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Identification of an endophytic *Rhizobium* in stems of *Populus*

Sharon L. Doty[1*], Megan R. Dosher[1], Glenda L. Singleton[1], Allison L. Moore[1], Benoit Van Aken[2], Reinhard F. Stettler[1], Stuart E. Strand[1], and Milton P. Gordon[3]

[1]College of Forest Resources, Box 352100, University of Washington, Seattle, WA 98195,
Email. sldoty@u.washington.edu;
[2]Department of Civil and Environmental Engineering, University of Iowa, Iowa City, IA 52242;
[3]Department of Biochemistry, Box 357350, University of Washington, Seattle, WA 98195, USA

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Abstract
We have discovered a *Rhizobium* species that lives within the stems of poplar (cottonwood; *Populus*). Poplar trees are deciduous, non-leguminous trees that are able to grow in nutrient-poor soil or gravel alongside rivers. Sequencing of 16S and 23S ribosomal RNA genes of the poplar endophyte revealed that the bacterium is *Rhizobium tropici*, with 99% identity to the type strain and 100% of the species-specific bases. This diazotrophic (nitrogen-fixing) species is known for its ability to nodulate an exceptionally wide range of legumes; however, its endophytic nature in non-legumes has not been described. In this paper, we describe the association of this bacterium with a variety of *Populus trichocarpa* × *P. deltoides* hybrids from both our laboratory and outdoors, and compare this unique isolate to the type strain of *Rhizobium tropici*, CIAT899.

Keywords: Cottonwood, diazotroph, *Rhizobium*, poplar

This paper is dedicated to the memory of poplar tree researcher, Professor Paul E. Heilman, an inspirational supporter of this project, who died on October 18, 2001.

1. Introduction

Nitrogen is an essential macronutrient for plant growth. Although the atmosphere is nearly 80% dinitrogen gas, only some microorganisms are able to fix (reduce) the dinitrogen into usable ammonia and nitrate. Plants can obtain nitrogen through decomposing organic material, from chemical fertilization, or through symbiotic relationships with nitrogen-fixing (diazotrophic) micro-organisms.

There are four major classes of symbiotic relationships between diazotrophs and plants (Crawford et al., 2000). One is the specific *Rhizobium*/legume interaction which involves the gram negative *Rhizobia* (*Rhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Sinorhizobium*) in root nodules of specific plant partners. The second class is the gram positive actinomycete, *Frankia*, in the root nodules of alder trees. The third class of diazotroph/plant relationships involves cyanobacteria with ferns, liverworts, cycads, and some dicots. A fourth class that has recently gained attention is the relationship between some non-leguminous plants and nitrogen-fixing endophytic or plant-associated bacteria (Galun, 2003).

A deviation from the first class is the interaction of *Rhizobia* with non-leguminous plants. *Rhizobium* was shown to attach to maize, wheat, rice, oat, and asparagus, although no nodulation or nitrogen fixation was evident (Noel et al., 1996). Noel and co-workers demonstrated that *R. leguminosarum* could promote growth of the non-legumes, lettuce and canola, even in the absence of the symbiotic plasmid. *Rhizobia* were also shown to promote the growth of sunflower, a non-legume, by improving water stress tolerance and increasing nitrogen uptake (Alami et al., 2000).

A plant growth-promoting substance, termed lumichrome, was isolated from rhizobia growing with alfalfa (Phillips et al., 1999). There are multiple growth promoting effects of *R. leguminosarum* bv *trifolii* on rice including increased root and shoot growth, increased grain yield, increased efficiency of fertilizer N use, and more efficient uptake of soil nutrients (Yanni et al., 2001). Thus, *Rhizobia* can increase the growth of non-leguminous plants in ways other than providing fixed nitrogen.

Besides *Rhizobia*, there are other nitrogen-fixing bacteria

*The author to whom correspondence should be sent.

associated with roots of non-legumes. These bacteria, loosely grouped together as plant growth promoting rhizobacteria (PGPR), can fix nitrogen and produce plant hormones in the rhizosphere of a variety of non-leguminous plants such as rice (Mehnaz et al., 2001), wheat (Hassan et al., 1998), sugarcane (James et al., 1994), palm trees (Reis et al., 2000) and maize (De Salomone et al., 1996).

These PGPR include *Azospirillum*, *Enterobacter* and *Acetobacter*. Many of these PGPR have plant protective effects as well (Selosse et al., 2004). For example, by secreting exopolysaccharides, some can alter the soil structure, leading to protection of the plant from water stress and temperature variations (Selosse et al., 2004).

