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Genome Survey and Characterization of Endophytic Bacteria Exhibiting a Beneficial Effect on Growth and Development of Poplar Trees▽†

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The association of endophytic bacteria with their plant hosts has a beneficial effect for many different plant species. Our goal is to identify endophytic bacteria that improve the biomass production and the carbon sequestration potential of poplar trees (*Populus* spp.) when grown in marginal soil and to gain an insight in the mechanisms underlying plant growth promotion. Members of the *Gammaproteobacteria* dominated a collection of 78 bacterial endophytes isolated from poplar and willow trees. As representatives for the dominant genera of endophytic gammaproteobacteria, we selected *Enterobacter* sp. strain 638, *Stenotrophomonas maltophilia* R551-3, *Pseudomonas putida* W619, and *Serratia proteamaculans* 568 for genome sequencing and analysis of their plant growth-promoting effects, including root development. Derivatives of these endophytes, labeled with *gfp*, were also used to study the colonization of their poplar hosts. In greenhouse studies, poplar cuttings (*Populus deltoides* × *Populus nigra* DN-34) inoculated with *Enterobacter* sp. strain 638 repeatedly showed the highest increase in biomass production compared to cuttings of noninoculated control plants. Sequence data combined with the analysis of their metabolic properties resulted in the identification of many putative mechanisms, including carbon source utilization, that help these endophytes to thrive within a plant environment and to potentially affect the growth and development of their plant hosts. Understanding the interactions between endophytic bacteria and their host plants should ultimately result in the design of strategies for improved poplar biomass production on marginal soils as a feedstock for biofuels.

Endophytic bacteria are bacteria that reside within the living tissue of their host plants without substantively harming it (19, 26). They are ubiquitous in most plant species, latently residing or actively colonizing the tissues. The diversity of cultivable bacterial endophytes is exhibited not only in the variety of plant species colonized but also in the many taxa involved, with most being members of common soil bacterial genera such as *Enterobacter*, *Pseudomonas*, *Burkholderia*, *Bacillus*, and *Azospirillum* (21, 23). Endophytic bacteria have several mechanisms by which they can promote plant growth and health. These mechanisms are of prime importance for the use of plants as feedstocks for biofuels and for carbon sequestration through biomass production. This is vital when considering the aim of improving biomass production of marginal soils, thus avoiding competition for agricultural resources, which is one of the critical socioeconomic issues of the increased use of biofuels.

Like rhizosphere bacteria, endophytic bacteria have been shown to have plant growth-promoting activity that can be due to the production of phytohormones, enzymes involved in growth regulator metabolism, such as ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, auxins, indole-3-acetic acid (IAA), acetoin, 2,3-butanediol, cytokinins (3, 13–15, 20, 30), or combinations thereof. They can also improve plant growth via the fixation of nitrogen (diazotrophy) (9, 38).

Typical examples of marginal soils include soils that have deteriorated due to the presence of heavy metals or organic contaminants. These are often soils with a history of industrial, military, or mining activities. Endophytic bacteria can assist their host plants in overcoming phytotoxic effects caused by environmental contamination (5, 11, 12, 36), which is of direct relevance for waste management and pollution control via phytoremediation technologies. When nonsterile poplar cuttings (*Populus trichocarpa* × *deltoides* cv. Hoogvorst) were inoculated with the endophyte *Burkholderia cepacia* VM1468, a derivative of *B. cepacia* Bu72 which possesses the pTOM-Bu61 plasmid coding for a constitutively expressed toluene degradation pathway, it was observed that in addition to decreasing the phytotoxicity and releasing toluene, strain VM1468 also considerably improved the growth of poplar trees in the absence of toluene (36). This observation, which was the first of its kind for poplar trees, prompted us to further study the poplar tree-associated beneficial endophytic bacteria in order to improve the overall performance of poplar trees, as it can enhance multiple applications, including biomass production, carbon sequestration, and phytoremediation. This was done by screening endophytic bacteria for their plant growth-promoting capabilities toward poplar trees by performing colonization studies with *gfp*-labeled strains, by examining their metabolic properties, and by initiating the genome sequencing of several strains.

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MATERIALS AND METHODS

Isolation of endophytic bacteria. Root and shoot samples were collected from the 10-year-old hybrid poplar tree H11-11 (*Populus trichocarpa* × *P. deltoides*) that had been growing in the presence of carbon tetrachloride (12 ppm homogeneously) for 8 years at an experimental site in Washington State. In addition, native willow (*Salix gooddingii*) material was collected from 5-year-old native plants that had been growing in the presence of both trichloroethylene (18 ppm) and carbon tetrachloride (12 ppm) for 5 years. Cuttings were removed from the plants with clippers that were washed with ethanol between cuts and placed in acetone-rinsed volatile organic analysis vials which were placed on ice for shipment from the field. Roots and shoots were treated separately. Fresh root and shoot samples were vigorously washed in distilled water for 5 min, surface sterilized for 5 min in a solution containing 1% (wt/vol) active chloride (added as a sodium hypochlorite [NaOCl] solution) supplemented with 1 droplet Tween 80 per 100 ml solution, and rinsed three times in sterile distilled water. A 100-μl sample of the water from the third rinse was plated on 869 medium (25) to verify the efficiency of sterilization. After sterilization, the roots and shoots were macerated in 10 ml 10 mM $MgSO_4$ using a Polytron PT1200 mixer (Kinematica A6). Serial dilutions were made, and 100-μl samples were plated on nonselective media in order to test for the presence of the endophytes and their characteristics.

16S rRNA gene amplification, amplified 16S rRNA gene restriction analysis (ARDRA), sequencing, and strain identification. Total genomic DNA of endophytic bacteria was isolated as described previously (7). 16S rRNA genes were PCR amplified using the standard 26F-1392R primer set (2).

For ARDRA, aliquots of the PCR products were digested overnight at 37°C with HpyCH4IV in 1× NEB buffer 1 (New England Biolabs, Beverly, MA). ARDRA patterns were grouped, and clones with representative patterns were selected for sequencing.

Purified PCR products (QIAquick columns) of 16S rRNA genes were sequenced using the Prism BigDye terminator sequencing kit (Applied Biosystems, Foster City, CA) with 100 ng of template DNA. The extended sequences were obtained with universal primers 26F and 1392R. Taxonomic classifications were determined according to Wang et al. (44) at the Ribosome Database Project II (http://rdp.cme.msu.edu/index.jsp). The sequences used for identification of the cultivable endophytes are available in the GenBank database (www.ncbi.nlm.nih.gov) under accession numbers EU340901 through EU340978.

