Microb Ecol (2016) 72:407–417
DOI 10.1007/s00248-016-0793-x

PLANT MICROBE INTERACTIONS

Functional Role of Bacteria from Invasive *Phragmites australis* in Promotion of Host Growth

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Received: 13 March 2016 / Accepted: 18 May 2016 / Published online: 3 June 2016
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Abstract We hypothesize that bacterial endophytes may enhance the competitiveness and invasiveness of *Phragmites australis*. To evaluate this hypothesis, endophytic bacteria were isolated from *P. australis*. The majority of the shoot meristem isolates represent species from phyla *Firmicutes*, *Proteobacteria*, and *Actinobacteria*. We chose one species from each phylum to characterize further and to conduct growth promotion experiments in *Phragmites*. Bacteria tested include *Bacillus amyloliquefaciens* A9a, *Achromobacter spanius* B1, and *Microbacterium oxydans* B2. Isolates were characterized for known growth promotional traits, including indole acetic acid (IAA) production, secretion of hydrolytic enzymes, phosphate solubilization, and antibiosis activity. Potentially defensive antimicrobial lipopeptides were assayed for through application of co-culturing experiments and mass spectrometer analysis. *B. amyloliquefaciens* A9a and *M. oxydans* B2 produced IAA. *B. amyloliquefaciens* A9a secreted antifungal lipopeptides. Capability to promote growth of *P. australis* under low nitrogen conditions was evaluated in greenhouse experiments. All three isolates were found to increase the growth of *P. australis* under low soil nitrogen conditions and showed increased absorption of isotopic nitrogen into plants. This suggests that the *Phragmites* microbes we evaluated most likely promote growth of *Phragmites* by enhanced scavenging of nitrogenous compounds from the rhizosphere and transfer to host roots. Collectively, our results support the hypothesis that endophytic bacteria play a role in enhancing growth of *P. australis* in natural populations. Gaining a better understanding of the precise contributions and mechanisms of endophytes in enabling *P. australis* to develop high densities rapidly could lead to new symbiosis-based strategies for management and control of the host.

Keywords *Bacillus amyloliquefaciens* · *Achromobacter spanius* · *Microbacterium oxydans* · Lipopeptide · Plant growth promotion

Electronic supplementary material The online version of this article (doi:10.1007/s00248-016-0793-x) contains supplementary material, which is available to authorized users.

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Introduction

Common reed (*Phragmites australis* (Cav.) Trin. Ex Steud.) is a perennial grass native to Eurasian wetlands and currently found on every continent [31]. Although the subspecies of *Phragmites* (*P. australis* spp. *americanus*) is native to North American wetlands [70], the invasive subspecies (*P. australis* spp. *australis*) has aggressively outcompeted it and many other native species. This macrophyte is highly productive, even in low nutrient environments and outcompetes other plants to create large monodominant populations that can decrease diversity of plants and animals [76, 79]. Its success in invasion has been related to its ecological range [47, 48], high genetic diversity [23], and high efficiency of dispersion, able to colonize different sites via seed dispersal and clonal growth through frequent tillering [55]. *Phragmites* is also effective

in taking up nitrogen from the soil, and the aboveground standing stocks of nitrogen may be 2–3 times higher in *Phragmites* stands [54]. This is consistent with our understanding that invasive plant-driven changes in soil N cycles (e.g., changes in soil microbial or mutualism communities) may play an important role during successful plant invasion [78].

Mutualisms between plants and their microbiomes are common and may facilitate the plant invasion processes [13]. Specifically, the competitive ability of invasive plants can be increased through mutualistic associations that increase growth, increase tolerance to salinity, reduce herbivory, or increase tolerance to drought stress [4, 6, 33, 66, 77]. For example, nitrogen-fixing endophytic bacteria are involved in the invasiveness of *Sorghum halepense* in soil with low concentration of nitrogen [69]. Plant microbes can live epiphytically (on the surface of the plant) or endophytically (within the plant), and bacteria and fungi that colonize the internal tissue of the plant showing no external sign of infection or negative effect on their host are called endophytes [72]. These microorganisms are beneficial to plants through auxin production [62], N_2 fixation, or increased mineralization of soil nutrients [46, 85] that results in plant growth promotion. They also can help plants increase tolerance to stresses, including soils contaminated by heavy metals [44]. Natural products synthesized by endophytic bacteria can induce resistance to plant pathogens [8] or biocontrol phytopathogens by lipopeptide resulting in plant growth [64]. Some endophytic bacteria help plants to acquire minerals from inaccessible sources solubilizing phosphorus as orthophosphate in cactus growing on rocky areas [49].

Understanding the relationship between *P. australis* and its microbial symbionts could lead to the development of methodologies for disrupting or manipulating the specific symbiotic relationships in order to reduce the invasive character of the host [39]. Endophytic fungi [5, 24] and bacteria [45, 50] have been isolated from leaves, roots, and rhizomes of *P. australis* but not yet from meristematic tissues. Characterization of the identity and functional role of microorganisms in invasive species are the first steps for the development of new management techniques [39], so this study characterized endophytic bacteria in *P. australis* subsp. *australis* meristematic tissues and tested their plant growth-promoting attributes under low nitrogen conditions. Our hypothesis was that endophytic bacteria in *Phragmites australis* subsp. *australis* contribute to promotion of plant growth under low nitrogen conditions.