Some diazotrophs live within the plant stem, such as the endophyte, *Gluconacetobacter diazotrophicus* (formerly *Acetobacter diazotrophicus*), of sugarcane. *Nif* (nitrogen fixing) mutants of this organism had a reduced growth promoting effect on sugarcane, suggesting that fixed nitrogen may be transferred to the plant under normal symbiotic conditions (Sevilla et al., 2001). This bacterium appears to be capable of secreting nearly half of its fixed nitrogen in a form that the plant can utilize (Cohjo et al., 1993).

The ability of *G. diazotrophicus* to fix nitrogen in the aerobic environment of the stem is attributed to "respiratory protection" whereby the extremely rapid respiration from metabolism of high levels of sucrose within the sugarcane stem leads to a microaerobic environment needed for the oxygen-sensitive nitrogenase enzyme (Flores-Encarnacion et al., 1999).

In the last ten years, there have been a number of reports on the isolation of endophytic fungi and bacteria that seem to have symbiotic relationships with the plants they colonize (Selosse et al., 2004). Some of the bacterial endophytes, including *Azoarcus*, *Herbaspirillum* and *Acetobacter*, are at least suspected of providing fixed nitrogen to the plant hosts (Reinhold-Hurek and Hurek, 1998). However, the endophytic nature of the bacteria makes quantification of nitrogen fixation difficult.

Poplar trees (cottonwoods and aspens) are highly productive deciduous trees with rapid development and are grown worldwide for use as lumber, paper, plywood, and fuel (Heilman, 1999). They have been associated with agriculture since ancient times, and have proved especially effective in windbreaks and in stabilizing banks of streams and canals (Stettler et al., 1996). A common tree of riverine environments, poplars are able to experience frequent flooding and deposition, tolerating weeks of inundation (Braatne et al., 1996; Stettler et al., 1996).

In this paper, we report the discovery of the association of *Rhizobium tropici* within cottonwood plants. Since there are several differences between the poplar endophyte and the type strain of *R. tropici*, we propose to name these new isolates by the subspecies name, *R. tropici* bv *populus*.

2. Materials and Methods

Bacterial strains

We received the type strain of *Rhizobium tropici*, CIAT899, as a gift from Dr. O. Mario Aguilar (La Plata, Argentina). *Agrobacterium tumefaciens* strain C58 was from Dr. E.W. Nester (University of Washington), and *Sinorhizobium meliloti* strain 1021 was from Dr. J. Leigh (University of Washington).

Isolation of the endophyte

Stems of *Populus trichocarpa* × *P. deltoides* clones were surface-sterilized with 20% bleach for 12 minutes, 10% iodophor for 2 minutes, and 70% ethanol for 5 minutes, or with 0.1% mercuric chloride, and rinsed several times with sterile water. The ends of the explants were removed, and the stems were incubated in the light on Murashige and Skoog medium (Murashige and Skoog, 1962) (MS; Caisson Laboratories Inc., Rexburg, Idaho). Resulting bacteria were then subcultured on YEM medium (0.5 g/l yeast extract, 10 g/l mannitol). The first isolate from *Populus trichocarpa* × *P. deltoides* clone H11-11 was termed strain PTD1 (*Populus trichocarpa* × *P. deltoides*).

Characterization of growth

Salt tolerance of the bacterium was assayed on YEM with a sodium chloride concentration range of 0.5% to 4% in 0.5% increments. Growth of strain PTD1 was compared with that of the type strain for *Rhizobium tropici*, CIAT899. A keto-lactose test was performed as described (Bernaerts and De Ley, 1963) to verify that the bacterium was not *Agrobacterium*.

Quantification of bacteria within the stems

Cuttings from cottonwood clones were surface-sterilized with detergent, 10% bleach, and 10% Iodophor. The dead ends of the cuttings were removed. The stems were then sectioned into nodes and internodes, and placed in Eppendorf tubes. After each sample was weighed, it was minced using a sterile scalpel in 1 ml of 1 mM HEPES buffer, pH 7. The minced tissue and buffer were transferred back to the Eppendorf tubes. Samples were diluted in more buffer, and 100 µl were plated on YMA or on YMA with 2.5% NaCl or on MS medium. After 2 days of incubation at 28°C, PTD1-like colony forming units were counted.

Identification of the endophyte

Genomic DNA was prepared using standard methods (Ausubel et al., 1995) with the following modifications. The cell lysis step was performed at 68°C for 30 min. Multiple phenol/chloroform extractions were done due to

the high amount of polysaccharides in the samples. PCR was performed using Epicentre (Madison, Wisconsin) PCR Pre-Mix buffer B and Taq polymerase (Promega; Madison, Wisconsin) in an Eppendorf thermocycler.

