

A diazotrophic, indole-3-acetic acid-producing endophyte from wild cottonwood

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Abstract An endophytic bacterium, wild poplar strain B (WPB), isolated from stems of wild cottonwood (*Populus trichocarpa*) was identified to *Burkholderia vietnamiensis* by analyzing the *recA* and rDNA genes. Phylogenetic analysis of the *nifHDK* cluster indicates that the WPB isolate shares high sequence similarity with known *B. vietnamiensis* strains. The nitrogenase activity of WPB was determined by a $^{15}\text{N}_2$ incorporation assay and an acetylene reduction assay. WPB was also monitored for production of indole-3-acetic acid (IAA), a phytohormone which can promote plant growth, when incubated with L-tryptophan. In addition, its plant growth promotion capacity was assessed by inoculating the WPB strain onto Kentucky bluegrass in nitrogen-free medium. Compared to uninoculated control plants, the plants inoculated with WPB gained more dry weight (42%, $p=0.01$) and more nitrogen content (37%, $p=0.04$) in 50 days.

Keywords Diazotrophic endophyte · Poplar trees · Plant growth promotion · *Burkholderia vietnamiensis* · IAA · Phylogenetic analysis · Turf grass · Nitrogen fixation · *Populus* · Auxin · Endophytes

Introduction

Dozens of endophyte isolates from wild black cottonwood (*Populus trichocarpa*) growing in its native habitat in Western Washington have been characterized (Doty et al. 2009). One of the isolates from the wild cottonwood, named WPB (wild poplar strain B), was closely related to the *Burkholderia cepacia* complex (Bcc) based on its 16S rDNA gene sequence. This isolate expressed highest nitrogenase activity in the acetylene reduction assay among all the isolates (Doty et al. 2009). More than thirty different *Burkholderia* species have been described (Coenye and Vandamme 2003) and the number of newly identified *Burkholderia* species has increased continuously (Perin et al. 2006). The *B. cepacia* complex (Bcc) is a group of genetically distinct, phenotypically similar bacteria including at least nine species (Chiarini et al. 2006). They share a high sequence similarity for the 16S rDNA gene (98–99%) but display a relatively low similarity in the *recA* gene sequence, enabling the Bcc to be differentiated and sorted into species (Payne et al. 2005). Moreover, all Bcc species possess unusually complex and plastic genomes consisting of two to four chromosomes which harbor insertion sequences that potentially increase the frequency of genetic mutation and recombination (Lessie et al. 1996). This characteristic may facilitate their adaptation to different environments and explain why strains of the Bcc have been found in habitats as diverse as soil, plant, water, rhizosphere, and animal surfaces (Coenye and Vandamme 2003).

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Bcc species are considered to be beneficial to agriculture. *B. vietnamiensis* is the only species able to fix atmospheric nitrogen among Bcc isolates (Gillis et al. 1995). *B. vietnamiensis* strain TVV75 has been shown to promote rice growth by means of both nitrogen fixation and plant hormone (indole-3-acetic acid, IAA) production (Tran Van et al. 2000). *B. vietnamiensis* strain G4 can degrade aromatic pollutants such as toluene, phenol, and chlorinated solvents such as trichloroethylene (Nelson et al. 1987). Strains of Bcc can also produce antifungal metabolites, including antibiotics and siderophores, and be useful in controlling growth of soil-borne fungal plant pathogens (Parke and Gurian-Sherman 2001). While Bcc isolates are increasingly exploited for biotechnological applications, commercial application has been hampered by the risk that Bcc may pose to human health (Chiarini et al. 2006). Resulting concerns about risks to human health limits their potential use in agriculture despite the findings that *B. cepacia* and *B. vietnamiensis* have been isolated from plant tissue or the rhizosphere of rice, maize, and sugarcane and been shown to be able to promote crop growth (Tran Van et al. 2000; Perin et al. 2006). Based on the wide existence of Bcc species in the natural environment, the U.S. Environmental Protection Agency (EPA) does not regulate the injection of the Bcc members into groundwater for bioremediation (EPA 2003).

