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Development of a catabolically significant genetic probe for polycyclic aromatic hydrocarbon-degrading *Mycobacteria* in soil

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Abstract

A gene probe for the detection of polycyclic aromatic hydrocarbon (PAH) induced *nidB* and *nidA* dioxygenase genes has been designed from *Mycobacteria* JLS, KMS, and MCS. The probe detects a catabolic gene involved in the initial steps of PAH biodegradation in mycobacteria. The gene probe is comprised of three PCR primer sets designed to detect the genes that code for two subunits of the PAH induced dioxygenase enzyme within PAH-degrading mycobacteria. The probe was built by combining three primer sets with a DNA extraction procedure that was designed to lyse the gram-positive mycobacteria cells while in the soil matrix and remove PCR inhibitors. The probe was tested on PAH contaminated soils undergoing bioremediation through landfarming and uncontaminated soils from the same site. The PAH gene probe results demonstrate that the dioxygenase genes can be detected in soils. Sequencing the *nidA* and *nidB* PCR products verified that the genes were detected in soil. Comparisons of the sequences obtained from the soil probe to seven known *nid* gene sequences from various PAH-degrading mycobacteria showed between 97 and 99% nucleotide matches with the *nidB* gene and 95 and 99% matches with the *nidA* gene.

Introduction

One of the most important mechanisms for the removal of organic environmental pollutants including polycyclic aromatic hydrocarbons (PAH) has been demonstrated to be bioremediation. *In situ* aerobic biodegradation of PAH has been shown to be one of the most effective remediation strategies for detoxification of PAH contaminated sites (Cerneglia et al. 1992a; Cerneglia 1992b; Cerniglia 1993; Ginn et al. 1996). Research has shown that microbial activity is a major part of the natural remediation of PAH (Cerneglia 1984; Ginn et al. 1996). Research has also shown that mycobacteria isolates are major contributors to the biodegradation of environmental pollutants including gasoline components and high and low molecular weight PAH (Bastiens et al. 2000; Churchill et al. 1999; Dean-Ross & Cerneglia 1996; Heitcamp et al. 1988; McLellan et al. 2002; Schneider et al. 1996). Although biodegradation has been demonstrated in laboratory studies, a continuing challenge to acceptance of bioremediation is demonstration that remediation of the compounds actually represents biological metabolism and transformation to non-toxic forms (Madsen 1991; National Research Council 1993). An important question in the use of bioremediation is the effectiveness of bioaugmentation and biostimulation. The ability to detect PAH degradation capabilities of microbes in soils will aid in making engineering decisions. These decisions

could involve the design of a biostimulation system or determining if bioaugmentation is necessary.

Developing gene probes for detection of organisms and genes responsible for biodegradation of target compounds will aid in demonstrating the presence of naturally occurring PAH-degrading organisms in contaminated environments. The approach is to show that indigenous soil microbes have the enzymes and metabolic systems capable of the required contaminant transformation (Williams et al. 1999). This is done by using nucleic acid tools that can detect genes that code for enzymes involved in the transformation process. These tools can survey the entire microbial community while other methods, such as culturing, detect less than 10% of the entire community (Stapleton & Steven 1998). In the past, probing techniques have focused on phylogeny and 16S ribosomal DNA analyses to detect biodegrading species in environmental samples (Hristova et al. 2001; Loffler et al. 2000; Muyzer et al. 1993; Olsen et al. 1986). This focus on 16S ribosomal DNA has also been true for PAH-degrading mycobacteria (Cheung & Kinkle 2001; Wang et al. 1996). Ideally these nucleic acid tools could focus on genes that code for enzymes that are directly involved in the degradation of the target compounds (Beller et al. 2002; Stapleton et al. 2000). In order to use PCR and nucleic acid sequencing as a tool for detection, the targeted gene sequence should be known and the function of the gene must be well characterized (Stapleton & Steven 1998).

