DETERMINATION OF THE PRESENCE OF THE CATABOLIC ALKANE MONOOXYGENASE GENE FROM SOIL MICROORGANISMS ISOLATED FROM COASTAL SAND DUNES

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ABSTRACT

Thirty-nine bacteria were isolated and characterized from sand dune soils in Guadalupe, CA. The soils had varying levels of petroleum contamination. The isolates were screened by polymerase chain reaction (PCR) for the presence of the alkane hydroxylase gene, *alk*B. Two sets of oligonucleotide primers were designed using the *Pseudomonas oleovorans alk*B gene sequence. Ten amplified DNA fragments of differing sizes from the first primer set were extracted from agarose gels and sequenced to confirm homology to the *Ps. oleovorans alk*B gene. None of the fragments sequenced were homologous to *Ps. oleovorans alk*B gene. The isolates were screened with a second set of primers containing degeneracies to account for variations in the *alk*B gene sequences. Six DNA fragments of similar size to the *Ps. oleovorans* fragment were amplified with this primer set. The fragments were from six isolates whose genera are known to degrade petroleum hydrocarbons. Further studies should include sequencing the six amplified DNA fragments to confirm homology to the *Ps. oleovorans alk*B gene.

INTRODUCTION

Biodegradation of petroleum hydrocarbons by microorganisms has been an area of interest for many years. Natural populations of microorganisms are one of the main ways petroleum and other hydrocarbon pollutants are removed from the environment (3). Petroleum released from geological deposits and decomposed plant material make hydrocarbons available to microbial communities (6). The prevalence of oil spills into the environment has increased interest in studying microbes involved in biodegradation of hydrocarbons. Many different aspects of these communities are studied including isolating and identifying the microorganisms present and determining what roles they play in the biodegradative processes.

One aspect of petroleum hydrocarbon degradation includes determining what enzymatic pathways the microorganisms utilize to degrade the hydrocarbons. In general, microorganisms degrade these hydrocarbons by first inserting molecular oxygen into the hydrocarbon structures. Most often this reaction is catalyzed by an oxygenase enzyme complex (6). For short chain *n*-alkanes (C_5 to C_{12}), an alkane monooxygenase enzyme begins the primary attack on the hydrocarbon (2). The general attack on the hydrocarbon is as follows:

$$\text{substrate-H} + O_2 + AH_2 \xrightarrow{\text{monooxygenase}} \text{substrate-OH} + A + H_2O$$ (AH_2 is an oxidizable co-substrate, usually NADH) (2)

The terminal oxidation of an *n*-alkane produces the corresponding *n*-alcohol (6).

Alkane oxidation by *Pseudomonas oleovorans* has been investigated in depth. Studies have revealed that *Ps. oleovorans* has two gene regions that contain the *alk* regulon located on the OCT-plasmid (OCT for *n*-octane utilization) (5). Alkane monooxygenase is comprised of a three-polypeptide protein complex that carries out alkane degradation (2, 5, 8). The first gene involved in alkane oxidation is *alk*B, an alkane hydroxylase, which is a cytoplasmic membrane protein (5, 8). Another gene, *alk*T, a rubredoxin reductase located in the cytoplasm, acts as an electron carrier between NADH and the hydroxylase (8). Figure 1 depicts the terminal oxidation of an *n*-alkane by *Ps. oleovorans* (2), with the alkane hydroxylase-catalyzed reaction occurring in the cytoplasmic membrane.