Populus species have been widely used for phytoremediation of contaminated soil and groundwater (Gordon et al. 1998; Doty et al. 2007), for the pulp paper industry, and has been proposed for the production of biofuel (Sims et al. 2006). Bacterial endophytes isolated from hybrid poplar trees (*P. trichocarpa* × *P. deltoides*) growing in nutrient-rich soil were found to be able to promote biomass production and root development of poplar trees (Taghavi et al. 2009). However, none of the isolates showed nitrogen-fixing capacity. The discovery of endophytic nitrogen-fixing Bcc species (WPB) in wild poplar trees may open the way to realizing the benefits of the species in non-agricultural applications. Understanding the nitrogen-fixing ability of the new Bcc isolate is an important step towards using the species for plant growth promotion. The objectives of this study were to identify the endophytic species and characterize its capacity for plant growth promotion with a focus on nitrogen fixation.

Materials and methods

WPB was one of the bacterial strains isolated from stems of *Populus trichocarpa* growing beside the Snoqualmie River research site at Three Forks Park in King County, Washington State (Doty et al. 2009).

Genomic DNA of the bacterium was extracted as described by 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 to remove the high amounts of polysaccharides in samples.

Genes of *recA*, *nifH*, *nifD*, and *nifK* were amplified by polymerase chain reactions (PCR). PCR was performed on DNA extracts in 25 µl with final concentrations of 1× PCR Pre-Mix buffer E (Epicentre, Madison, WI), 100 nM of forward and reverse primers, 5 U of Taq DNA polymerase (Fermentas), and 1 µL of template DNA. The reaction mixture was held at 95°C for 5 min followed by 34 cycles of amplification at 95°C for 30 s, 55°C (58°C for *recA* amplification) for 30 s and 72°C for 60 s, with a final step of 72°C for 5 min in Mastercycler thermalcycler (Eppendorf, Westbury, NY). A 1-kb fragment of *recA* gene of WPB was amplified with the primers BCR1 and BCR2 primers (Mahenthalingam et al. 2000) for species identification. Three PCR reactions were conducted for *nif* gene identification. A 1.2-kb fragment comprising almost entire *nifH* gene, the intergenic spacer region, and the 5' end of *nifD* gene was amplified with the primers IGK (Poly et al. 2001) and NDR1 (Valdes et al. 2005). A 1.1-kp fragment of *nifK* gene was amplified with the primers *nifKf* and *nifKr* (Minerdi et al. 2001). Based on obtained *nifD* and *nifK* sequences, WPB_ *nifDf* (5'-CGG CGC GCC AAG CAT TTG GGT ACC-3') and WPB_ *nifKr* (5'-GCT GCC GGC CTT GCC GTC CC-3') were designed and used for amplifying a 2.2-kb fragment covering *nifD* gene, transgenic spacer region, and 5' end of *nifK* gene.

PCR products were subjected to electrophoresis in 0.8% agarose gel. Target bands were collected from the agarose gel and DNA extracted from it using the QIAEXII gel extraction kit (Qiagen, Valencia, California). DNA fragments were cloned using the pGEM T Easy kit (Promega, Madison, Wisconsin) in general accordance with the manufacturer's instructions. Sequencing was conducted using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and an ABI 3730XL sequencer (Applied Biosystems) at the Department of Biochemistry sequencing facility of the University of Washington. Sequence data have been submitted to Genbank under the accession numbers EU563933–EU563935.