Materials and Methods

Isolation, Metabolic Characterization, and Plant Growth-Promoting Attributes of Endophytic Bacteria

Young stems (~15 cm length) of five individuals of *P. australis* subsp. *australis* were collected from a population on the roadside in a moist wooded area at the junction of Dudley Road and Ryders Lane on the Cook campus of Rutgers University in New Brunswick, NJ. Tissues were superficially disinfected with a 2 % sodium hypochlorite solution for 7 min and then rinsed three times with sterile water. Tissue fragments (2 × 2 mm) were placed onto plates containing 10 % trypticase soy agar (TSA) and 1 % yeast extract + 1 % sucrose (YES) media. Ten replica plates (10 fragments/plate) were made for each culture medium. The plates were kept at room temperature and growth was assessed periodically. Bacteria strains were stored at −80 °C in the Department of Plant Biology and Pathology, Rutgers University.

Genomic DNA from bacteria was isolated using QIAamp DNA Mini Kit (QIAGEN®). The identification of endophytic bacteria was done sequencing the 16S rDNA region (Electronic Supplementary Material Table 1). Enterobacterial repetitive intergenic consensus (ERIC)-PCR fingerprinting profiles [36] were obtained. Then, 16S rDNA was sequenced for 12 strains (A9a, A12a, A28, A30, B1, B2, B3, B5, B9, B19a, B42b, and B25) coming from three ERIC-PCR groups. All sequences were deposited in GenBank under accession numbers: KP860304–KP860314. Sequences were identified by comparison to GenBank accessions using BLASTn (http://www.ncbi.nlm.nih.gov).

Seventy-one carbon source utilization tests and 23 chemical-sensitivity tests of bacteria were performed using GNIII microplates (96 wells) from Biolog Corporation (USA) to metabolic characterization of bacteria. The data were transformed into a binary matrix (0 = no active metabolism, 1 = active metabolism). Using the binary matrix, the similarity between the metabolic profile of strains was calculated using the Jaccard coefficient with NTSySpc 2.11T (Applied Biostatistics, Setauket-USA).

Functional traits involved in promoting plant growth were determined by standard procedures: production of indoleacetic acid (IAA) was assayed with Salkowski's reagent [41]; phosphate solubilization ability of the strains was tested by spotting bacteria on GL-rich medium [74]; production of cellulase, protease, amylase, and esterase was determined according to Carrim et al. [14]; nitrogenase activity was determined by the acetylene reduction assay (ARA) [29] and growth of strain in N-free glucose medium [58].

Testing for Antibiosis Against Filamentous Fungi and Lipopeptide Production

Some species of bacteria are known to inhibit growth of pathogenic fungi (*Fusarium oxysporum*, *Alternaria alternata*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*) and non-pathogenic (*Aspergillus flavus*), so we tested for antagonistic associations using our microbes. Antibiosis between endophytic bacteria and fungi was evaluated in 10 % TSA. Discs containing mycelium of fungi were inoculated in the center of

Table 1 Evaluation of functional traits of endophytic bacteria from *Phragmites australis*

Strain	IAA ($\mu g\ mL^{-1}$)	$PO4^{-2}$ Sol.	Enzyme	Norris agar	Acethylene reduction (μmol)[b]	Antagonistic activity against (%)				
						FO	AL	BO	CO	AF
B. amyloliquefaciens A9a	3.78 ± 0.15	–[a]	cellulase, lipase, protease, and amylase	+[a]	737.0 ± 14.7	33.3 ± 2.0[c]	55.9 ± 2.5	53.8 ± 1.1	54.1 ± 0.4	25 ± 1.3
A. spanius B1	0	–	lipase	+	640.0 ± 35.5	–	–	–	–	–
M. oxydans B2	11.75 ± 0.28	–	protease	+	691.0 ± 3.6	–	–	–	–	–

FO Fusarium oxysporum, *AL Alternaria alternata*, *BO Botrytis cinerea*, *CO Colletotrichum gloeosporioides*, *AF Aspergillus flavus*

[a] (+) growth in medium; (−) no growth

[b] Data represents the means ± standard deviation from three replicates. We injected 500 μL of sample into chromatograph. The means are different by Duncan test $p < 0.05$

[c] Percentage of fungal growth inhibition compared to the growth obtained in control plates. Data represent the mean ± standard deviation from three replicates

a plate, and bacteria were transferred to two equidistant points in the plate. Controls were inoculated only with the fungi. Three replicates were made for each fungus, and the plates were incubated at lab-ambient temperature. Zones of inhibition between bacteria and fungal colonies were measured at 2-day intervals. Antagonism was expressed as a percentage of mycelial growth compared to control treatment.

