Cloning Protocol and Sequence Analysis of the *alkB* Gene in *Gordonia otitidis*

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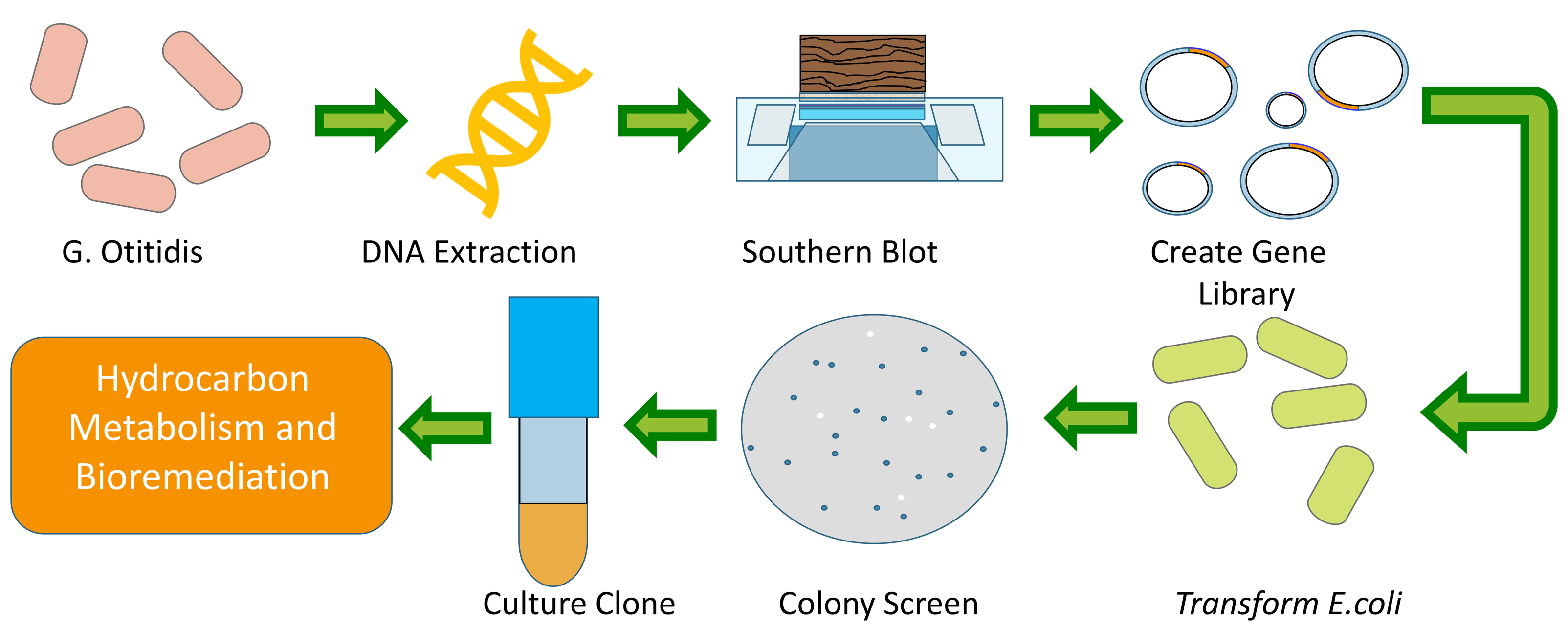
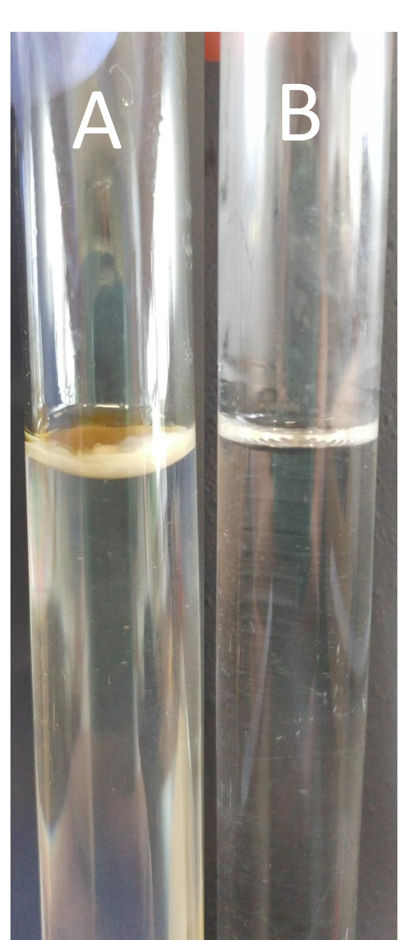
Overview