The universal 16S primers were 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492 (5'-GGTTACCTTGTTACGACTT-3') that amplified a 1.5 kb fragment. Sequences for these primers were kindly provided by Jen Hebb. The sequence of the 23S primers were derived from *Rhizobium tropici* sequence (forward primer: 5'-CGAAACGCGGACCAGGCCAG-3'; reverse primer: 5'-CTTCGCCTACACCTACCGGC-3') and amplified a 362 bp product. The thermocycler program was 94°C for 15 s, 44°C for 15 s, 72°C for 30 s, and it was repeated for 24 cycles. Initial experiments were done using Taq polymerase and colony suspensions, but later confirmatory experiments utilized Pfu polymerase and purified genomic DNA. The samples were subjected to electrophoresis in a 2% agarose gel. The DNA was purified using an electroeluter (International Biotechnologies, Inc), precipitated in ethanol, and resuspended in sterile water. The PCR products were sequenced by the Seattle Biomedical Research Institute (Seattle, Washington).

PCR amplification of nifH *genes*

DNA was purified from CIAT899, PTD1, and from *S. meliloti*, a gift from Professor J. Leigh (Microbiology Dept., UW). Initial experiments were conducted using the Universal *nifH* primers and PCR conditions described by Franke et al. (1998). *R. tropici* CIAT899 and *S. meliloti* DNA were used as positive controls. The 390 bp PCR product of CIAT899 was subcloned into pGEM T Easy (Promega; Madison, WI) and sequenced. The sequence was only 96% identical to the published *R. tropici nifH* sequence (Accession Number AY079181; not strain CIAT899). PCR primers to amplify the *nifH* gene were then designed based on our CIAT899 sequence: FP 5'-GGCATCGGCAAGTCCACCACCTCAC-3' and RP 5'-CAACCAACACCCGGCTCCGGGCCCCCGGAC-3'. These primers were tested on DNA from CIAT899, *S. meliloti* and PTD1.

Since the universal primers did not seem to amplify nitrogenase sequence from PTD1, we used a more stringent method that utilized two successive PCR with nested primers. PCR primers to amplify the highly conserved region of the nitrogenase subunit H gene (*nifH*) were designed as described (Burgmann et al., 2004). The first set of degenerate primers (*nifH*-universal, for A site and reverse site) – forward primer 5'-GCIWTITAYGGNAARGGNGG-3' and reverse primer 5'-GCRTAIABNGCCATCATYTC-3' – amplified a 464 bp region of the nitrogenase gene (Burgmann et al., 2004). *Rhizobium meliloti* genomic DNA was used as a test template for the Epicentre Fail-Safe PCR buffer series. Several different buffers (A, B, E, F, and G) supported good amplification of the *nifH* product. Epicentre Pre-Mix Buffer A was then selected for the PCR of PTD1 genomic DNA. The thermocycler program was 5 min at 95°C and then 11 s at 94°C, 8 s at 48°C, and 10 s at 72°C for 40 cycles, followed by a polishing step for 10 min at 72°C. The sample was then used in the nested reactions.

The series of nested primers described in Burgmann et al. (2004) amplified a 371 bp subregion of the *nifH* gene. The PCR products were purified from the agarose using the Qiaex II Gel Extraction Kit (Qiagen; Valencia, CA), and ligated into the cloning vector, pGEM T Easy. Sequencing of the inserts in both directions was performed by the UW Biochemistry Sequencing Facility (Seattle, WA) using primers for the T7 and SP6 regions of the vector.

3. Results

Discovery of the poplar endophyte

Stems of the cottonwood hybrid, *Populus trichocarpa* × *P. deltoides* clone H11-11, were collected from our growth room, surface-sterilized, and grown in tissue culture on Murashige and Skoog medium (Murashige and Skoog, 1962). Frequently, a bacterium with prodigious exopolysaccharide grew on the medium surrounding the poplar stems but was not observed on other laboratory plants such as *Leucaena leucocephala*, *Nicotiana tabacum*, *P. tremula* × *P. alba* 717-1B4, and *Robinia pseudoacacia*. Therefore, it was suspected that the bacterium was not a general contaminant, but was specific to the hybrid cottonwoods. Surprisingly, these cottonwood explants remained healthy and grew for months, proliferating despite the heavy load of surrounding bacteria (Fig. 1). This suggested that the bacterium may be symbiotic or neutral rather than pathogenic. Out of numerous plates of poplar explants, the bacterial growth was nearly always of one colony type.