Screening for metabolic properties. Bacteria were screened for their carbon utilization in Schatz minimal salt medium (31) supplemented with different carbon sources, which were added at 2 g/liter. As positive controls, strains were grown in Schatz minimal salt medium supplemented with C-mix (per liter of medium: 1.3 ml glucose 40%, 0.7 ml lactate 50%, 2.2 ml gluconate 30%, 2.7 ml fructose 20%, and 3 ml 1 M succinate). To determine IAA production, strains were grown in Schatz medium plus C-mix supplemented with 100 μg/ml L-tryptophan. IAA concentrations were determined by the Salkowsky reaction (24). In order to test for growth on toluene as the sole carbon source, bacteria were plated on Schatz medium and incubated for 7 days at 30°C in sealed 10-liter vessels with the addition of 600 μl toluene. To test for autotrophy and nitrogen fixation, bacteria were inoculated in Schatz medium without a carbon or nitrogen source (omit NH_4NO_3), respectively. ACC deaminase activity was tested, with ACC as the sole nitrogen source (43). For reproducibility, all experiments were done in triplicate, starting from isolated colonies.

Inoculation of poplar trees with endophytic bacteria and analysis of plant growth. Inocula (250-ml culture) were prepared by growing endophytic bacteria in 1/10-strength 869 medium (25) at 30°C on a rotary shaker until a cell concentration of 10^9 CFU/ml was reached (optical density at 660 nm [OD_{660}] of 1). The cells were collected by centrifugation, washed twice in 10 mM $MgSO_4$, and suspended in 1/10 of the original volume (in 10 mM $MgSO_4$) to obtain an inoculum with a cell concentration of 10^{10} CFU/ml. Per microbial strain tested, seven cuttings from poplar (*Populus deltoides* × *P. nigra*) DN-34 of approximately 30 cm were weighed and placed in a 1-liter beaker containing 0.5 liter of a half-strength sterile Hoagland's nutrient solution (5), which was refreshed every 3 days. The cuttings were allowed to root for approximately 4 weeks until root formation started. Subsequently, a bacterial inoculum was added to each jar at a final concentration of 10^8 CFU/ml in half-strength Hoagland's solution. After 3 days of incubation, cuttings were weighed and planted in nonsterile sandy soil and placed in the greenhouse with a constant temperature of 22°C and 14 h light-10 h dark cycle with photosynthetic active radiation of 165 mmol/m^2s. After 10 weeks, plants were harvested, and their total biomass, their increase in biomass, and the biomass of different plant tissues were determined. Data were also collected from noninoculated control plants. Growth indexes were calculated as (Mt − M0)/M0 after 10 weeks of growth in the presence or absence of endophytic inoculum, where M0 is the plant's weight (g) at week 0 and Mt is the plant's weight (g) after 10 weeks. The statistical significance of the results was confirmed at the 5% level using the Dunnett test.

To determine the effects of endophytic bacteria on the rooting of poplar DN-34, cuttings were treated as described above, except that the endophytic inoculum was added from day 1.

Construction and imaging of green fluorescent protein (GFP)-labeled endophytes. In order to follow endophytic colonization of poplar trees, we successfully labeled *Enterobacter* sp. strain 638, *Pseudomonas putida* W619 (36), and *Serratia proteamaculans* 568 with *gfp* using *Escherichia coli* S17-1/λpir (pUT::miniTn5-Km-*gfp*) (37) as a donor in conjugation (35). Kanamycin (100 μg/ml)-resistant transconjugants were selected on 284 minimal medium (32) complemented with a carbon mix (a mixture of glucose, gluconate, lactate, succinate, and acetate was added at 0.05% [wt/vol] each) and subsequently tested for *gfp* expression under UV light.

The stability of the *gfp* insertions was verified by growing individual transconjugants for 100 generations on nonselective 869 medium (25). One hundred individual colonies were grown on nonselective medium and subsequently replica plated on 869 minimal medium supplemented with kanamycin, after which they were tested for *gfp* expression. All *gfp*-labeled strains gave transconjugants that stably maintained the insertion (less than 1% loss of the *gfp* marker after growth for 100 generations under nonselective conditions). No *gfp*-expressing derivatives of *Stenotrophomonas maltophilia* R551-3 and *Methylobacterium populi* BJ001 were obtained. *S. maltophilia* R551-3 was found to possess natural resistance to kanamycin and tetracycline, the two antibiotics available to select *gfp*-containing minitransposons. For *M. populi* BJ001, neither transformation nor horizontal gene transfer could be successfully used to introduce the *gfp*-containing minitransposon.

To determine the sites of miniTn5-Km2-*gfp* insertion, genomic DNA was isolated (7) and digested with HpyCH4IV, which has no recognition site between the 5′ end of *gfp* and the upstream end of the miniTn5 transposon part. After digestion, HpyCH4IV was heat inactivated for 20 min at 65°C. Subsequently, the restriction fragments were ligated to a Y-shaped linker cassette, which was obtained by annealing two oligonucleotides with sequences 5′-TTTGGATTTG CTGGTCGAATTCAACTAGGCTTAATCCGACA-3′ and 5′-CGTGTCGGA TTAAGCCTAGTTGAATTTATTCCTATCCCTAT-3′ as described previously (42). The ligation mixture was purified using the GFX PCR DNA and gel band purification kit (GE Healthcare Biosciences) and used as a template for linear amplification, using a single complementary primer pointing outward from the 5′ end of *gfp* (GFP primer, 5′-GAAAAGTTCTTCTCCTTTAC-3′). The linear amplification results in the repair of the Y-shaped linker cassette only for those fragments that contain the *gfp* insertion region. Subsequently, PCR was performed using the GFP primer plus the linker primer (5′-GGATTTGCTGGTC GAATTCAAC-3′), which will only hybridize to the repaired linker. For each of the transconjugants tested, this resulted in the amplification of a single DNA fragment, indicative of a single insertion of miniTn5-Km2-*gfp*. Sequence analysis allowed for determining the location of the miniTn5-Km2-*gfp* insertions as follows: position 341748 of the *P. putida* W619::*gfp*7 chromosome within ORF326, which encodes a putative heavy metal-translocating P-type ATPase (CadA like); position 8455 of plasmid pENT628-1 of *Enterobacter* sp. strain 638::*gfp*7 in the noncoding region of a two-component regulatory system; and position 27524 of the 46,804-bp plasmid pSER568-1 of *S. proteamaculans* 568::*gfp*1 within ORF4938, which encodes a putative HNH endonuclease. None of the insertions seemed to have occurred in functions with obvious roles in plant-microbe interactions.

The Axiovert 200 M (Zeiss) fluorescence microscope, equipped with an AxioCam MRm charge-coupled-device camera and ApoTome, was used to visualize *gfp*-labeled bacteria on the surface of and inside the plant tissue. Images were acquired with Zeiss AxioVision software. The colors on the image are pseudocolors. The green channel was acquired with Zeiss filter set 10 (excitation, 450 to 490 nm; beam splitter, 510 nm; emission, 515 to 565 nm), and the red channel was acquired with Zeiss filter set 00 (excitation, 530 to 585 nm; beam splitter, 600 nm; emission, >615 nm).

