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Sugarcane Growth Promotion by the Endophytic Bacterium *Pantoea agglomerans* 33.1

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The promotion of sugarcane growth by the endophytic *Pantoea agglomerans* strain 33.1 was studied under gnotobiotic and greenhouse conditions. The green fluorescent protein (GFP)-tagged strain *P. agglomerans* 33.1::pNKGFP was monitored *in vitro* in sugarcane plants by microscopy, reisolation, and quantitative PCR (qPCR). Using qPCR and reisolation 4 and 15 days after inoculation, we observed that GFP-tagged strains reached similar density levels both in the rhizosphere and inside the roots and aerial plant tissues. Microscopic analysis was performed at 5, 10, and 18 days after inoculation. Under greenhouse conditions, *P. agglomerans* 33.1-inoculated sugarcane plants presented more dry mass 30 days after inoculation. Cross-colonization was confirmed by reisolation of the GFP-tagged strain. These data demonstrate that 33.1::pNKGFP is a superior colonizer of sugarcane due to its ability to colonize a number of different plant parts. The growth promotion observed in colonized plants may be related to the ability of *P. agglomerans* 33.1 to synthesize indoleacetic acid and solubilize phosphate. Additionally, this strain may trigger chitinase and cellulase production by plant roots, suggesting the induction of a plant defense system. However, levels of indigenous bacterial colonization did not vary between inoculated and noninoculated sugarcane plants under greenhouse conditions, suggesting that the presence of *P. agglomerans* 33.1 has no effect on these communities. In this study, different techniques were used to monitor 33.1::pNKGFP during sugarcane cross-colonization, and our results suggested that this plant growth promoter could be used with other crops. The interaction between sugarcane and *P. agglomerans* 33.1 has important benefits that promote the plant's growth and fitness.

The existence of endophytic bacterial communities has been recognized for over a hundred years (32). Initially, these microorganisms were considered to be neutral with regard to their effects on host plants; more recently, however, their positive impact has been verified in a broad range of crops (66), in which they may contribute directly to plant growth by promoting nutrient availability, biological nitrogen fixation, and the production of phytohormones (38, 68). Indirectly, they may also reduce microbial populations that are harmful to the plant, acting as agents of biological control through competition, antibiosis, or systemic resistance induction (60, 71).

Members of the *Enterobacteriaceae* family (*Gammaproteobacteria*) are frequently described as rhizosphere colonizers of sugarcane and other grasses (84). This class includes *Enterobacter* spp. (8, 49), *Klebsiella* spp. (49), and *Enterobacter cloacae* and *Pantoea agglomerans* (formerly *Erwinia herbicola*) (62). Many studies have reported the endophytic presence of *Enterobacteriaceae* members in various crop species (76). *P. agglomerans* has been described to be an important corn and wheat endophyte (64), and it has also been isolated from potato stems (2), rice seeds (64, 65), and citrus leaves (1). Many studies have shown the potential of *Pantoea* spp. for systemic resistance induction (37, 44, 56) and protection against pests and plant-pathogenic microorganisms (4, 9, 28, 35, 58). Additionally, these bacteria may induce plant growth by increasing the nitrogen supply in nonsymbiotic associations (2, 7, 69, 79, 80), solubilizing phosphorus (45, 46, 72), and stimulating phytohormone production (78, 85).

Much of the current research in the field focuses on the capacity of endophytes to colonize several different agronomically important hosts. The term for this phenomenon in endophytes, cross-colonization, is adopted more commonly from the plant pathogen literature (23). Zakria et al. (84) demonstrated that a *Pantoea* sp. (strain 18-2) from sweet potato and an *Enterobacter* sp. (strain 35-1) from sugarcane are able to endophytically colonize rice and promote its growth. *Klebsiella pneumoniae* 342, isolated from corn and subsequently labeled with the green fluorescent protein (GFP), was able to colonize and *Catharanthus roseus* and *Citrus sinensis* (42).

The introduction of a potentially beneficial bacterium with cross-colonization capacity requires careful monitoring. One of the oldest techniques for monitoring microorganisms in an environment is to plate the organism on solid medium in a laboratory; this technique has several limitations, especially the considerable time required to obtain results. Recently, molecular approaches have been developed to monitor bacterial species in host environments. For example, tagging with related gene markers, such as GFP, has been particularly useful for following bacterial infection pathways and for characterizing tissue and organ colonization (14, 27, 74, 75). The quantitative PCR (qPCR) technique, commonly employed to quantify and study clinical (5, 17, 57), phyto-

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TABLE 1 Plasmids and strains used in this work