Acetylene reduction assay (ARA) of the isolate was performed according to the protocol described by Kessler and Leigh (1999). Sixteen milliliters of Ashby's broth [200 mg/L K₂HPO₄, 200/L mg MgSO₄·7H₂O, 200 mg/L NaCl, 100 mg/L K₂SO₄, 5 g/L CaCO₃, 2 mL/L trace mineral solution and 2 mL/L vitamin solution (Xin et al. 2008)] containing 3% sucrose was added into each 27-mL Balch test tube. Twenty microliters of cultures (OD₆₀₀=0.5–1.0), incubated in YPD (1% yeast extract, 2% peptone, and 2% dextroses) broth for 24 h followed by 1× Ashby's

medium—3% (w/v) sucrose for 24 h, were added into the Ashby's broth before the sealing of the test tubes. Acetylene gas was injected into head space (11 mL) of the test tubes at a final concentration of 4% (v/v) and incubated for 1–7 days at 30°C with shaking. Headspace of test tubes were filled with Argon/CO₂ (80%/20%), N₂, or air, respectively. The ethylene peak was identified using a gas chromatograph with a column at 85°C containing a mixture of Poropak N and Poropak Q attached to a flame ionization detector. Positive nitrogen fixation activity of bacterial cultures was demonstrated by increased ethylene concentration over time.

Nitrogen fixation activity was also examined using the ¹⁵N₂ incorporation assay. Cultures were diluted to 10 mL to achieve the same optical density (OD₆₀₀=0.5) with Qubit nitrogen-free medium (Qubit, Kingston, ON) containing 3% sucrose in 27-mL Balch tubes. Headspace of the test tubes was filled with Argon/CO₂ (80%/20%). NH₄Cl was added to negative controls at 5 g/L. Pure ¹⁵N₂ isotope gas was obtained from Isotec (Miamisburg, OH) and washed with sterile 1 M hydrochloric acid water to remove possible ¹⁵NH₃ contamination before being dosed to the test tubes. A yeast strain (*Saccharomyces cerevisiae*) isolated from Baker's yeast (Lesaffre yeast corporation, Milwaukee, WI) was used as a negative control. ¹⁵N₂ was dosed to the headspace of the tubes to 30% (v/v) at time zero. After 3 days, the same ¹⁵N₂ dosage was repeated for each tube and 5 mL of air was injected in the headspaces. After 6 days, cultures were centrifuged and cell pellets were collected and lyophilized using a freeze-drying unit at -50°C overnight. Ground, dry samples were weighed and delta ¹⁵N was analyzed at the stable isotope facility at the University of Alaska in Fairbanks.

To quantify the production of IAA, WPB was grown in YMA medium (2 g/L mannitol, 400 mg/L yeast extract, 500 mg/L K₂HPO₄, 200 mg/L MgSO₄·7H₂O, 100 mg/L NaCl, pH=6.8) with or without 0.1% (w/v) L-tryptophan for 1, 2, 5, and 7 days and 1.5 mL of the cells were pelleted by centrifugation at 10,000×g for 5 min. One milliliter of supernatant was mixed with 2 mL of Salkowski reagent (2 mL of 0.5 M FeCl₃+98 mL 35% HClO₄; Gordon and Weber 1951), and the intensity of pink color developing in the mixture after 30 min was quantified by a Hach DR/4000 spectrophotometer (Hach, Loveland, CO) at a wavelength of 530 nm. Cell pellets were dried at 100°C overnight and weighed for normalizing IAA production. Similarly, a series of IAA standard solutions dissolved in YMA medium with known concentrations was subjected to the same method to set up the calibration curve.

Effect of WPB on plant growth was tested in vivo on Kentucky bluegrass (*Poa pratensis* L.). Seven young seedlings of Kentucky bluegrass about 20 days old, germinated from surface sterilized seeds, were planted in

sterile sand in the upper compartment of each 400-mL two-compartment plastic chamber with the bottom compartment (200 mL) for medium storage (Rodriguez et al. 2008). The WPB culture in ATCC nitrogen-free medium (50 mg/L K₂HPO₄, 150 mg/L KH₂PO₄, 200 mg/L MgSO₄·7H₂O, 20 mg/L CaCl₂, and 2 mL/L trace mineral solution, pH=7.0) was used as inoculum for three chambers in quantities about 5×10⁸ cells per chamber. Another four chambers of plants without WPB inoculation served as controls. All plants were inoculated at room temperature under fluorescent light with 16/8 h light/darkness. Murashige and Skoog medium (1×; MS; Caisson Laboratories Inc., Rexburg, Idaho) was supplied to the bottom apartment of each chamber in the first 10 days, and then 1× ATCC nitrogen-free medium was supplied during the incubation period.