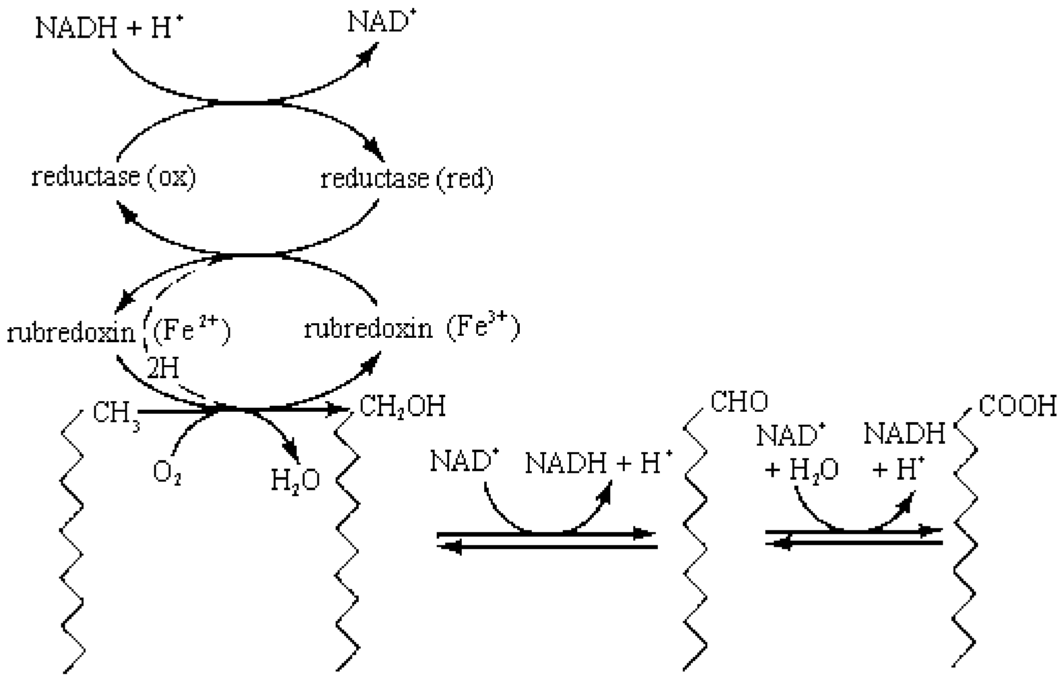

Figure 1. Terminal oxidation of an *n*-alkane by *Pseudomonas oleovorans.* (2)

Previous studies (9, 10) similar to this one involved determining the presence of *alk*B in isolates from Arctic environments using the *Ps. oleovorans alk*B primers. Multiple DNA fragment amplification was observed in the studies and Southern hybridization was used to confirm homology to the *Ps. oleovorans alk*B gene. Many of the amplified fragments did not hybridize with the probe. These primers are specific for the *Ps. oleovorans alk*B gene on the OCT plasmid, and seem to work only with the gene from the OCT plasmid.

The objective of this investigation was to examine bacteria isolated from coastal sand dunes for the presence of the alkane hydroxylase gene, *alk*B. Isolates from sand dune soils were screened by PCR to determine the presence of *Ps. oleovorans alk*B, and suspected positive DNA fragments were sequenced. Isolates were also screened with a second set of primers to determine the presence of the *alk*B gene.

MATERIALS AND METHODS

Isolation of the Petroleum Degrading Bacteria

Soil samples were taken at a six-inch depth from the Guadalupe Dune site. Multiple samples of each type were drawn from pristine (S1), mildly contaminated (S3), and heavily contaminated (S5) sites. Three bacteria (S3MD4A, S3MD4B, and S3MD5) were isolated using the spread plate method on a Minimum Diluent Agar (MDA) as the isolation medium. MDA is composed of 50 mM KH_2PO_4, 50 mM Na_2HPO_4, 80 mM NH_4Cl, 0.036 mM $FeSO_4$, 0.40 mM $MgSO_4$, 0.50 mM $CaCl_2$, 0.008% yeast, and 0.500 mL filter-sterilized diluent. The other thirty-six bacteria were isolated using the spread plate method on a Soil Diluent Agar (SDA) as the isolation medium. SDA is composed of a soil extract solution (using contaminated soil and tap water) along with filter-sterilized diluent (0.500 mL), agar (15 g/L), 38 mM $(NH_4)_2SO_4$, 7.4 mM K_2HPO_4, 0.008% yeast extract, and filter-sterilized cyclohexamide solution (10 mL/L). After twenty-one days, colonies were picked from the MDA/SDA based on differences in colony morphology. Isolates were transferred to Trypticase Soy Agar (TSA) for growth before DNA extraction using the Fast Prep method (Bio 101, Alta Vista, CA). Gram stains, catalase tests, oxidase tests, and glucose fermentation tests were performed on the isolates.