Genomic DNA from bacteria was used to amplify surfactin, inturin A, bacillomycin, and fengycin genes. The oligonucleotides and program sets used in the PCR amplifications are listed in Table 1. Amplified fragments were purified and sequenced. Sequences were analyzed using BLASTx and BLASTn algorithms available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Crude extract containing lipopeptides was obtained according to Zhao et al. [87]. The lipopeptide precipitate was resuspended in 10 mL of methanol to yield the crude extract of lipopeptide (CEL). CEL was analyzed by thin layer chromatography (TLC) [27]. The CEL in methanol was characterized by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry [37]. Positive ion detection and the reflector mode were used. Data were acquired at reflector positive mode from 800 to 4.000 and the data were compared with the references (see Electronic Supplementary Material Table 3).

Plant Growth Promotion under Low Nitrogen Conditions

P. australis seeds obtained from populations on Sandy Hook Island, New Jersey, were stratified at 4 °C for 6 months then planted for germination in trays containing vermiculite. The trays were placed in the greenhouse at 17:16 °C day/night cycle, 75 % relative humidity, and a photoperiod of 15 h (300 μE) for 12 days. Trays were irrigated daily with tap water. The bacteria strains were grown in Luria-Bertani (LB) broth at room temperature under stirring 150 rpm. Seedlings were immersed in the bacterial suspension ($OD_{600} = 0.8$) for 2 h. Control seedlings were immersed in sterile LB. The seedlings were then transplanted to 3-in. pots containing vermiculite/soil mix (3:1) 2× autoclaved (1 atm and 121 °C) for 1 h. Soil mix was purchased from Fafard® Growing Mix 2. Every 15 days, the plants were sprayed with bacterial suspension ($OD_{600} = 0.8$) or sterile LB media (control). The experimental design was completely randomized and built with 17 repetitions for each treatments: control (without bacteria inoculation) and three bacteria species inoculated separately. A nutrient solution (Hoagland's nutrient nitrogen-free solution) was added every 15 days until the percolation of the solution. The pots were placed in the greenhouse at 17:16 °C day/night cycle, 75 % relative humidity, and a photoperiod of 15 h (300 μE). The plants were harvested and rinsed in tap water 45 days after inoculation. Shoot length was measured for each plant before they were stored in a dryer at 70 ° C until a constant dry weight. The dry weights of aboveground and belowground plant parts were determined using an analytical balance. Growth promotion efficacy (GPE %) was estimated to elucidate the relative effect of tested strains on plant according to Almoneafy et al. [3] using the following formula: GPE (%) = $[(G_T - G_C)/G_C] \times 100$. Where, GPE refers to growth promotion efficacy, G_T refers to growth parameter in bacteria-treated group, and G_C refers to growth parameter in control group.

In order to quantify bacteria-associated nitrogen fixation in plants, we conducted isotopic N15 gas assimilation experiments with multiple air controls. Three plants from each treatment in the greenhouse experiment were collected and placed in a gas chamber filled with 33 mL of 15 N-labeled gas (N_2, NH_4, NO_3 and NO_2; Sigma-Aldrich Lot MBBB0968V). One plant from each treatment was placed in ambient air to confirm the absence of 15 N assimilation. After a 10-day incubation period under 12 h alternating light/dark cycle, we removed the plants from the chamber and washed them. We oven dried all samples for 24 h at 80 °C to prepare for mass spectroscopic 14:15 N ratio analysis. After drying, we sent 0.9–1.0 g of dried

material to the Stable Isotope/Soil Biology Laboratory at the Odum School of Ecology at the University of Georgia for analysis.

Statistical Analysis

Statistical evaluation of growth parameters and assimilation 15 N gas was performed by ANOVA and the differences between treatment were estimated by Duncan's test (SPSS version 20, IBM, USA).

Results

Isolation, Metabolic Characterization, and Plant Growth-Promoting Attributes of Endophytic Bacteria

The rate of isolation of bacteria from the sample fragments was higher (*T* test, $p<0.001$) in 1 % YES medium (94.0 %) compared to 10 % TSA (34.8 %). We isolated 45 strains and their genetic diversity was verified by ERIC-PCR. The molecular identification via blast 16S rDNA revealed that ERIC-PCR groups represented three different species. The largest group of strains was identified as *Bacillus amyloliquefaciens* (99 % identity with sequences deposited in GenBank; phylum = *Firmicutes*). Other groups of strains were obtained with high identity to sequences of *Achromobacter spanius* (phylum = *Betaproteobacteria*), and *Microbacterium oxydans* (phylum = *Actinobacteria*), respectively. *B. amyloliquefaciens* was isolated frequently (75.5 %), followed by *A. spanius* (15.5 %) and *M. oxydans* (8.8 %).

