PCR and 16s rDNA sequencing for identifying and comparing Gram-negative bacteria

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Abstract

16s rDNA genes encode a ribosomal RNA. Each species' unique sequence of this universal gene can be used as a means for identifying different bacterial species. Several species of Gram-negative pathogenic bacteria were used in the initial study. The bacteria were: *Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica, Proteus vulgaris,* and *Klebsiella pneumoniae.* DNA was isolated from each organism and was amplified by PCR, followed by purification and DNA sequencing. The resulting sequences were then analyzed to allow for identification of the bacteria. In the final stage of this study, environmental samples were collected; the bacteria were isolated and identified using this technique. The molecular techniques used in this experiment were performed with the intention of illustrating the usefulness of 16s rDNA sequencing for undergraduate laboratory work in microbiology.

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Introduction

Identification of bacteria is important for basic biological research and applied clinical microbiology. Traditional methods of identifying bacteria, including culturing, can be time consuming and unreliable. Molecular based methods of sequencing provide quick, reproducible identifications. One reason that the 16s rDNA gene sequencing is so widely used to identify bacteria is that this gene is highly conserved, since it is involved in essential processes in the cell, and there are multiple copies of 16s rDNA in a bacterial cell making it easy to amplify (Navarro, et al., 2002) (Jeng, et al., 2001). The goal of this project was to test techniques for identifying Gram-negative bacteria using this gene for molecular sequencing (Roussaux, et al, 2001), and to examine how useful these techniques can be for undergraduate students.

Materials and Methods

I. DNA extraction

The Gram-negative bacteria were plated on Hektoen Enteric (HE) Agar and cultured overnight in a 37° C incubator. These cultures were then used immediately or stored in a refrigerator for no longer than two weeks prior to subsequent bacterial DNA extraction. The Prepman isolation kit (Applied Biosystems, Foster City, CA) was used for isolation of bacterial DNA. (Anonymous, 2000). A dense inoculum of bacteria was taken from isolated colonies on a culture plate and added to 200 µL of Prepman Ultra Sample Preparation Reagent® (Applied Biosystems, Foster City, CA) in a 2.0 mL microcentrifuge tube. This mixture was then vortexed for 10 to 30 seconds to aid in lysing cell membranes and releasing the DNA. Next, the sample was heated in a thermal cycler for 10 minutes (94° C). Tubes were then centrifuged for 3 minutes at 16,000 x g. The supernatant was transferred to a new tube, and the bottom waste pellet was discarded. For several initial trials with the control cultures, an alternative to this procedure was performed by adding bacteria to 200 µL of water instead of Prepman reagent) and then the lysate was used for PCR.

II. PCR

The PCR mix consisted of 1 µL of the bacterial DNA in a clean 500 µL microcentrifuge

tube, 1 µL each (20 picomoles) rP2 primer 5'ACGGCTACCTTGTT-ACGACTT-3', and fD1 primer 5'-AGAGTTTGATCCTGGCTCAG-3',
12.5 µL of HotStar Taq master PCR mix (Qiagen, Valencia, CA) and 9.5 µL of distilled water was added to the reaction mix. This resulting mixture was placed in a PCR machine, which was programmed with the following parameters:

TEMPERATURE (C)		TIME (min)	CYCLES
denature	94	15	1
denature	94	2	20
anneal	57	0.5	20
extension	72	2	20
final extension	72	10	1

III. Analysis of PCR Product

The PCR product was run in a 2.5% agarose electrophoresis gel, made with 0.5x TBE buffer and Gelstar® stain to confirm the presence of product. Samples showing an appropriately sized band were saved for purification and DNA sequencing. Pictures were taken using a Kodak DC100 camera and Kodak Digital Science ID software for MacIntosh Computers, using a Sybr Green filter with three-second exposure time.

IV. PCR Purification and Sequencing

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and eluted with water. Purified products were placed in a -20°C freezer until they could be sent for sequencing. DNA sequences were analyzed by submission to the ribosomal database project website, –http://rdp.cme.msu.edu/html, Michigan State University, for identification.

V. Environmental Sampling

Environmental sampling was performed by sampling twelve different sites: door handles, toilet seats, vending machines, dollar bills, human throats, shoe soles, and keyboards from Sattgast Hall.

Samples were swabbed using a sterile cotton swab and plated on Mueller-Hinton, EMB, or MacConkey agar. DNA Extraction from bacterial cultures, PCR, PCR purification, and sequencing were performed as described earlier.