Plasmid or strain	Description	Reference or source
Plasmids		
pNKBOR	Kan^r mini-Tn*10*	Rossignol et al. (64)
pNKGFP	Kan^r mini-Tn*10 gfp*	Ferreira et al. (25)
pUC18	Amp^r	Yanisch-Perron et al. (82)
pCM88	Tet^r *gfp*	Marx and Lidstrom (48)
pSMC21	Ap^r Kan^r *gfp*	Kuchma et al. (40)
pUC4K	Kan^r	Taylor and Rose (73)
pJTT	Kan^r *cry1 Ac7*	Downing et al. (21)
pGEM-T Easy	Amp^r	Promega
pWM1013	Kan^r *des red*	Brandl and Mandrell (11)
Strains		
DH5α λ *pir*	*recA1 endA1 hsdR1 relA1* λ::*pir*	Kolter et al. (39)
33.1	Strain from *Eucalipto grandis*	Procópio (59)
33.1::pNKBOR	33.1 harboring pNKBOR	This work
33.1::pNKGFP	33.1 harboring pNKGFP	Ferreira et al. (25)

pathogenic (6, 15, 47, 54, 81), and endophytic (41) bacteria, has also been used for these purposes.

Sugarcane tissue culture has been used to remove pathogens from desirable material (34), and it has been used in breeding programs to reduce cloning time and rapidly obtain large amounts of material with the desired genotype. For this reason, we studied the possibility of plant growth promotion during sugarcane seedling acclimation. We aimed to reduce the time of seedling development under greenhouse conditions, therefore reducing the costs of seedling production. A plant growth-promoting strain of *P. agglomerans*, strain 33.1, previously isolated from *Eucalyptus grandis*, was evaluated during its interactions with sugarcane, with a focus on its potential for cross-colonization and promotion of sugarcane growth under nursery conditions during seedling acclimation.

MATERIALS AND METHODS

Microorganisms and growth conditions. *Pantoea agglomerans* 33.1, isolated from *Eucalyptus grandis* plants (58), and its derivative tagged strain, 33.1::pNKGFP (25), as well as *Escherichia coli* derivatives were routinely grown at 28°C and 37°C, respectively, on Luria-Bertani (LB) medium (67). The tagged strain 33.1::pNKBOR was obtained according to the methodology described by Ferreira et al. (25). All of the strains (Table 1) were stored in 20% glycerol at −80°C. The plasmids were propagated and isolated from *E. coli* DH5α *pir* or Top10 and purified with a plasmid miniprep kit (Mobio) according to the manufacturer's recommendations.

Plant material. The sugarcane seedlings (varieties SP80-1842 and SP80-3240) were initially cultivated under *in vitro* conditions. The seedlings were supplied by Sabrina Moutinho Chabregas (CTC, Piracicaba, SP, Brazil).

Cross-colonization under gnotobiotic assays. For plant inoculation, the bacterial cells were transferred to 50 ml of LB medium supplemented with kanamycin (100 μg/ml) and incubated at 28°C under shaking conditions (150 rpm) until the late log phase. The cells were harvested (by centrifugation at 4,500 × *g* for 15 min) and washed with phosphate-buffered saline (PBS) at pH 6.5, and the cell density was adjusted to 10^5 CFU/ml. The SP80-1842 sugarcane seedlings were cultured in 50-ml tubes containing 7 ml of agar-free MS medium (51) with the endophytic bacteria at 28°C under 16-h photoperiods. In the control plants, PBS was used in place of the bacterial suspension. Eighteen days after bacterial inoculation (DAI), three plants per treatment were sampled for fluorescence microscopy (FO) and scanning electron microscopy (SEM) analysis. The reisolation and qPCR procedures were performed on 5 plants at 5 and 14 DAI.

Fluorescence microscopy. Roots and aerial fresh tissues were cut and immediately observed with visible light and fluorescence microscopy (Axiophot II; Zeiss, Germany) under a green (fluorescein isothiocyanate [FITC], 510 nm) fluorescence filter. Both images were combined, using the overlay module in the MetaVue program (Universal Imaging Corporation), to identify the bacterial cells.

SEM. Plant tissues were cut (approximately 2 cm^2), fixed in buffered Karnovsky solution (2% glutaraldehyde, 0.001 M $CaCl_2$, 2.5% paraformaldehyde) at 4°C, and rinsed three times (10 min for each rinsing) with a 0.05 M cacodylate buffer at pH 7.2. The samples were infiltrated with a 30% glycerol solution for 30 min and treated with an osmium tetroxide solution in a 1% cacodylate buffer for 2 h. The samples were then washed three times in distilled water, dehydrated with acetone (in a series of 30, 50, 70, 90, and 100% acetone dilutions), dried to the critical point using a dryer (CPD 030; Balzers), mounted on aluminum stubs, and coated with gold (MED 010; Balzers). The metal-coated samples were examined using SEM (Leo; Zeiss).