PCR Amplification

16S rDNA

PCR was performed using PAF and 531R primers (1) corresponding to positions 8 and 531 on *Escherichia coli* 16S rDNA. This resulted in an amplified DNA fragment of approximately 530 base pairs. Final concentrations of the reaction mix were 10 mM Tris-HCl (pH 8.0), 1 mM dNTP, 20 μg/mL BSA, 2.5 mM $MgCl_2$ and 2 U Amplitaq (PE Applied Biosystems). Reaction temperatures and cycling were done as follows: 94º C for 2 min, forty cycles of 94º C for 30 sec, 56º C for 30 sec, 72º C for 30 sec, and one cycle of 72º C for 7 min. All PCR products were visualized on a 1.5% agarose gel.

***alk*B gene**

PCR was performed with two different primer sets to determine the presence of the catabolic gene for alkane monooxygenase (*alk*B). Primers *alk*B-F (5′ TGGCCGGCTACTCCGAT-GATCGGAATCTGG 3′) and *alk*B-R (5′ CGCGTGGTGATCCGAGTGCCGCTGAAGGTG 3′) were used corresponding to positions 703 and 1572 on *Ps. oleovorans* ATCC 29347 16S rDNA, resulting in amplification of a DNA fragment of approximately 870 bp (9, 10). The second set of primers *alk*B-F477 (5′ AGGGTACNGTARATTCTTTATTGAGCAT 3′) and *alk*B-R911 (5′ AGRYTTARASACGATGRKRGCCGTATTCC 3′) were designed using Lasergene Software Suite (DNASTAR, Inc. Madison, WI), corresponding to positions 477 and 911 on *Ps. oleovorans* ATCC 29347 16S rDNA. This resulted in an amplified DNA fragment of approximately 430 bp (7). Final concentrations of the reaction mix were 10mM Tris-HCl (pH 8.0), 800 mM dNTP, 2.0 mM $MgCl_2$ and 2.0 U Amplitaq (PE Applied Biosystems). Reaction temperatures and cycling were done as follows: 96º C for 2 min, thirty-five cycles of 94º C for 1 min, 55º C for 1 min, 72º C for 1 min, and one cycle of 72º C for 7 min (9). The annealing time was modified to 53.5° C after optimization trials. All PCR products were visualized on a 1.2% agarose gel.

Nucleotide Sequencing

16S rDNA

Amplified 16S DNA fragments from isolated strains were concentrated using Microcon centrifugal filter devices (Millipore Corporation, Bedford, MA). DNA sequencing was performed using the Dye Terminator Cycle Sequencing Ready Reaction with polymerase FS (PE Applied Biosystems). The 16S rDNA sequences were used to conduct searches in non-redundant DNA databases (BLAST).

***alk*B gene**

DNA amplified using the *alk*B primers was concentrated using Microcon centrifugal filter devices (Millipore Corporation, Bedford MA). DNA fragments chosen for sequencing were then extracted from an agarose gel following the protocol of the QIA Gel Extraction Kit (Qiagen, Chatsworth, CA). DNA sequencing was performed using the Dye Terminator Cycle Sequencing Ready Reaction with polymerase FS (PE Applied Biosystems). The amplified sequences were then used to conduct searches in non-redundant DNA databases (BLAST).

