Lab 3: Identification of Bacteria in an Environmental Sample

CE773

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Introduction

This lab introduced students to the process of identifying microorganisms present in an environmental sample. When performing such an identification, two main methods exist: culture-dependent and culture-independent. A culture-dependent process was selected for use during this lab. While both types of identification methods are scientifically valid, this lab should help ascertain why selecting the correct method for the given circumstances may be the most important step in any microbial identification process.

Methods

The lab began with the inoculation of liquid cultures. Five plates with individual isolated colonies present were chosen from the plates that were prepared during lab two. In order to inoculate a liquid culture, the blunt end of a sterile cotton swab was lightly pressed against on individual colony. Then, the same end of the swab was submerged and shaken inside a sterile microcentrifuge tube containing 1 mL of nutrient broth. Next, the solution was vortexed for 5 seconds and added to another tube containing 9 mL of nutrient broth—again, the solution was vortexed. This process was performed a total of five times, once for each plate chosen. Then the tubes were placed in an incubator shaker table and incubated overnight at 30 °C.

Between day one and day two of lab, DNA was extracted from each of the pure liquid cultures by the TA. The extraction was performed using a DNA extraction kit (Qiagen DNA Stool Mini Kit Catalog #51504).

After DNA extraction, the samples were ready for the Polymerase Chain Reaction (PCR). PCR was to be performed with two primer pairs, 341F/518R and 341F/907R. For each primer pair, a Mastermix was prepared in a 2 mL microcentrifuge tube. Each Mastermix contained 35

µL buffer, 7 µL dNTP mix, 3.5 µL primer F, 3.5 µL primer R, 3.5 µL Taq polymerase and 290.5 µL water. During the creation of each Mastermix, the solution was vortexed after the addition of each reagent. Once the Mastermixes were prepared, 49 µL of Mastermix was placed in each of the twelve 0.5mL, thin walled PCR reagent tubes—341F/518R Mastermix in tubes 1 thru 6 and 341F/907R Mastermix in tubes 7 thru 12. Then, 1 µL of each template DNA or 1 µL of autoclaved water (used for control) was added to the tubes in the following manner:

Primer Pair 341F/518R	Primer Pair 341F/907R
Tube 1: Isolate 1 DNA	Tube 7: Isolate 1 DNA
Tube 2: Isolate 2 DNA	Tube 8: Isolate 2 DNA
Tube 3: Isolate 3 DNA	Tube 9: Isolate 3 DNA
Tube 4: Isolate 4 DNA	Tube 10: Isolate 4 DNA
Tube 5: Isolate 5 DNA	Tube 11: Isolate 5 DNA
Tube 6: Negative Control (water only)	Tube 12: Negative Control (water only)

Finally, the capped, labeled tubes were centrifuged briefly and placed into the Thermocycler to begin the PCR program, which consisted of the following steps:

Step 1: 15 minutes at 94 °C
Step 2: 1 minute at 94 °C
Step 3: 1 minute at 55 °C
Step 4: 1 minute at 72 °C
Step 5: Repeat Steps 2 thru 4 (29 times)
Step 6: 10 minutes at 72 °C
Step 7: 24 hours at 4 °C

Following PCR, the TA purified the PCR products with a PCR purification kit (Qiagen QIAquick PCR Purification Kit Catalog #28104) and poured the gel for the electrophoresis process. Gel electrophoresis was used for quality assurance and quality control. Samples of PCR product were placed on the gel, along with DNA ladders (MBI Fermentas GeneRuler #SM0333), 5 µL each, for positive control. Before being placed on the gel, each sample was mixed with a loading dye to enable the sample to sink through the TAE buffer covering the gel. A 1 µL sample of PCR product was mixed with 5 µL of loading dye. The ladders (L), PCR

products (P) and negative controls (N) were placed in the gel wells as illustrated in the following figure.

Row 1: 341F/518R

L P1 P2 P3 P4 P5 N L

Row 2: 341F/907R

L P1 P2 P3 P4 P5 N L

Once properly loaded, the gel was covered and hooked up to the power source to run negative to positive. The power source was set to run at 90 mA and 100V for 50 minutes. After the time elapsed, a working stain solution containing 10 µL of 10,000xSYBR Safe Stain and 90 µL of 1xTAE buffer was provided in order to stain the gel. The gel was placed in a stain tray, covered in stain solution, covered with foil, and placed on the shaker table for 30 minutes at slow speed. Finally, the stained gel was ready for Image Analysis. Also, as another method of obtaining DNA concentration data, the samples were analyzed using a Nanodrop Spectrophotometer ND-1000.

After checking the quality of our PCR products, an equal sample of each product was sent to be sequenced at the KU DNA Sequencing Facility. Once sequences were obtained, they were "proofread" (a handful of base pairs were removed from the start and finish of each sequence) and entered into the online Genbank database for identification.

Results

Our group was able to obtain seven identifiable sequences from our ten samples of PCR product. The following list displays the identification obtained from the Genbank database for each sample.