Mycobacteria JLS, KMS, and MCS were isolated from soils collected at the Libby, MT, Superfund site; a PAH-contaminated site undergoing bioremediation (Hurst et al. 1996; Miller et al. 2004). These mycobacteria have demonstrated the ability to degrade high molecular weight PAH including benzo(a)pyrene and pyrene. These organisms have been classified as fast growing mycobacteria with the ability to mineralize pyrene as much as 60% *in vitro* (Miller et al. 2004). The PAH degradation trends demonstrated by these organisms are similar to the PAH degradation kinetics of microcosm studies of PAH contaminated soils collected from the same site (Hurst et al. 1996). The finding of pyrene degradation in soil supports laboratory studies using IR and SR-FTIR technologies on magnetite where *M*. JLS was shown to degrade pyrene (Holman et al. 2002). These findings suggest that the *Mycobacteria* JLS, KMS, and MCS are indigenous soil microbes that play a role in biodegradation of PAH at this site.

The enzymes involved in the metabolism of PAH by *Mycobacteria* JLS, KMS, and MCS have been evaluated with the goal of using the information to create gene probes. One of the key enzymes that catalyze the initial steps in the metabolism of PAH is dioxygenase (Khan et al. 2001). Dioxygenase enzymes attack the aromatic structure of PAH by adding two oxygen atoms to the PAH molecule forming dihydrodiols (Bakermans & Madeson 2001; Heitcamp et al. 1988). A dioxygenase system involved in the initial steps of PAH degradation has been found in several known PAH degrading mycobacteria (Brezna et al. 2003). Production of these enzymes increases during PAH degradation. The enzyme is a multicomponent protein including a smaller subunit, NidB, and a larger subunit, NidA. The genetic sequences for these proteins have been discovered to reside on the bacterial genome and are not closely related to other PAH induced dioxygenase enzymes found in pseudomonad species (Churchill et al. 1999; Khan et al. 2001). Dioxygenase *nidA* and *nidB* genes have been found on *Mycobacteria* JLS, KMS, and MCS and the phylogenies of these genes have been found to cluster with other *nid* dioxygenase genes found in *M. gilvum* (AF548348 and AF548347), *M. frederiksbergense* (AF548346 and AF548345), *M. flavescens* (AF548344 and AF548343), *M. vanbaeelinii* (AF249302 and AF249301). PCR probes designed to specify dioxygenase genes isolated from pseudomonad species did not bind to dioxygenase genes in mycobacteria (McLellan et al. 2002). The order for the mycobacteria large and small subunits has been found to be unique among mycobacteria. The small subunit (*nidB*) is first, and the large subunit (*nidA*) follows in the 5′ to 3′ direction on the bacterial genome (Kahn et al. 2001; Miller et al. 2004; Moser & Stahl 2000).

The *nidA* and *nidB* genes are ideal for the development of a gene probe. They are genes from organisms that are able to degrade PAH in soil and genes that code for enzymes that are integral to the degradation pathway of PAH in these organisms. The *nidA* and *nidB* gene sequences have been used to create a gene probe for the detection of PAH degrading mycobacteria dioxygenase systems in soils undergoing bioremediation in

landfarms at the Libby, Montana, Champion International, Superfund site (U.S. EPA 1997). To the knowledge of the authors, no information exists in the literature on the development of soil gene probes for the detection of dioxygenase genes from indigenous soil mycobacteria.

The objective of the experiments described here is the development of the mycobacteria dioxygenase gene probe by demonstrating three phases of development, design, build, and test. The design phase of the gene probe is the design of PCR primers that are specific to PAH degrading mycobacteria *nid* dioxygenase genes. The build phase is the development of a procedure for DNA extraction from soil and PCR that is capable of extracting and purifying DNA from the mycobacteria in soil and amplifying the *nid* genes from the extraction. The test phase involved testing the specificity of the probe and testing the probe on soils. The gene probe was used to test soil collected from the Libby, Montana site. Soils from the site included contaminated soil undergoing bioremediation through landfarming and uncontaminated pristine soil collected from the same area. The probe was tested by extracting DNA from the soil, running PCR using three *nid* specific PCR primer sets, and for positive PCR reactions sequencing the *nidB* and *nidA* PCR products. The resulting sequences were compared to sequences for *nid* genes from PAH-degrading mycobacteria.