RESULTS

*alk*B primers corresponding to *Pseudomonas oleovorans* ATCC 29347

Multiple DNA fragment amplification was observed with these primers. The positive control, *Ps. oleovorans* ATCC 29347, exhibits two fragments after its PCR amplification. The first fragment is the 870 bp amplification, while the second fragment is approximately 280 bp. Isolates were designated positive if amplification resulted from the multiple PCRs performed. The positive amplification did not have to be the same size as the *Ps. oleovorans* DNA fragment. None of the suspected "positive" fragments were the same size as the one from *Ps. oleovorans.*

Figures 2, 3, and 4 illustrate the multiple band amplification found in *Ps. oleovorans* and the isolates. The bright amplifications were taken to be a positive result for *alk*B, and the were the DNA fragments that underwent gel extraction and sequencing to verify homology to *Ps. oleovorns alk*B.

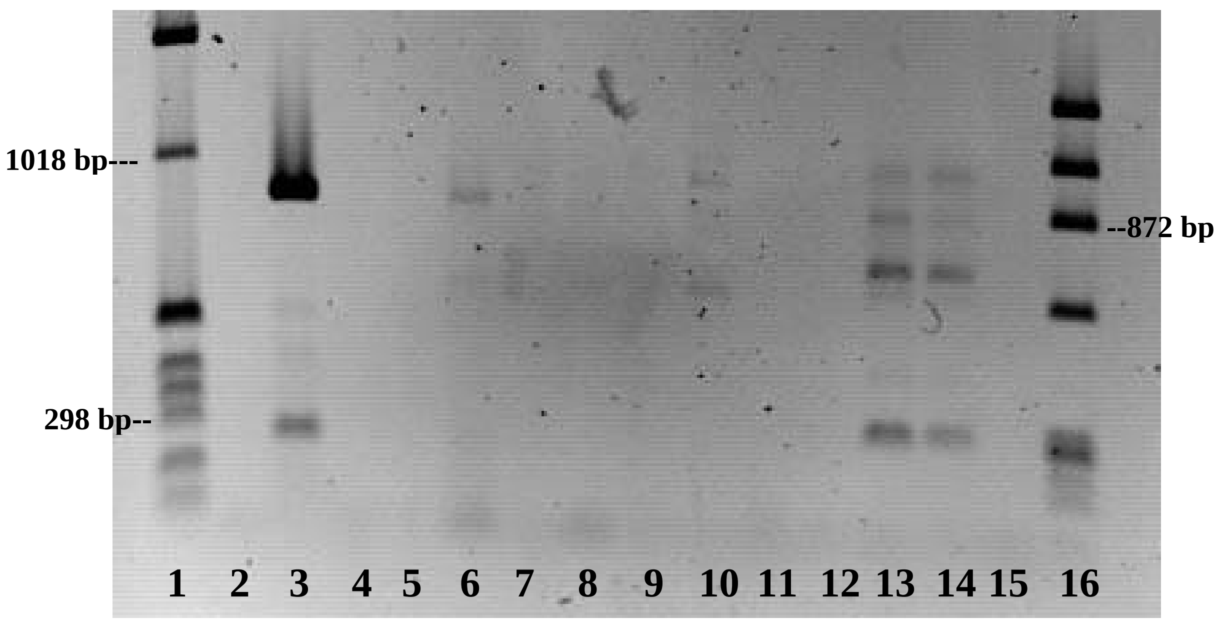

Figure 2. Detection of *alk*B in S1 isolates by PCR with *Ps. oleovorans* primers. 1.2% agarose gel electrophoresi Lanes: 1, 1 Kb DNA Ladder; 2, closed control; 3, *Ps. oleovorans* (*alk*B-positive control); 4, S1RS2; 5, S1RS3A; 6, S1RS4; 7, S1RS6; 8, S1RS7; 9, S1RS8; 10, SIRS9; 11, S1RS10; 12, S1RS11; 13, S1RS12; 14, S1RS16; 15, open control; 16, φX174/Hae*III* Ladder.