Metabolic profiles of *B. amyloliquefaciens* (strains A9a, B2, and B5), *A. spanius* B1 and *M. oxydans* B2 were done using GEN III Microplates. There were no differences in the metabolic profiles among the three strains of *B. amyloliquefaciens* (A9a, B3, and B5); however, profiles among the three species were very different. *B. amyloliquefaciens* A9a and *M. oxydans* B2 had higher metabolic similarity (Jaccard coefficient 0.6951), using 49 and 55 different carbon sources, respectively, from Biolog Inc. (Hayward, CA, USA). *A. spanius* B1 used only 27 carbon sources and its profile showed a similarity coefficient under 0.5 with A9a (Jaccard coefficient 0.4000) and B2 (Jaccard coefficient 0.4578). The three species demonstrated variation in susceptibility to chemical compounds. Although *B. amyloliquefaciens* A9a grew in all evaluated salt concentrations (1, 4, and 8 % NaCl), this strain was sensitive to troleandomycin, minocycline, lincomycin, niaproof 4, vancomycin, tetrazolium violet, and blue tetrazolium. *A. spanius* B1 and *M. oxydans* B2 showed no metabolic activity on 8 % NaCl. Strain B1 was the most resistant to chemical compounds, being sensitive to only 1 % sodium lactate, minocycline, potassium tellurite, and sodium bromate.

Important functional traits for promoting plant growth were detected in *B. amyloliquefaciens* A9a, *A. spanius* B1, and *M. oxydans* B2 (Table 1). The three strains were able to grow in nitrogen-free media and to reduce acetylene. *M. oxydans* B2 stood out for IAA synthesis (11.75 ± 0.28 μg/mL); almost three times the concentration synthesized by *B. amyloliquefaciens* A9a (3.78 ± 0.15 μg/mL). IAA was not detected in the culture supernatant from *A. spanius* B1. *B. amyloliquefaciens* A9a secreted amylase, protease, cellulase, and esterase, while *A. spanius* A1 and *M. oxydans* B2 showed activity only for lipase and protease, respectively. None of the three species solubilized phosphate.

Antagonism against Fungi and Characterization of Bacterial Lipopeptides

B. amyloliquefaciens A9a inhibited the growth of pathogens in vitro (Table 1). Inhibition ranged from 25 % ± 1.3 to 55.9 % ± 2.5 for *A. flavus* and *A. alternata*, respectively. The mycelium treated with lipopeptides suffered morphological changes showing development of swollen, pigmented chlamydospore-like structures. TLC separation showed at least four spots in the *B. amyloliquefaciens* A9a extract, suggesting that four distinct lipopeptides were present. *A. spanius* B1 and *M. oxydans* B2 did not show antibiosis against fungi.

Key genes in lipopeptide synthesis were amplified and detected from *B. amyloliquefaciens* (A9a, B3, and B5) and *M. oxydans* B2 DNA using specific primers (Electronic Supplementary Material Table 1). *ipa14*, *fen*D, and *itu*C were detected in both bacterial species, and *srf*AA, *bmy* ,and *itu*D were amplified only from *B. amyloliquefaciens* strains. The fragment sizes and sequences of lipopeptide genes were very similar between *B amyloliquefaciens* (A9a, B3, and B5) and *M. oxydans* B2 (Electronic Supplementary Material Table 2). We did not detect any lipopeptide genes in *A. spanius* B1 with any primer sets (Electronic Supplementary Material Table 1).

The nucleotide sequences of the amplified fragments had high homology (98–100 %) to the lipopeptide genes from *B. amyloliquefaciens* strains (Electronic Supplementary Material Table 2): surfactin (*srf*AA); iturin A (*ipa*14, *itu*C, *itu*D, and *bmy*); and fengicin (*fen*D). The sequence of translated amino acids showed high homology (98–100 % identity BlastX) and had conserved domains with lipopeptide synthesis enzymes in *B. amyloliquefaciens*. We used two other *B. amyloliquefaciens* strains—B3 and B5—to amplify and sequence the genes involved in the synthesis of lipopeptides. Amplification and sequencing of these fragments demonstrated that they were similar to those obtained for the strain A9a. These data and ERIC-PCR fingerprinting corroborate the identity of B3 and B5 as *B. amyloliquefaciens*.

The antifungal activity of the CEL from *B. amyloliquefaciens* A9a was confirmed in vitro using 0, 4, and 40 μg/mL in media (Fig. 1). Reduction in fungal growth was concentration-

Table 2 Results of experiments to evaluate endophytic bacteria for growth promotion of *Phragmites australis* plants

Inoculants	Aboveground dry matter (mg)	GPE* (%)	Aboveground length (cm)	GPE (%)	Underground dry matter (mg)	GPE (%)	Total dry matter (mg)	GPE (%)
Control	0.0062 ± 0.0015a[a]	–	3.31 ± 0.66a		0.0078 ± 0.0030a		0.0140 ± 0.0042a	
Bacillus amyloliquefaciens A9a	0.0114 ± 0.0041b	83.8	6.50 ± 2.36b	96.4	0.0195 ± 0.0103b	150.0	0.0309 ± 0.0109b	120.7
Achromobacter spanius B1	0.0130 ± 0.0055b	109.7	6.53 ± 2.22b	97.2	0.0219 ± 0.0106b	180.8	0.0350 ± 0.0144b,c	150.0
Microbacterium oxydans B2	0.0185 ± 0.0048c	198.4	7.42 ± 1.93b	124.2	0.0265 ± 0.0087b	239.7	0.0450 ± 0,0129c	221.4

[a] Data are presented as the mean ± standard deviation (SD) of 17 replicates of plants. Values in the same column followed by the same lowercase letter did not differ significantly at 99 % (Duncan)

*Growth promotion efficacy (GPE %)

dependent. *A. alternata* and *C. gloeosporioides* were more sensitive to the extracts in culture media. Mycelial growth of both species was completely inhibited at the highest concentration used (40 μg/mL). The growth of other plant pathogens ranged between 88.98 and 96.82 % with 40 μg/mL of extract. The activities of the extracts of *A. spanius* B1 and *M. oxydans* B2 were not evaluated because the cultures did not show antibiosis and we could not detect the presence of lipopeptides via TLC [27].

