv. LCP85384. In addition, the inoculation of sugarcane plants with the knockout mutants for the *nifH* or *anfH* genes did not show a negative effect on the plant growth promotion, which means that both nitrogenases are active for the conditions tested.

The *Kosakonia* genus has been recently defined from a group of strains that were previously classified as *Enterobacter* (Brady et al. 2013). As mentioned, some of these strains have been reported to be plant-associated and to be plant-growth promoters (Kämpfer et al. 2005; Witzel et al. 2012; Brock et al. 2013; Bergottini et al. 2015). In particular, the strains *Kosakonia* sp. NN145S and NN143E were reported to be able to promote the growth of sugarcane plants and that the BNF was one of the mechanisms involved (Lin et al. 2012). In addition, strain *Kosakonia* sp. R4-368, isolated from Jatropa plants, was able to reduce acetylene and promote the growth of its plant host (Madhaiyan et al. 2013). A bioinformatic analysis of strain R4-368 genome only showed the presence of the *nif* regulon, and knockout mutants in the *nif*H, *nif*D, and *nif*K genes lost their nitrogenase activity and their ability to promote plant growth (Madhaiyan et al. 2013). Alternatively, strains *K. radicincitans* DSM16656^T and UMEnt01/12, *Kosakonia* sp. NN145S and NN143E, *K. oryzae* Ola51T and YD4 have been shown to produce FeMo- and Fe-nitrogenases (Lin et al. 2012; Ekandjo et al. 2018; Li et al. 2017). Nevertheless, the role of the different nitrogenases in the PGP of their host is unknown for these strains.

In this study, we demonstrate the cultivar-specific PGP effects of the diazotroph endophytic strain *Kosakonia* sp. UYSO10 on setts of one of the most common sugarcane cultivars grown in Uruguay. In addition, we demonstrate that both nitrogenases present in strain UYSO10 are functional and active in the conditions tested and that the Fe-nitrogenase is not regulated by the Mo levels in the growth media. Additionally, the plant growth promotion assays demonstrated that the BNF is involved in the plant growth promotion of sugarcane plants by *Kosakonia* sp. UYS010. All of these results stress the potential of the strain *Kosakonia* sp. UYSO10 to serve as a model system to study plant growth promotion.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals N/A.

Informed consent N/A.

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