In order to identify the bacterium, a sample of the bacterium was subjected to PCR with primers to amplify segments of the 16S and 23S ribosomal genes. Control PCR reactions in which no colony was added did not result in a PCR product. Sequencing and BLAST searches (Altschul et al., 1997) of the poplar bacterium PCR products revealed a match to the 16S gene of *Rhizobium tropici* (99% identity; 1301/1308 bases) and a match to the 23S gene of *Rhizobium tropici* (99% identity; 322/325 bases). The 16S sequence from the poplar bacterium included the six bases that differentiate *R. tropici* from other rhizobia (George et al., 1994) (Fig. 2). We then tested for the presence of the bacterium using 16S ribosomal gene sequencing of bacteria isolated from three other *P. trichocarpa* × *P. deltoides* clones (184–402, 15–29, and 50–197) from our growth room, and all three matched *R. tropici*.

We also tested *P. deltoides* (Eastern Cottonwood) clone ST66 (provided by John Blake, Savannah River and propagated in our plant growth room) for the presence of the bacterium. Again, surface-sterilization of stems and plating of the explants on MS medium yielded predominantly one colony type. No bacterial growth occurred from the leaves or petioles. The 16S gene was amplified and sequenced, and the results were a 98% match (735/745 bases) with *R. tropici.*

Figure 1. Stem sections from *P. trichocarpa* × *P. deltoides* hybrids were surface-sterilized and plated on MS medium. Bacteria grew out from the stems, covering the whole plate, yet did not have any deleterious effects on the vigor of the emerging shoots.

To determine if the bacterium was limited to poplars in our growth room, branches from clone H11-11 grown at an outdoor site in a nearby city, and from another *P. trichocarpa* × *P. deltoides* hybrid, clone 184–402, from Oregon (Steve Strauss, OSU) were harvested, surface-sterilized, and grown in culture. The same bacterial species (as verified by sequencing of the 16S and 23S rDNA) grew from these independent sources. These independent isolations confirmed the unique relationship between the bacterium and these poplars.

All further experiments were done using the first isolate from *P. trichocarpa* × *P. deltoides* clone H11-11, and termed strain PTD1 (*Populus trichocarpa* × *deltoides*) to distinguish it from the other isolates.

Localization of the endophyte

To determine which parts of the poplar stems harbored the endophyte, we segregated surface-sterilized stems into nodes and internodes. The bacterium was more often isolated from nodes than from internodes, and from middle rather than from the youngest and oldest stem sections. Overall, there was too much variability from plant to plant and also seasonal differences, such that a meaningful bacterial count was not possible. In one experiment comparing upper and lower sections of the plant, there were

CGAGCGCCNC GCAAGGGGAG CGGCAGACCG GTGAGTAACG CGTGGGAATC TACC**T**TTT**G**C
TACGGAATAA CGCAGGGAAA CTT<u>G</u>TGCTAA TACCGTATGT GTCCTTCGGG AGAAAGATTT
ATCGG**CA**A**GA** GATGAGCCCG CGTTGGATTA GCTAGTTGGT GGGGTAAAGG CCTACCAAGG
CGACGATCCA TAGCTGGTCT GAGAGGATGA TCAGCCACAT TGGGACTGAG ACACGGCCCA
AACTCCTACG GGAGGCAGCA GTGGGGAATA TTGGACAATG GGCGCAAGCC TGATCCAGCC
ATGCCGCGTG AGTGATGAAG GCCCTAGGGT TGTAAAGCTC TTTCACCGGA GAAGATAATG
ACGGTATCCG GAGAAGAAGC CCCGGCTAAC TTCGTGCCAG CAGCCGCGGT AATACGAAGG
GGGCTAGCGT TGTTCGGAAT TACTGGGCGT AAAGCGCACG TAGGCGGATC GATCAGTCAG
GGGTGAAATC CCAGGGCTCA ACCCTGGAAC TGCCTTTGAT ACTGTCGATC TGGAGTATGG
AAGAGGTGAG TGGAATTCCG AGT<u>G</u>TAGAGG TGAAATTCGT AGATATTCGG AGGAACACCA
GTGGCGAAGG CGGCTCACTG GTCCATTACT GACGCTGAGG TGCGAAAGCG TGGGGAGCAA
ACAGGATTAG ATACCCTGGT AGTCCACGCC GTAAACGATG AATGTTAGCC GTCGGGCAGT
ATACTGTTCG GTGGCGCA<u>GC</u> TAACGCATTA AACATTCCGC CTGGGGAGTA CGGTCGCAAG
ATTAAAACTC AAAGGAATTG ACGGGGGCCC GCACAAGCGG TGGAGCATGT GGTTTAATTC
GAAGCAACGC GCAGAACCTT ACCAGCCCTT GACATCCTGT GTTACCTCTA GAGATAGGGG
GTCCACTTCG GTGGCGCAGA GACAGGTGCT GCATGGCTGT CGTCAGCTCG TGTCGTGAGA
TGTTGGGTTA AGTCCCGCAA CGAGCGCAAC CCTCGCCCTT AGTTGCCAGC ATTTAGTTGG
GCACTCTAAG GGGACTGCCG GTGATAAGCC GAGAGGAAGG TGGGGATGAC GTCAAGTCCT
CATGGCCCTT ACGGGCTGGG CTACACACGT GCTACAATGG TGGTGACAGT GGGCAGCGAG
CACGCGAGTG TGAGCTAATC TCCAAAAGCC ATCTCAGTTC GGATTGCACT CTGCAACTCG
AGTGCATGAA GTTGGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG AATACGTTCC
CGGGCCTTGT ACACACCGCC CGTCACACCA TGGGAGTTGG TTGGAACCGA AGGTAGTGCG