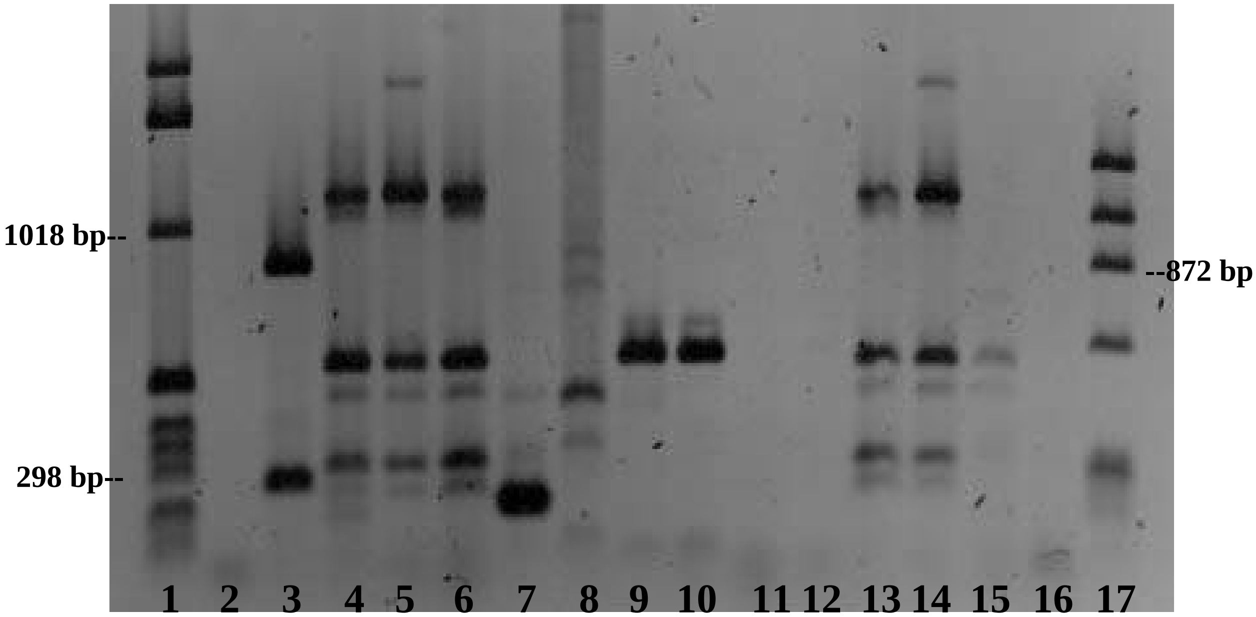

Figure 3. Detection of *alk*B in S3 isolates by PCR using *Ps. oleovorans* primers. 1.2% agarose gel electrophoresis. Lanes: 1, 1 Kb DNA Ladder; 2, closed control; 3, *Ps. oleovorans* (*alk*B-positive control); 4, S3LS1; 5, S3EH1; 6, S3EH2; 7, S3EH3; 8, S3EH4; 9, S3EH7; 10, S3EH8; 11, S3RS2; 12, S3RS6; 13, S3MD4A; 14, S3MD4B; 15, S3MD5; 16, open control; 17, φX174/Hae*III* Ladder.