Materials and methods

Cultures

M. JLS cultures were grown in dilute Luria broth (LB) medium in 200 ml flasks, shaking at 220 rpm at 25 °C. Dilute LB medium consisted of one part LB (Difco, Becton Dickinson, Sparks, MD) to nine parts by volume of Basal Salts media (2.38 g $(NH_4)SO_4$, 0.28 mg $FeSO_4*7H_2O$, 10.69 mg $CaCl_2*7H_2O$, 0.25 g $MgSO_4*7H_2O$, 0.50 g NaCl, 1.42 g Na_2HPO_4, 1.36 g KH_2PO_4, pH 6.5 in one liter water). Five-day-old cultures were used for analysis and various inoculations. The five-day cultures correspond to observations of stationary phase for *Mycobacteria* JLS, KMS, and MCS (Miller et al. 2004). Other cultures used for probe specificity experiments were also grown for 5 days under the same conditions and in the same medium. These cultures included *M. vanbaleenii* (Khan et al. 2002), *Erwinia herbicola, Pseudomonas pv. syringae* B728a, *Pseodomonas auriginosa, Bacillus pamilus, Bacillus megaterium, Burkholderia cepacia, Brevibacterium acetylicum, Clavibacter michiganensis insidiosum, Pseodomonas putida* isolate Corvallis, two strains of *Escherichia coli* (XL-1 blue and DH5 alpha), *Lactococcus lactis, Lactobacillus casei,* and the Methyl *tert*-Butyl Ether-Degrading Strain PM1 (Hristova et al. 2001). These bacteria were chosen based on availability and include gram positive and soil-isolated organisms. pseudomonad species were tested because some pseudomonad strains have shown PAH metabolizing capabilities.

Soils

The soil collected from the Champion International Superfund Site in Libby, MT, was PAH contaminated soil that had been treated in land treatment unit 2 (LTU2) and was collected in 2002. This soil was sandy loam (54% sand, 34% silt, and 12% clay). The soil had a pH of 7.48, an EC of 3.9 mhos/cm, and 2.7% organic carbon. A background (uncontaminated) soil was also collected from nearby the LTUs. The soil was passed through a 1.7-mm sieve and stored in the dark at 4 °C. The sand control was standard Ottawa sand from EM Science (Darmstadt, Germany). Sand was sterilized by autoclaving at 121 °C for 60 min, stored at room temperature for 24 h to allow fungal and bacterial spore germination and sterilized again by autoclaving for 60 min.

Polymerase Chain Reaction (PCR)

PCR oligonucleotide primers were ordered from Qiagen Operon (Alameda, Ca), and all PCR reagents were purchased from MBI Fermentas, Inc. (Amherst, NY). All PCR was performed using an Eppendorf Mastercycler^R thermocycler and 0.20-ml tubes. PCR primers were designed based on homology between the known mycobacteria *nid* dioxygenase genes. The primers (Table 1) were designed to target four regions on the mycobacteria genome: the entire *nidA* gene, the first half of the *nidA* gene, the entire *nidB* gene and a section of the genome constituting part of the *nidB* and *nidA* genes and including the region between the genes. When mycobacteria genomic DNA was used as a

Table 1. Summary of PCR primers used in dioxygenase studies

Primer name	Primer	Primer set target
nidA forward	ATGACCACCGAAACAACCGGA	*nidA* 1st half of subunit
nidA 735–714 reverse	AATGTTAGACACGAAGTGCCG	*nidA* 1st half of subunit
nidA reverse	TCAAGCACGCCCGCCGAATGC	*nidA* entire subunit
nibB forward	ATGAACGCGGTTGCGGTCGAT	*nidB* entire subunit
nidB reverse	CTACAGGACTACCGACAGGTT	*nidB* entire subunit
nidB 270–290 forward	GCGGACCCGGCACTTCGTGTC	*nidB-nidA* region
nidA 171–151 reverse	AAACACCCAGGCGCGCCCGAA	*nidB-nidA* region