Results

Gel analysis of PCR products showed a band of the expected size (~1350 bp) for each of the five species: *P. vulgaris, P. aeruginosa, K. pneumoniae, E. coli* and *S. enterica* (Figure 1). Products were purified and submitted for sequence analysis.

Sequence analysis of the known samples correctly identified *P. vulgaris, P. aeruginosa, K. pneumoniae*, and *S. enterica.* All positive results were obtained for samples ran with the fd1 primer (see appendix). The sample labeled *E. coli* was identified, by sequencing, as *P. vulgaris.*

Appropriate-sized DNA bands were also obtained following PCR from four of the twelve environmental plates. Samples from tonsils, teeth, money and bottom of a shoe were amplified using PCR and purified for sequencing. (Figure 2)

Six purified samples were sent to sequence and two samples came back with positive matches to database sequences. The sample from the bottom of a shoe came back positive for *Bacillus megaterium* and *Staphylococcus pasteuri. B. megaterium* had a 78% match and *S. pasteuri* came back with a 29% match. (see appendix)

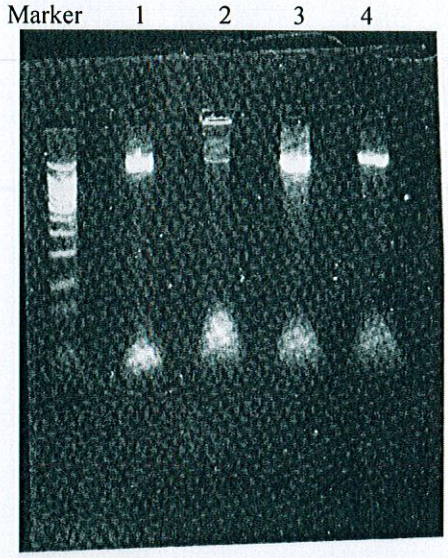

Figure: Gel electrophoresis of bacterial control cultures showing positive bands for the 16s rDNA gene for 1. *K. pneumoniae*, 2. *P. vulgaris*, 3. *P. aeruginosa*, and 2. *S. enterica*. Marker used: 100 bp Marker

Discussion

Initially, I had problems amplifying DNA from the known bacteria. This was probably due to problems encountered when DNA was prepared by lysing the cells with water instead of the Prepman reagent. Insufficient DNA may have been collected because of nucleases and PCR-inhibiting molecules, characteristic of Gram-negative bacteria. There are other techniques for isolating DNA from bacteria in a PCR-ready form (Duarte, et al., 1998), and these techniques could be used in future trials to examine the effectiveness of these alternate methods.

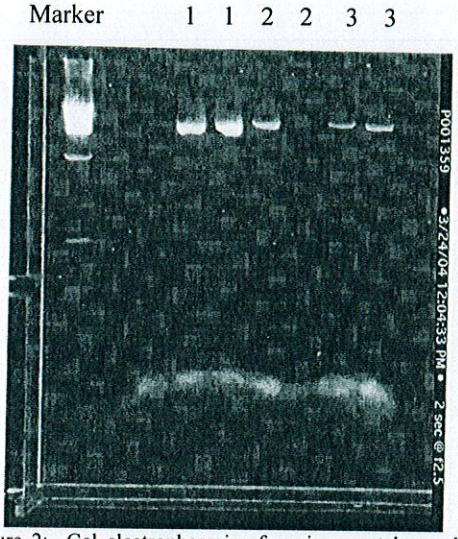

Figure 2: Gel electrophoresis of environmental samples, showing positive bands for 16s rDNA for the following sample sites: 1. teeth, 2. tonsils, and 3. a shoe sole. Marker: *λBstEII*

PCR products ran on the agarose gels all gave a positive result for at least one sample of the amplified DNA. This is not necessarily very encouraging, because many gels were run before generating appropriate samples for sequencing. In the clinical and research setting, there would be no time to run up to nine or ten gels to get a confirming band of product. Other complications may have come from experimental error, such as possible mistakes made with PCR recipe, or programming errors for the thermal cycler, although the possibility of those mistakes was minimized by careful protocol checks.

A high proportion of environmental samples were successfully amplified by the PCR. However, it was difficult to isolate bacteria from many environmental samples. Only four samples yielded isolated colonies after two days incubation and three days of room temperature growth. Mueller-Hinton agar should have allowed growth of many different species of bacteria, and EMB or MacConkey agar should have allowed for the growth of many species of Gram-negative bacteria. A possible explanation for this result might be that sampling surfaces were not swabbed sufficiently, or that the areas may have just been cleaned.