Figure 2. 16S ribosomal gene sequence of the poplar endophyte. The bases in bold differentiate *R. tropici* from several other *Rhizobium* species. Underlined bases differentiate the poplar bacteria from *R. tropici* (accession #D11344).

ten-fold more colony-forming-units from the lower stem than from the upper stem. In some experiments which compared nodes and internodes, there were colony-forming units from the nodes but none from the internodes of the upper stem.

Antibiotic resistance of PTD1

PTD1 was originally isolated on MS plant growth medium containing 30 g/l sucrose and the antibiotics, cefotaxime at 500 µg/ml, kanamycin at 100 µg/ml, timentin at 250 µg/ml, and Plant Preservative Mixture (Plant Cell Technology). Since it displayed resistance to so many antibiotics, we tested for its resistance to others. PTD1 was also resistant to chloramphenicol at 25 µg/ml, ampicillin at 100 µg/ml and carbenicillin at 100 µg/ml. On this MS medium, PTD1 produced a prodigious exopolysaccharide. To determine if the exopolysaccharide was involved in providing protection from the antibiotics, we grew the strain on several different media to find ones on which the bacterium did not produce the exopolysaccharide (Fig. 3, Table 1).

The exopolysaccharide production seemed to be highest when sugar (sucrose or mannitol) was present and when sodium chloride levels were low. When PTD1 was grown on these media that did not support the production of the exopolysaccharide, it was no longer resistant to cefotaxime or timentin, yet was still resistant to the other antibiotics. Thus, the thick layer may serve a partially protective role for this bacterium.

We compared the antibiotic resistance of PTD1 to that of the type strain of *Rhizobium tropici*, CIAT899, and of *Agrobacterium tumefaciens* strain C58. Like strain PTD1, strain CIAT899 was resistant to cefotaxime, chloramphenicol, carbenicillin and ampicillin; however, unlike strain PTD1, it was sensitive to kanamycin and timentin. C58 was sensitive to all of the antibiotics tested.

Table 1. Exopolysaccharide is produced on media with mannose or sucrose and low salt. Strain PTD1 was streaked from a frozen stock onto a variety of media, and exopolysaccharide production was scored visually.

Media	Sugar	Added NaCl	Exopoly-saccharide
YEM	+	–	+++
MS (sucrose/plant media)	+	+/– (low)	++
LB (Yeast ext/Tryptone/NaCl)	–	+	–
TYC	–	–	–
MGL (LB/mannitol)	+	+	–

Table 2. PTD1 is more salt tolerant than CIAT899. Growth of *R. tropici* strains on YEM containing increasing levels of sodium chloride was scored visually.

Percent NaCl	CIAT899	PTD1
0	++++	++++
2	++	++++
2.5	+	++
3.0	–	+

Salt and acid tolerance

Both CIAT899 and PTD1 exhibited rapid growth (two days on rich medium to form distinct colonies of 1–2 mm) and similar colony morphology. Since *R. tropici* was reported to be salt tolerant (Ulrich and Zaspel, 2000) and acid tolerant (Riccillo et al., 2000), we assayed for these characteristics in PTD1. Growth occurred on yeast extract/mannitol medium containing up to 3% sodium chloride, slightly higher than the range for *R. tropici* (2.5%) (Table 2). PTD1 grew normally up to 1.5% sodium chloride; at higher levels, the colonies were smaller. In comparison, strain CIAT899 did not show any growth at 3% NaCl, and only some growth (but no distinct colonies) at 2.5% NaCl.