After being harvested, plant roots were rinsed with 10 mM $MgSO_4$. Visualization of *gfp* expression proved to be difficult due to autofluorescence from the plant tissue itself and was achieved by counterstaining the tissue section with 0.05% methyl violet for 30 s, which caused the plant cells to fluoresce red under near-UV light (11).

Genome sequencing. Genome sequencing was carried out at the Joint Genome Institute (DOE, Walnut Creek, CA) for *Enterobacter* sp. strain 638 (http://genome.jgi-psf.org/finished_microbes/ent_6/ent_6.home.html), *P. putida* W619 (http://genome.jgi-psf.org/finished_microbes/psepw/psepw.home.html), *S. proteamaculans* 568 (http://genome.jgi-psf.org/finished_microbes/serpr/serpr.home

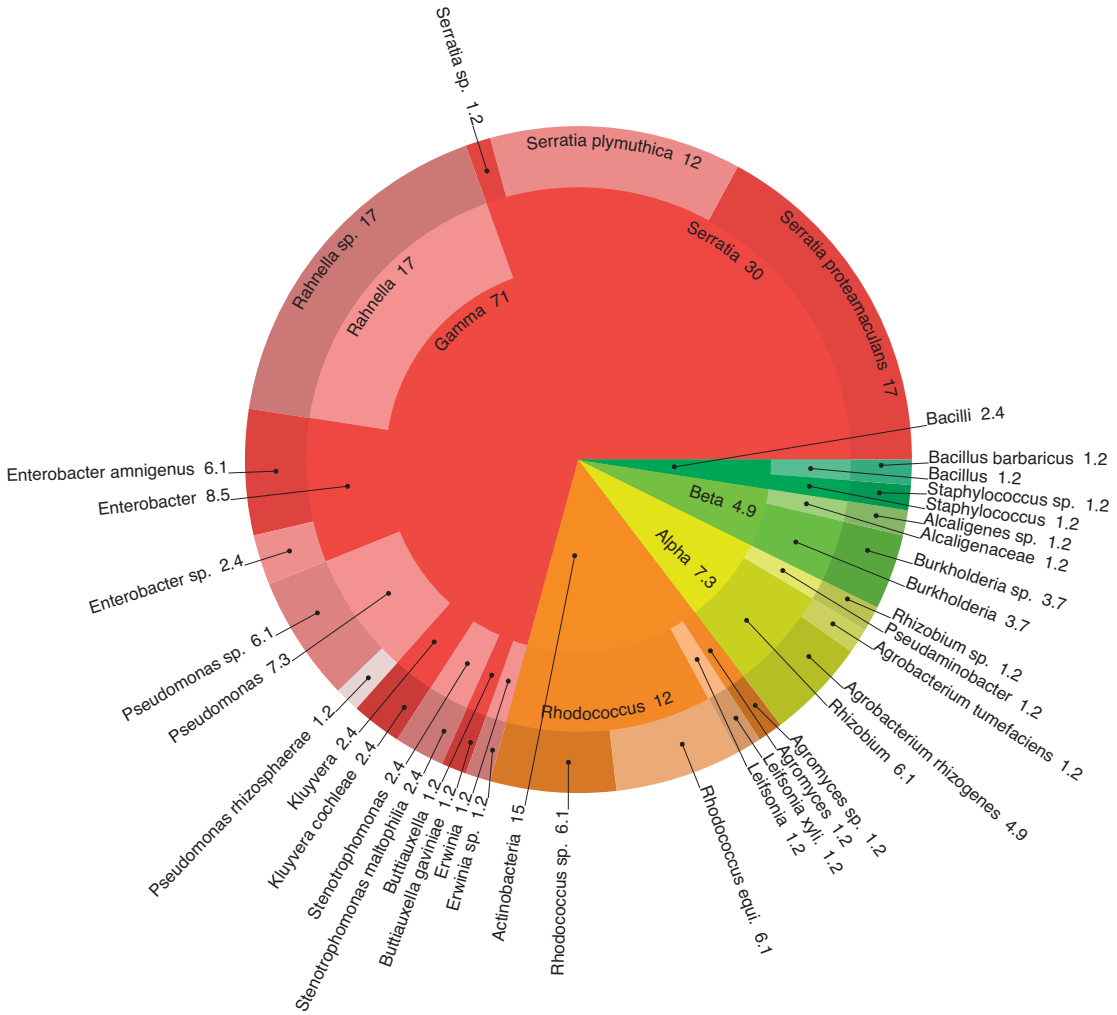

FIG. 1. Taxonomic breakdown of 16S rRNA gene sequences of the cultivable endophytic community composition as isolated from hybrid poplar H11-11 and willow trees. Taxonomic classifications were determined according to Wang et al. (44). The central pie shows percentages by phyla; each outer annulus progressively breaks these down to finer taxonomic levels, with families, genera, and species in the outermost annuli. Numbers indicate the relative abundance, expressed as a percentage, of the different taxonomic groups.

.html), and *S. maltophilia* R551-3 (http://genome.jgi-psf.org/finished_microbes/stema/stema.home.html). Metabolic functions were identified using the integrated microbial genomes (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi) system (22) and with the basic local alignment search tool (BLAST) (1). Metabolic pathways were constructed using PRIAM predictions mapped on the KEGG database (http://www.genome.ad.jp).

RESULTS

Isolation and characterization of endophytic bacteria from poplar and willow trees. Endophytic bacteria were isolated under aerobic conditions from surface-sterilized root and stem samples taken from hybrid poplar tree H11-11 and native willow (*Salix gooddingii*) that were grown in a silty loam soil with groundwater below it that was contaminated with carbon tetrachloride or trichloroethylene and carbon tetrachloride, respectively. Based on morphological characteristics, 78 strains were selected for identification. Their total genomic DNA was extracted and used to amplify the 16S rRNA gene. ARDRA with HpyCH4IV was used to determine closely related clones, after which representative 16S rRNA genes were sequenced for species identification. A detailed breakdown of the cultivable endophytic community composition is presented in Fig. 1. The majority of the isolated strains (71%) belonged to *Gammaproteobacteria*, with *Serratia* spp., *Serratia plymuthica*, *Serratia proteamaculans*, and *Rahnella* spp. being the most frequently found. Other dominant gammaproteobacteria included *Pseudomonas* spp. and *Enterobacter* spp. The *Actinobacteria* (15% of the population) were dominated by *Rhodococcus* spp. The presence of *Stenotrophomonas maltophilia* is also noteworthy, as this species represents an increasing medical issue of multidrug resistance (6) and seems to be well adapted to live in association with both human and plant hosts.