template the primer set targeting the first half of *nidA* produced a product 735 bp long in *Mycobacteria* JLS, KMS, and *M. vanbaleenii* and 744 bp in MCS. The primer set targeting the entire *nidA* gene produced the products 1368 bp in *M.* JLS, *M.* KMS, and *M. vanbaleenii,* and 1377 bp in *M.* MCS. The *nidB* primer set produced a product 510 bp long, and the *nidB* to *nidA* primer set produced a product 471 bp long in *M.* JLS, *M.* KMS, and *M. vanbaleenii,* and 480 bp in *M.* MCS. The *nidA* and *nidB* primer sets were designed from homology with *M. vanbaleenii* and the length of the products was confirmed with the sequencing of *Mycobacteria* JLS, KMS, and MCS (Miller et al. 2004). The *nidB* to *nidA* primer set was designed based on 100% homology with seven known mycobacteria dioxygenase *nid* genes.

The PCR cycling conditions were; a four minute hotstart at 94 °C, cooling to 4 °C for addition of Taq polymerase, 40 cycles of 94 °C denaturation for one minute, 60 °C annealing, 72 °C lengthening for one minute (2 minutes for the *nidA* forward and reverse primers), at the end of cycling 72 °C for 5 min and storage at 4 °C. The reagent concentrations were 27.5–29.5 μl sterile deionized water, 5 μl of 10× PCR buffer with and without ammonium sulfate, 2 μl of 1 mM dNTP, 8 μl of 25 mM $MgCl_2$ solution, 2 μl of each primer solution (10 μM), 0.5 μl of Taq polymerase (diluted in 9.5 μl of water prior to addition), and 3 μl of template solution. Templates were isolated genomic DNA, cell cultures grown under conditions stated above, or DNA soil extractions. All PCR experiments included isolated purified genomic DNA from *Mycobacteria* JLS, MCS, or KMS as positive controls and blank samples without template added as negative controls on the PCR reaction. All PCR products were observed using electrophoreses of the PCR products on 1.1% agarose gels stained with ethidium bromide. The gels were marked by loading 7 μl of Gene Ruler™ 100 bp DNA Ladder Plus (0.1 mgDNA/ml) from MBI Fermentas.

Sequencing and analyses

Nucleotide sequencing was performed by automated sequencing using dye-labeled universal terminators on an Applied Biosystem (ABI) 373 DNA sequencer (Foster City, CA). Nucleotide sequences were obtained from both strands of each PCR product. The dioxygenase sequences were aligned using BLAST (National Center for Biotechnology Information). These aligned sequences were then compared to the seven *nidB* and the seven *nidA* genes in the genbank database. These *nid* genes included *nidA* genes from *M. gilvum* strain BB1 (AF548347), *M. frederiksbergense* strain FAn9T (AF548345), *M. flavescens* strain PYR-GCK (AF548343), *M. vanbaleenii* (AF249301), *M.* JLS (AY330098), *M.* MCS (AY330102), *M.* KMS (AY330100). These *nid* genes included *nidB* genes from *M. gilvum* strain BB1 (AF548348), *M. frederiksbergense* strain FAn9T (AF548346), *M. flavescens* strain PYR-GCK (AF548344), *M. vanbaleenii* (AF249302), *M.* JLS (AY330098), *M.* MCS (AY330101), *M.* KMS (AY330099). Substantial regions of the *nid* genes (over 400 bp in the *nidB* and over 700 bp in the *nidA*) were compared and the percentage presented in the results is the number of nucleotide matches.

DNA extraction from soil

During DNA extraction and purification from soils co-extraction of humic substances inhibits taq polymerase in PCR (Smalla et al. 1993; Tsai

& Olsen 1992). Bead beating combined with freeze thawing has been found to be most effective at lysing and extracting DNA from gram-positive bacteria. Purification of this extracted DNA by passing the extracted material through resins has been reported to be the most effective technique for purifying DNA extracts from soil for PCR reactions (Zhou et al. 1996). Sterilized sand was extracted as a blank sample to verify the extraction procedure did not contaminate the samples.

