Bioaugmentation with Engineered Endophytic Bacteria Improves Contaminant Fate in Phytoremediation

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Received July 7, 2009. Revised manuscript received October 21, 2009. Accepted November 3, 2009.

Phytoremediation of volatile organic contaminants often proves not ideal because plants and their rhizosphere microbes only partially degrade these compounds. Consequently, plants undergo evapotranspiration that contaminates the ambient air and, thus, undermines the merits of phytoremediation. Under laboratory conditions, endophytic bacteria equipped with the appropriate degradation pathways can improve in planta degradation of volatile organic contaminants. However, several obstacles must be overcome before engineered endophytes will be successful in field-scale phytoremediation projects. Here we report the first in situ inoculation of poplar trees, growing on a TCE-contaminated site, with the TCE-degrading strain *Pseudomonas putida* W619-TCE. In situ bioaugmentation with strain W619-TCE reduced TCE evapotranspiration by 90% under field conditions. This encouraging result was achieved after the establishment and enrichment of *P. putida* W619-TCE as a poplar root endophyte and by further horizontal gene transfer of TCE metabolic activity to members of the poplar's endogenous endophytic population. Since *P. putida* W619-TCE was engineered via horizontal gene transfer, its deliberate release is not restricted under European genetically modified organisms (GMO) regulations.

Introduction

Bioaugmentation, the introduction of natural or genetically modified microorganisms to improve the remediation of contaminated groundwater, has successfully been applied to remediate groundwater polluted with chlorinated solvents (*1, 2*) and so has become a widely accepted practice. However, this technology requires maintaining hydraulic control of the groundwater and continuously injecting nutrients, inducers, or oxygenating compounds. Phytoremediation is a more cost-efficient alternative for the remediation of such contaminated groundwater, but the evapotranspiration of volatile contaminants and their partial degradation products still undermine its merits (*3*). Laboratory studies show that the in planta metabolism of organic compounds can be strongly improved by inoculating plants with endophytic bacteria that can decompose the contaminants (*4*−*6*). Bioaugmentation with endophytic bacteria would have several benefits over traditional bioaugmentation, that is, simply enriching the soil with a consortium of pollutant-degrading bacteria. In the former case, the bacteria reside in a contained environment, making them less susceptible to predation; the plant provides nutrients to the bacteria, thus supporting their growth and establishment. However, several remaining problems must be resolved before endophytes can be successful in field-scale phytoremediation projects (*7, 8*). For example, during bioaugmentation, the inoculated bacteria would have to compete against the endogenous, well-adapted microbial population. Here, horizontal gene transfer could play an important role in enhancing the metabolic capabilities of the endogenous endophytes (*6*); rather than integrating a new bacterium into a stable community, the degradation pathway is transferred among members of the community.

To test whether the in planta bioaugmentation of phytoremediation with endophytic bacteria will enhance the remediation of organic solvents, we chose a site where the groundwater was contaminated with TCE in concentrations up to 100 mg L^{-1}. To control the groundwater TCE plume, hybrid poplar trees *Populus deltoides* × (*Populus trichocarpa* × *Populus deltoides*) cv. Grimminge were planted in the spring of 2006 perpendicularly to the plume. Two years later, in situ evapotranspiration measurements on six poplar trees (taken in May 2008) revealed that a significant amount of TCE was evaporating through the poplar leaves [an average of $(8.0 \times 10^{-2}) \pm (0.3 \times 10^{-2})$ ng of TCE cm^{-2} h^{-1}; Table 1]. As a case study to lower this amount, we inoculated three trees with *P. putida* W619-TCE. We selected this strain as the ideal candidate because it is a root endophyte of poplar that was equipped, via natural gene transfer, with the pTOM-Bu61 plasmid coding for constitutive TCE degradation (*6*). According to European legislation, the constructed *Pseudomonas putida* W619-TCE is considered as a non-genetic modified organism (non-GMO) and so can be used in field applications. Its parental strain, the nonpathogenic *P. putida* W619, of which the genome was recently sequenced (http://genome.jgi-psf.org/finished_microbes/psepw/psepw.home-