Screening of endophytic bacteria for plant growth-promoting properties. Selected cultivable endophytic gammaproteobacteria found in poplar trees, isolated as part of this study or previously described and which represent the major phylogenetic groups as identified in Fig. 1, were tested for their capacity to improve the growth of their host plants. The selected strains were the poplar endophytes *Enterobacter* sp. strain 638 (this study), *S. maltophilia* R551-3 (36), *P. putida* W619 (36), *S. proteamaculans*

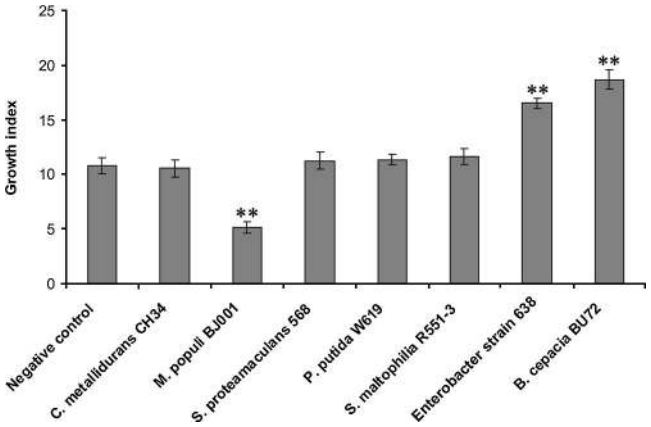

FIG. 2. Growth indexes for poplar cuttings inoculated with different endophytic bacteria. Growth indexes were determined 10 weeks after the inoculating and planting of the cuttings in sandy soil. Per condition, seven plants were used. Plants were grown in the greenhouse. Noninoculated plants were used as references. Bars indicate standard errors. Growth indexes were calculated as (Mt − M0)/M0 after 10 weeks of growth in the presence or absence of endophytic inoculum. M0, plant's weight (g) at week 0; Mt, plant's weight (g) after 10 weeks. The statistical significance of the increased biomass production of inoculated plants, compared to that of noninoculated control plants, was confirmed at the 5% level (**) using the Dunnett test.

568 (this study), and *M. populi* BJ001 (40, 41). *Burkholderia cepacia* Bu72, an endophyte originally isolated from yellow lupine which was found to have plant growth-promoting effects on poplar trees (36), and *Cupriavidus metallidurans* CH34 (also referred to as *Ralstonia metallidurans* CH34) (27), a typical soil bacterium with no known plant growth-promoting effects, were included as positive and neutral controls, respectively. Also, noninoculated cuttings were used as controls.

After root formation in hydroponic conditions and subsequent endophytic inoculation, the poplar DN-34 cuttings were planted in a marginal sandy soil and allowed to grow for 10 weeks, after which the plants were harvested and their biomasses were determined. After 10 weeks of growth, poplar trees inoculated with *M. populi* BJ001 had less new biomass than the controls (Fig. 2) ($P < 0.05$). Poplar cuttings inoculated with *Enterobacter* sp. strain 638 ($P = 0.018$) and *B. cepacia* BU72 ($P = 0.042$) showed statistically better growth than the control plants (Fig. 2), as was reflected by their growth indexes. The plant growth-promoting effects of *Enterobacter* sp. strain 638 and *B. cepacia* BU72 were reproducible in independently performed experiments.

Under the greenhouse conditions tested, no differences in growth indexes were found between those of the noninoculated control plants and those for plants inoculated with *S. maltophilia* R551-3, *P. putida* W619, and *S. proteamaculans* 568; their growth was comparable to that observed for plants inoculated with *C. metallidurans* CH34. Also, control plants and plants inoculated with the endophytic bacteria appeared healthy, except for plants inoculated with *M. populi* BJ001, which showed signs of stress, including chlorosis of the leaves.

Effects of endophytic bacteria on poplar root development. During hydroponic growth of poplar DN-34 cuttings and before endophytic inoculation occurred, difficulties with root formation were observed. After endophytic inoculation and subsequent growth in soil, we noticed that the root systems of inoculated poplar cuttings were often denser with many fine roots compared to those of the noninoculated control plants. To further test the effects of endophytic bacteria on root development, rooting experiments were performed in the presence and absence of *gfp*-labeled derivatives of *S. proteamaculans* 568, *P. putida* W619, and *Enterobacter* sp. strain 638. Root formation was very slow for noninoculated plants. In contrast, for cuttings that were allowed to root in the presence of the selected endophytes, root formation was initiated within 1 week, and shoot formation was more pronounced compared to that of the noninoculated plants (Fig. 3A). After 10 weeks, root formation for the noninoculated controls was still poor; however, for plants inoculated with *S. proteamaculans* 568, roots and shoots were well developed (Fig. 3B). Similar effects on root and shoot development were repeatedly noticed for plants inoculated with *P. putida* W619 and *Enterobacter* sp. strain 638 (see Fig. S-1A and B in the supplemental material).

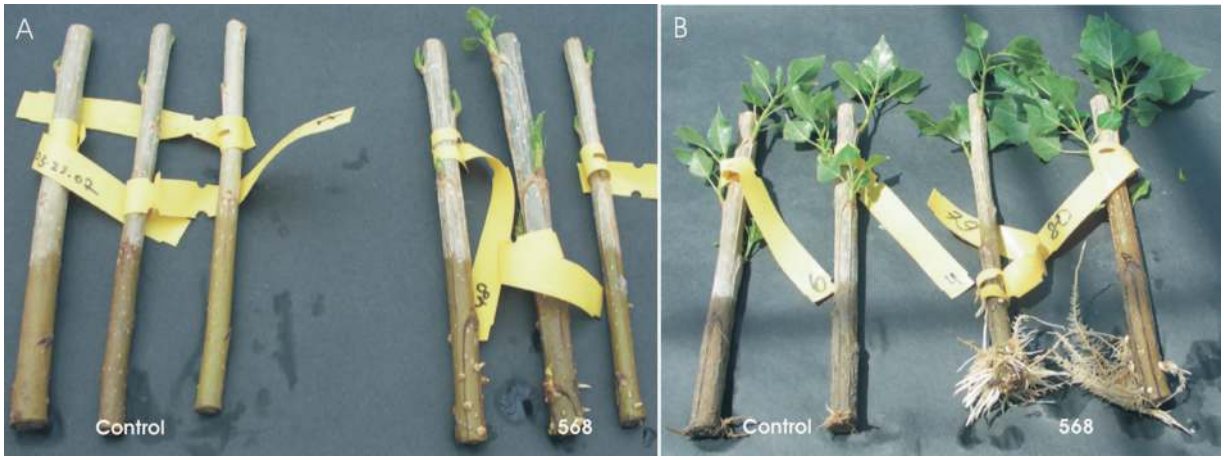

FIG. 3. Effects of *S. proteamaculans* 568 on the rooting and shoot formation of poplar DN-34. Plants were incubated hydroponically in half-strength Hoagland's solution in the absence (control) or presence (568) of strain 568. Root and shoot development are presented after 1 week (A) and 10 weeks (B).