After 50 days, grass tissue was collected from the chamber and separated into shoot (above sand surface) and root (below sand surface) segments. A small portion of the tissue was used to isolate endophytes, and all remaining tissue was dried, weighed, and assessed for nitrogen content in a CHN2400 Analyzer (Perkin Elmer, Waltham, MA). DNA of isolated microorganisms was extracted and the *nifH* gene amplified using primer set IGK and NDR1. PCR products were subjected to electrophoresis in a 0.8% agarose gel. Target bands were collected from the gel and DNA extracted using the QIAEXII gel extraction kit. Extracted DNA was sequenced without being cloned.

Results and discussion

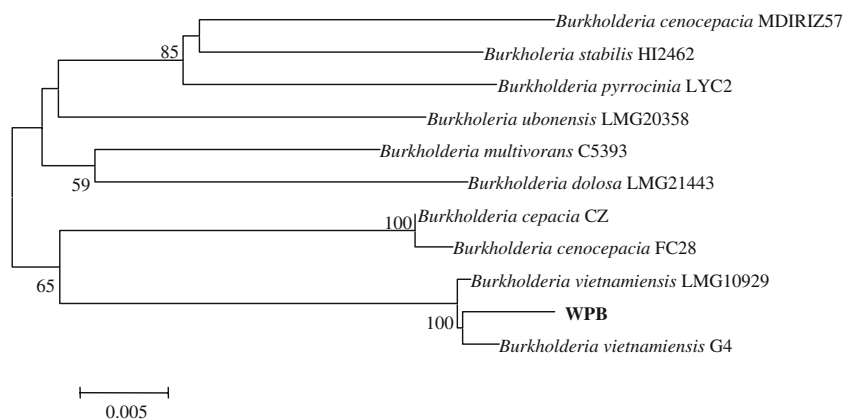
Phylogenetic analysis of *recA* gene and *nifHDK* gene cluster

A 1,043-bp *recA* gene fragment was amplified from WPB genomic DNA and then sequenced. Phylogenetic analysis of the *recA* sequence showed that the WPB isolate was most closely related to the species *Burkholderia vietnamiensis* (Fig. 1). Based on the high *recA* gene similarity (over 99%) between the WPB isolate and *B. vietnamiensis* strains, the wild poplar isolate was determined to be *B. vietnamiensis* strain WPB.

The nucleotide sequence was determined for a 3,853-bp region of the genome of *B. vietnamiensis* WPB which contains *nifH*, *nifD*, *nifK* genes and two intergenic spacer regions. The WPB isolate shared high similarity of *nif* gene sequences with the *B. vietnamiensis* strain G4: 99.1% for *nifH*, 98.9% for *nifD*, and 96.9% for *nifK*.

The sequences of two intergenic regions of the *B. vietnamiensis* WPB showed high similarities to the same regions of the *B. vietnamiensis* G4. The two strains share identical *nifH*–*nifD* intergenic regions and 97% identity for the *nifD*–*nifK* intergenic region. In the 82-bp *nifH*–*nifD*

Fig. 1 Phylogenetic tree of the *recA* gene. The tree was constructed with a total of 958 positions using a neighbor-joining distance matrix (Saitou and Nei 1987). The evolutionary distances were computed using the method by Jukes and Cantor (1969). Bootstrap values (1,000 tree interactions) are indicated at the nodes



intergenic region, one repeated and inverted sequence (GCCCCGCCGCGGGCGCGCGGGGGC) that could act as a putative transcription terminator was found at a position 17 bases beyond the stop codon of *nifH*. There was no such sequence found in the 77-bp *nifD*–*nifK* intergenic region. Analysis of regions immediately upstream of *nifD* and *nifK* revealed the presence of Shine and Dalgarno sequences (GGAG and AGGAG), the putative ribosome-binding sites. No transcription start site was found in the two intergenic spacer regions. This result suggested that the *B. vietnamiensis* WPB *nifHDK* genes were transcribed as an operon similar to that of other *Burkholderia* strains described previously (Minerdi et al. 2001).