TABLE 1. TCE Evapotranspiration of Experimental Trees before and after *in situ* Inoculation with *P. putida* W619-TCE

	TCE evapotranspiration (10^{-2} ng cm^{-2} h^{-1})	
tree	before inoculation (May)	3 months after inoculation (August)
1	8.9 ± 0.8	7.8 ± 1.8
2	7.1 ± 1.2	7.2 ± 1.0
3	8.4 ± 1.5	6.8 ± 0.5
4[a]	**7.9 ± 0.9**	**0.9 ± 0.1**
5	**8.1 ± 1.3**	**0.9 ± 0.4**
6	**7.5 ± 0.8**	**0.5 ± 0.1**

[a] *P. putida* W619-TCE inoculated trees are indicated with boldface type. In situ TCE evapotranspiration was determined for three independent noninoculated control poplar trees and three poplar trees inoculated with *P. putida* W619-TCE. For each tree, three TCE evapotranspiration measurements were performed simultaneously. The amount of evapotranspired TCE was calculated per hour and unit of leaf area. Values are mean ± standard error of three replicates.

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10.1021/es901997z
Published on Web 11/13/2009

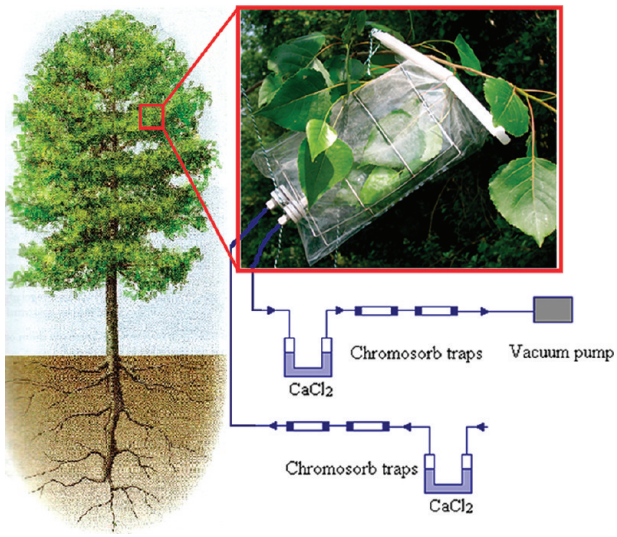

FIGURE 1. Schematic setup to measure in situ TCE evapotranspiration. Gas sampling pumps (ADC BioScientific) were connected to Teflon sampling bags (Chemware Laboratory products) via Teflon tubes and Chromosorb 106 traps. To avoid water condensing in the traps, a column with $CaCl_2$ was placed between the sampling bags and the Chromosorb traps. Further, a column with $CaCl_2$ and Chromosorb 106 traps also was placed before the inflow of the sampling bags to ensure a TCE-free inflowing air stream. For each condition (noninoculated and inoculated) three trees were investigated, and for each one analyzed, three independent measurements were performed. Twigs with up to 12 leaves were placed into the sampling bag that was made gastight around the twig, and an airflow of 5 L h^{-1} was created during 3 h. After sampling, we collected the leaves in plastic bags and stored them at 4 °C until analysis of the leaves' surface area. The Chromosorb 106 traps were analyzed by gas chromatography−mass spectrometry (GC-MS) with an ATD400 automatic thermal desorption system, an Auto System XLL gas chromatograph, and a Turbo mass spectrometer (Perkin-Elmer). The amount of evapotranspired TCE was calculated per hour and unit of leaf surface.