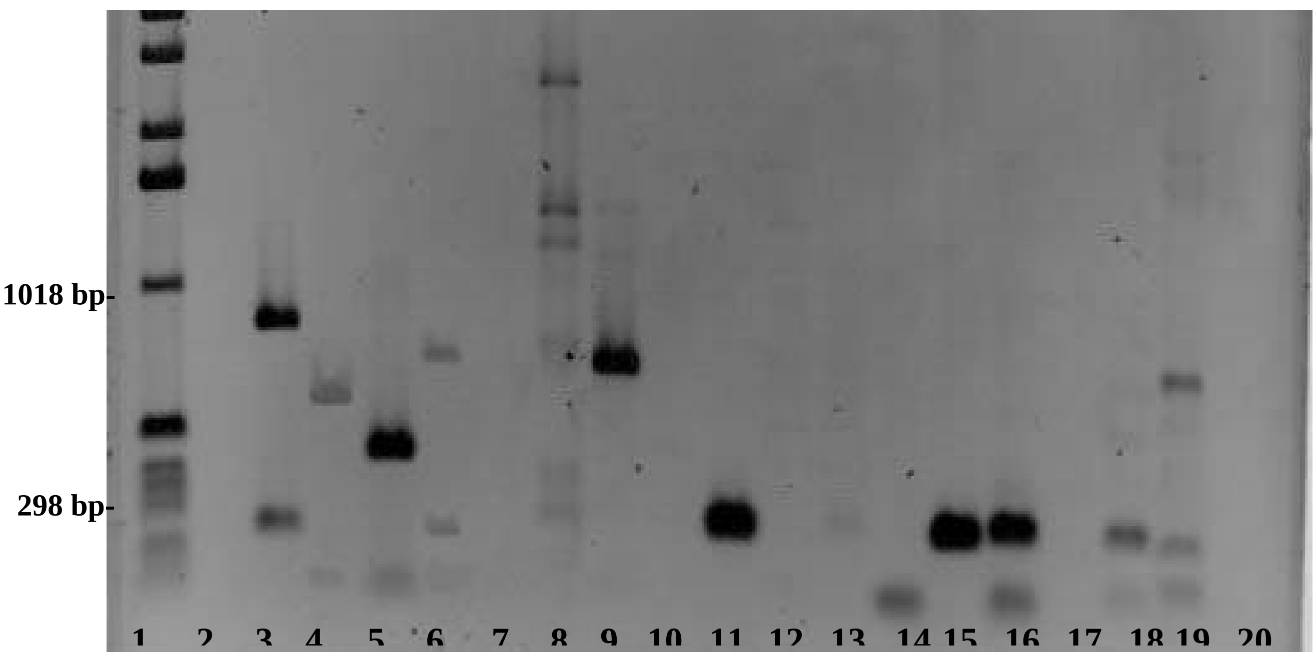

Figure 4. Detection of *alk*B in S5 isolates by PCR using *Ps. oleovorans* primers. 1.2% agarose gel electrophoresis. Lanes: 1, 1Kb DNA Ladder; 2, closed control; 3, *Ps. oleovorans* (*alk*B-positive control); 4, S5MM1; 5, S5MM2; 6, S5MM3; 7, S5MM4A; 8, S5MM5; 9, S5MM6; 10, S5MM7; 11, S5MM9; 12, S5MM11; 13, S5MM12; 14, S5MM13; 15, S5MM14; 16, S5RS1B; 17, S5RS2; 18, S5RS3; 19, S5RS4; 20, open control.

Table 1 lists the isolates having DNA fragments successfully isolated and sequenced. No DNA fragment sequences were homologous to the *Ps. oleovorans alk*B gene. Attempts to sequence fragments from isolates S3EH1, S3EH3, and S3EH4 were unsuccessful. Due to the unexpected results of the initial round of sequencing, sequences were not obtained for the rest of the suspected positive PCR fragments.

Table 1. BLAST ID results of apparent positive *alk*B isolates

Isolate	BLAST ID	E value
Ps. oleovorans(870 bp)	*P. oleovorans* TF4-1L (+OCT) plasmid *alk*B gene	0.0
Ps. oleovorans (280 bp)	*Bordatella bronchioseptica ris*A and *ris*S genes	3E-10
S1RS12 (280 bp)	*Streptomyces coelicolor* cosmid M11	3.7
S1RS16 (280 bp)	*Streptomyces coelicolor* cosmid M11	4.1
S3LS1	*Caenorhabditis elegans* cosmid F59A3	0.14
S3EH2	*Arabidopsis thaliana* DNA chromosome 4	0.85
S3EH7	*Streptomyces coelicolor* cosmid 3C3	0.007
S3EH8	*Streptomyces coelicolor* cosmid 3C3	0.007
S3MD4A	*Pseudomonas aeruginosa nir*C gene	0.53
S3MD4B	*Arabidopsis thaliana* DNA chromosome 4	0.86
S3MD5	*M. musculus Sry* locus	0.37
S5MM1	*Paracoccus denitrificans* NADH dehydrogenase	4E-47