DNA was extracted using a combined freeze thaw, vortex with glass beads and chromatographic removal of PCR inhibitors in PCR inhibitor binding resins. The materials used for this procedure were obtained from the Ultra Clean^{TM} Soil DNA Isolation Kit from MoBio^{R} Laboratories, Inc. (Solana Beach, CA), and the Soil Master^{TM} DNA Extraction kit from Epicentre^{R} (Madison, WI). The procedures used were a combination of standard protocols for these kits with some modifications. Soil samples (0.30 g wet weight) were placed in the Ultra Clean^{TM} Soil DNA Isolation Kit bead beater tubes. The tubes were placed at −80 °C for 10 min and then immediately thawed in a water bath at 60 °C. The tubes were secured to a vortex machine and vortex mixed for 10 min at maximum speed. They were then centrifuged at maximum speed for 30 s, the supernatant was placed in a clean microcentrifuge tube, centrifuged again for 30 s, and the supernatant was placed in a clean microcentrifuge tube. One ml of cold (−20 °C) isopropyl alcohol was added to precipitate the nucleic acids. Following incubation at 6 °C for 10 min the tubes were centrifuged for 10 min at maximum speed. The alcohol was decanted and the pellet was suspended in 180 μl of sterile deionized water. Residual protein was precipitated and inhibitors removed using the standard protocol for the Soil Master^{TM} DNA Extraction kit from Epicentre^{R}

Table 2. Summary of the specificity of *nidB* and *nidA* genes

Bacteria	*nidB* forward *to nidB* reverse		*nidA* forward *to* nidA reverse	
	Number of PCR products	PCR product size(s), bp	Number of PCR products	PCR product size(s), bp
M. KMS	1	510	1	1368
M. JLS	1	510	1	1368
M. MCS	1	510	2	1390 and 1000
M. *vanbaleenii*	1	510	1	1368
Erwinia herbicola	0	N/A	2	> 3000 and 1350
Pseudomonas pv. *Syringae* B728a	0	N/A	1	3000
Pseudomonas auriginosa	0	N/A	0	N/A
Bacillus pamilus	0	N/A	0	N/A
Bacillus megaterium	0	N/A	1	150
Burkholderia cepacia	0	N/A	0	
Brevibacterium acetylicum	0	N/A	1	500
Clavibacter mich. *insidiosum*	1	Weak product at 500	2	1350 and 500
Pseudomonas putida isolate Corvallis	0	N/A	1	Weak product at 500
E-coli (XL-1 blue)	0	N/A	1	Weak product at 500
E-coli (DH5 alpha)	0	N/A	5	Weak products at > 3000, 1400, 700, 500, < 500
L. *lactis*	0	N/A	0	N/A
Lb. *Casei*	0	N/A	0	N/A
PM-1	0	N/A	0	N/A

starting with the protein precipitation step (Epicentre 2003). The final purified DNA pellet was suspended in 50 μl of TE buffer.

Results and discussion

Primer design and specificity experiments

The specificity of PCR using primers designed to target *nidA* and *nidB* primer sets was tested using the primer sets that targeted the entire *nidA* and *nidB* genes. After 5 days all cultures showed visible growth, and spectrometry measurements suggested similar concentrations to the mycobacteria cultures. PCR was performed from aliquots of the bacterial cultures. PCR products showed that *nidB* primers were more specific than *nidA* primers (Table 2). It is not known whether any of the organisms tested besides the mycobacteria can mineralize PAH. Although the PCR products were much less intense than those produced by mycobacteria, *Clavibacter michiganensis insidiosum* produced products approximately the same size as the *nidB* and *nidA* genes for both primer sets. Clavibacter are gram positive and they are often found in the environment (Marcell & Beattie 2002), however no known data exists on the ability of these organisms to degrade PAH. Other PCR products produced from *nidA* primers were not near the size of the *nidA* gene and they were often less intense suggesting the possibility of inaccurate priming of the PCR primers.