1. Sequence no good
2. Citrobacter youngae partial 16S rRNA gene, strain CECT 5335
3. Comamonas testosteroni partial 16S rRNA gene, strain TacK2
4. Comamonas testosteroni partial 16S rRNA gene, strain TacK2
5. Citrobacter sp. F3-2 16S ribosomal RNA gene, partial sequence
6. Control
7. Sequence no good
8. Sequence no good
9. Comamonas testosteroni partial 16S rRNA gene, strain WAB1871
10. Comamonas testosteroni partial 16S rRNA gene, strain DSM 6781
11. Citrobacter youngae partial 16S rRNA gene, strain CECT 5335
12. Control

Both sets of samples, 341F/518R and 341F/907R, returned similar identifications—each set contained *Citrobacter* and *Comomonas* bacteria.

Discussion

Although the end results of the identification were similar, the "scores" obtained from the Genbank database were much lower for the best matches found for the 341F/518R products. This was expected, since the lower number of base pairs in these samples, compared to the 341F/907R samples, should leave more uncertainty present in the identification. If a sample has more base pairs, the database is able to present a narrower list of possible matches, each having a higher "score".

The three sequences that were "no good" were probably either contaminated with multiple sequences of DNA or not enough DNA was present before PCR. If the sample were contaminated with DNA from another organism before PCR, both strands would be amplified,

resulting in an unreadable DNA image. The same type of image could result from not enough DNA being present to amplify. In this case, the jumbled graph would be the appearance of the base pair present in the Mastermix. These are just a couple of the possible pitfalls present in a PCR-based analysis (Wintzingerode et al., 1997).

While our culture-dependent method produce two different types of bacteria, this is likely much less than the actual amount of unique bacteria present in our samples. Culture-dependent methods can only account for a small fraction of the microbial life present in a community (Ward et al., 1990). Studies of food fermentation and probiotics have shown that incorporating culture-dependent and culture-independent methods can give a better picture of the actual microbial community dynamics present in a sample (Pulido et al., 2005; Temmerman et al., 2003). So, incorporating a culture-independent method into our experiment likely would have lead to significantly more bacteria being identified.

Conclusion

Bacterial identification is not a cut-and-dried process. Much thought must be put into the method(s) that will be used to identify the bacteria. Culture-dependent methods tend to identify fewer bacteria than culture-independent methods. However, culture-independent methods have their downfalls as well, such as detecting spore-forming bacteria (Mincer et al., 2005). In general, a mixed approach utilizing both methods will likely yield the most comprehensive results.

References

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Wintzingerode, F., Göbel, U.B., Stackebrandt, E. 1997. Determination of Microbial Diversity in Environmental Samples: Pitfalls of PCR-based rRNA Analysis. 21 (3), 213-229.

Questions

1. For each reactant added to the PCR mastermix, what is its purpose?

   Template DNA contains the target DNA to be copied.
   Primers anneal to the single-stranded DNA after denaturing.
   The Taq polymerase assists in DNA replication.
   The buffer provides a suitable chemical environment for optimum activity and stability of the DNA polymerase.
   $MgCl_2$ provides the divalent cations needed for proper bonding.
   The dNTPs are the building blocks from which the DNA polymerases synthesizes a new DNA strand.

2. Some researchers use a different annealing temperature in their PCR program. Why?

   If a researcher has very low DNA template concentrations, it may be necessary for him/her to lower the annealing temperature in order to obtain detectable PCR product.

3. When performing PCR reactions and gel electrophoresis, it is important to always run controls. For the PCR reaction, there is a negative control. For the electrophoresis, the ladder serves as a positive control.

   After electrophoresis, the following results were obtained. Explain what might have happened:

A. The lane loaded with the negative control has a small, faint band (i.e. the negative is positive).

   The negative control may have been contaminated with DNA prior to PCR.

B. The lane loaded with the ladder does not have anything, and the entire gel is blank (i.e. the positive is negative).

   The electrophoresis could have been run for way too long, causing everything to disappear. Or, maybe the terminals were connected in reverse.

C. The lane loaded with the ladder does not have anything, but the lanes loaded with the samples display one band for each lane.

   Maybe the negative control was used accidentally instead of the ladder. Or, maybe the ladder is bad.

4. After PCR amplification of total community DNA using a specific primer set, why is it usually necessary to either clone or run DGGE on the products before sequencing them?

   Cloning or DGGE are generally used to resolve the different forms of the gene in order to ensure the amplified genes are sequenced.

5. You wish to identify whether organisms capable of autotrophic growth using the Calvin cycle exist in various soil samples. This pathway requires a unique enzyme, *ribulose bisphosphate carboxylase*. Describe one way you could do this that does not involve either culturing or microscopic methods.

   I would determine the target gene required to produce the enzyme. The I would use fluorescent in situ hybridization (FISH) to see if the target gene is present in the sample.

6. Give an example, not already listed in Ch. 18, of how environmental genomics has discovered a known metabolism in a new organism, although that organism has never been cultured.

   The nearly complete assembly of the genome of an uncultured bacterium, Kuenenia stuttgartiensis, revealed unique metabolic adaptations associated with anaerobic ammonium oxidation.

   M. Strous, E. Pelletier, S. Mangenot, T. Rattei, A. Lehner, M.W. Taylor, M. Horn, H. Daims, D. Bartol-Mavel and P. Wincker et al., Deciphering the evolution and metabolism of an anammox bacterium from a community genome, Nature 440 (2006), pp. 790–794.