Southern blot analysis. The number of insertions of the pNKGFP fragment in the 33.1::pNKGFP genome was confirmed using Southern blotting. For this technique, probes were generated by PCR, using primers (PPNKF [5′-CCT TCA TTA CAG AAA CGG C-3′] and PPNKRII [5′-GGT GAT GCG TGA TCT GAT CC-3′]) designed by the OligoPerfect Designer program (Invitrogen) on the basis of the pNKBOR sequence.

These primers amplify a 362-bp sequence located between the insertion sites (ISs) that correspond to a portion of the Kan^r gene and a conserved plasmid fragment. The probe specificity was evaluated using DNA from the strains, plasmids (Table 1), and plants. The PCR mixture contained 1 μl of the samples (10 ng), 0.2 M primers PPNKF and PPNKRII, 3.75 mM $MgCl_2$, 0.2 mM a deoxynucleoside triphosphate mixture, and 2.5 U of *Taq* DNA polymerase (Fermentas) combined with 1× buffer for a final volume of 25 μl. The PCR program consisted of an initial denaturation step at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 30 s. The final step was a 10-min extension at 72°C. The amplified products were separated by electrophoresis with a 1.2% agarose gel and stained with ethidium bromide.

The probes were labeled using the random-primed DNA labeling method, and the hybridization was confirmed with a Gene Images AlkPhos (alkaline phosphatase) direct labeling and detection system (GE Healthcare) according to the manufacturer's instructions.

qPCR. Sugarcane seedlings inoculated either with or without 33.1::pNKGFP were sampled, and the roots and aerial parts of these plants were separated. To separate the bacterial cells from the sugarcane surface, the root was incubated in 2 ml of PBS buffer at 28°C for 2 h, and the bacterial cells were harvested by centrifugation. Total bacterial DNA was extracted as described by Araújo et al. (1). After this surface sterilization, the total DNA of the root and aerial plant parts was extracted by the cetyltrimethylammonium bromide method, according to Doyle and Doyle (22).

The qPCR analysis was performed in a 25-μl final volume, which contained 12.5 μl of the master mix of Platinum SYBR green qPCR SuperMix-UDG (Invitrogen) and the PPNKF and PPNKRII primers (10 μM each). Aliquots of the master mix (20 μl) were dispensed in the wells, and 5 μg of DNA (50 ng/μl) was added as a PCR template. The qPCR cycles consisted of a denaturation step at 94°C for 5 min, 40 cycles at 94°C for 30 s, and a final step at 61°C for 15 s. The plasmid fragment quantification was performed using an iCycler iQ real-time PCR instrument (Bio-Rad Laboratories Inc.). Four replicates in duplicate were used, and a standard curve was obtained for every run using a known copy number (10^5 to 10^8) of the linearized plasmid pNKGFP.

Sugarcane growth promotion and defense protein production. The micropropagated sugarcane seedlings (varieties SP80-1842 and SP80-3240) were transferred to plastic pots containing the organic substrate PlantMax Horticultura (Eucatex). The transferred plants were first acclimated in a humid chamber at 28°C for 7 days. The bacterial wild-type 33.1 and tagged 33.1::pNKGFP strains were inoculated into the substrate (10^8