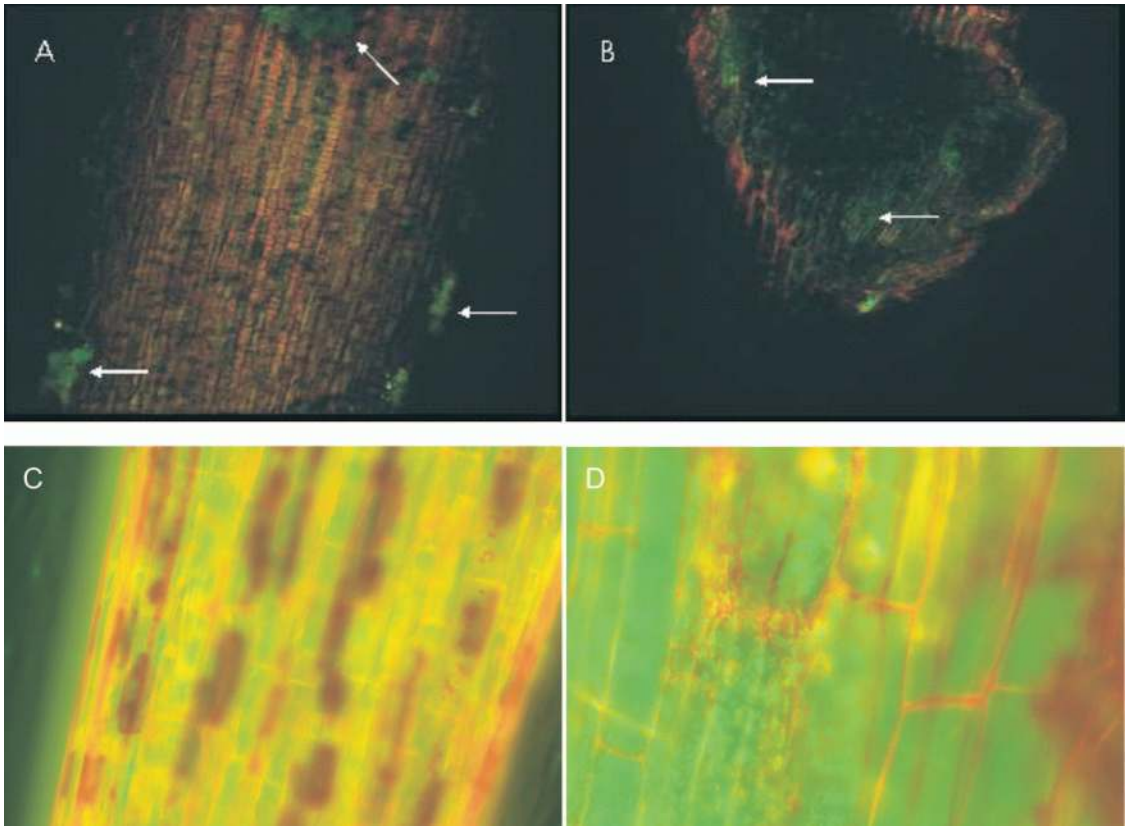

FIG. 4. Endophytic colonization of the poplar DN-34 roots by *gfp*-labeled derivatives of *S. proteamaculans* 568, *Enterobacter* sp. strain 638, and *P. putida* W619. (A) Colonization of the surface of a poplar root by a *gfp*-labeled derivative of *P. putida* W619. The picture was taken by fluorescence microscopy. Arrows indicate the positions of microcolonies on the root surface. (B) Interior view of a translateral section of a poplar root colonized by a *gfp*-labeled derivative of *P. putida* W619. The picture was taken with the help of the apotome feature of the fluorescence microscope. Arrows indicate the zones of dense interior colonization. (C and D) Interior views of a section of a poplar root colonized by a *gfp*-labeled derivative of *S. proteamaculans* 568 (C) and *Enterobacter* sp. strain 638 (D). The root tissue was stained with 0.05% methyl violet to decrease autofluorescence.

Fluorescence microscopy was used to visualize the internal colonization of the plant roots by the *gfp*-labeled strains, confirming their endophytic behavior (Fig. 4). For *P. putida* W619, the formation of microcolonies on the root surface was observed in addition to internal colonization (Fig. 4A and B). Interestingly, these colonies were absent on the root surfaces of plants inoculated with *S. proteamaculans* 568 and *Enterobacter* sp. strain 638, where only internal colonization was observed (Fig. 4C and D). No *gfp* expression was detected for roots from noninoculated control plants (results not shown).

Bacterial properties hypothesized to be important for colonization and plant growth promotion. In order to better understand their plant growth-promoting properties, we screened endophytic bacteria for properties related to phytohormone production and the metabolism of plant growth-regulating compounds, as well as the utilization of different carbon sources, including plant biomass-derived sugars and plant metabolites. These compounds included IAA production from tryptophan and the metabolism of phenylacetic acid, 4-aminobutyrate (GABA), and ACC, which plants produce as a precursor of stress ethylene. *Burkholderia vietnamiensis* Bu61, a derivative of the environmental strain *B. vietnamiensis* G4 that constitutively expresses toluene and trichloroethylene degradation (33, 34), was included for comparison with *B. cepacia* Bu72. The results are presented in Table 1.

None of the endophytes tested was able to grow autotrophically or able to fix nitrogen. The *Burkholderia* strains were able to grow in ACC as the sole nitrogen source. These strains, as well as *S. proteamaculans* 568 and *P. putida* W619, were also able to metabolize phenylacetic acid and GABA. GABA could also support the growth of *M. populi* BJ001. All strains produced IAA, as was determined using the method of Salkowski (17), with the highest levels observed for *P. putida* W619 and *M. populi* BJ001. Some interesting differences were observed when comparing the *Burkholderia* strains: in contrast to the environmental strain *B. vietnamiensis* Bu61, *B. cepacia* Bu72 was able to utilize arbutin, salicin, pectin, trehalose, and cellobiose, compounds typically found in poplar and willow trees. Also, the other endophytic strains tested seem well adapted to utilize a broad spectrum of plant-derived compounds as carbon sources. Only *Enterobacter* sp. strain 638 and *S. proteamaculans* 568 were able to grow on lactose as the sole carbon source, which is consistent with the presence of a *lacZ* gene on their genomes.