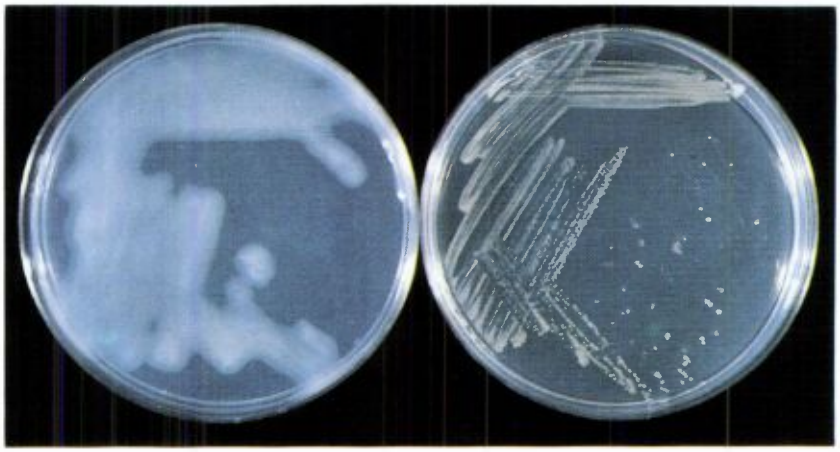

Figure 3. Exopolysaccharide production by the poplar endophyte, PTD1, is growth medium-specific. PTD1 was streaked onto yeast extract mannitol agar (YMA; left plate) or onto tryptone yeast extract/calcium chloride agar (TYC; right plate) and incubated at 28°C for 2 days. The exopolysaccharide was clearly more prominent on the YMA plate than on the TYC plate.

PTD1 grew well on medium at pH 4.5 as reported for *R. tropici*. One of the reported strains of *R. tropici* was heat tolerant (van Rhijn et al., 1993); however, PTD1 grew well at room temperature and 30°C but not at 37°C, so it does not correspond to that strain of *R. tropici*.

Amplification of a fragment of the nitrogenase subunit, nifH

To further compare the poplar endophyte with CIAT899, we amplified a segment of the gene encoding the NifH subunit of nitrogenase, the enzyme responsible for nitrogen fixation. Our first attempts, using the universal *nifH* primers (Franke et al., 1998) with DNA of PTD1 yielded numerous faint PCR products but the sequence of the purified DNA fragments did not match that of the *nifH* gene. These same primers, however, amplified *nifH* from CIAT899 and from *Sinorhizobium meliloti*, as confirmed by sequence analysis. We then designed primers using our derived *nifH* sequence from CIAT899 and used these primers with genomic DNA of PTD1, yet still no amplification resulted except from the CIAT899 control. Therefore, the *nifH* genes of CIAT899 and PTD1 must differ substantially in the regions where these CIAT899-specific primers and the universal *nifH* primers bind.

We then switched to using the nested PCR technique described by Burgmann et al. (Burgmann et al., 2004) for the amplification of a wider variety of nitrogenase sequences from the environment. This technique allows an additional level of specificity by utilizing two different sets of primers, one set internal to the other. When we used this nested PCR technique, we obtained numerous faint bands from the first set of PCR primers with PTD1 DNA but clear PCR product of the correct size (approximately 371 bp) after the different nested reactions. We subcloned these products into pGEM T Easy and sequenced the inserts. The sequence analysis from the PTD1 PCR products revealed a close identity between to the reported *nifH* sequence of *Rhizobium tropici* (98%; 158/161 bases; accession AY079181). The full experiment was repeated on an independent DNA preparation of PTD1 using the first universal *nifH* primer set followed by the B site primer set for the nested reaction. The 371 bp PCR product was purified, subcloned, and sequenced with the same results as before, confirming the validity of the PCR. We directly compared the PTD1 *nifH* sequence with our sequence of CIAT899 *nifH* and found significant identity (99%; 350 bases). A comparison of the two *nifH* sequences is shown in Fig. 4.

