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Application of PCR-based Restriction Fragment Length Polymorphisms (RFLP) of Genes Encoding 16S rRNA in the Study of Bacterial Diversity in Antibiotic Bioreactors

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Abstract: The PCR-based restriction fragment length polymorphisms (PCR-based RFLP) is a method applied in molecular biology which leverage the differences in cells' homologous DNA molecules from different locations on sites of action of restriction enzyme. RFLP acts as a molecular marker and is specific to a single clone or combination of restriction enzyme. This method involves the PCR amplification of 16S rRNA genes, a highly conserved region with variable regions for bacterial species differentiation. The PCR method uses two primers to amplify the 16S rRNA genes isolated from total bacterial community DNA. The amplicons from the PCR are subjected to restriction enzymes digest. The resulting restriction fragments are resolved according to their size using gel electrophoresis. The patterns are visualized and used to classify the bacterial community into diverse groups. Representative amplicons of each group are sequenced to determine their identity. In this study, PCR-based RFLP analysis was employed to study the effect of different antibiotics at varying concentrations on bacterial community in various bioreactors. Following the standard procedures, the method was able to classify and give the identity of 254 selected bacterial isolates from sludge samples of the various reactors into 7 groups, which includes Pseudomonas gessardii group, Staphylococcus saprophyticus group, Acinetobacter sp. group, Bacillus sp. group, Myroides marinus group, and Enterobacter sp. From their different patterns and subsequent sequencing. Generally, our result shows that PCR-RFLP is a veritable tool for studying and identifying the diversity of bacterial communities and can be employed in studying the recombination rate providing genetic distance between loci.

Keywords: *Bioreactors, Bacteria Diversity, Genes, PCR-RFLP, 16S rRNA*

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I. INTRODUCTION

Bioreactors, especially the anaerobic membrane bioreactors, are commonly used to remove antibiotic residues from wastewater (1). The function of the reactors is based on the principle that antibiotics are degraded or transformed by the metabolic functions of microbes within the system. This is usually enhanced by membrane filtration to restrain biomass and improve treatment efficacy. Other molecular tools currently provide sophisticated adjuncts to the culture-dependent techniques. One such strategy is the combination of PCR and rRNA-based phylogeny, which has been effectively used in elucidation of microbial environments and identifying uncultured organisms. The PCR-based Restriction fragment length polymorphism (RFLP) analysis was one of the early techniques to be extensively used for detecting variation at the DNA sequence level. The principle of PCR-RFLP is based on resultant patterns generated after the digestion of restriction enzymes of DNA molecules obtained from different individuals (2).

Restriction fragment length polymorphism uses the specificity of restriction endonucleases to generate different fragments of variable length in DNA samples across different individuals or organisms. Analysis by RFLP played an important role in genome mapping and genetic disease analysis. With the recent advances in molecular biology and genomics, the use of RFLP has been reduced due to its time-consuming steps, expensive and cumbersome features (3). There have been so many reports on the application of PCR-

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based RFLP in studying the diversity of microbial communities in environmental samples, soil environments microbial diversity (4), and also applied in the analysis of medical samples (5).

Our study is on the application of PCR-based restriction fragment length polymorphism analysis of the 16S rRNA gene, a universally present and highly conserved region, which contains variable regions that allow for differentiation between species (6), in bioreactors designed to monitor the removal of antibiotic residues, and their effects on bacterial diversity. This report focuses on the diversity of the bacterial community by applying PCR-RFLP analysis. The possibly large number of fragments will be resolved by using polyacrylamide gels to produce a community-specific pattern (7, 8). Table 2: Sequence of 16S rRNA primers used in this study

II. METHODOLOGY

The study was on bioreactors designed to remove varying amounts of different types of antibiotics in sludge samples. Table 1 shows the estimation of the antibiotic-resistant ratio in the sludge samples from each reactor, at six (6) different concentration levels (mg/l) (Table 1). Sludge samples were taken from each of the reactors according to standard procedures. Isolation and purification of bacterial strains with distinct morphology were carried out on tryptic soy agar and nutrient agar plates, respectively, with or without antibiotics incorporated.