TABLE 1. Screening of endophytic bacteria and related strains for their metabolic properties and functions that affect plant hormone balances and growth[a]

Metabolic property or function[b]	OD_{660} of:						
	568	638	Bu61	Bu72	W619	R551-3	BJ001
Autotrophy	−	−	−	−	−	−	−
Nitrogen fixation	−	−	−	−	−	−	−
D-Mannitol	+++	++	++	+++	+++	++	+++
Lactose	+	+++	−	−	−	−	−
Sucrose	++	+	++	++	+++	+	++
Arbutin	+++[c]	++	−[c]	++	+++[c]	+	++
Salicin	++	+	−	+++	++	++	++
Pectin	−	−	−	+/−	−	−	−
Trehalose	+++	++	−	+++	++	−	++
D-Mannose	++	+	+++	++	++	++	++
L-Arabinose	++	++	+++	+++	++	−	++
Xylose	−	++	+++	++	++	+	++
Maltose	+	++	−	−	++	+/−	++
Cellobiose	++	++	−	++	++	−	++
Chitin	+	+/−	++	++	++	++	++
GABA	++	−	+++	+++	++[d]	−	++
PAA	++	−	+++	+++	++[e]	−	−
ACC	−	−	+	+	−	−	−
Glucose	++	+	+++	++	++	+	+
Gluconate	+++	++	+++	++	+++	+++	++

[a] The endophytic bacteria *S. proteamaculans* 568, *Enterobacter* strain 638, *B. cepacia* Bu72, *P. putida* W619, *S. maltophilia* R551-3, and *M. populi* BJ001 and the soil bacterium *B. vietnamiensis* Bu61 were tested for their metabolic properties. Cultures were inoculated at OD_{660} of 0.01. Growth of the cultures was determined by an increase of OD_{660}, which was followed until the cultures had reached stationary growth phase or after 5 days. +++, OD_{660} of >0.8; ++, OD_{660} of 0.5 to 0.8; +, OD_{660} of 0.25 to 0.5; +/−, OD_{660} of 0.1 to 0.25; −, OD_{660} of <0.1. Experiments were carried out as independent triplicates.

[b] Autotrophy or nitrogen fixation was tested by growing the strains on minimal growth medium that lacked a carbon or nitrogen source, respectively. ACC deaminase activity (ACC) was determined by testing the growth on 1-aminocyclopropane-1-carboxylic acid as either a carbon or nitrogen source. GABA and PAA metabolism were determined by testing bacterial growth on these compounds as either a carbon or nitrogen source. The IAA concentrations produced by strains 568, 638, Bu61, Bu72, W619, R551-3, and BJ001 were 3.23, 3.90, 2.57, 2.38, 29.39, 2.98, and 9.38 μg/ml, respectively. These concentrations were determined using the method of Salkowski (17) and calculated for a culture OD_{660} of 1.0.

[c] Brown color formation in the medium.

[d] Orange color formation in the medium.

[e] Yellow color formation in the medium.

Preliminary analysis of the genome sequences of endophytic bacteria for plant growth-promoting functions. Genome sequencing of *Enterobacter* sp. strain 638, *S. maltophilia* R551-3, *P. putida* W619, and *S. proteamaculans* 568 was exploited for properties related to phytohormone production and the metabolism of plant sugars and growth-regulating compounds. A schematic overview of the pathways required for the synthesis of the plant growth-promoting compounds and their distribution among these endophytes is presented in Fig. 5.

Acetoin and 2,3-butanediol synthesis. It was shown that a blend of volatile compounds, especially 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol, emitted by rhizobacteria can enhance plant growth (29, 30). Acetolactate synthase (AlsS) and acetolactate decarboxylase (AlsD) catalyze the two-step conversion from pyruvate to acetoin, which can be converted into 2,3-butanediol, either by the bacteria or by the host plant. The *alsDS* acetoin synthesis pathway was present in *S. proteamaculans* 568 and *Enterobacter* sp. strain 638, which also can convert acetoin into diacetyl, a compound whose role in plant growth promotion is unknown. The acetolactate decarboxylase gene *alsD* was lacking in *S. maltophilia* R551-3 and *P. putida* W619.

IAA synthesis. Endophytic bacteria also enhance plant growth through the synthesis of the plant auxin IAA, whose production was the most pronounced for *P. putida* W619. IAA synthesis from tryptophan can occur via three alternative pathways (Fig. 5), two of which were present in strain W619. The first pathway involves a tryptophan-2-monooxygenase (IaaM) that oxidizes tryptophan to indole-3-acetamide and an indoleacetamide hydrolase (IaaH) that produces IAA. Genome analysis revealed that although a putative *iaaH* gene was present in all strains except *Enterobacter* sp. strain 638, the putative tryptophan 2-monooxygenase could only be found in *P. putida* W619 and *B. vietnamiensis* Bu61. *P. putida* W619 further has the complete pathway to synthesize IAA via tryptamine and indole-3-acetaldehyde and lacks the tryptophan 2,3-dioxygenase (KynA), thus driving the conversion of excess tryptophan to IAA synthesis instead of tryptophan metabolism. Consistent with this high level of IAA production is the presence of four genes encoding putative auxin carriers on the W619 genome.

ACC metabolism. Putative ACC deaminase genes were found in *P. putida* W619, *Enterobacter* sp. strain 638, and *S. proteamaculans* 568. However, none of them coded for a protein that possessed the conserved amino acid signature characteristic for a genuine ACC deaminase (16) (see Fig. S-2 in the supplemental material). The lack of ACC deaminase activity was confirmed by the inability of these strains to grow on ACC as their sole nitrogen source (Table 1). Consistent with its growth on ACC, *B. vietnamiensis* Bu61 coded for a putative ACC deaminase with the correct signature.

γ-Aminobutyrate and phenylacetic acid metabolism. *S. proteamaculans* 568 and *P. putida* W619 metabolized GABA and contained the complete pathway for GABA uptake and degradation. From the strains that failed to grow on GABA, *S. maltophilia*