.html), is closely related to *P. putida* KT2440, an organism that is generally recognized as safe (GRAS certified) (*9*). Furthermore, inoculating poplar with *P. putida* W619 under greenhouse conditions promoted plant growth 4-fold, decreased the activities of glutathione reductase in the roots, and superoxide dismutase in the roots and the leaves, and considerably lowered stomatal resistance, all indicative of the poplar's improved fitness (*10, 11*).

Experimental Section

In Situ Evapotranspiration. *P. putida* W619-TCE was cultured in 869 medium (*12*), and 40 L of this bacterial suspension [10^8 colony-forming units (cfu) mL^{-1}] was added to the roots of the three experimental trees via drainage tubes, installed when the trees were planted. At the same time, the control trees received 40 L of 869 medium without bacteria. Three months after inoculation (mid-August), the in situ evapotranspiration of TCE was determined for three independent noninoculated and three inoculated poplar trees. For each tree, we simultaneously performed three TCE evapotranspiration measurements. The system designed for these measurements was based on one used by Strycharz and Newman (*13*) and is shown in Figure 1. The amount of evapotranspired TCE was calculated per hour and unit of leaf area.

Isolation of Cultivable Bacterial Strains. To isolate cultivable bacteria, samples were taken of different plant compartments at the same time as we measured the in situ evapotranspiration for the noninoculated control poplars and those inoculated with *P. putida* W619-TCE. Rhizosphere-soil (2 g) and roots (1 g) were sampled and stored in sterile Falcon tubes (50 mL) filled with 20 mL of sterile 10 mM $MgSO_4$; and, stem, twig, and leaf samples were transferred into separate sterile plastic bags until surface sterilization and maceration of the tissue in the laboratory (*4, 14*). For every compartment, samples were taken from three different trees and analyzed separately.

Characterization of Cultivable Isolated Bacteria.

Genotypic Characterization. After purification, total genomic DNA of all morphologically different bacteria was extracted by use of a DNeasy blood and tissue kit (Qiagen). Aliquots (1 μL) of the extracted DNA were directly used for polymerase chain reaction (PCR) without further purification.

The BOX1 primer (5′-CTACGGCAAGGCGACGCTGACG-3′) was used for BOX-PCR DNA fingerprinting, which was carried out as described earlier (*4, 15*). The obtained PCR products were separated by gel electrophoresis in a 1.5% agarose gel and visualized by Gel Red nucleic acid gel staining and UV illumination.

The universal 1392R (5′-ACGGGCGGTGTGTRC-3′) and the bacteria-specific 26F (5′-AGAGTTTGATCCTGGCTCAG-3′) primers were used for prokaryotic 16S rRNA gene amplification, which was carried out as described by Taghavi and co-workers (*4, 15*). PCR products of the 16S rDNA amplification were directly used for amplified 16S rDNA restriction analysis (ARDRA) and sequencing.

For ARDRA, aliquots of these PCR products were digested for 2 h at 37 °C with 1 unit of the 4-base-specific restriction endonuclease *Hpy*CH4 IV in 1× NEB buffer 1 (New England Biolabs). The digestion products obtained were examined electrophoretically in a 1.5% agarose gel and visualized by Gel Red nucleic acid gel staining and UV illumination. ARDRA patterns were grouped, and strains with representative patterns were selected for sequencing, which was performed as described earlier (*4, 15*).

Sequence Match at the Ribosome Database Project II was used for nearest-neighbor and species identification. In order to verify the identification, a neighbor-joining analysis was performed. Prior to this analysis, the sequences were aligned by use of Clustal X (*16*). A neighbor-joining tree was constructed with PAUP*4.0b10 (*17*), by use of default settings. In order to assess branch supports, bootstrap values were calculated with 2000 pseudoreplicates.