Table 1: Reactors Antibiotic Concentration at the Six (6) Different Stages

STAGE	BLANK (mg/l)	SPM (mg/l)	OTC (mg/l)	STM (mg/)	TGC (mg/)
1	0	0	0	0	0
2	0	0.1	0.1	0.1	0.1
3	0	1	1	1	1
4	0	5	5	5	5
5	0	50	50	50	10
6	0	200	200	200	50

Key: SPM -Spiramycin; OTC -Oxytetracycline; STM -Streptomycin; TGC -Tigecycline

➢ *DNA Extraction from Bacterial DNA*

Genomic DNA was isolated from 254 selected bacterial isolates from the different reactors using the TIANamp bacteria DNA kit (TIANGEN Biotech Co., Beijing). This, according to the manufacturer's instructions, involves the pipetting of 5 mL bacterial culture suspension in a microcentrifuge tube and centrifuging for 1 minute at 10,000 rpm. Using the provided solutions in the kit and following the manufacturer's instructions, without any modification, the DNA samples were extracted. The concentration and quality of the DNA were determined by the use of NanoDrop ND-1000 (Nanodrop, USA). Extracted nucleic acid was stored at −80 °C before analysis.

➢ *PCR Amplification of Bacteria 16S rRNA Genes*

PCR amplification of the 16S rRNA genes was carried out using bacterial universal primers 27F and 1492R (Table 2.0) for the target of the conserved region of the 16S rRNA of the genomic DNA. The standard 50µl PCR mixture (Takara, Dalian, China) included 1× PCR buffer containing 1.5 mM MgCl2, 200 mM of each deoxynucleoside triphosphate (dNTP), 10 pmol of each primer, 1.25 U of TaKaRa© rTaq polymerase, and 1µl of DNA template. Polymerase chain reaction conditions consisted of initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, annealing temperature at 55°C for 1 min, and extension at 72 °C for 1 min 30 s, and completed with a final extension at 72°C for 10 min. Sterile water was used as the negative control.

Table 2: Sequence of 16S rRNA Primers Used in this Study

Primer	Specificity	Sequence	Reference
27F	Universal	AGAGTTTGATCCTGGCTCAG	Lane, 1991
1492R	Universal	TACGGYTACCTTGTTACGACTT	

➢ *Gel Electrophoresis*

At the end of the reaction, the amplified products were analyzed by electrophoresis in 0.8% (weight/volume) Agarose gel prepared using 0.8% agarose (Invitrogen) in Tris-Borate (TBE) buffer solution (pH 8.5) in a 500 mL Erlenmeyer flask. Following the standard protocol, the gel is prepared using the appropriate comb size, and the gel is placed in an electrophoretic tank. A volume of 5 µL of the PCR product was mixed with 1 µL of 6× loading dye, and the DNA ladder was loaded too. A voltage of between 90V and 100V was applied for 30 - 45 minutes with an alternating current. The gel was taken out and then photographed on a TransUV illuminator (Biorad) using Quantity One Gel Imaging software.

The amplified DNA fragments were cut and purified from the Agarose gels using the TIANgel© Midi purification kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions.

➢ *Restriction Fragment Length Polymorphism (RFLP) Grouping of Amplicons*

Amplified products were grouped according to the analysis of Hae III restriction fragment length polymorphism (RFLP) patterns using BioNumerics version 6.01 (Applied Maths, Sint-Martens-Latem, Belgium). Amplified products were digested using the Hae III (TaKaRa© Dalian, China). The reaction mixture contains 1µL of Hae III enzyme, 2µL of 10×M buffer, 20 µl of sterilized distilled water, and 1µg of purified DNA substrate. The mixture was incubated at 37˚C

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for 6 h. The product was analyzed on 2% agarose as described previously.