MALDI-TOF mass spectrometry of CEL from *B. amyloliquefaciens* A9a indicated presence of clusters of peaks (Electronic Supplementary Material Table 3). One cluster of peaks in the range of 1016.60 to 1111.47 *m*/*z* ratio corresponded to iturin and bacillomycin isoforms and homologues. Another cluster of peaks was observed in the range of 1435.73 to 1529.74 *m*/*z* ratio corresponding to fengycin isoforms and homologues. The third cluster of peaks corresponded to surfactin (1016.60 to 1043.53 *m*/*z*). One peak (939.5 *m*/*z*) represents kurstakins, the lowest molecular weight group of lipopeptides. The peak at 1421.7 *m*/*z* may be identified as bacitracin. The possible assignments for 33 m/z peaks are presented in Supporting information Table 3. Most of the detected peaks are iturin and fengycin variations and these corresponded to approximately 64 % of detected peaks. *B.*

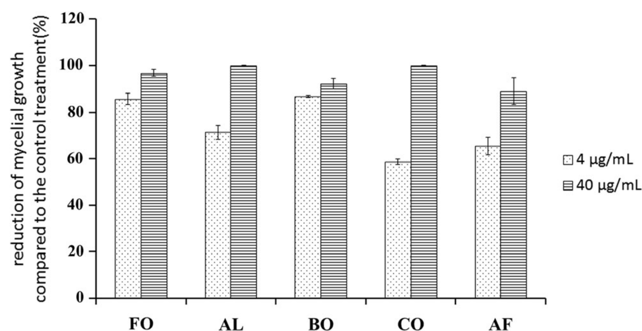

Fig. 1 Fungicidal activity of the crude extract of lipopeptide from *B. amyloliquefaciens* A9a. *FO Fusarium oxysporum*, *AL Alternaria alternata*, *BO Botrytis cinerea*, *CO Colletotrichum gloeosporioides*, *AF Aspergillus flavus*. *Bars* standard deviation. The differences between the average concentrations are statistically different (*T* test 99 % confidence level)

amyloliquefaciens C6c showed the co-production of different homologous compounds for each lipopeptide family (Electronic Supplementary Material Table 3). Nineteen peaks were not identified by the mass *m*/*z* because they did not correspond to any published *m*/*z* masses.

Although lipopeptides were not detected from CEL of *A. spanius* B1 and *M. oxydans* B2 by TLC analysis, MALDI-TOF showed a few peaks of lipopeptides. The peaks 939.5 (kurstakin) and 1488.7 (fengycin) *m*/*z* were detected in extracts from *A. spanius* B1 and *M. oxydans* B2. *M. oxydans* B2 showed a third peak 1502.8 *m*/*z* (fengycin).

Unidentified peaks were similar between the three species (827.4, 926.4, 1052.6 *m*/*z*), between *B. amyloliquefaciens* A9a and *A. spanius* B1 (852.4 and 997.4 *m*/*z*), and between *A. spanius* B1 and *M. oxydans* B2 (911.4, 1359.7, and 1688.8 *m*/*z*). These results suggest that in some cases, these may be producing the same or similar compounds; however, this needs to be confirmed by further chemical analyses.

Growth Promotion of *P. australis* by Endophytic Bacteria

Our greenhouse experiment showed a significant effect on plant growth when seedlings were inoculated with endophytic bacteria (Table 2). Aboveground stem height of inoculated plants was 96.4–124.2 % higher than that of the control treatment (Duncan <0.01). All bacterial treatments increased plant root biomass of treated plants over the control treated with only media (Duncan <0.01). Similar results were obtained for the total biomass. Plants inoculated with *A. spanius* B1 and *M. oxydans* B2 resulted in an increase of 109.7 and 198.4 %, respectively, in accumulation of aboveground dry matter. The greatest increase of total biomass was observed in plants inoculated with *M. oxydans* B2 (221.4 %), followed by *A. spanius* B1(150.0 %) and *B. amyloliquefaciens* A9a (120.7 %).