TCE Degradation Capacity. Headspace gas chromatography was used in order to test TCE degradation capacity of the reisolated cultivable bacteria. For this experiment bacteria were grown at 30 °C in 40 mL of Schatz medium (*18*) with addition of 100 mg L^{-1} TCE and in Schatz medium supplemented with C-mix (per liter of medium: 0.52 g of glucose, 0.35 g of lactate, 0.66 g of gluconate, 0.54 g of fructose, and 0.81 g of succinate) and 100 mg L^{-1} TCE. Samples of 10 mL were taken at the beginning of the experiment and after 3 days, and were transferred in 20 mL headspace vials to which 4 g of NaCl was added to stop all bacterial activity. Samples were analyzed by headspace (Teledyne Tekmar HT3) gas chromatography (Trace GC Ultra, Interscience). The volatilization of TCE was taken into account by measuring control samples (without addition of bacteria), and degradation was calculated as a percentage of the nonvolatilized fraction.

To verify that the pTOM plasmid of the inoculated strain was responsible for the TCE degradation capacity, the presence of the pTOM degradation plasmid was tested by PCR with *tomA4*-specific primers 3323F (5′-GTT GCC CTC AAA CCC TAC AA-3′) and 3780R (5′-AGG GGC TGA ATG TTG AGT TG-3′). Cycling conditions consisted of one denaturation cycle of 95 °C for 5 min; followed by 35 cycles of 94 °C for 1 min, 60 °C for 30 s, and 72 °C for 1.5 min; and completed with an extension cycle of 8 min at 72 °C. The PCR products

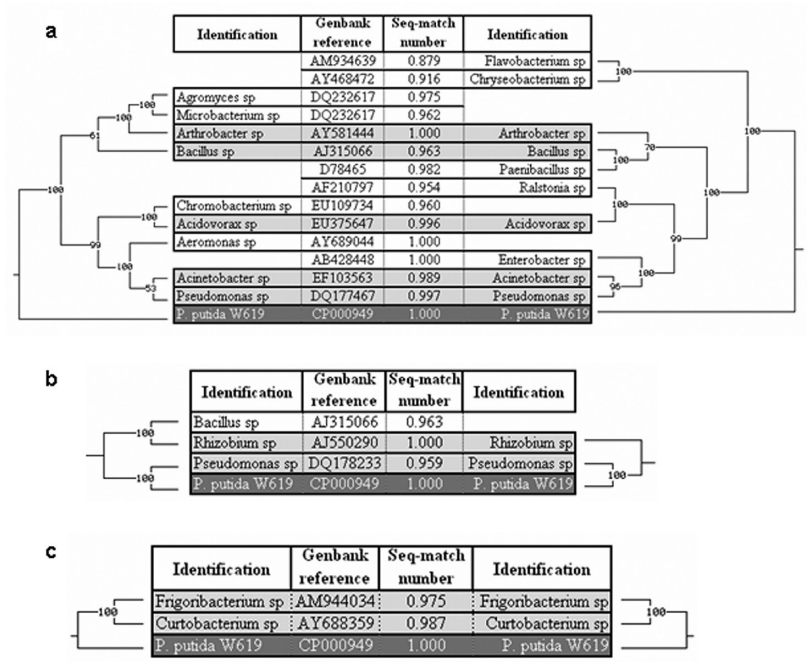

FIGURE 2. Neighbor-joining trees of 16S rRNA genes of the isolated bacteria. (a) Rhizosphere-soil bacteria, (b) root endophytes, and (c) stem endophytes associated with noninoculated (left) and inoculated (right) poplar were isolated and identified. The 16S rRNA gene-based identification, the accession number of the closest related strain, and the sequence match number are shown. For comparison, strain *P. putida* W619-TCE (denoted by dark gray) was included. Bacterial strains that are present in both inoculated and noninoculated control plants are highlighted in light gray. To construct neighbor-joining trees, data from the three inoculated trees were combined, as were the data from the noninoculated trees.

obtained were examined by electrophoresis in a 1.5% agarose gel and visualized by Gel Red nucleic acid gel staining and UV illumination.