It was commonly observed for diazotrophs that nitrogenase genes located on one operon have multiple transcripts due to different transcription and post-processing patterns of the *nifHDK* cluster (Dominic et al. 1998). The repeated and inverted sequence on the *nifH*–*nifD* intergenic region could terminate transcription downstream of *nifH* under certain conditions. The finding of the transcription terminator suggests that *nifH* mRNA and *nifHDK* mRNA would be the dominant *nif* transcripts of WPB.

Assays for function of nitrogen fixation genes

The expression of nitrogenase genes of the WPB *B. vietnamiensis* strain was examined using a $^{15}\text{N}_2$ incorporation assay, considered a definitive method to confirm ability to fix nitrogen. Results show that under diazotrophic conditions, the isotope ratio of $^{14}\text{N}/^{15}\text{N}$ in the WPB biomass was 13.7, which indicates a 20-fold ^{15}N enrichment in WPB compared to the isotope ratio of 272 in natural air. In the presence of NH_4Cl , no enrichment was observed in the WPB biomass (isotope ratio of 270.4). There was no significant ^{15}N enrichment for the negative control, *S. cerevisiae* strain, under both diazo-

trophic conditions (isotope ratio of 271.2) and the presence of NH_4Cl (isotope ratio of 268.8).

In acetylene reduction assays, ethylene production rates for WPB were observed to be higher under aerobic conditions than under anoxic conditions (Fig. 2). Increased ethylene production rates after the 1-mL air injection into the tubes confirmed that the presence of oxygen can improve the nitrogenase activity. It is widely accepted that purified nitrogenase, regardless of its source, is rapidly and irreversibly inactivated by oxygen (Marchal and Vanderleyden 2000). However, some free living, aerobic, nitrogen-fixing bacteria have been observed that require a minimal concentration of oxygen to support their nitrogen-fixing activity, a process requiring at least 16 mol of ATP for reduction of 1 mol of dinitrogen gas (Marchal and Vanderleyden 2000). Even facultative anaerobic nitrogen-fixing bacteria such as *Klebsiella pneumoniae* show an increased nitrogen-fixing capacity in the presence of a limiting amount of oxygen (Poole and Hill 1997). The

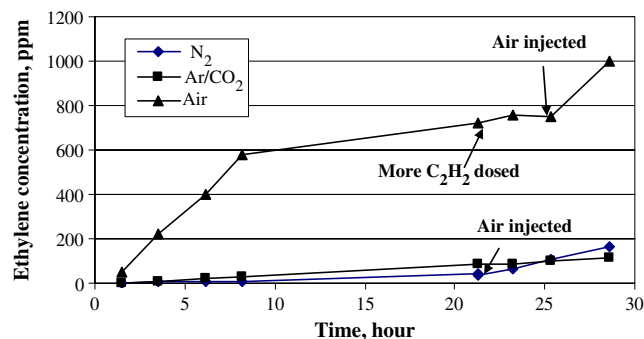


Fig. 2 Acetylene reduction assay for the *B. vietnamiensis* WPB with different headspace gases. WPB was incubated in the Ashby's nitrogen-free medium plus sucrose at 30°C with shaking in Balch tubes that were filled with different headspace gases. After injection of acetylene gas to the headspace, headspace gases were sampled and produced ethylene gas was quantified using a GC at several time points during the following 28 h. Extra acetylene gas and air were also injected to different tubes during the experiment

ARA results of this study are in line with the conclusion that the nitrogen-fixing process may slow down due to the lack of ATP when oxygen is absent.