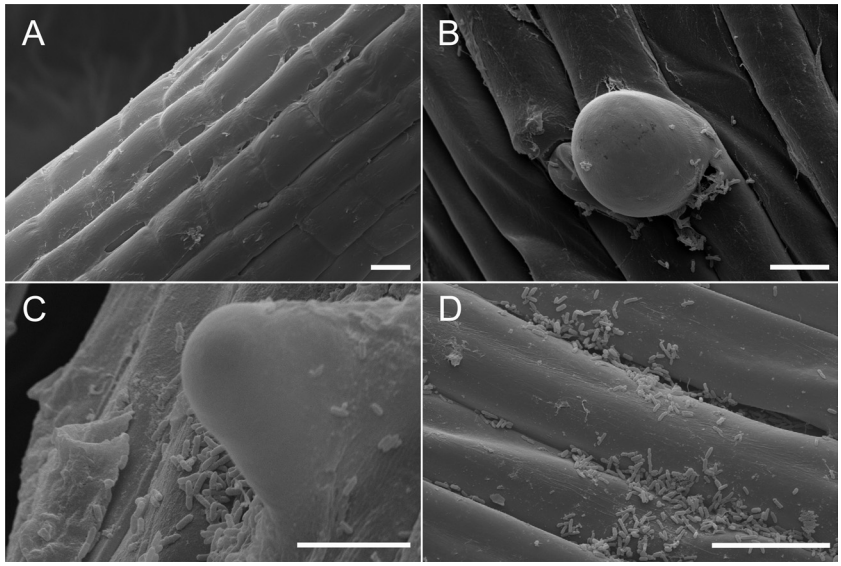

FIG 1 Sugarcane infection and root surface biofilm formation by 33.1 and 33.1::pNKGFP. Control (A) and 33.1 (B) bacterial cells during root infection through rooting fissures, indicated by the white arrow (B), and biofilm formation by 33.1 (C) and 33.1::pNKGFP (D) on the sugarcane root surface. The samples were collected at 3 days after inoculation. Magnifications, ×500 (A), ×1,500 (B and D), and ×3,000 (C). Bars, 20 μm (A and D) and 10 μm (B and C).

CFU/plant), and the pots were then transferred to a greenhouse at 28°C. The substrate of the control pots was inoculated with PBS buffer that did not contain any bacterial cells. The dry mass, related defense protein production, and bacterial cross-colonization of the plants were evaluated at 30 DAI.

Forty plants from each treatment were sampled, washed, and divided into root and aerial parts. The vegetative tissues were weighed and dried at 55°C for 5 days to reach a constant weight.

Enzymatic activity was assessed using separate total protein extractions from the leaves and roots as described by Ferreira-Filho et al. (26). The amount of protein present in the supernatant was measured using the Bradford procedure (10), with bovine serum albumin (BSA) as the standard. Four replicates were used for the measurements of enzymatic activity.

The endoglucanase and chitinase activities from the crude plant extract were assayed using Remazol brilliant blue carboxymethylcellulose (RBB-CMC) and carboxymethyl-chitin-Remazol brilliant violet (CM-chitin-RBV) from Loewe Biochemica GmbH (Sauerlach, Germany), respectively. The enzyme activity was measured as the absorbance per milliliter of the substrate reaction per hour (29).

Bacterial reisolation. The densities of *P. agglomerans* in the rhizosphere and inside the sugarcane tissues were evaluated at 4 and 15 DAI in the gnotobiotic assay and at 30 DAI in the greenhouse assay. To promote the detachment of bacteria from the roots, the plant samples were placed in a new sterile tube containing 2 ml of PBS and agitated at 120 rpm for 1 h. The cell suspension was diluted and plated on 5% tryptic soy broth for bacterial quantification. The seedlings were washed in running tap water and surface disinfected using serial rinsing in 70% ethanol and 2% hypochlorite as described by Araújo et al. (1). To confirm the efficiency of the disinfection process, aliquots of the sterile distilled water used in the last washing were spread onto 5% tryptic soy (TS) agar medium and examined for surface contaminants after a 3-day incubation at 28°C. The surface-disinfected samples were macerated in a PBS buffer, and the appropriate dilutions were plated onto 5% TS agar supplemented with kanamycin (100 μg/ml) and benomyl (50 μg/ml). The cultivable bacterial density associated with sugarcane was also evaluated under greenhouse conditions. For this process, the macerated samples were plated on TS agar without the selective antibiotic, and the number of tagged strains among the indigenous community was measured by exposing the plates to UV light.

Bacterial plant growth promotion mechanisms. (i) IAA production. The indoleacetic acid (IAA) production of the 33.1 strain was assessed using the quantitative method developed by Bric et al. (12). The absorbance (optical density at 520 nm) values obtained were interpolated from a standard curve to determine the IAA concentration. Two independent experiments were performed in triplicate.

(ii) Phosphate solubilization. The ability of strain 33.1 to solubilize inorganic phosphate was evidenced by a halo obtained after cultivation of the bacteria on culture medium supplemented with two inorganic phosphate sources, $Ca_3(PO_4)_2$ and $Al(PO_4)$, at 28°C for 72 h, as described by Verma et al. (80) and Hara and Oliveira (31), respectively. An estimation of the halo size (cm) divided by the colony size (cm) generated a solubilization index (SI) that was used to quantify the phosphate solubilization.

(iii) Biological nitrogen fixation (BNF). The ability of the strain to fix atmospheric nitrogen was assessed as described by Döbereiner et al. (19). To confirm the ability of the strain to fix atmospheric nitrogen, seven consecutive streaks were performed in the semisolid nitrogen-free nitrogen-fixing biomass (Nfb) medium (19). The nitrogenase activity was measured after 5 days of incubation using an acetylene reduction assay (33). The chromatography gas analysis was completed at the Post-Harvest Laboratory, ESALQ-USP. The samples were incubated with 10% acetylene for 2 h at 28°C. The nitrogenase activity was expressed in nanomoles of ethylene per hour.