The specificity experiment suggested that a battery of PCR primer sets derived from the known sequences for the *nid* genes was necessary in order to create a probe that is specific to *nid* dioxygenase genes. In addition the PCR products that were suspected to be *nid* dioxygenase genes were sequenced for confirmation. This approach was used because, although some bacteria produced multiple PCR products at low stringency conditions with one of the *nid* primer sets, only the mycobacteria produced single intense PCR products of the expected sizes in both reactions. The multiple products may be the result of low stringency priming. Low stringency priming occurs when the primers bind to regions of DNA that are not exactly homologous to the primer. Low stringency priming or mis-priming does not necessarily represent homology between the primer and the target DNA. Since low stringency conditions needed to be maintained in order to produce products from the extraction, products were verified by using multiple primer sets and sequencing the products.

For probing purposes the *nidA* primer set targeting the entire gene was replaced with the *nidA* forward to *nidA* 735–714 reverse primer set to increase PCR efficiency by decreasing the total product length. Also, the *nidB* to *nidA* primer set was created to take advantage of the homology between *M. gilvum* (AF548348 and AF548347), *M. frederiksbergense* (AF548346 and AF548345), *M. flavescens* (AF548344 and AF548343), *M. vanbalinii* (AF249302 and AF249301), and the *Mycobacteria* JLS, KMS, and MCS. This *nidB* to *nidA* primer set was also designed to take advantage of the unique gene order of the dioxygenase subunits. These two new primer sets combined with the *nidB* primer set comprised the dioxygenase gene probe. A positive probe resulted when PCR for each primer set produced intense products in the expected size range specified by each respective primer set on the *nid* dioxygenase gene system and confirmed by sequencing the most intense products and comparing those sequences to known *nid* dioxygenase genes from mycobacteria.

Probe testing on soils

The *nid* dioxygenase probe was tested on LTU and background soils. Lack of contamination in the extraction and in the PCR reagents was verified by including PCR reactions without template added and PCR reactions with extractions from sterilized clean sand as template. Neither of these samples produced products with any of the three primer sets. The results of the probe test indicate that the *nid* dioxygenase gene probe detected *nid* dioxygenase genes in the LTU2 soil (Figures 1–3). The detection is evident by the intense PCR products that are approximately 510 bp for the *nidB* primer set, 471 bp for the *nidB* to *nidA* primer set and 735 bp for the *nidA* primer set.

The test on background soil did not conclusively demonstrate that the mycobacteria were present in the soils (Figures 1–3). Background soil is uncontaminated soil that is not undergoing bioremediation but is expected to represent the LTU2 soil before contamination and bioremediation. The presence or absence of mycobacteria in

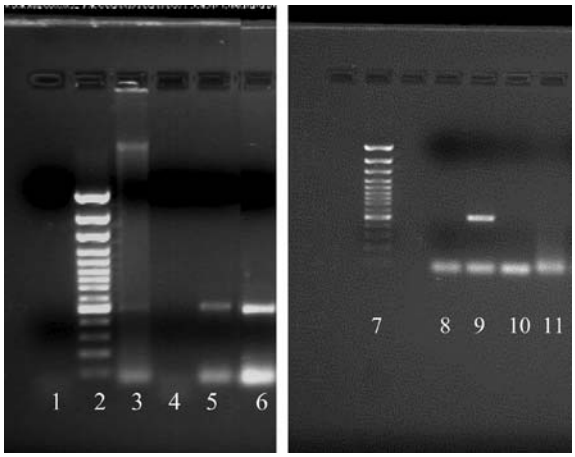

Figure 1. Soil gene probe, n*idB* target region. 1. Sterile sand extraction, 2. 100 bp DNA Ladder plus, 3. *M*. JLS DNA, 4. PCR blank (no template), 5. LTU soil extraction, 6. LTU soil extraction, 7. 100 bp DNA Ladder plus, 8. PCR blank (no template), 9. *M*. JLS DNA, 10. Sterile sand extraction, 11. background soil extraction.

this soil is unconfirmed. PCR on the background soil extraction using the *nidB to nidA* primer set produced the expected PCR product (471 bp). PCR on the background soil using the *nidA* primers produced faint multiple PCR products. PCR on the background soil using the *nidB* primer set produced no PCR products.