In a 15 N2 gas assimilation experiment (Table 3), mass spectroscopic analysis showed that *P. australis* incorporated 15 N into tissues. Highest incorporation into leaves and stems was observed in plants inoculated with *A. spanius* B1. Highest incorporation into roots was observed in plants inoculated

Table 3 15 N isotopic analysis results for 15 N-enriched and non-enriched plants after 10-day growth

Treatment	Leaves		Stem		Roots	
	δ15 N vs (‰)	Atoms % 15 N	δ15 N vs (‰)	Atoms % 15 N	δ15 N vs (‰)	Atoms % 15 N
Control	12.86 ± 0.77* aA	0.3712 ± 0.0003 aA	15.32 ± 1.48 aA	0.3720 ± 0.0005 aA	6.38 ± 2.37 aB	0.3688 ± 0.0008 aB
B. amyloliquefaciens A9a	22.28 ± 1.10 bA (73**)	0.3746 ± 0.0004 bA	16.98 ± 3.18 aB	0.3723 ± 0.0011 aB	10.03 ± 1.18 (57) bC	0.3701 ± 0.0004 bC
A. spanius B1	25.77 ± 4.50 bA (100)	0.3759 ± 0.0016 bA	29.24 ± 4.56 aA	0.3771 ± 0.0017 aA	17.03 ± 1.01 (167) cB	0.3727 ± 0.0004 cB
M. oxydans B2	21.30 ± 3.06 bA (65)	0.3742 ± 0.0011 bA	16.33 ± 13.14 aA	0.3724 ± 0.0047 aA	19.21 ± 1.66 (201) cA	0.3734 ± 0.0006 cA
Plants without 15 N	4.68 ± 0.38	0.3700 ± 0.0001	1.84 ± 0.37	0.3700 ± 0.0001	2.66 ± 0.26	0.3700 ± 0.0001

*Values are means ± standard deviation (SD); different letters in the same row (uppercase) and column (lowercase) indicate significant difference at $P < 0.05$ (Duncan test)

**Increase % in relation to control: [(GT − GC)/GC] × 100. Where, GT refers 15 N in plant inoculated group and GC refers to 15 N in control group

with *M. oxydans* B2 and *A. spanius* B1. Bacterial inoculation with *B. amyloliquefaciens* and *M. oxydans* did not change N incorporation into stems over what was seen in non-inoculated controls. However, bacterial inoculation with *B. amyloliquefaciens* and *M. oxydans* increased N incorporation into leaves over what was seen in non-inoculated controls. Plants that had not been exposed to 15 N2 gas showed minimal 15 N content.

Discussion

Isolation, Metabolic Characterization, and Plant Growth-Promoting Attributes of Endophytic Bacteria

Roots and leaves host different endophytic species distinguished by the structure and composition of the organ-specific communities [12, 40, 52]. Root endophytic bacteria are reasonably well studied with respect to location and interactions with the host; comparatively little is known about endophytes associated with shoot meristems [63]. The shoot meristems are an important tissue responsible for the growth and development of new leaves and stems. We reason also that endophytes that colonize shoots are more likely to be transmitted with seeds than endophytes that remain in roots. Thus, aerial organ endophytes may be critical microbes in terms of survival value and competitive enhancement.

Endophytes examined in this study were obtained from *Phragmites* from a single collection site. Our results showed that cultivable endophytic bacteria community composition in shoot meristematic tissues consisted of: *B. amyloliquefaciens* (*Firmicutes* 75.5 %), *A. spanius* (*Proteobacteria* 15.5 %), and *M. oxydans* (*Actinobacteria* 8.8 %). The metabolic profiles of both Gram-positive strains, *B. amyloliquefaciens* and *Microbacterium oxydans*, were more similar to each other (0.6951 Coef Jaccard) than to the Gram-negative bacterium *A. spanius*. *B. amyloliquefaciens* A9a could be considered a halophilic bacterium since it was capable of growth at high concentrations of NaCl.

The three species of bacteria we isolated from shoots of *P. australis* have not previously been identified in communities of *Phragmites* endophytes. There are a few papers regarding endophytic bacteria of *P. australis*, but these papers have used roots and rhizomes for isolation of bacteria [43, 71]. Various biotic and abiotic factors influence the composition of the microbiota associated with plants [9, 26]. We propose that the kind of tissue used and the environmental conditions have effects on the community composition of endophytes in *P. australis* [43, see 71].

B. amyloliquefaciens was the most frequently isolated endophyte among the bacteria isolated. This bacterium is a Gram-positive, motile, spore-forming, rod-shaped bacterium that is present in the soil or in plant hosts as an endophyte. It has been classified into two subspecies *B. amyloliquefaciens* subsp. *plantarum* (for plant-associated strains) and *B. amyloliquefaciens* subsp. *amyloliquefaciens* (for soil strains) [10]. Our *Phragmites* isolate is *B. amyloliquefaciens* ssp. *plantarum*. *B. amyloliquefaciens* is a bacterium that is known to stimulate plant growth and produce secondary metabolites that suppress plant pathogens [16, 84]. *A. spanius* is a Gram-negative, oxidase- and catalase-positive, rod-shaped bacterium that has been previously found to colonize the rhizoplanes and internal tissues of annual ryegrass [15, 28]. The genus *Microbacterium* contains species that are Gram-positive rods and have been found to be endophytic in tomato roots [53] and in maize grains [88].