IAA production

WPB was shown to produce 5.7 mg IAA per g dry cell after 7-day incubation with 0.1% L-tryptophan. No IAA production was detected for WPB after 7-day incubation without the addition of L-tryptophan. Also, no detectable IAA was produced by the negative control, *S. cerevisiae* strain, even with the addition of 0.1% L-tryptophan.

Tryptophan as a strong stimulation for the release of IAA has also been reported for other endophytic bacteria (Omay et al. 1993; Hung et al. 2007; Taghavi et al. 2009). Some bacteria and fungi lack the ability to synthesize tryptophan, essential for protein synthesis, and must obtain it from plant or microbial sources (Radwanski and Last 1995). The observed requirement of external tryptophan addition for the IAA synthesis suggests that WPB may be either unable to synthesize tryptophan or unable to produce tryptophan at required levels for the IAA synthesis. This situation can be interpreted as an example of a mutually advantageous plant–microbe interaction: the plant provides tryptophan for WPB and WPB, in return, converts extra tryptophan to IAA for promoting the plant growth.

Grass growth promotion assay

The effects of WPB on plant growth were assessed under nitrogen-limiting conditions by measuring shoot and root dry weight and total nitrogen content of Kentucky bluegrass over a 50-day period (Table 1). Kentucky bluegrass is one of the most important forage and turf grasses in temperate climates (Barcaccia et al. 1997). The species employed in this study exhibits obligate apomixis, an asexual form of reproduction. Asexual reproduction ensures that grasses growing from different seeds are

genetically extremely similar, consequently growing similarly under similar environmental conditions. Although both the WPB-inoculated and uninoculated control plants grew during the testing period, the plants inoculated with WPB gained significantly more biomass (42%) than the controls in terms of both shoot and root. Differences on the root dry weight were significant ($P=0.01$), with 72% increase for the plant with WPB inoculation. The total nitrogen of the inoculated plants was 37% greater than that for the control plants ($P=0.04$). These results indicate that the WPB inoculum enhanced the growth of Kentucky bluegrass under nitrogen-limiting conditions.

After 50 days, both plants inoculated with WPB and the controls were subjected to endophyte isolation. No endophytes were isolated from the control treatment plants while a number of colonies were isolated from plants inoculated with WPB. Determination of the organism was confirmed on the basis of determining the *nifH* sequence for one of these reisolated strains. This finding confirms that the *Populus* isolate was able to colonize Kentucky bluegrass.

The intimate beneficial interactions between plants and *B. vietnamiensis* suggest use of the bacterium for plant growth promotion and phytoremediation of aromatic polluted environments. The isolation of the *B. vietnamiensis* from wild *Populus* demonstrates that the strain is adapted to the environment inside *Populus*. Its effects on plant growth promotion suggest WPB could interact with *Populus* in a positive way. Future research should determine if the WPB isolate is able to promote growth of *Populus*, fix nitrogen within the plant, and pass some of the fixed nitrogen to the plant.

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Table 1 Growth promotion of Kentucky bluegrass by WPB during a 50-day period

		Shoot		Root		Whole plant	
		Dry weight, g/plant	N content, mg/plant	Dry weight, g/plant	N content, mg/plant	Dry weight, g/plant	N content, mg/plant
WPB-inoculated plants	Average	0.172	3.88	0.100	1.02	0.272	4.90
	Standard deviation	0.022	0.76	0.008	0.04	0.030	0.80
Control plants	Average	0.134	3.04	0.058	0.53	0.192	3.57
	Standard deviation	0.013	0.51	0.018	0.21	0.023	0.50
ANOVA <i>p</i> -value		0.03	>0.05	0.01	0.01	0.01	0.04
% increase compared to control plants		28	28	72	93	42	37