Statistical Analysis. The TCE evapotranspiration data sets employed were analyzed by use of linear mixed-effect models (*19*) to correct for the correlation between different twigs of the same plant (measurements were made on three twigs per plant). TCE degradation was compared statistically by use of one-way analysis of variance (ANOVA) (per medium) and posthoc multiple comparison testing (Tukey-Kramer). When necessary, transformations were applied to approximate normality and/or homoscedasticity. The statistical analyses were performed by the ANOVA and MIXED procedure in SAS 9.1.3.

Results and Discussion

In Situ Evapotranspiration. Three months after inoculation (mid-August), the in situ evapotranspiration of TCE was determined from the leaves of the control and inoculated poplar trees. The respective values had an average of $(7.2 \times 10^{-2}) \pm (0.3 \times 10^{-2})$ ng of TCE cm^{-2} h^{-1} for the control trees and $(0.8 \times 10^{-2}) \pm (0.1 \times 10^{-2})$ ng of TCE cm^{-2} h^{-1} for the inoculated trees, representing a 9-fold reduction ($p = 0.0002$) in TCE evapotranspiration for the inoculated trees (Table 1). In contrast, TCE evapotranspiration through the leaves of the noninoculated control trees was similar to that seen before inoculation (Table 1), indicating that reduced evapotranspiration was not related to differences in the plant's growth cycle.

Characterization of Cultivable Isolated Bacteria.

Genotypic Characterization. To verify that the inoculated *P. putida* W619-TCE had been integrated into the plant-associated microbial community, cultivable bacteria from the rhizosphere-soil, roots, stems, twigs, and leaves of the three inoculated and three noninoculated experimental trees were isolated and identified via BOX- and ARDRA-DNA fingerprinting and 16S rRNA gene sequencing.

In the rhizosphere-soil, the bacterial communities of inoculated and noninoculated poplars were characterized by common species (*Pseudomonas*, *Arthrobacter*, *Bacillus*, *Acinetobacter*, and *Acidovorax* spp.), as well as species exclusively found in noninoculated trees (*Agromyces*, *Microbacterium*, *Chromobacterium*, and *Aeromonas* spp.) or the inoculated ones (*Flavobacterium*, *Chryseobacterium*, *Paenibacillus*, *Ralstonia*, and *Enterobacter* spp.) (Figure 2a). The total numbers of cultivable isolates found in the rhizospheres of the noninoculated trees (3.5×10^7 cfu/g of rhizosphere-soil) and the inoculated trees (5.0×10^7 cfu/g of rhizosphere-soil) were similar and were dominated by *Pseudomonas* spp. (Figure 3a). Interestingly, the *Pseudomonas* spp. in the rhizospheres were phylogenetically distinct from *P. putida* W619-TCE (Figure 2a), indicating that after 3 months the inoculated strain was not established in the rhizosphere. Most of the remaining cultivable rhizospheric bacteria were represented by *Acinetobacter* and *Flavobacterium* spp., respectively, for the noninoculated and inoculated poplar trees. This difference suggested that inoculation with *P. putida* W619-TCE changed the composition of the rhizospheric community.

The cultivable members of the endophytic community in the roots of noninoculated poplar trees encompassed *Bacillus*, *Rhizobium*, and *Pseudomonas* spp., while roots from the inoculated poplars contained only *Rhizobium* and *Pseudomonas* spp. (Figure 2b). With 16S rRNA gene sequencing, the *Pseudomonas* spp. from both endophytic communities were undistinguishable from *P. putida* W619. However, BOX-PCR DNA fingerprinting analysis revealed that the *Pseudomonas* spp. isolated from the roots of the three inoculated trees indeed corresponded to the inoculated *P. putida* W619-TCE, while the *Pseudomonas* spp. isolated from the roots of three noninoculated trees showed different BOX-PCR patterns (Figure 4). We noticed a strong increase in the total number of cultivable root isolates, from 9.4×10^4 cfu (g fresh weight)$^{-1}$ for the noninoculated trees to 3.2×10^7 cfu (g fresh weight)$^{-1}$ for the inoculated ones (Figure 3b). Since *Pseudomonas* spp. dominated both endophytic root communities, this increase most likely reflected an enrichment of *P. putida* W619-TCE