Antagonism against Pathogens and Characterization of Lipopeptides of Endophytes

B. amyloliquefaciens and other closely related *Bacillus* sp. have been used to successfully control many plant pathogenic fungi [83, 86]. *B. amyloliquefaciens* A9a may be a defensive endophyte of *Phragmites*. Based on our results, *B. amyloliquefaciens* A9a exhibited high antibiosis activity against several phytopathogenic fungi. This bacterium is known to produce antifungal lipopeptides that may be secreted into the growth medium [41]. It is generally accepted that

these compounds help the host defend against pathogens, inducing systemic responses in plants [34] or acting directly on the pathogen as an “antibiotic” [60]. The lipopeptides produced by numerous *Bacillus* spp. are classified into three families depending on their amino acid sequences: surfactins; iturins (iturins, mycosubtilin and bacillomycins); and fengycins [59]. These molecules are synthesized by non-ribosomal peptide synthetase (NRPS) encoded by operons in bacteria [65]. The genes involved in lipopeptide synthesis have been previously detected in *B. amyloliquefaciens* [35] and their sequences are well conserved among different strains of this species [17].

The antagonistic activity of A9a correlated with antifungal activity of CEL (Table 1). The antifungal activity of the extract was concentration-dependent, completely inhibiting growth of *A. alternata* and *C. gloeosporioides* at 40 μg/mL. Our MALDI-TOF analysis of CEL exhibited peaks that may be attributed to the sodium and potassium adducts of lipopeptides [19]. By comparing the mass with the mass numbers reported for the lipopeptide complexes from other *Bacillus* strains (Electronic Supplementary Material Table 3), we found four lipopeptide families from A9a CEL: iturin, surfactin, fengycin, and kurstakins. Kurstakin was discovered in *B. thuringiensis* and considered to be a biomarker of this species. However, it was later detected in other species of *Bacillus* [7]. We did not find reports of synthesis of kurstakin by *B. amyloliquefaciens*, but we do know that purified kurstakins can display antifungal activities [30]. We detected one MALDI-TOF peak corresponding to bacitracin, an antibiotic synthesized by both *B. subtilis* and *Bacillus licheniformis* [22]. Bacillomycin, a member of the iturin family, has been reported together with fengycin to have strong antifungal activity, being responsible for the main antagonistic activity of the commercially available biological control strain *B. amyloliquefaciens* FZB42 against *F. oxysporum* [75]. Surfactins have weak antifungal activity but are known for their biosurfactant, hemolytic, antiviral, and antibacterial activities [11]. Fengycins are less hemolytic than either surfactins or iturins and possess strong antifungal capacity, specifically against filamentous fungi [19, 51].

Interestingly, *fen*D, *ipa*14, and *itu*C were also amplified from the *M. oxydans* B2 DNA indicating that this species may also be able to synthesize fengycin and iturin. We detected by MALDI-TOF only fengycin from the *M. oxydans* CEL. The failure to detect lipopeptide genes in *A. spanius* B1 could be due to the absence of the genes in this bacterium or simply failure of the primers to match lipopeptide genes of this species. Our results showed that detection of lipopeptide genes by PCR may not represent the actual capacity of synthesis and/or secretion of these compounds. In this study, we detected via PCR two sequences of genes (*itu*C and *ipa*14) in *M. oxydans* B2, but we were unable to detect either lipopeptide by TLC or by MALDI-TOF analysis.

MALDI-TOF analysis indicated kurstakin and one peak of fengycin in *A. spanius* B1 and *M. oxydans* B2 extracts, and a second peak in *M. oxydans* B2 corresponding to fengycin. We did not find reports of synthesis of kurstakin by these species. The amount of secreted lipopeptide was not sufficient to inhibit fungal growth in vitro in our antibiosis experiment. These results lead us to believe that *M. oxydans* B2 may have non-functional genes or the growth conditions were not favorable for lipopeptide operon expression. The oligos used for Gram-positive bacteria (B. *amyloliquefaciens* A9a and *M. oxydans* B2) did not work in Gram-negative bacteria (*A. spanius* B1).

The Role of Increased Nitrogen Assimilation in Growth Promotion of *P. australis* by Endophytic Bacteria

Endophytic infections often lead to enhanced plant productivity, either by producing plant growth hormones, enhancing nutrient acquisition, synthesizing metabolites that restrict vertebrate or invertebrate herbivory, or reducing disease susceptibility [68]. Our three microbes show some of these same capabilities. Endophytic fungi [5, 24] and bacteria [45, 50] are associated with leaves, roots, and rhizomes of *P. australis*. The functional roles of this community in adaptability and growth promotion has not been fully determined, although, fungal endophytes belonging to genus *Stagonospora* (isolated from *Phragmites* seeds) were shown to enhance reed biomass [21].

Invasive species often have a competitive advantage over the native community from the moment when the invaders enter the environment. One of the main factors for successful plant invasion is that invading plants often affect nitrogen cycles via direct and/or indirect ways, such as changes in soil microbial communities, litter decomposition rates, and/or soil physicochemical properties [78].