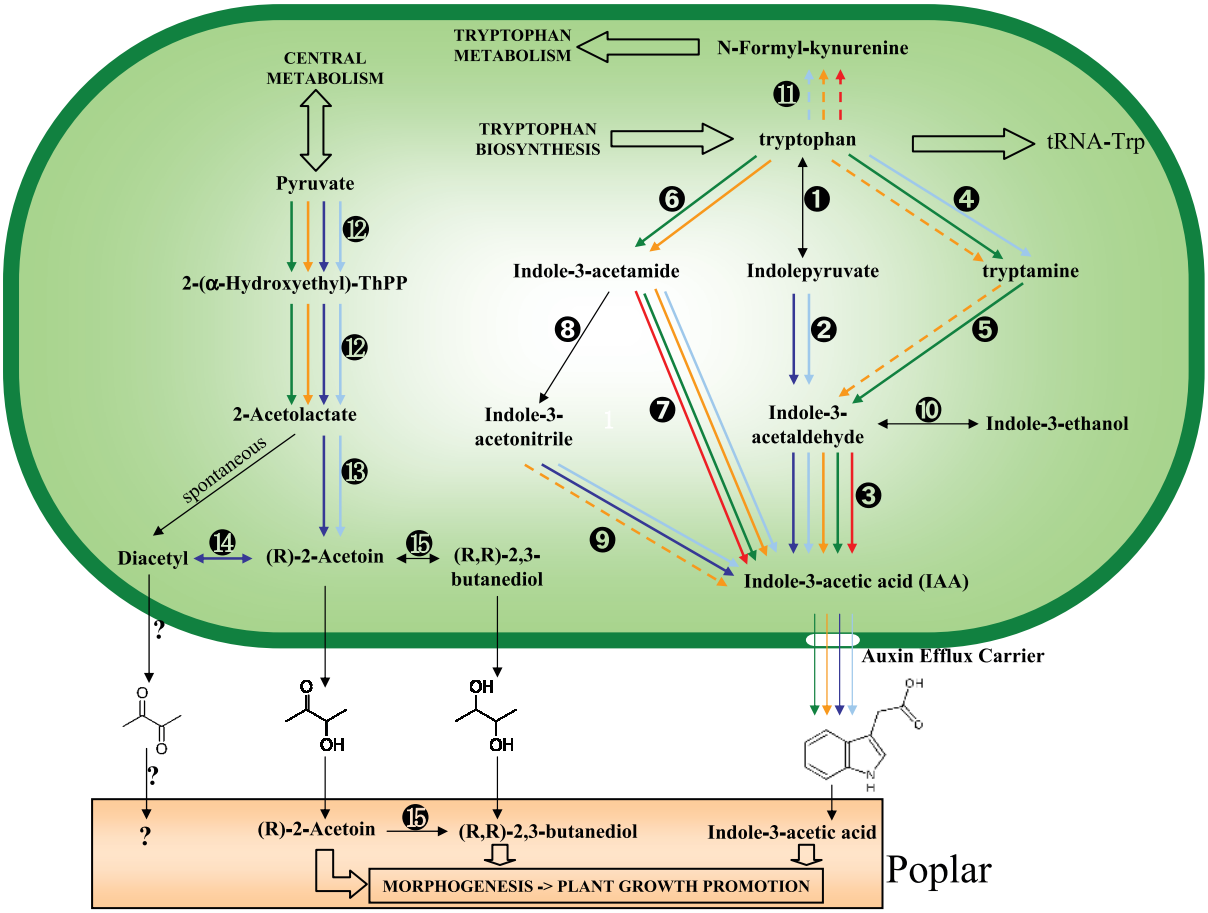

FIG. 5. Metabolic pathways involved in the production of plant growth hormones (IAA, diacetyl, acetoin, and 2,3-butanediol) found on the genomes of selected endophytic bacteria. The metabolic pathways were constructed using PRIAM predictions mapped on the KEGG database (http://www.genome.ad.jp). For each organism, differently colored arrows are used to indicate the presence of the putative pathways: *S. maltophilia* R551-3 (red), *P. putida* W619 (green), *B. vietnamiensis* G4 (orange), *Enterobacter* sp. strain 638 (dark blue), and *S. proteamaculans* 568 (light blue). Black arrows indicate known pathway steps that could not be identified. Dashed arrows correspond to the presence of putative enzymes (PRIAM E value below 10^{-30}). In the tryptophan-dependent IAA synthesis, the enzymes involved are tryptophan transaminase Lao1 (1), indolepyruvate decarboxylase IpdC (2), indole-3-acetaldehyde dehydrogenase DhaS (3), tryptophan decarboxylase Dcd1 (4), amine oxidase (5), tryptophan 2-monooxygenase IaaM (6), deaminase IaaH (7), nitrile hydratase (8), nitrilase YhcX (9), and indole-3-acetaldehyde reductase AdhC (10). Besides the production of IAA, the main pathway for tryptophan metabolism is via tryptophan 2,3-dioxygenase KynA (11). In butanoate metabolism, the production of acetoin from pyruvate is catalyzed by the acetolactate synthase AlsS (12) and the acetolactate decarboxylase AlsD (13). The genome of *Enterobacter* sp. strain 638 encodes the acetoin dehydrogenase ButA (14) that is able to catalyze the conversion of acetoin into diacetyl. It is unknown if this compound has plant growth stimulatory effects. The 2,3-butanediol dehydrogenase ButB (15) involved in the conversion of acetoin into 2,3-butanediol was not found encoded on the genomes of the endophytic bacteria but is present on the poplar genome.

R551-3 lacked the γ-aminobutyrate transaminase (EC 2.6.1.19), while the γ-aminobutyrate permease (COG1113) was absent in *Enterobacter* sp. strain 638. *S. proteamaculans* 568, *P. putida* W619, and *B. vietnamiensis* Bu61 were also able to grow on phenylacetic acid, which is consistent with the presence of *paa* operons on their genomes.

PTS sugar uptake systems. Genes coding for phosphotransferase (PTS) sugar uptake systems were dominantly present in *Enterobacter* strain 638 and *S. proteamaculans* 568, and their phylogenetically assigned substrate specificity seems to be consistent with their sugar metabolism (Table 1). *Enterobacter* strain 638 and *S. proteamaculans* 568 possessed PTS belonging to the α-glucoside family (for the uptake of glucose, *N*-acetylglucosamine, maltose, glucosamine, and α-glucosides; seven and four copies for strains 638 and 568, respectively), β-glucoside family (for the uptake of sucrose, trehalose, *N*-acetylmuramic acid, and β-glucosides; seven and five copies, respectively), fructose family (for the uptake of fructose, mannitol, mannose, and 2-*O*-α-mannosyl-D-glycerate; two copies in both strains), and lactose family (for the uptake of lactose, cellobiose, and aromatic β-glucosides; six and three copies, respectively). A copy of the glucitol family (for the uptake of glucitol and 2-methyl-D-erythritol) was only found in *S. proteamaculans* 568. Both *P. putida* W619 and *S. maltophilia* R551-3 possessed a single gene coding for a PTS from the fructose family.

DISCUSSION

The cultivable endophytic bacteria from poplar and willow trees share many closely related strains, the majority of which

belong to the *Gammaproteobacteria* (Fig. 1). The dominance of gammaproteobacteria is consistent with previous observations of the endophytic community diversity in poplar trees growing on a benzene-, toluene-, ethylbenzene-, and xylene-contaminated site (28). However, in contrast to previous studies, we also observed a significant number of *Rhodococcus* spp. (12% of the cultivable strains), including *Rhodococcus equi*. As noticed previously (36), we found that the highest number of endophytic bacteria reside in the roots, with their numbers declining in the stems, shoots, and leaves (results not shown).