The sequences of 4 out of the 5 positive control samples generated high quality matches to the expected organism: *P. vulgaris* 78%, *Pseudomonas aeruginosa* 79%, *K. pneumoniae* 79% and *S. enterica* 82%. Unexpectedly, the *E. coli* sample generated a sequence that matched *P. vulgaris* most closely. Experimental error was most probably to blame, perhaps a mislabeled tube sent to Northwoods DNA for sequencing.

Sequencing environmental samples yielded inconsistent results. Only two of the twelve tubes generated high-quality sequence matches to database organisms. One reason may have been that the environmental isolates were not streaked sufficiently to generate pure cultures. Evidence supporting this hypothesis included the sequencing chromatograms, which showed mixed peaks, indicative of mixed samples.

Conclusions

This experiment proved to be a consistent way of identifying bacteria. Despite its tie-ups and technical faults, this would be an excellent method for undergraduates to investigate. They would be able to perform modern molecular techniques, get a handle for the theory behind them, and examine the pitfalls of research and laboratory experiments in modern science. If using this technique as a lab exercise for undergraduate students, it would be wise to isolate the DNA using the Prepman kit, and use universal primers such as fd1 and rP2 to minimize frustrations.

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Web sites used:

http://www.ridom.de/rdna/index.shtml

http://www.teriin.org/division/bbdiv/mb/rbi.htm

http://rdp.cme.msu.edu/html/

DNA sequences from known bacteria:

Organism Identification: *Klebsiella pneumonia*

GCCTAACACATGCAAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCG
GACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAAC
GGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCAC
GCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAG
GCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACG
GTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCT
GATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTT
GGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAG
CACCGGCTAACTTCGTGCCAGCAGCCNCGGTAATACGAANGGTGCAAGCGTTAATC
GGAATTACTGGGCGTAAAGCNCGCCTANGTGGTTCACAAGTTGGATGTGAAATCCC
CNGGCTCAACCTGGGAACTGCATCCCAAACTACTG

Organism Identification: *Pseudomonas aeruginosa*

AGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGC
CTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAATGTCGCAAG
ACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAG
CTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGA
TGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAA
GGCCTTCGGGTTGTAAAGCACTTTCANCGGGGANGAAGGCGATGANGTTAATAACC
TNGTCGATTNGACGTTACCC

Organism Identification: *Proteus vulgaris*

GGCCTAACACATGCAAGTCGAGCGGTAACAGAAAGAAAAGCTTGCTTTCTTGCTGA
CGAGCGGCGGGACGGGTGAGTAATGTATGGGGATCTGCCCGATAGAGGGGGATAAC
TACTGGAAACGGTGGCTAATACCGCATGACGTCTACGGACCAAAGCAGGGGCTCTT

CGGACCTTGCGCTATCGGATGAACCCATATGGGATTAGCTAGTAGGTGAGGTAATG
GCTCACCTAGGCAACGATCTCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGA
CTGAGACACGGCCCANACTCCTACNGGAGGCAGCAGTGGGGAATATTGCACAATGG
GCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTAGGGTTGTAAAG
TACTTTCNNCGGGGAGGAAGGTGATAAAGTTAATACCTTTNTCAATTGACGTTACCC
GCAGAANAANCCCCGGCTNACTCCATNCCATCNGCCGCGGNAATACGGAAGGTGCA
AGCNTTAATCNGAATTACTGGGCCTAAAGCCCNCGCAGCGGTNGATTCAGTCNAAT
GTTAAANCCCCGAACTTAACNTGGGAATTGCCTCTGNAACTGGGTGGCTAGAGTCNT
GTNNAAGGGNGGAGAANTCANTCTAACCCT