The dominant PCR products that were present in the agarose gels for the test on LTU2 soil were presumed to be amplified *nid* genes from native PAH degrading mycobacteria in the soil. This presumption was verified by extracting and sequencing two of these PCR products. *Mycobacteria* JLS, KMS, and MCS were isolated from the same soil samples (Miller et al. 2004). It is therefore a reasonable assumption that these three distinct PAH degrading organisms may still survive in the soil. Other uncultured PAH-degrading mycobacteria may also survive in the soil. Therefore, it was suspected that the PCR products from the probe of LTU soil could have been several *nid* genes from several different organisms. Difficulties in sequencing these PCR products were expected and were encountered. The sequencing yielded indeterminate regions notably after the first 21 nucleotides of the *nidA* gene there is a 9 bp insertion in MCS that is not present in the *nid* genes from other mycobacteria. As suspected the *nidA* sequences from the LTU soil probe was indeterminate in this region. Although there were indeterminate regions, substantial regions of the *nidB* and *nidA* gene sequences were obtained, including a 420 bp region for *nidB* and a 722 bp region for *nidA*. These regions were compared to the *nid* gene sequences from seven known PAH-degrading mycobacteria. The results indicate a 97–98%

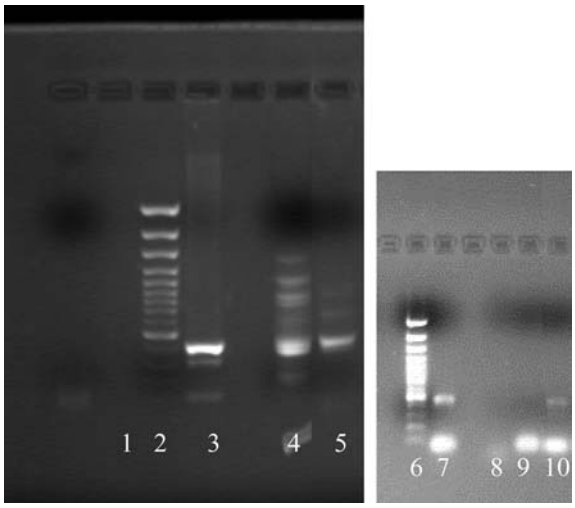

Figure 2. Soil gene probe, n*idB* to *nidA* target region. 1. Sterile sand extraction, 2. 100 bp DNA Ladder plus, 3. *M*. JLS DNA, 4. LTU soil extraction, 5. LTU soil extraction, 6. 100 bp DNA Ladder plus, 7. *M*. JLS DNA, 8. blank (no template), 9. Sterile sand extraction, 10. background soil extraction.

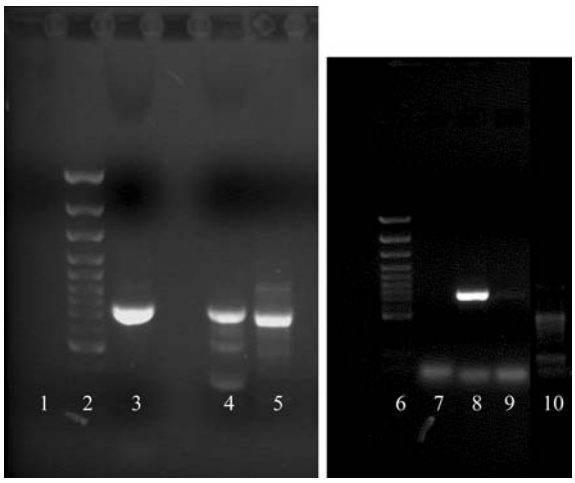

Figure 3. Soil gene probe, *nidA* target region. 1. sterile sand extraction, 2. 100 bp DNA Ladder plus, 3. *M*. JLS DNA, 4. LTU soil extraction, 5. LTU soil extraction, 6. 100 bp DNA Ladder plus, 7. blank (no template), 8. *M*. JLS DNA, 9. sterile sand extraction, 10. background soil extraction.