References

- Ausubel F, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1995) Short protocols in molecular biology. Wiley, Hoboken, NJ
- Barcaccia G, Mazzucato A, Belardinelli A, Pezzotti M, Lucretti S, Falcinelli M (1997) Inheritance of parental genomes in progenies of *Poa pratensis* L. from sexual and apomictic genotypes as assessed by RAPD markers and flow cytometry. *Theor Appl Genet* 95:516–524. doi:10.1007/s001220050592
- Chiarini L, Bevivino A, Dalmastrì C, Tabacchioni S, Visca P (2006) *Burkholderia cepacia* complex species: health hazards and biotechnological potential. *Trends Microbiol* 14:277–286. doi:10.1016/j.tim.2006.04.006
- Coenye T, Vandamme P (2003) Diversity and significance of *Burkholderia* species occupying diverse ecological niches. *Environ Microbiol* 5:719–729. doi:10.1046/j.1462-2920.2003.00471.x
- Dominic B, Chen YB, Zehr JP (1998) Cloning and transcriptional analysis of the *nifUHDK* genes of *Trichodesmium* sp. IMS101 reveals stable *nifD*, *nifDK* and *nifK* transcripts. *Microbiology-SGM* 144:3359–3368
- Doty SL, James CA, Moore AL, Vajzovic A, Singleton GL, Ma C, Khan Z, Xin G, Kang JW, Park AY, Meilan R, Strauss SH, Wilkerson J, Farin F, Strand SE (2007) Enhanced phytoremediation of volatile environmental pollutants with transgenic trees. *Proc Natl Acad Sci USA* 104:16816–16821. doi:10.1073/pnas.0703276104
- Doty SL, Oakley B, Xin G, Kang JW, Singleton GL, Khan Z, Vajzovic A, Staley JT (2009) Diazotrophic endophytes of native black cottonwood and willow. *Symbiosis* 47:23–33
- EPA (2003) *Burkholderia cepacia* complex, significant new use rule. *Fed Regist* 68:35315–35320
- Gillis M, Vanvan T, Bardin R, Goor M, Hebbar P, Willems A, Segers P, Kersters K, Heulin T, Fernandez MP (1995) Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov for N₂-fixing isolates from rice in Vietnam. *Int J Syst Bacteriol* 45:274–289
- Gordon SA, Weber RP (1951) Colorimetric estimation of indoleacetic acid. *Plant Physiol* 26:192–195. doi:10.1104/pp.26.1.192
- Gordon M, Choe N, Duffy J, Ekuon G, Heilman P, Muiznieks I, Ruszaj M, Shurtleff BB, Strand S, Wilmoth J, Newman LA (1998) Phytoremediation of trichloroethylene with hybrid poplars. *Environ Health Perspect* 106:1001–1004. doi:10.2307/3434144
- Hung PQ, Kumar SM, Govindsamy V, Annapurna K (2007) Isolation and characterization of endophytic bacteria from wild and cultivated soybean varieties. *Biol Fertil Soils* 44:155–162. doi:10.1007/s00374-007-0189-7
- Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: Munro HN (ed) *Mammalian protein metabolism*. Academic, New York, pp 21–132
- Kessler PS, Leigh JA (1999) Genetics of nitrogen regulation in *Methanococcus marisplaudis*. *Genetics* 152:1343–1351
- Lessie TG, Hendrickson W, Manning BD, Devereux R (1996) Genomic complexity and plasticity of *Burkholderia cepacia*. *FEMS Microbiol Lett* 144:117–128. doi:10.1111/j.1574-6968.1996.tb08517.x
- Mahenthalingam E, Bischof J, Byrne SK, Radomski C, Davies JE, Av-Gay Y, Vandamme P (2000) DNA-based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis*, *Burkholderia multivorans*, *Burkholderia stabilis*, and *Burkholderia cepacia* Genomovars I and III. *J Clin Microbiol* 38:3165–3173
- Marchal K, Vanderleyden J (2000) The “oxygen paradox” of dinitrogen-fixing bacteria. *Biol Fertil Soils* 30:363–373. doi:10.1007/s003740050017