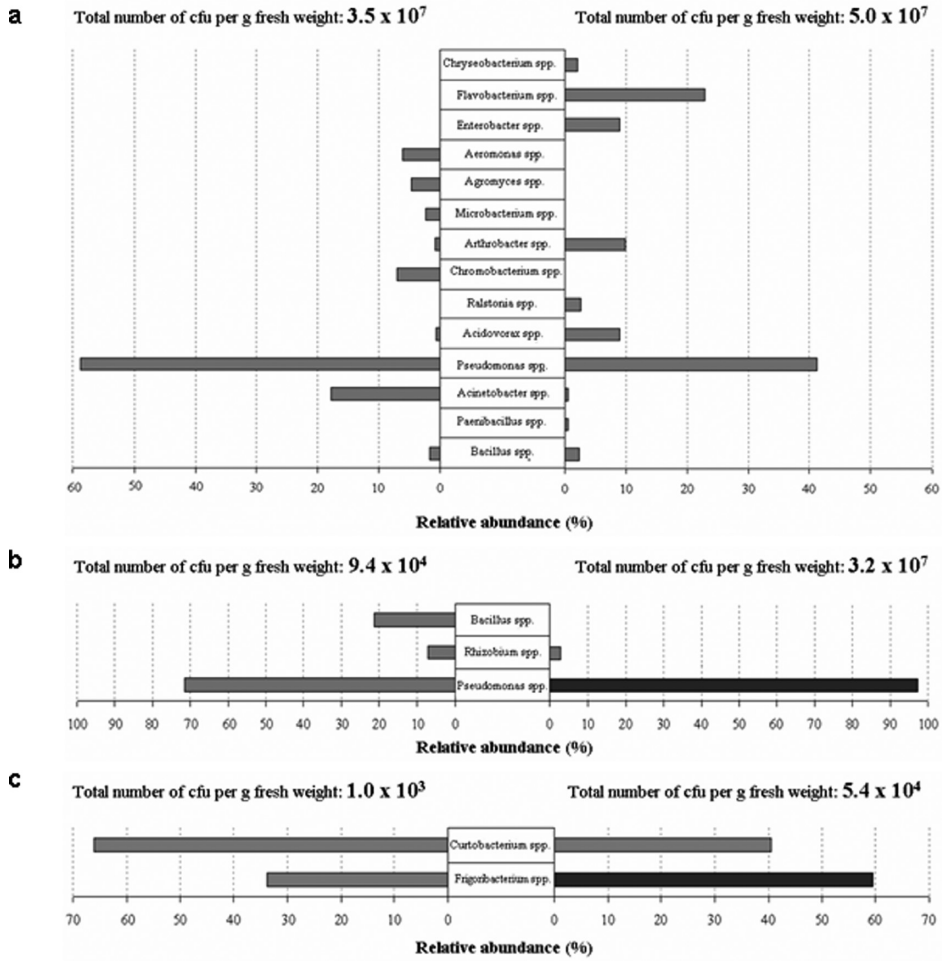

FIGURE 3. Relative abundance of cultivable bacteria isolated from poplar. The relative abundance of (a) rhizosphere-soil bacteria, (b) root endophytes, and (c) stem endophytes of noninoculated (left) and inoculated (right) poplar is expressed as a percentage of the total number of cultivable isolates per gram fresh weight present in (a) rhizosphere-soils, (b) roots, or (c) stem. Black bars represent the bacterial strains that possess the pTOM-Bu61 plasmid and can degrade TCE. To analyze the diversity, data from the three inoculated trees were combined, as were the data from the noninoculated trees.

in the roots of inoculated trees. This conclusion was confirmed by the presence of the pTOM-Bu61 plasmid among the *Pseudomonas* spp. in the roots of all inoculated trees (see below), while the plasmid was absent from the *Pseudomonas* spp. residing in the roots of the control poplars.