Statistical analysis. The statistical analyses of the data were conducted using the SAS program (SAS Institute Inc., Cary, NC) and were designed to account for the random design and subfactorial nature of the qPCR and reisolation data. To quantify the bacteria, the obtained data were log transformed to stabilize the variance. The bars in the figures represent the means ± standard errors of four replicates. The asterisks (* and **) in the figures indicate significant differences ($\alpha = 0.05$ and 0.01, respectively) according to Student's *t* test.

RESULTS

Microscopy analysis of sugarcane interactions with the 33.1 and 33.1::pNKGFP strains. The behavior of the *P. agglomerans* 33.1 and 33.1::pNKGFP strains during their interactions with micropropagated sugarcane plants was evaluated using SEM. No bacteria or fungi were observed in the control treatments on any of the

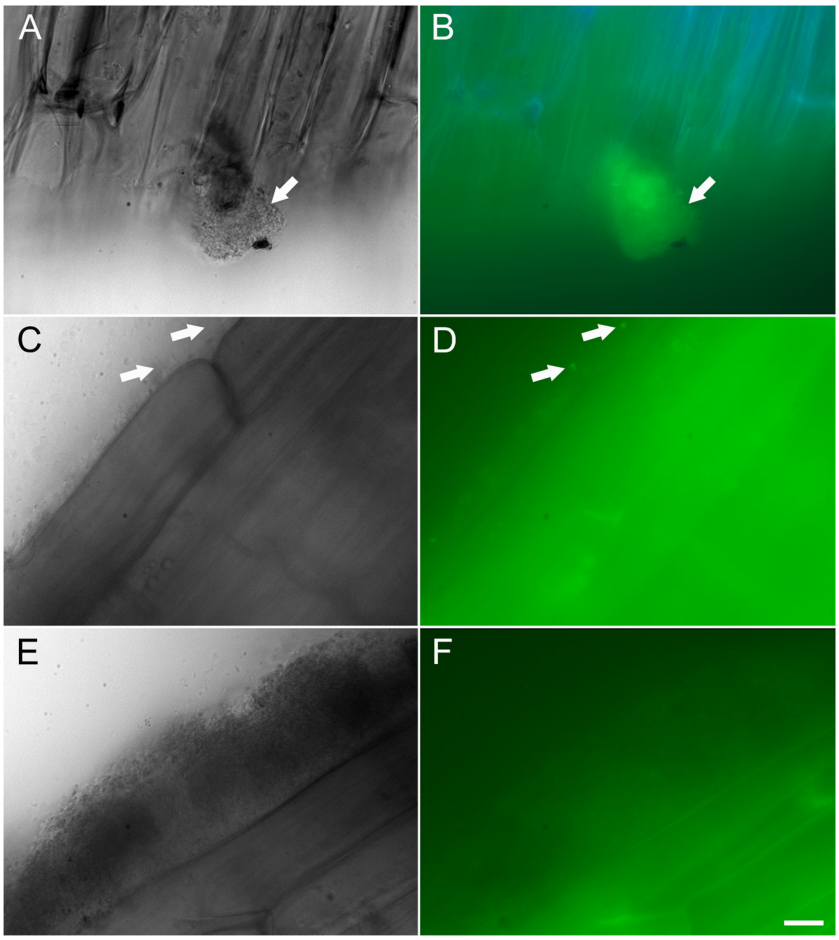

FIG 2 Visualization of 33.1::pNKGFP cells during biofilm formation on the sugarcane root surface using light microscopy at 5 DAI (A), 10 DAI (C), and 18 DAI (E) and fluorescence microscopy under an FITC (510 nm) filter to visualize the decrease in fluorescence at each respective time (B, D, and F). Arrows, the 33.1::pNKGFP bacterial cells that expressed GFP. Magnification, ×200; bar, 10 μm.

observed samples (Fig. 1A). The 33.1 and 33.1::pNKGFP cells aggregated on the root surface (Fig. 1B to D).

The formation of a biofilm by 33.1::pNKGFP on the sugarcane root surfaces was observed at all of the sampled points, as described above (Fig. 2A, C, and E), but bacterial fluorescence was observed only at 5 DAI (Fig. 2B). We observed a fluorescence reduction after this period but not a reduction in the bacterial biofilm aggregate (Fig. 2D and F). The sugarcane samples also showed autofluorescence; however, the presence of a few 33.1::pNKGFP cells fluorescing in the bacterial aggregation is clearly visible. Otherwise, in the MS medium suspension, bacterial fluorescence was not affected in the aggregated cells (data not shown). We did not observe fluorescent bacterial cells inside the sugarcane tissues, probably due to the loss of bacterial fluorescence observed in the biofilm aggregation.