DNASTAR®-designed *alk*B degenerate primers

Amplification of multiple fragments did not occur using these primers. A single 430 bp DNA fragment amplified in all positive cases. Out of the thirty-nine isolates, six isolates produced DNA fragments of the same size as *Ps. oleovorans.* Table 2 lists these isolates and their 16S rDNA matches in the GenBank database.

Table 2. Identification of *alk*B positive isolates (degenerate primers).

ISOLATE	BLAST ID	E Value
S1RS12	Rhodococcus sp.	0.0
S5RS3	Alcaligenes sp.	0.0
S5MM1	Unidentified alpha-proteobacterium	E-173
S5MM2	Phyllobacterium myrsinacearum	0.0
S5MM3	Bacillus halmapalus DSM 8723	0.0
S5MM5	Microbacterium terrae	0.0

Figure 5 illustrates the single-band DNA fragment amplification with the six positive isolates and *Ps. oleovorans*. 20 µL of DNA, as opposed to 5 µL of DNA, was loaded into each lane of Figure 5 to achieve visualization of positive amplifications.

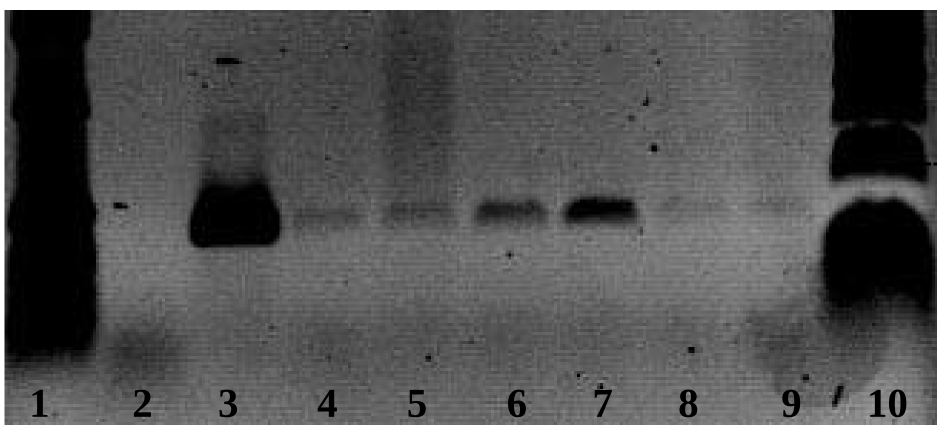

Figure 5. Positive isolates amplified by PCR with degenerate primers designed to detect the presence of *alk*B. 1.2% agarose gel electrophoresis. Lanes 1, 1 Kb DNA Ladder; 2, closed control; 3, *Ps. oleovorans* (*alk*B-positive control); 4, S1RS12; 5, S5MM1; 6, S5MM2; 7, S5MM3; 8, S5MM5; 9, S5RS3; 10, φX174/Hae*III* Ladder.

DISCUSSION

_alk_B primers corresponding to _Ps. oleovorans_ ATCC 29347

The investigation into the presence of the *alk*B gene had intriguing results. The positive control, *Ps. oleovorans*, consistently had two amplified fragments of DNA at 870 bp and ~280 bp. The isolates had a variety of DNA fragments amplified with this primer set. Figure 3 (S3 isolates) contains a very good representation of this phenomenon. Almost all of the S3 isolates were suspected as positive for the *alk*B gene, yet none of these DNA fragments analyzed by sequencing were actually positive for the gene. None of the suspected positive isolates resulted in an amplification of DNA fragments with similar size to the *Ps. oleovorans alk*B gene.