4. Discussion

We have described the isolation of a bacterium with fast growth and prodigious exopolysaccharide production that was present in the stems of several different cottonwoods from different sources. The 16S and 23S ribosomal DNA sequences of the endophyte closely matched that of

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PTD1      GATCCAAAAG CGGACTCCAC CCGCCTGATC TTGAACTCGA AGGCGCAGGA
CIAT899   GATCCCAAAG CCGACTCCAC CCGCCTGATC TTGAACTCGA AGGCGCAGGA

PTD1      CACCGTGCTG CATCTAGCGG CACAGGAAGG TTCGGTGGAA GACCTCGAAC
CIAT899   CACCGTGCTG CATCTAGCGG CACAGGAAGG TTCGGTGGAA GACCTCGAAC

PTD1      TCGAGGACGT GCTCAAAATC GGCTATAAGG GCATCAAGTG CGTAGAGTCC
CIAT899   TCGAGGACGT GCTCAAAATC GGCTATAAGG GCATCAAGTG CGTAGAGTCC

PTD1      GGGGGCCCGG AGCCGGGTGT TGGTTGCGCC GGCCGCGGCG TCATCACCTC
CIAT899   GGGGGCCCGG AGCCGGGTGT TGGTTGCGCC GGCCGCGGCG TCATCACCTC

PTD1      CATCAACTTC CTTGAGGAAA ACGGCGCCTA CGACAATGTC GACTATGTCT
CIAT899   CATCAACTTC CTTGAGGAAA ACGGCGCCTA CGACAATGTC GACTATGTCT

PTD1      CCTATGACGT GCTCGGCGAC GTCGTGTGCG GCGGCTTCGC AATGCCGATC
CIAT899   CCTATGACGT GCTCGGCGAC GTCGTGTGCG GCGGCTTCGC AATGCCGATC

PTD1      CGCGAGAACA AGGCCCAGGA AATCTACATC GTCATGTCCG GCGAGATGAT
CIAT899   CGCGAGAACA AGGCCCAGGA AATCTACATC GTCATGTCCG GCGAAATGAT

PTD1      GGCG
CIAT899   GGCT
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Figure 4. Comparison of partial *nifH* genes for nitrogenase reductase from *R. tropici* bv *populus* strain PTD1 (this work) and *R. tropici* strain CIAT899 (this work). Differences between PTD1 and CIAT899 are in bold type and underlined.

Rhizobium tropici. Additionally, both bacteria possess similar phenotypic characteristics such as acid and salt tolerance, fast growth, high production of exopolysaccharide, and resistance to antibiotics. Because of these similarities, we propose that they are of the same species. However, due to the differences in resistance to some antibiotics, differences in maximum salt tolerance levels, and different nitrogenase sequences, we propose that PTD1 belongs to a different subspecies. Therefore, we named our original isolate, strain *R. tropici* bv. *populus* strain PTD1 for *Populus trichocarpa* × *deltoides* isolate one.

Phylogenetic trees based on *nifH* sequences or 16S ribosomal sequences usually agree (Franke et al., 1998). *R. tropici* CIAT899 has a single copy of the *nifH* gene (Martinez et al., 1985), and the pSym plasmid was shown to be sufficient to transfer the ability to promote an effective symbiotic process to *Agrobacterium* transconjugants (Martinez et al., 1987; van Rhijn et al., 1993), demonstrating that all the nitrogen fixation and nodulation genes are on this pSym plasmid. When primers that amplified the *nifH* gene of CIAT899 failed to amplify the *nifH* gene of PTD1, we also attempted to amplify another pSym-encoded gene, *nodD*, that is involved in regulation of nodulation. Primers that amplified *nodD* of *R. tropici* failed to amplify any product from PTD1. The sequence of the internal portion of the *nifH* gene of PTD1 did closely match (99%) that of CIAT899.

Perhaps the divergence in sequence is near the regions of the first set of universal *nifH* primers rather than in the internal region that was cloned and sequenced. It appears that the poplar isolate has the chromosome of *R. tropici* (based upon the results of the 16S and 23S sequencing) but perhaps a different symbiotic plasmid. Further study of other pSym-localized genes is needed to determine if PTD1 received the plasmid by horizontal transfer from another *Rhizobium* species.

The host range of *R. tropici* (previously termed *R. leguminosarum* bv *phaseoli* type II; also known as *Rhizobium* genospecies Q) is unusually broad. This species can nodulate plants across 6 families and 18 legume genera (Perret et al., 2000) including the common bean, *Phaseolis vulgaris* (Martinez-Romero et al., 1991), *Leucaena leucocephala* (a tropical tree) (Martinez-Romero et al., 1991), *Macroptilium* (siratro) (Vargas et al., 1990), alfalfa (van Berkum et al., 1998), several *Acacia* tree species (Khbaya et al., 1998; Lafay and Burdon, 1998; Lafay and Burdon, 2001), soybeans introduced to Paraguay (Chen et al., 2000), *Robinia pseudoacacia* (black locust tree) in Europe (Ulrich and Zaspel, 2000), and *Medicago ruthenica* (a forage crop) in Inner Mongolia (van Berkum et al., 1998).