Monitoring of 33.1::pNKGFP sugarcane cross-colonization using qPCR and reisolation. To develop a qPCR technique to monitor *P. agglomerans* 33.1::pNKGFP in sugarcane, the specificity of the designed set of primers was first tested using conventional PCR. The primers PPNKF and PPNKRII were tested against plasmids and bacterial strains (Table 1) that were specific to the tagged strain (Fig. 3A). No amplification was observed for the DNA of sugarcane without 33.1::pNKGFP inoculation (data not shown). The 33.1::pNKGFP strain presented only one insert of the plasmid (Fig. 3B); therefore, it was used for the quantification of *P. agglomerans* in the sugarcane seedlings.

Using this qPCR technique under gnotobiotic conditions, we observed a higher number of 33.1::pNKGFP cells in the rhizo-

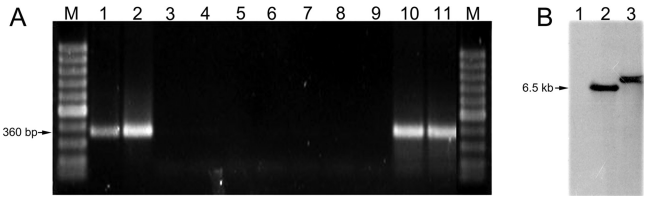

FIG 3 Specificity of conventional PCR with the PPNKF and PPNKRII primer set for the detection of *P. agglomerans* 33.1::pNKGFP. (A) Lanes: 1, plasmid pNKBOR; 2, plasmid pNKGFP; 3, plasmid pUC18; 4, plasmid pCM88; 5, plasmid pSMC21; 6, plasmid pUC4K; 7, plasmid pJTT; 8, plasmid pGEM-T Easy; 9, plasmid pWM1013; 10, 33.1::pNKBOR; 11, 33.1::pNKGFP; M, 100-bp DNA ladder as a molecular marker (Fermentas). The PCR product, indicated by the arrow, is 360 bp. (B) Integration of the pNKGFP fragment into the *P. agglomerans* 33.1 chromosome, confirmed with Southern blot analysis of the plasmid and chromosomal DNAs cut with EcoRI and probed with the 360-bp amplicon fragment obtained from a PCR using primers PPNKF and PPNKRII. Lanes: 1, 33.1; 2, plasmid pNKGFP; 3, 33.1::pNKGFP.

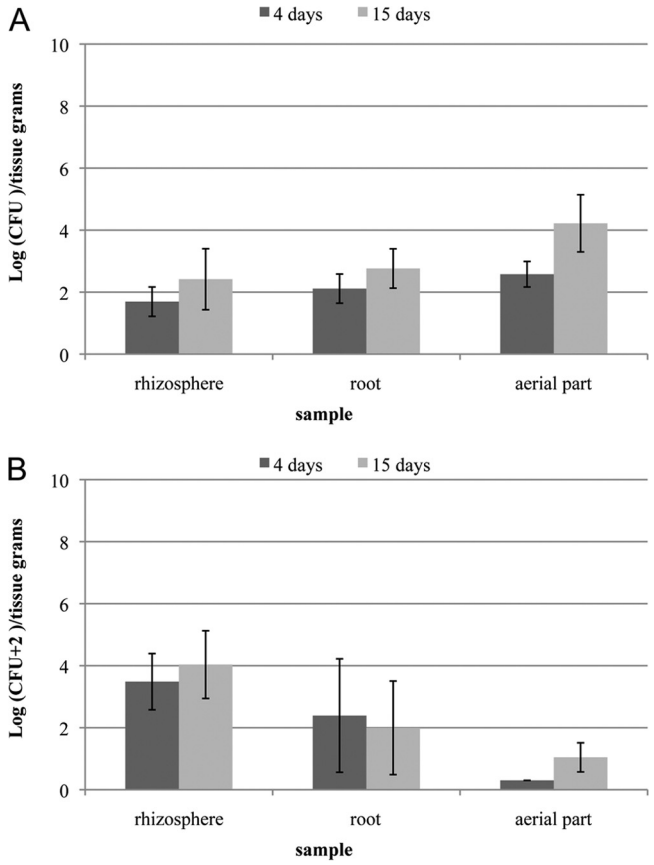

FIG 4 *P. agglomerans* 33.1::pNKGFP density during sugarcane cross-colonization, measured using qPCR (A) and reisolation (B) at 4 and 15 DAI. The abundance data, in CFU/g of tissue, were log transformed to stabilize the variance. The results are the means of the four replicates for each sample. The bars represent the standard error of each treatment.

sphere and in sugarcane tissues at 15 DAI. The strain had multiplied and moved from sugarcane roots to aerial parts by 15 DAI, increasing the bacterial cell density in aerial tissues and in root tissues (Fig. 4A). The number of bacterial cells detectable with qPCR is similar to that detectable with reisolation, and both techniques showed an increase in the number of bacterial cells in the rhizosphere and aerial plant parts (Fig. 4B).