The similarity of communities was estimated by visual comparison of the electropherograms by band-matching the different fragment patterns to produce a similarity matrix. For each RFLP pattern, one or two amplified products were sequenced by Invitrogen, Inc. (Shanghai, China) using a BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) on an ABI 3730 capillary sequencer (Applied Biosystems). The phylogenetic analysis of 16S rRNA gene sequences with Ribosomal Database Project II release 9.49 and the GenBank database using the BLAST program was carried out to identify the various bacterial groups.

III. RESULTS

The result of the experiment shows a successful extraction of DNA fragments from the various bioreactor compartments. The NanoDrop readings showed a substantial amount and high purity of the DNA fragments isolated. The PCR amplification of the selected DNA fragments from the TransUV illuminator (Biorad) using Quantity One Gel Imaging software shows a successful amplification of the 16S rRNA genes (Plate 1) from all the selected bacterial isolates.

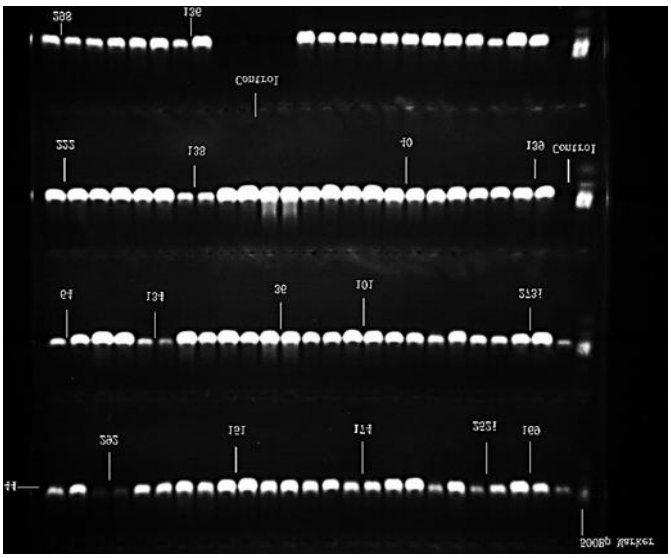

Plate 1: Amplified 16S rRNA Genes of the Bacterial Isolates in 1% Agarose Gels, with a 500bp DNA Marker

The Hae III digest of the amplified PCR products gave restriction fragment length polymorphism (RFLP) patterns that helped in the grouping of the bacterial isolates according to their analysis (Plate 2). The visual comparison of the electropherograms shows the similarity of the bacterial communities and the subsequent sequencing of one or two representatives of each pattern, classifying the bacterial isolates into 7 groups of the 254 isolates, which include *Pseudomonas gessardii* group, *Staphylococcus saprophyticus* group, *Acinetobacter* sp. group, *Bacillus* sp. group, *Myroides marinus* group, and *Enterobacter* sp. Group. Table 3 shows the frequency of the bacteria isolates distribution in the various reactor compartments. The electropherograms show the 100bp markers that gave an estimate of the fragment size, assisting in the grouping. The numberings on the plate show some of the members of each unique pattern in the community (Figure 2).

IV. DISCUSSION

The bioreactors in this study were primarily designed to remove antibiotics in a wastewater system. Antibiotics of various types and concentrations were added to the bioreactors, and their effect on the diversity in the bacterial population was monitored over time. Our focus in this study is on how PCR-based restriction fragment length polymorphism (PCR-RFLP) was applied to classify the bacterial diversity of the bioreactors in monitoring the effect of the antibiotics on the bacterial population. The PCR-based RFLP uses the specificity of restriction endonucleases to generate different fragments of variable length of DNA samples across different organisms (9). In this study, analysis of 16S rRNA genes of bacterial isolates obtained from the different bioreactor sludge samples was used to determine the bacterial diversity within the microbial community.