It is interesting to speculate that a *Rhizobium* species with such an unusually broad host range may have also adapted to be an endophyte of a variety of non-legumes. A study of the endophytic host range of this bacterium is in order.

The bacteria within poplar stems were more often isolated from areas with a growing bud than in long internodal regions. The morphology and physiology in these two locations would be quite different, and as yet, we have no decisive answer as to why the bacteria would colonize one but not the other. It may be that, since the nodes are more metabolically active than the longer internodes, they may be more of a carbon sink and thus have more sucrose for the bacteria to grow on. The "sink versus source" concept may also help to explain why the bacterium was found in higher numbers in the nodes of the middle of the plant as opposed to the youngest and oldest regions, or the reason may be that the bacterium is in the process of moving upward in the plant. We are currently making a GFP-labeled version of the bacteria so that we can track its entry and migration through the plant and determine its final distribution pattern.

Cottonwoods establish naturally in nutrient poor areas where other tree species are absent. These hardy trees colonize gravelly and sandy areas with no clear source of organic material for obtaining fixed nitrogen (Stettler et al., 1996). It therefore seems reasonable that such a colonizing tree adapted to nutrient-poor riparian environments with flooding and deposition regimes would harbor nitrogen-fixing bacteria within its stem. We have successfully grown poplar cuttings in the absence of nitrate and ammonium for months without signs of nitrogen deprivation (Doty, unpublished), and we are currently attempting to quantify any nitrogen fixation that may be occurring within the plants and to determine which microorganisms may be responsible.

After more than 40 years of research on diazotrophic bacteria associated with grasses and crops, there have been few conclusive reports of fixed nitrogen transfer that do not involve the classic legume-root nodule interaction (Giller and Merckx, 2003). Methods developed to study nitrogen fixation in root nodules do not work well with endophytic bacteria (Kahn et al., 2002). When legumes are exposed to $^{15}N_2$ gas, for example, the presence of ^{15}N label in plant tissues that do not harbor the bacteria (ie. leaves) offers good evidence that there was fixed nitrogen transfer from the bacteria (in the root nodules) to the rest of the plant. In the endophytic association, however, there is not this clear differentiation of tissues with and without the bacteria (Kahn et al., 2002). Although bacterial nitrogen fixation has been quantified when associated with non-legumes, it has been difficult to prove that a substantial amount of fixed nitrogen is actually being incorporated by the plant (Giller and Merckx, 2003; Kahn et al., 2002).

However, there is a great deal of circumstantial evidence that fixed nitrogen may be transferred to non-legumes. For example, there is a report of a variety of endophytic bacteria in African sweet potato that have nitrogenase genes (Reiter et al., 2003). Sweet potato is known for its ability to grow in nitrogen-poor soils. A high number of diazotrophs have been isolated from tropical plants such as

banana, pineapple, coffee, and sugarcane (Cruz et al., 2001; Jimenez-Salgado et al., 1997; Reiter et al., 2003; Sevilla et al., 2001). A clearly documented case of nitrogen transfer to a non-legume from diazotrophic bacteria is that of *Gluconacetobacter diazotrophicus* of sugarcane (Sevilla et al., 2001). It was demonstrated that significant amounts of fixed nitrogen were incorporated in sugarcane and that this effect did not occur when the plants were inoculated with *nif-* mutant bacteria. The availability of new techniques for the analysis of diazotrophic endophytic bacteria will hopefully begin a new understanding of the contribution of these bacteria to plants.

From studying the endophytic bacteria in native *P. trichocarpa* in a riparian environment (Doty, unpublished), it seems clear that there is a wide diversity of microorganisms within poplar, and likely in other woody plants. Recently another endophytic bacterium of poplar was isolated, *Methylobacterium populi* sp. nov., a *P. deltoides* × *nigra* DN34 isolate (Van Aken et al., 2004a). This pink-pigmented, facultatively methylotrophic bacterium has the unusual ability to utilize methane as the sole source of carbon and energy (Van Aken et al., 2004a). It is also able to degrade nitro-substituted explosives, an ability that may, when combined with its host poplar trees, help in the remediation of contaminated sites (Van Aken et al., 2004b). A recent report by Ganley et al. (2004) demonstrated that there is a substantial biodiversity of fungal endophytes within western white pine trees (*Pinus monticola*). These endophytes may play a protective role in non-host resistance (Ganley et al., 2004). Clearly, a broader study of the endophytic microorganisms in trees is needed.

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