A bacteria, *Gordonia otitidis,* was previously isolated from soil at the University of the Fraser Valley Abbotsford campus based on its ability to metabolize used motor oil. The current study is involved in the characterization of a gene thought to be involved in hydrocarbon metabolism, and therefore, essential for the bioremediation of motor oil. Very little is known about *Gordonia otitidis* with respect to its ability to metabolize oil; therefore, the study of this bacteria may provide a unique metabolic pathway not previously characterized. Characterization of this pathway requires cloning and sequencing of genes associated with hydrocarbon metabolism. *alk*B (an alkane monooxygenase), has been selected as a candidate for further study. Part of the gene has been cloned and sequenced, and we now wish to clone the remainder of the gene along with its regulatory region, which will allow us to study expression levels under conditions that stimulate increased transcription.



We have developed a Southern blot procedure (see above) to identify restriction enzymes that can be used in the cloning of the gene into *Escherichia coli*. Following the generation of a gene library, transformants will be screened for the presence of the *alk*B gene using a PCR generated probe from the previously cloned section of this gene. A secondary cloning procedure involving PCR amplification of the 5' region of *alkB* with the upstream region has also been developed. Cloning this fragment will allow future analysis of the promoter region of *alkB* and determination of transcriptional regulation mechanisms.

Cell Cultures



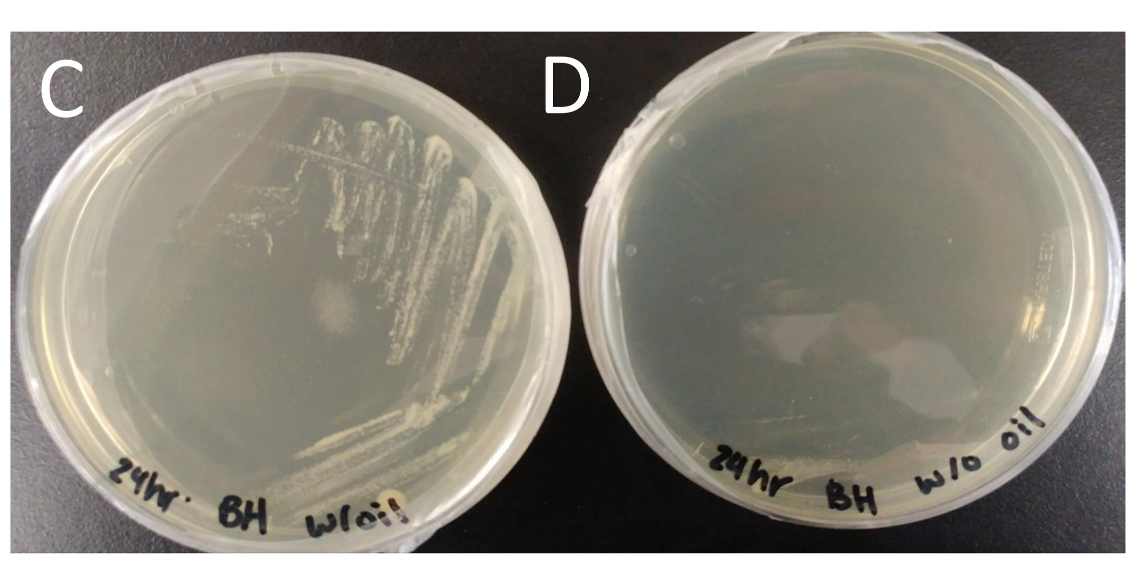


Figure 1 *Gordonia otitidis* cultures
A) BH minimal salt broth with 100 µL sterile used motor oil **B)** BH minimal salts media without motor oil **C)** TSA streaked with 24 hour culture *G. otitidis* in BH media with 100µL sterile used motor oil. **D)** TSA streaked with 24 hour culture *G. otitidis* in BH media without motor oil as carbon source