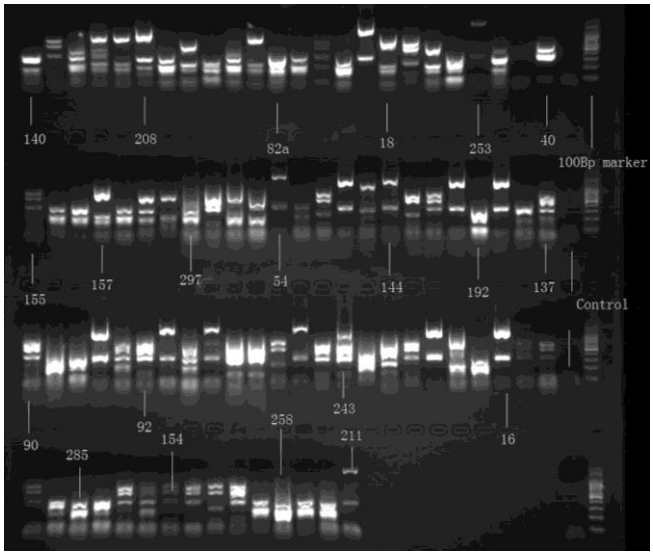

Plate 2: *Hae III* Fragments of PCR Amplicons Digest of 16S rRNA Genes of Selected Bacterial Strains in 1.5% Agarose Gels; with 100bp DNA Marker

LEGEND
Isolate code
140 - *Proteus mirabilis* group
157 - *Pseudomonas gessardii* group
144 - *Staphylococcus saprophyticus* group
192 -*Acinetobacter* sp. group
156 - *Bacillus* sp. group
208 - *Myroides marinus* group
243 - *Enterobacter* sp. Group

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Table 3 : Frequency Distribution of Selected Bacterial Strains Isolated from the Bioreactors SPM -Spiramycin; OTC -Oxytetracycline; STM -Streptomycin; TGC -Tigecycline

Genus or Species	No of Isolates from					Total No of Isolates
	Blank	SPM	OTC	STM	TGC	
Acinetobacter sp.	31	2	4	3	3	43
Bacillus sp.	24	2	3	2	2	33
Enterobacter hormaechei	9	1	3	2	2	17
Enterobacter sp.	26	3	3	2	2	36
Myroides marinus	15	1	2	2	1	21
Proteus mirabilis	30	3	4	1	2	40
Pseudomonas gessardii	21	1	3	2	3	30
Staphylococcus saprophyticus	27	2	1	2	2	34
TOTAL	**183**	**15**	**23**	**16**	**17**	**254**

The result of our study showed that genomic DNA was successfully isolated from 254 bacterial isolates, and their concentrations and purity were determined by the NanoDrop. The PCR-based RFLP was carried out, and its analysis revealed a wide range of bacterial diversity within the reactors. This technique was able to select for similarity in pattern, which depicts the different bacterial groups in the reactor. After the sequencing and phylogenetic analysis, 7 bacterial groups were classified from the 254 isolates, including *Pseudomonas gessardii* group, *Staphylococcus saprophyticus* group, *Acinetobacter* sp. group, *Bacillus* sp. group, *Myroides marinus* group, and *Enterobacter* sp. Group. These isolates are very similar to bacterial groups identified in a pharmaceutical wastewater applying the PCR-RFLP analysis [10]. Aside from being used as a molecular technique to study microbial wastewater communities (11), they are also used in the study of microbial diversity in soil environments (4) and medical samples of protozoan parasites (5).

V. CONCLUSION

The application of PCR-RFLP in our study is useful in estimating the phylogenetic diversity and composition of communities in the different reactors. This method makes it practicable to include a larger population of microbial communities in determining their abundance in various environments. It gives insight into determining the number and abundance of numerically dominant ribotypes within a community. The method produces distinct community patterns that can be used to evaluate the similarity of the different bacterial communities. This approach should provide a simplistic means to study microbial diversity and to monitor changes in microbial community structure that may occur in response to environmental perturbations such as the inclusion of antibiotics within a reactor.

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