Endophytic bacteria from poplar trees, representative of the dominantly observed genera *Enterobacter*, *Serratia*, *Stenotrophomonas*, and *Pseudomonas*, were tested for their capacities to improve growth of their poplar host. In addition, they were screened for the production or metabolism of plant growth-promoting compounds, phytohormones, and sugars. A better understanding of their plant growth-promoting capabilities was further obtained by initiating the sequencing of their genomes.

Enterobacter sp. strain 638 had the most-pronounced beneficial effect on the development and growth of poplar cuttings. This result was not only repeatable in our hands with *P. deltoides* × *P. nigra* DN-34 but also with the hybrid poplar clone 0P367 (*Populus deltoides* × *P. nigra*) (significance level, $P < 0.05$) (L. Newman, unpublished results). On the other hand, while no significant plant growth-promoting effect was observed for *P. putida* W619 with *P. deltoides* × *P. nigra* DN-34, strain W619 significantly (significance level, $P < 0.01$) promoted the growth of another hybrid poplar tree [*Populus deltoides* × (*P. trichocarpa* × *P. deltoides*) cv. Grimminge] (N. Weyens, J. Boulet, D. Adriaensen, J.-P. Timmermans, E. Prinsen, S. Van Oevelen, J. D'Haen, K. Smeets, D. van der Lelie, S. Taghavi, and J. Vangronsveld, submitted for publication). Also, the promiscuous plant growth-promoting effect of *B. cepacia* Bu72 on poplar trees (this study and reference 36) and yellow lupine (5) is noticeable. Therefore, before the application of this concept to other poplar cultivars, preliminary studies to confirm the plant growth-promoting effect of the selected endophyte are required.

The plant growth-promoting effect of *Enterobacter* sp. strain 638 might be explained by the presence of the putative *alsDS* pathway for acetoin synthesis (Fig. 5), a potent plant growth-promoting compound (29, 30). As for the rhizosphere bacterium *Bacillus amyloliquefaciens* FZB42 (8), it was unclear which function catalyzes the conversion of acetoin into 2,3-butanediol. We assumed that acetoin (produced and released by *Enterobacter* sp. strain 638 and *S. proteamaculans* 568) can enter the poplar cells, where it can be converted into 2,3-butanediol. *Enterobacter* sp. strain 638 also possesses a putative acetoin reductase for synthesis of diacetyl, whose role in plant growth promotion is unknown.

None of the other traits linked to plant growth regulation were identified in *Enterobacter* sp. strain 638: the strain produces low levels of IAA, is unable to fix nitrogen, and lacks the pathways to metabolize ACC, GABA, and phosphonoacetic acid (PAA). Sequence analysis, however, revealed that *Enterobacter* sp. strain 638 contains a 157.7-kb plasmid, pENT638-1, which is related to F plasmids found in other *Enterobacteriaceae*. Plasmids of this family are involved in host interaction and virulence, such as the pFra plasmid of the plague microbe *Yersinia pestis* (18). In pENT638-1, the pFra pathogenicity island has been replaced by a 23-kb putative genomic island (flanked by an integrase gene and having a GC content that is significantly different than that of the rest of the plasmid). This island contains a group of open reading frames with strong homology to hypothetical proteins of *Azotobacter vinelandii* AvOP, as well as to a putative *srfABC* operon, which is also present in a horizontally acquired region of *Salmonella* spp. and which is believed to be involved in virulence (46). Adjacent to this region, a putative *ndvB* (8,532-bp) gene was located. *ndvB*, which codes for a protein involved in the production of β-(1→2)-glucan, is required by *Sinorhizobium meliloti* for bacterial invasion of the nodule (10). Many other genes involved in plant invasion were present on pENT638-1—genes coding for proteins with an autotransporter domain (type V secretion) or virulence domains (agglutinin, pertactin, or adhesin). In addition, plasmid pENT628-1 carries many *relBE* toxin/antitoxin systems, often located in the proximity of regions that presumably play a role in the successful interaction between *Enterobacter* sp. strain 638 and its host.

S. proteamaculans 568 is interesting, as it is, in contrast to *Enterobacter* sp. strain 638, able to metabolize GABA and PAA, two compounds involved in regulating plant responses to stress. *S. proteamaculans* 568 is, like *Enterobacter* sp. strain 638, able to produce 2-acetoin but lacks the acetoin reductase for the bidirectional conversion of acetoin and diacetyl. Furthermore, it lacks the putative plant invasion functions found on plasmid pENT628-1. A further comparison between both *Enterobacteriaceae* strains should provide a better understanding of the observed differences in their plant growth-stimulating potentials.

In the *Enterobacteriaceae*, sugar uptake dominantly occurs via PTS systems, and both *S. proteamaculans* 568 and *Enterobacter* strain 638 contain PTS systems that are consistent with their sugar metabolism (Table 1). *S. proteamaculans* 568 also contains a copy of PTS from the glucitol family. The high number of PTS genes found in the *Enterobacteriales* compared to those found in the *Pseudomonadales* and the *Xanthomonadales* is well known (4). Consistent with this observation is that both *P. putida* W619 and *S. maltophilia* R551-3 possessed a single gene coding for a PTS from the fructose family. This PTS family is most prevalent in proteobacteria and is believed to have evolved into other PTS systems (4).

P. putida W619 seems to be well adapted to influence the phytohormone balances of its host: the strain appears to produce high levels of IAA and is able to metabolize PAA and GABA. Elevated levels of GABA and PAA, a nonindolic auxin that can account for up to one-half of the total bioassayable auxin activity in plant extracts (45), can inhibit plant growth. The complexity of the phytohormone balance points toward the existence of a complex mechanism that fine-tunes the interactions between *P. putida* W619 and other endophytes and their poplar hosts. For instance, the negative effects on poplar development observed after inoculation with *M. populi* BJ001 might reflect a disturbance of this balance, e.g., caused by unnaturally high numbers of this bacterium during inoculation.

Many endophytic bacteria, such as *S. maltophilia* R551-3, are closely related to pathogenic microorganisms whose genomes have been or are in the process of being sequenced. Future genome annotation and comparative genomics of endophytic bacteria and phylogenetically closely related, nonendophytic

microorganisms should result in the identification of the subset of genes necessary for a successful endophytic colonization of poplar trees. Understanding the interactions between endophytic bacteria and their host plants, facilitated by the published genome sequence of *Populus trichocarpa* (39), should ultimately result in the design of strategies for improved poplar biomass production as a feedstock for biofuels and bioremediation.

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