- Minerdi D, Fani R, Gallo R, Boarino A, Bonfante P (2001) Nitrogen fixation genes in an endosymbiotic *Burkholderia* strain. *Appl Environ Microbiol* 67:725–732. doi:10.1128/AEM.67.2.725-732.2001
- Nelson MJK, Montgomery SO, Mahaffey WR, Pritchard PH (1987) Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. *Appl Environ Microbiol* 53:949–954
- Omay SH, Schmidt WA, Martin P, Bangerth F (1993) Indoleacetic-acid production by the rhizosphere bacterium *Azospirillum brasilense* Cd under *in vitro* conditions. *Can J Microbiol* 39:187–192
- Parke JL, Gurian-Sherman D (2001) Diversity of the *Burkholderia cepacia* complex and implications for risk assessment of biological control strains. *Annu Rev Phytopathol* 39:225–258. doi:10.1146/annurev.phyto.39.1.225
- Payne GW, Vandamme P, Morgan SH, LiPuma JJ, Coenye T, Weightman AJ, Jones TH, Mahenthalingam E (2005) Development of a *recA* gene-based identification approach for the entire *Burkholderia* genus. *Appl Environ Microbiol* 71:3917–3927. doi:10.1128/AEM.71.7.3917-3927.2005
- Perin L, Martínez-Aguilar L, Castro-González R, Estrada-de Los Santos P, Cabellos-Avelar T, Guedes HV, Reis VM, Caballero-Mellado J (2006) Diazotrophic *Burkholderia* species associated with field-grown maize and sugarcane. *Appl Environ Microbiol* 72:3103–3110. doi:10.1128/AEM.72.5.3103-3110.2006
- Poly F, Monrozier LJ, Bally R (2001) Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. *Res Microbiol* 152:95–103. doi:10.1016/S0923-2508(00)01172-4
- Poole RK, Hill S (1997) Respiratory protection of nitrogenase activity in *Azotobacter vinelandii*—roles of the terminal oxidases. *Biosci Rep* 17:303–317. doi:10.1023/A:1027336712748
- Radwanski ER, Last RL (1995) Tryptophan biosynthesis and metabolism—biochemical and molecular-genetics. *Plant Cell* 7:921–934
- Rodríguez RJ, Henson J, Van Volkenburgh E, Hoy M, Wright L, Beckwith F, Kim YO, Redman RS (2008) Stress tolerance in plants via habitat-adapted symbiosis. *Intern Soc Microb Ecol* 2:404–416
- Saitou N, Nei M (1987) The neighbor-joining method—a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
- Sims REH, Hastings A, Schlamadinger B, Taylor G, Smith P (2006) Energy crops: current status and future prospects. *Glob Change Biol* 12:2054–2076. doi:10.1111/j.1365-2486.2006.01163.x
- Taghavi S, Garafola C, Monchy S, Newman L, Hoffman A, Weyens N, Barac T, Vangronsveld J, van der Lelie D (2009) Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growth and development of poplar trees. *Appl Environ Microbiol* 75:748–757. doi:10.1128/AEM.02239-08
- Tran Van V, Berge O, Ke SN, Balandreau J, Heulin T (2000) Repeated beneficial effects of rice inoculation with a strain of *Burkholderia vietnamiensis* on early and late yield components in low fertility sulphate acid soils of Vietnam. *Plant Soil* 218:273–284. doi:10.1023/A:1014986916913
- Valdes M, Perez NO, Estrada de los Santos P, Caballero-Mellado J, Pena-Cabrales JJ, Normand P, Hirsch AM (2005) Non-*Frankia* Actinomycetes isolated from surface-sterilized roots of *Casuarina equisetifolia* fix nitrogen. *Appl Environ Microbiol* 71:460–466. doi:10.1128/AEM.71.1.460-466.2005
- Xin G, Gough HL, Stensel HD (2008) Effect of anoxic selector configuration on SVI control and bacterial population fingerprinting. *Water Environ Res* 80:2228–2240. doi:10.2175/106143008X325700