Table 3. Comparison of sequences from the LTU soil probe to known PAH-degrading mycobacteria *nid* genes

Mycobacteria	Comparison of LTU soil probed *nidB* gene to known *nidB* genes		Comparison of LTU soil probed *nidA* gene to known *nidA* genes	
	Ratio*	Percentage matched	Ratio*	Percentage matched
M. KMS	410/418	98	721/722	99
M. JLS	410/418	98	415/722	99
M. MCS	413/418	98	668/703	95
M. vanbaleenii nidB	406/418	97	721/722	99
M. gilvum strain BB1 nidB	414/418	99	710/716	99
M. frederiksbergense strain FAn9T nidB	407/418	97	709/716	99
M. flavescens strain PYR-GCK	406/418	97	718/722	99

*The ratio is number of nucleotide matches: number of nucleotides compared.

nucleotide identity match for the *nidB* gene and a 95–99% identity match for the *nidA* gene (Table 3). The identity matches indicate that the positive gene probe results indicated in the electrophoreses gels of the PCR reactions do in fact represent detection of *nid* genes from PAH degrading mycobacteria in the LTU soil. The percentage of identity matches between the soil probe products and the known PAH degrading mycobacteria *nid* genes is similar to the natural identity between known PAH degrading mycobacteria *nid* genes within this soil system (Miller et al. 2004).

The results of this dioxygenase gene probe test indicate that this probe can be developed into useful tools in monitoring bioremediation. The probe can be expanded for many uses including quantitative real time PCR and functional gene probe arrays (Beller et al. 2002; Stapleton et al. 2000). In order to use the probe for monitoring of bioremediation, the probe can be combined with quantitative PCR or real time PCR techniques. The template quantifying capabilities of real time PCR can be used with the same soil DNA extraction techniques and knowledge of *nid* gene sequences used in this study to allow quantification of *nid* dioxygenase DNA in soil samples for monitoring of *in situ* soil bioremediation. A significant obstacle to using quantitative PCR for this purpose will be quantifying the effect of the soil extraction procedure on the original relative amounts of template DNA in soil. The authors are currently conducting research to extend the current procedure to develop a quantitative gene probe.

The genetic information and PCR primer sets can be an invaluable addition to the creation of functional gene probe arrays for the detection of soil bioremediation capabilities and for monitoring biodegradation activities. The *nid* dioxygenase probe can be combined with dioxygenase probes derived from the pseudomonad dioxygenases. The *nid* genes that are functionally specific to PAH metabolism can be combined with similar genes for catabolic enzymes involved in the transformations of environmental pollutants such as methyl *tert*-butyl ether, trichloroethene, toluene and other common pollutants to characterize bioremediation capabilities in soils and sediments (Hristova et al. 2001; Stapleton et al. 2000; Woo et al. 2000). This array will expand the classification abilities of gene probes for detection of PAH biodegrading organisms in soils and other environmental matrices including sediments and biofilms (Moser & Stahl 2000). The probe array can be performed using multiple PCR reactions or using microarray technology (Wu et al. 2001).

Conclusions

The *nid* dioxygenase gene probe can be used to aid in detecting the presence of PAH degrading mycobacteria in soils. Detection of these organisms gives information on the biodegradation and bioremediation capabilities of the indigenous microbial communities of contaminated soils. The *nid* probe was designed to be specific to PAH degrading mycobacteria. The probe was built as a soil probe by using a soil DNA extraction tech-

nique that was able to extract PCR quality DNA from mycobacteria in a soil matrix. The test results demonstrate that the *nid* dioxygenase soil gene probe is capable of identifying *nid* genes in soils undergoing bioremediation. These soils are the same soils undergoing bioremediation of PAH and from which the *Mycobacteria* JLS, KMS, and MCS were isolated. The probe has been verified by sequencing the PCR products and comparing the sequences to known PAH-degrading mycobacteria *nid* genes. The test verification indicates a high degree of similarity (95–99%) between the probe and the *nid* dioxygenase genes of known PAH degrading mycobacteria.

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