The major problem with sequencing was the wrong DNA fragment was selected for extraction and sequencing. Instead of choosing a fragment the same size as *Ps. oleovorans*, the most abundant DNA fragment was selected for extraction and sequencing. None of the DNA fragments analyzed contained part of the *Ps. oleovorans alk*B gene. Other DNA fragments that were not sequenced may have contained the *alk* B gene, but poor results from previous isolates made this seem improbable. This is the reason the rest of the isolates from S5 soils were not sequenced. The initial assumption was that different organisms may contain *alk*B gene fragments of differing sizes. This was in error and further experiments should be conducted with DNA fragments of 870 bp (or 280 bp) selected for extraction and sequencing. These primers were possibly amplifying DNA fragments with no homology to *Ps. oleovorans alk*B.

The *Ps. oleovorans alk*B primers were designed to detect the *alk*B gene on the OCT plasmid (5, 7, 8, 9, 10). They contain no degeneracies, making the PCR conditions more stringent. The annealing temperature was lowered to account for the stringency of the primers. The result was multiple DNA fragment amplification, none of which appeared to be the actual

*Ps. oleovorans alk*B gene after sequencing analysis. Only one DNA fragment, from S5MM1, may be related to the alkane degradation. It was homologous to a NADH dehydrogenase enzyme. Preliminary steps of alkane degradation require NADH to provide electrons for the hydroxylase enzyme. However, this is accomplished by *alk*T, not *alk*B. A second set of primers with degeneracies was therefore used to detect the presence of the *Ps. oleovorans alk*B gene.

DNASTAR® designed *alk*B degenerate primers

Six isolates each produced a single DNA fragment that was the same size as the amplified DNA fragment of *Ps. oleovorans.* It is important to note that none of the amplified DNA fragments were extracted or sequenced. Five of these isolates were found in the S5 soil sample, the higher *n*-alkane sample. This makes sense considering the likelihood that this gene may reside on a plasmid (3, 6, 8, 9, 10). Plasmid transfer would be likely to occur in a highly contaminated soil sample, conferring this gene to a variety of organisms. If the DNA is in this mobile form it can be transferred within the microbial population via conjugation or transformation (3).

The largest problem with these primers was the low yield of DNA in amplification. Only one amplified DNA fragment from S5MM3 was positive in the repeated trials. The other five amplified DNA fragments were not always visualized on the agarose gel after PCR amplification. Figure 5 reveals how faint some of the amplifications were, even with 20μL of sample loaded into each lane for purposes of visualization.

The 16S rDNA GenBank matches for the six isolates (Table 2) are all expected genera for soil microorganisms (3, 4, 6). Three of the isolates are common soil microorganisms (S1RS12, S5RS3, and S5MM3), and the other three are known soil microorganisms (S5MM1, S5MM2 and S5MM5). *Alcaligenes, Bacillus,* and *Rhodococcus* are common genera among soil microorganisms known for petroleum degradation (3, 6). *Proteobacteria*, *Phyllobacterium*, and *Microbacterium* are also soil microorganisms (4). The diversity of genera represented by the six isolates further supports the probability that the *alk*B gene resides on a plasmid.

In summary, it appears that some of the isolates from the Guadalupe sites contain the alkane hydroxylase biodegradative enzyme. These organisms should thus be capable of degrading short chain *n*-alkanes present in petroleum.

Further studies

To further this investigation, PCR must be performed again using the *Ps. oleovorans* degenerate primers. The PCR cycles should be increased to forty-five to increase the amount of amplified DNA product. The size of the amplified DNA fragment compared to *Ps. oleovorans* is not conclusive proof of homology. Sequencing of amplified products should be performed to confirm that they are homologous to the *Ps. oleovorans alk*B gene. Plasmid extractions could be performed on any positive isolates to see if the isolate contains a plasmid since biodegradative genes are commonly found on plasmids. If the plasmid extraction is successful, PCR should be performed on purified plasmid DNA to determine if the plasmid or the genome contains the *alk*B gene.

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