PCR Gene Fragment Analysis of *alkB*

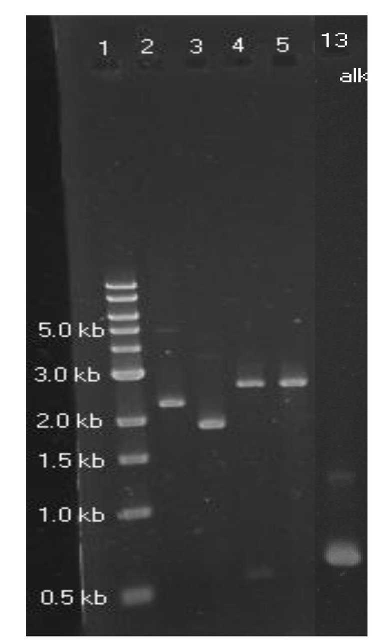
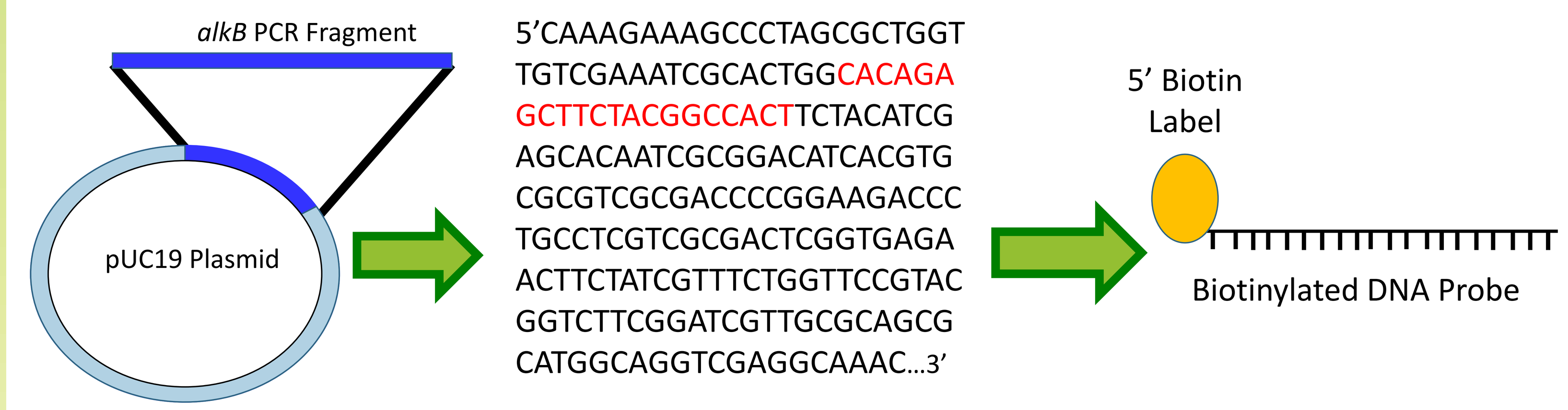


Figure 2 *alkB* PCR clone Miniprep isolation
Lane 1: 1kb Ladder
Lane 2: *alkB* PCR fragment in pUC19
Lane 3: pUC 19
Lane 4: *alkB* PCR clone SalI digestion
Lane 5: pUC19 SalI digestion
Lane 13: *alkB* PCR Product

alkB fragment sequenced by alphaDNA inc. and analyzed using NCBI BLAST to create DNA probe to be used for Southern blot and colony screening procedure.