For all trees tested, the cultivable members of the endophytic communities in the stems of noninoculated and inoculated trees consisted of *Frigoribacterium* and *Curtobacterium* spp. (Figure 2c); no *P. putida* W619-TCE was isolated. However, we gained good evidence that inoculation with *P. putida* W619-TCE altered community composition, even in parts of the plant where it was not established. Thus, along with an increase in the total number of isolates from 1.0×10^3 cfu (g fresh weight)$^{-1}$ for the noninoculated poplar trees up to 5.4×10^4 cfu (g fresh weight)$^{-1}$ for the inoculated poplar trees, there was a shift to *Frigoribacterium* spp. as the dominant species (Figure 3c).

No cultivable endophytic bacterial strains were isolated from the twigs and leaves of the control or inoculated trees.

TCE Degradation Capacity. To verify that the reduced TCE evapotranspiration observed after inoculation was related to bioaugmentation with *P. putida* W619-TCE, the TCE degradation capacities of representative members of all isolated strains were determined by headspace gas chromatography (*15*) and compared to that of *P. putida* W619-TCE. Bacteria were grown in Schatz medium (*18*) with and without adding a carbon mix and supplemented with 100 mg L^{-1} TCE. Only the endophytic *Pseudomonas* and *Frigoribacterium* spp. (black bars in Figure 3b,c) isolated, respectively, from the roots or stems of the inoculated trees could constitutively degrade TCE with efficiencies similar to *P. putida* W619-TCE (Figure 5). For all three inoculated trees, both species were capable of degrading TCE and even became the dominant members of their respective endophytic communities, suggesting that the capacity to degrade TCE offers a selective advantage. For all individual isolates of both *Pseudomonas* and *Frigoribacterium* spp., the presence of the pTOM-Bu61 plasmid was confirmed by PCR (against its *tomA4* gene). Since no TCE degradation phenotype nor pTOM-Bu61-like plasmids were found among the cultivable members of the different endophytic and rhizosphere communities of the noninoculated poplar trees, we could attribute the TCE degradation capacity of endophytic communities from the roots and stems of the inoculated trees directly to bioaugmentation with *P. putida* W619-TCE. This strain became well-established and dominated the roots' endophytic community. Furthermore, the *Frigoribacterium* spp. (Figures 2c and 3c) present as an endogenous endophyte in the stem received the pTOM-Bu61 plasmid via horizontal gene transfer, and thereafter became the dominant member of its endophytic community. This was generally observed for the endophytic communities of all three inoculated poplars.

However, the *Pseudomonas* and *Frigoribacterium* spp. lost their TCE degradation capacity within 20 generations when cultivated under nonselective conditions (meaning in rich

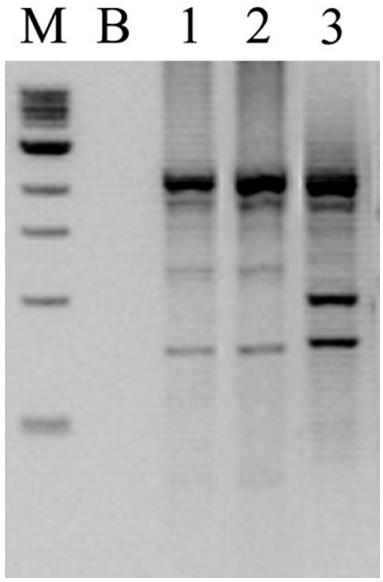

FIGURE 4. BOX-PCR fingerprints of the different *Pseudomonas* strains. (Lane 1) *P. putida* W619-TCE; (lane 2) *Pseudomonas* spp. isolated from roots of poplar trees inoculated with *P. putida* W619-TCE; (lane 3) *Pseudomonas* spp. isolated from roots of noninoculated poplar trees; (lane M) 1 kB DNA marker; (lane B) blank.