In the greenhouse assay, 33.1::pNKGFP was also able to colonize acclimated sugarcane when added to the substrate. A greater number of tagged bacterial cells was observed in the rhizosphere than in the aerial parts (Table 2). The density of the indigenous bacterial community was not affected by the addition of 33.1::pNKGFP. A similar number of indigenous bacterial cells was observed in the rhizosphere in all treatments, either with or without the addition of bacterial strain 33.1 or 33.1::pNKGFP.

Plant growth promotion. After substrate inoculation, *P. agglomerans* 33.1 significantly promoted the biomass accumulation of the sugarcane plants (Fig. 5A and B). In variety SP80-1842, this result was observed in only aerial plant tissues, whereas for variety SP80-3240, increased biomass accumulation was observed in root tissues as well (Fig. 5B). For both sugarcane varieties, we observed that bacterial inoculation induced chitinase and cellulase production in root tissues (Fig. 5D).

The 33.1 strain was able to solubilize two different phosphate sources. Calcium phosphate was hydrolyzed to a greater extent by the 33.1 strain than was aluminum phosphate, with SI values of 3.4 and 1.72, respectively. Even during phosphate solubilization, 33.1 showed the capacity to produce IAA, generating approximately 100 μg/ml under the assessed conditions.

Nitrogen fixation by strain 33.1 was confirmed in all seven consecutive inoculations in the Nfb medium. All of the incubations developed a halo under the medium surface; however, very little nitrogenase activity (0.03 nmol/h ethylene) was detected by acetylene reduction.

DISCUSSION

P. agglomerans (*Erwinia herbicola*) is a cosmopolitan bacterium that lives in diverse environments, including soil (55, 83), water (50), insects (18), and humans (16). This species has been found endophytically in many important crops (34), acting as a plant growth promoter (2, 45, 46, 69, 78, 80, 85), biocontrol agent (4, 9, 58), and even a systemic resistance inducer (37, 44, 56). In this study, we evaluated the interactions of *P. agglomerans* with sugarcane, aiming to confirm the bacterium's capacity to cross-colonize this crop and to increase the growth and fitness of sugarcane seedlings. Strain 33.1 was selected due to its identification as a growth promoter in its original host, *E. grandis* (59). The mechanisms associated with plant growth promotion and cross-colonization in this bacterium had, however, not been previously described.

The 33.1 and 33.1::pNKGFP strains both interacted similarly with sugarcane, forming aggregates around the roots and infecting their hosts through radicular fissures. Frequently, endophytic bacteria live in the soil, and prior to systemic colonization, the bacterial cells attach to the roots close to the fissures caused by lateral root emergence (13). Compant et al. (14) also observed the presence of *Burkholderia* sp. strain PsJN at lateral root emergence sites, suggesting that crack entry colonization occurred in grapevine plantlets in a manner similar to the phenomenon previously ob-

TABLE 2 Density of sugarcane-associated bacteria measured using reisolation

	Bacterial density (CFU/g of sample)			
Sample	33.1::pNKGFP density[a]	Control[b]	33.1[b]	33.1::pNKGFP[b]
Rhizosphere	2.9×10^5 a	8.6×10^5 A	12.4×10^5 A	18.4×10^5 A
Root	4.3×10^2 b	7.1×10^5 A	10.5×10^5 A	4.5×10^5 AB
Aerial part	2.2×10^1 b	2.2×10^3 B	1.4×10^3 B	1.5×10^3 B

[a] The 33.1::pNKGFP density was measured at 30 DAI. Values with the same letter within a column are not significantly different ($P > 0.005$) according to Tukey's test, which was conducted with the SAS program (release 9.1). The results are the means of four replicates of each sample.

[b] The bacterial density was measured at 30 DAI with 33.1 or 33.1::pNKGFP. The treatment control contained an inoculation of PBS medium without bacterial cells. Values with the same letter within a column are not significantly different ($P > 0.005$), according to Tukey's test conducted with the SAS program (release 9.1). The results are the means of four replicates of each sample.