Efficiency Test for Biotinylated Probe

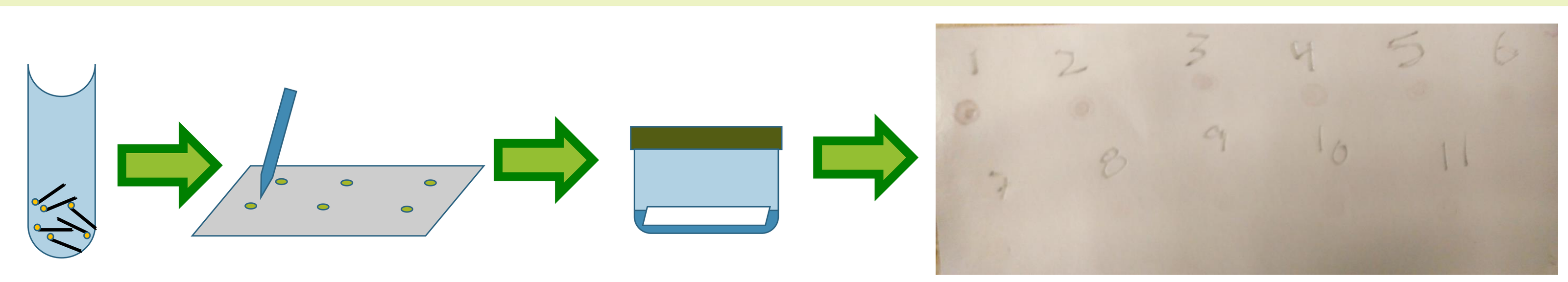
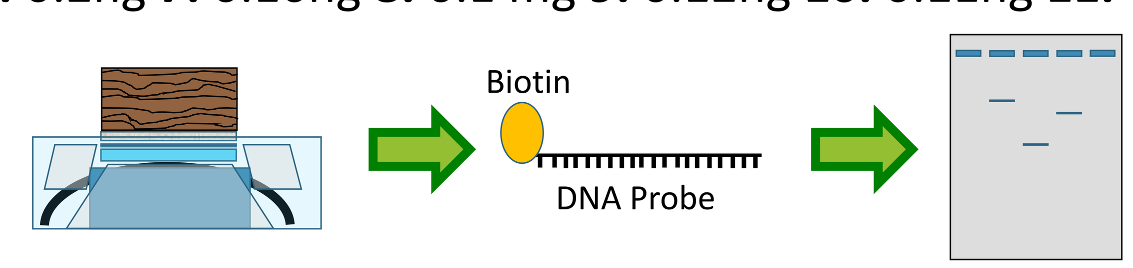


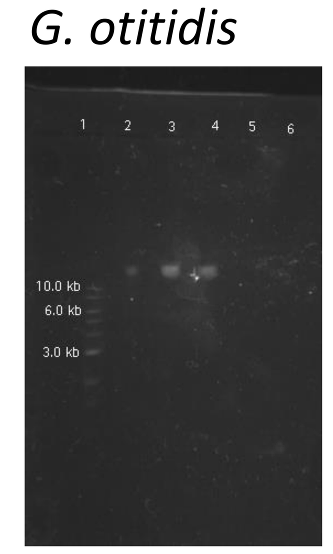
Figure 3 Detection Efficiency
Biotinylated probe detection with streptavidin-alkaline phosphatase conjugate and BCIP substrate. **1.** 1.0ng **2.** 0.66ng **3.** 0.5ng **4.** 0.33ng **5.** 0.25ng **6.** 0.2ng **7.** 0.16ng **8.** 0.14ng **9.** 0.12ng **10.** 0.11ng **11.** 0.1ng



The biotinylated probe will be used to detect restriction fragments containing the *alkB* gene in a southern blot. The fragments will be isolated and transformed to create clones containing the entire *alkB* gene for future mechanistic studies of oil metabolism.

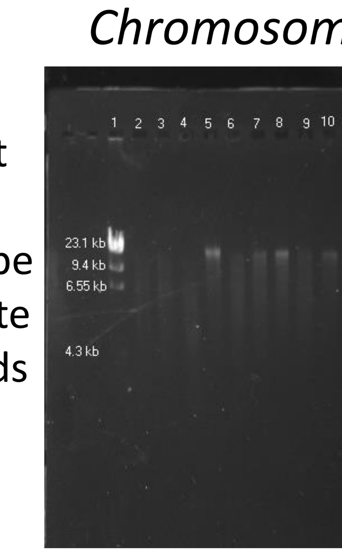
DNA Extraction and Digestion

Figure 4 *DNA Isolation from G. otitidis*



Lane 1: 1kb Ladder
Lane 2: *G. otitidis* test tube
Lane 3: *E. coli* test tube
Lane 4: *G. otitidis* plate
Lane 5: *E.coli* w/ beads
Lane 6: *G. otitidis* w/ beads

Figure 5 *Restriction Digest of Chromosomal G. otitidis DNA*



Lane 1: λ HindIII Ladder
Lane 2: *EcoRI*
Lane 3: *EcoRV*
Lane 4: *HindIII*
Lane 5: *XbaI*
Lane 6: *ClaI*
Lane 7: *BamHI*
Lane 8: *SacI*
Lane 9: *PstI*
Lane 10: *SalI*

Highest chromosomal DNA extraction yield using *G. otitidis* test tube culture. XbaI, SacI, and SalI create largest fragments, increasing likelihood of restriction fragment containing entire *alkB* gene.