The results of our isotopic nitrogen gas assimilation experiment (Table 3) show that all three bacteria substantially increased the amount of isotopic nitrogen that accumulated in *Phragmites* plants over absorption into plants where bacteria were not applied. The bacteria we tested all possess the enzymatic capability to absorb nitrates, nitrites, nitrogen oxide and ammonia molecules that may be impurities in the isotopic nitrogen gas used in our experiments [18]. Thus with our data, it is not possible to determine if increased accumulation of isotopic nitrogen in the plants is due to nitrogen fixation or enhanced absorption of 15 N-labeled ammonia, nitrate or nitrous oxide present in the molecular nitrogen. What is clear from our data is that all three microbes increase accumulation of isotopic nitrogen into plants.

Results of the acetylene reduction tests (Table 1) indicate that all three bacteria are capable of nitrogen fixation, and it is possible that at least some of the 15 N accumulation in plant tissues could be the result of nitrogen fixation by the applied

microbes. However, we do not have any direct evidence that the observed growth promotion of *Phragmites* is the result of nitrogen fixation by the microbes we applied to plants. Instead, we hypothesize that increased efficiency of nutrient absorption from rhizosphere nutrient pools contributes to the growth promotional effects we observed in our experiments (Table 2) [see 67]. A similar mechanism for microbially enhanced nitrogen accumulation in plants was proposed by Hurek et al. [32] where it was found that kallar grass bearing rhizobacteria that were incapable of nitrogen fixation nevertheless enhanced nitrogen accumulation into plants. Endophytes that colonize surfaces of roots may increase nitrogen available to the plant through enhanced scavenging of organic nitrogen and perhaps other forms of nitrogen in the rhizosphere [80]. In *Phragmites*, root microbes may collaborate with plants to increase nitrogen supply by secreting proteases around roots to degrade microbial enzymes forming peptide fragments that may be absorbed by roots [1, 2]. It has also been shown that microbes that associate with roots may colonize surfaces of roots and also become internalized within root cells where they degrade as roots develop [61, 80, 81]. Continuous internal re-colonization of growing root meristems by surface microbes could result in a continuous transfer of nutrients from microbial community pools outside roots to microbes within root cells and ultimately to the host root. Microbially enhanced transfer of nutrients to host roots from rhizosphere nutrient pools could explain the plant growth promotion observed in our experiments. Results of growth promotional experiments (Table 2) and nitrogen absorption experiments (Table 3) are consistent with this hypothesis. The growth promotion efficiency (GPE) of the three strains of bacteria in order of magnitude was B2 > B1 > A9a. Examination of isotopic absorption data shows that absorption in order of magnitude in leaves was B1 > A9a > B2; absorption in stems was B1 > A9a > B2; absorption in roots was B2 > B1 > A9a. Only in roots do we see a correlation between increased absorption of isotopic nitrogen and the observed GPE. This is consistent with a scenario where enhanced absorption of rhizosphere nutrients plays a role in the growth promotion we observed in our experiments. The growth promotional microbes that we evaluated may in essence be functioning through facilitation of the transfer of nutrients from the rhizosphere to roots. Additional experiments would be needed to further evaluate this proposed nitrogen-scavenging mechanism.

There is some evidence that the way invasive *Phragmites* manages nitrogen may in fact be key to its competitive nature. Invasive *P. australis* outperforms native species in environments with both high and low soil nitrogen levels [25, 42]. Interestingly, its most aggressive growth can occur in low oxygen environments; for example, in swamp soils, where *P. australis* has been shown to utilize organic nitrogen from rhizospheres of roots to a greater extent than its competitors [56, 57]. Enhanced absorption of organic nitrogen could be the result of the collaborative symbiosis with root microbes [80]. Nitrogen sources could also include increasing nitrogen availability to *Phragmites* through associative nitrogen fixation [20, 73]. Additional studies are needed to evaluate whether associative nitrogen fixation is an important source of nitrogen for the plant.

In terms of nitrogen management, microbes may play other roles. For example, under low oxygen conditions, as is often seen in water-soaked soils where *Phragmites* grows, ammonia may accumulate to toxic levels in plant tissues [38, 82]. The ability of plants to cope with and manage high levels of ammonia and other nitrogenous compounds may be important in efficient metabolism and growth of *P. australis*. In this respect, it may be significant that bacteria often possess transporters for absorption of ammonium and other nitrogenous compounds. It is conceivable that some *Phragmites* endophytes could function in part in detoxification of ammonia or ammonium from tissues of *Phragmites*. By absorption of potentially toxic levels of ammonia from the immediate environment of the plant and converting it to forms that are better assimilated by the host such as nitrates or amino acids, endophytes may effectively increase nitrogen use efficiency in the host and avoid oxidative stress related to ammonia toxicity.

Acknowledgments The authors are grateful for funding and other support to the US Geological Survey, the John E. and Christina C. Craighead Foundation, USDA-NIFA Multistate Project W3147, The New Jersey Agricultural Experiment Station, The Federal University of Mato Grosso (UFMT), Department of Plant Biology and Pathology of Rutgers University, The Brazilian National Council for Scientific and Technological Development (CNPq) for Post Doctoral Fellowship, and the International Institute of Science and Technology in Wetlands (INAU). Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the US Government. This article is a contribution of the USGS Great Lakes Science Center.

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