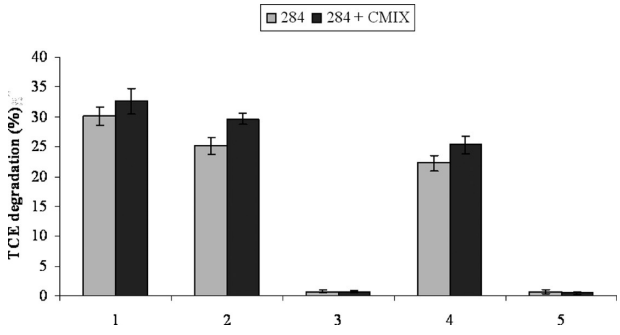

FIGURE 5. Bacterial TCE degradation of bacteria 1−5 followed by headspace chromatography. (1) *P. putida* W619-TCE; (2) *Pseudomonas* spp. isolated from the roots of poplar trees inoculated with *P. putida* W619-TCE; (3) *Pseudomonas* spp. isolated from the roots of noninoculated poplar trees; (4) *Frigoribacterium* spp. isolated from the stems of poplar trees inoculated with *P. putida* W619-TCE; (5) *Frigoribacterium* spp. isolated from the stems of noninoculated poplar trees. Bacteria were grown in Schatz medium with addition of 100 mg L^{-1} TCE and in Schatz medium supplemented with C-mix and 100 mg L^{-1} TCE. Samples were taken at the beginning of the experiment and after 3 days. The volatilization of TCE was taken into account by measuring control samples (without addition of bacteria), and degradation was calculated as a percentage of the nonvolatilized fraction. Values are mean ± standard error of three biological independent replicas.

medium without TCE). This loss of degradative capacity has also been recently demonstrated by Barac et al. (*20*) for a toluene phytoremediation site; thus, once the contaminants' concentration decreased below the detection limit and no longer exerted selection pressure, the endophytic community is restored to its original situation and loses its degradation potential.

We demonstrated that in situ inoculation into poplars of endophytic bacteria with the appropriate degradation pathway remarkably lowered TCE evapotranspiration. Endophytic bacteria able to degrade TCE had a selective advantage and were able to dominate the community, and once this pathway was no longer utilized it was rapidly lost by the bacteria. The concept of using natural or engineered endophytes for remediation purposes is broadly applicable. Many endophytic strains are related closely to environmental strains that carry, on mobile DNA elements, degradation pathways for a broad spectrum of organic contaminants; hence, it may be relatively straightforward to construct, via conjugation, non-GMO endophytic bacteria with a la carte degradation properties.

Considering that one of the major obstacles to the implementation of phytoremediation is the evapotranspiration of volatile contaminants (*21*), we believe that improved phytoremediation via bioaugmentation will offer a safe, breakthrough approach to the large-scale application of phytoremediation of organic contaminants. Another point making this work very special and original is that we introduced a bacterial strain that was modified under laboratory conditions back into the environment, which is in general very difficult to achieve.

Acknowledgments

This research was funded by the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen) for N.W. It also was supported by the UHasselt Methusalem Project 08M03 VGRJ. D.v.d.L., L.N., and S.T. are funded by the U.S. Department of Energy, Office of Science, BER, Project KP1102010 under Contract DE-AC02-98CH10886, and by Laboratory Directed Research and Development funds (LDRD09-005) at the Brookhaven National Laboratory under contract with the U.S. Department of Energy. Special thanks are due to Jos Kaelen and Tim Joris for constructing the in situ evapotranspiration sampling system. We thank Jenny Put and Jan Czech for GC analysis and Avril Woodhead for carefully reviewing the manuscript.

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ES901997Z