PCR Amplification of Secondary *alkB* fragment

Primers designed for amplification of upstream and 5' region of *alkB* gene. Downstream region previously cloned by *Moreton, M*.

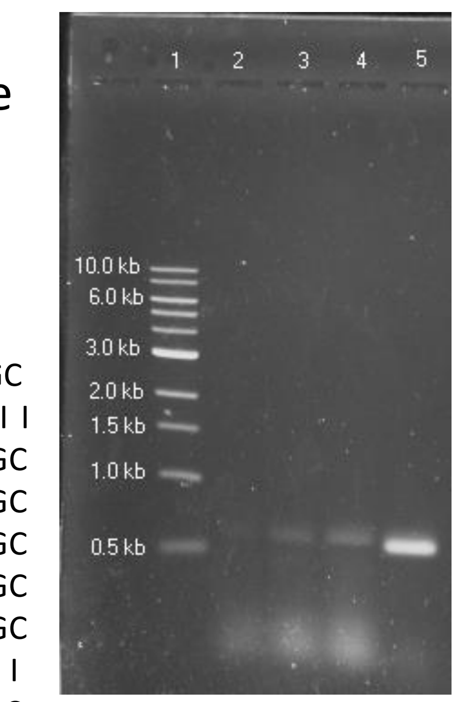
Figure 6 Primer Alignment
Forward primer alignment based on conserved region of carbohydrate diacid transcriptional regulator gene directly upstream of *alkB*. Designed using protein sequence HPIRERAG

```
G. sp. 852002-10350_   CACCCCATCAGGGAGCGAGCAGGC
       SCH5691597      || ||||||    ||||||||||||||
NZ_LZTC01000089.1      CATCCCATTCGGGAGCGAGCAGGC
NZ_LZO01000109.1       CATCCCATTCGGGAGCGAGCAGGC
NZ_LZTD01000092.1      CATCCCATTCGGGAGCGAGCAGGC
NZ_BAF01000117.1       CATCCCATTCGGGAGCGAGCAGGC
NZ_LDTZ01000022.1      CATCCCATTCGGGAGCGAGCAGGC
                       || ||   |   | || ||   ||   |||
NZ_JZDQ02000007.1      CACCCGCTGCGTGAGCGGGCCGGC
```

Reverse primer designed using good quality sequence in *alkB* PCR fragment at *alkB* locus 493-517 bp.

PCR Sequence:
5' CGGCCACTTCTACATCGAGCACAA 3'
Reverse Complement:
5' TTGTGCTCGATGTAGAAGTGGCCG 3'

Figure 7 PCR Amplification



Lane 1:1kb Ladder
Lane2: 3µL DNA template PCR
Lane 3: 4µL DNA template PCR
Lane 4: 5µL DNA template PCR
Lane 5: Positive Control (Moreton, M. *alkB* PCR amplification)

Amplification increased efficacy as volume of DNA template increased. Molecular weight of amplicon is approximately 600bp, corresponding with target amplicon of 629bp. Amplified region must be confirmed with cloning and sequencing.

Conclusion

This ultimate goal of this study is to provide the ability to study the regulation of the *alk*B gene in response to exposure to a toxic substance in the environment, such as oil. As discussed, we have studied two methods in attempts to clone the regulatory region of the *alk*B gene. We have successfully sequenced the primary *alkB* PCR fragment and identified it as a 338bp region of the *alkB* gene located centrally at nucleotide position 488-826. The DNA probe designed from this sequence will be used in future southern blot detection and colony screening of *alkB* gene clones. We have also been successful in generating the promoter region of *alk*B using PCR from an upstream region. This product will now be cloned allowing us to proceed to the next step in our studies. Potential use of this gene fragment includes the cloning of the GFP gene under control of the *alk*B promoter to be used as a reporter gene providing a quantitative measurement of gene expression following exposure to an environmental toxin. Both the full *alkB* and upstream region clones will allow future study of *alkB* regulation and further comprehension of hydrocarbon metabolism for use in bioremediation.

Acknowledgements

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References

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