Organism Identification: *Proteus vulgaris*
CGCCTAACACATGCAAGTCGAACGGTAACAGGNAAGCAGCTTGCTGCTTCGCTGAC
GAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTA
CTGGAAACGGTGGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCG
GGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTTGTTGGTGAGGTAACGGCT
CACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTG
AGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCG
CAAGCCTGATGCACCATGCCGCGTGTATGAAGAAGCCTTCGGGTTGTAAAGTACTTT
CAGCGGGGANGAAAGTGTGTGGTTAATAACCGCAGCAATTGACGTTACCCGCAGAA
NAANCACCNGCTAACTCCNTGCCAGCACCNCGGTAATACNGAAGGTGCAAGCNTTN
ATCNGAATTACTGGGCGTAAAGCGCACGCNNGCGGTCTGTCCAGTCCGATGTGAAA
TCCCCCGGCTCAACCTGGGAACTGCCTTCCAAACTGGCANGCTTGAGTCNTGTAAAA
GGGGGTANAATTTCNGGTGTANCGGTGAAATGCNTAAANATCTTGAAGAATACCCG
TGGCGAAAGNGGCCCCTGGACCAAAACGACCTCAANTGCCAAAACTGGGGANCNA
AANGAATAATANCCCGTATCCCCCCCTAAACANNTNTACTTNAANTTTTCCCCTANN
CTGCNTCCCAACTAACCCTTAATAAACCCNCGGGAATCCCCNCCAGGTTAAAACCAT
AATTTANGGGCCCCCCAACCGNGGACTTGGNTTNTTTTTCAN

Organism Identification: *Salmonella enterica*
ATGATCACAAAGTGGAAACGCCCTCCCGAAGTTAAGCTACCTACTTCTTTTGCAACC
CACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTAG
CATTCTGATCTACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCC
AATCCGGACTACGACATACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTT
TGTATATGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCATGATGACTTGA
CGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCTA
ACCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTC
ACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCACAGTTCCCGAAGGCACC
AATCCATCTCTGGAAAGTTCTGTGGATGTCAAGACCAGGTAAGGNTCTTCGCGTTGC
ATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGT
TTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGATTTAACGCGTTAGCTCCGGAAGC
CACGCCTCAAGGGCACAACCTCCAAATCGACATCGTTTACCGCGTGGACTACCAGG
GTATCTAACCTGTTTGCTCCCCACGCTTTCCCACCTGACGTCAGTCTTTGTCAGGGGG
CCCCTTCCCACCGTATTCCTCCGAACTCTACCATTTCACCGCTNCACCTGGGATTTAC
CCCCTCTACANAAACTANNCTGCCANTTTCAAAGNANTTCCAGGTGAACCCGGGATT
TACATCCAATTGACAAACCCCCGCGGGGCTTNNCCCNGTATTCCAATAAACTGGNCC
CCCTATNCCCGCTCTGGNCGAATTACCGGGNTTTT

Sequence data from Environmental Samples:

Organism Identification: *Bacillus megaterium*

TCAGATCACAAAAGGTAAGCGCCCTCCCGAAGTTAAGCTACCTACTTCTTTTGCAAC
CCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTA
GCATTCTGATCTACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTC
CAATCCGGACTACGACAGACTTTATGAGTTCCGCTTGCTCTCGCGAGGTCGCTTCTCT
TTGTATCTGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTG
ACGTCATCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCTTTGAGTTCCCGCCAT
TACGCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACAT
TTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGCGTTCCCGAAGGC
ACTCCTCTATCTCTAAAGGATTCGCTGGATGTCAAGANTANGTAAGGTTCTTCNCGT
TGCATCNAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAANTCATTTG
AGTTTTAACCTTGCGGCCGTACTCCCCANGCGGTCGATTTAACGCGTTAGCTCCNGA
AGCCACAGTTCAAGACCACAACCTCTAAATCGACATCGNTTACAGCGTGGACTACC
AGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCCACCTGAACGNCAGTCTTTGTCCA
NGGGGGCGCTTCCCNCCNGTATTCCCCCNCATCTCTACCANT

Organism Identification: *Staphylococcus pasteuri*

CAGATCACAAAGTGGTAACGCCCTCCCGAAGTTAAGCTACCTACTTCTTTTGCAACC
CACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTGG
CATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCC
AATCCGGACTACGACGCACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTT
TGTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCATGATGACTTGA
CGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGACCTA
ATCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTC
ACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCACAGTTCCCGAAGGCACA
AATCCATCTCTGGATTCTTCTGTGGATGTCAAGACCAGGTAAGGTTCTTCGCGTTGC
ATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGT
TTTAACCTTGCGGCCGTACTCCCCAGGCGGTCTACTTAACGCGTTANCTCCGGAACC
ACGCCTCAAGGGCACAACCTCCAAGTAGACATCGTTTACGGCGTGGACTACCANGG
TATCTAACCTGTTTGCTCCCCACGCTTTCNCANCTGACGTCAGTCTTTTGTNCANGGG
GGCGCCNTCCCNCCCGTATTNCTCCNGANATCTAC