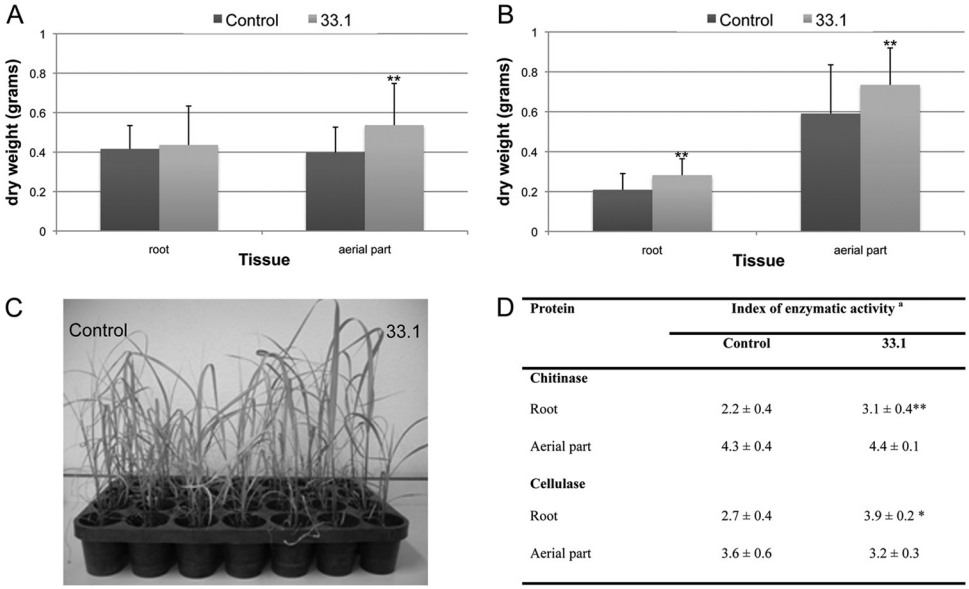

FIG 5 Sugarcane plant growth promotion by *P. agglomerans* 33.1. The strain was added to the substrate of acclimated variety SP80-1842 (A) and SP80-3240 (B) sugarcane. The data are the average dried weights at 30 DAI. The bars represent the standard error of each treatment. **, values in the same tissue and varieties are significantly different ($\alpha = 0.01$) from the control according to Student's *t* test. (C) Sugarcane variety SP80-1842 without (left) and with (right) 33.1 inoculation. (D) Sugarcane resistance protein production. [a], the index of enzymatic activity was calculated on the basis of the absorbance/ml of substrate/h. * and **, values are significantly different from the control treatment ($\alpha = 0.05$ and 0.01, respectively) according to Student's *t* test.

served with the same strain in potato plants (61). These results suggest that endophytic bacteria, which are able to colonize different host plants, could use the same approach in their first colonization step. Many endophytic species have a broad range of hosts; *Herbaspirillum seropedicae*, for example, has been found in a wide variety of crops, including sorghum, sugarcane, corn, and other grasses (3, 53). An endophyte isolated from a specific host family may colonize other plants, even plants in other families, suggesting a lack of specificity to a particular host (84). For example, *Klebsiella pneumoniae* 342 isolated from corn was able to colonize wheat, rice, *Arabidopsis thaliana*, and *Medicago sativa* (20). *Azoarcus indigens* isolated from a grass pasture also colonized rice and sorghum (24, 61, 70), and a *Burkholderia* sp. isolated from onion colonized grapevine (15) and potato (52).

Using qPCR and reisolation techniques, we demonstrated that *P. agglomerans* 33.1, which was previously isolated from *Eucalyptus* plants, was able to grow in sugarcane seedlings after systemic colonization. Endophytic bacteria are frequently found at a high concentration in roots, and there is often a gradient of bacterial abundance from the roots to the stem and leaves (43). These endophytes are hypothesized to colonize the root surface before colonizing the host itself (30, 36, 63). Our data indicated that 33.1::pNKGFP has a preference for the rhizosphere over the root and aerial parts of sugarcane, demonstrating typical endophytic behavior during colonization. Under greenhouse conditions, this strain did not affect the density of the endogenous bacterial community, but it was able to induce dry mass accumulation by IAA production and phosphate solubilization.

Additionally, *P. agglomerans* 33.1 stimulated the production of chitinase and endoglucanase by root tissues, with which the bacterial cells had more contact and stayed for a longer time. During plant invasion, bacterial cells may produce hydrolytic enzymes that are recognized by the plant, triggering the plant's resistance system. Trotel-Aziz et al. (77) reported an increase in the resistance of vines against *Botrytis cinerea* due to the augmentation of chitinases and other compounds induced by the inoculation of the *P. agglomerans* strain PTA-AF1.

We demonstrated that 33.1, an endophytic bacterium that promotes the growth of *E. grandis*, was able to cross-colonize and promote the growth of sugarcane. This bacterium also induced the synthesis of chitinase and endoglucanase enzymes, which are associated with plant protection against pathogens. Furthermore, new genes may be transferred to 33.1 to improve on its wide range of benefits to host plants. Our results contribute to the understanding of the interactions between plants and endophytes and demonstrate the viability of *P. agglomerans* 33.1 as an inoculant to improve sugarcane productivity.

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