Journal of Natural Sciences Research www.iiste.org
ISSN 2224-3186 (Paper) ISSN 2225-0921 (Online)
Vol.3, No.14, 2013

Molecular Identification of Soil Bacteria by 16srDNA Sequence

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ABSTRACT

In this current study, 16S rDNA (genotypic) identification technique is focused on identification of conventionally unidentifiable isolates those are unevaluated in isolated by employing molecular techniques and Bioinformatics in uploading and retrieving isolate gene sequences which are rapid, reliable and accurate in differentiation of various soils isolates. This study is an automaton of 16Sr DNA gene sequence that allows a queue comparison analysis of published sequences deposited in the microbial genome database was used. Polymerase chain reaction (PCR) amplification of 16SrDNA gene using the consensus bacterial primer and separation of the resulting polymer chain reaction amplicon by cloning, temperature gradient electrophoresis are major ecological techniques that are used in the description of soil bacteria. The isolated gene was cloned using PTZ57r or T cloning Vector amplified using 16SF and 16SR primer transformed in DH5α Cells resulting PCs 16s Plasmid hybrid. The primer 16S F_2 obtained from M13 forward primer was used and aligned using BLAST and submitted to EMBL+GENEBANK+DDBJ+ PDB. 99% similarity was observed and later it was analyzed with the existing sequence in ribosomal database project II. RDP classifier was used for confirmation with 100 % similarity. The bacteria were identified as Burkholderia cenocepacia when the sequence was submitted and retrieved via the World Wide Web and new sequence compared with those held in the database using the basic local alignment tool (BLAST). A segment of 734 out of 736 nucleotide of 16S rDNA gene of Burholderia Cenocepacia is the region of choice for primer construction because of proximity that provides a successful discrimination in strains of Burholderia Cenocepacia in soil. 16S rDNA gene account to 99% similarity score in molecular typing and identification of bacteria which concerns deposition of sequences into established microbial genomic database

Key Words: *Burkholderi; Bacterial transformation; Characterization; DNA based techniques*

1.0 INTRODUCTION

Microorganisms are heterotrophic and can be classified as decomposers and synthetic microorganisms. These microorganisms play a major role in the consumption and production process. Besides, these microorganisms have been used in medical aspects in conjunction with various techniques in molecular biology and genetic engineering. Various methods have been used for classification of the microorganism which includes biochemical identification depends on the biochemical reactions, conventional test and recombinant DNA technique have potentially been used as complimentary to phenotype distinction. In conjunction, with polymerase chain reaction along with restriction fragment length polymorphism (RFLP), an intensive molecular biology and molecular techniques have been employed as an aid to bacterial taxonomy and identification. The DNA base and hybridization, quantitatively define species by composition in G+C contents; clearly distinguish between the genomes that are unrelated several methods are employed to identify the microorganisms. Kits and landmark AP120E strip test which involves micromethod employing 20 different biochemical tests generates Septyl (7) Digit code of 18 to 21 hours basing on the biochemical reaction plus a screening oxidase test.. According to previous studies where an intergenic spacer region (ISR) the Species-specific primers are used (Rachman *et al,* 2003), a gene can be amplified, cloned with a suitable vector and its size estimated and analyzed by gel electrophoresis. Polymerase chain reaction (PCR) and electrophoresis are molecular techniques applied in recent for evaluation of environmental microorganisms. The analysis provides an accurate identification of bacteria and for confirmation is observed after the gene has been sequenced and show similarities with the sequence deposited in Gene Bank and its proteins can show the conserved domain of peptidase family during the analysis of 16SrDNA protein which is highly conserved among the heterozygous bacteria. Basing on previous research that was carried out (Jayarao *et al* 1992), 16SrDNA technique was applied and demonstrated potential application in finger printing in differentiation and identification of 12 bacterial species isolates from cows. Polymerase chain reaction as a molecular identification technique was used in the

identification of 40 clinical strains of *Serratia marcescens* and *Pseudomonas aerugenosa* when amplified spacer primers of 16S and 23S genes in prokaryotic rDNA and rRNA genetic loci were used and confirmed resolved products by electrophoresis (Kur *et al,* 1995). In a recent study carried out aiming at the detection and identification of typical *Leuconostoc* species, polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method was used. The method utilized a set of specific primers for amplification of the 16S rDNA region of typical *Leuconostoc* species. PCR-RFLP method enables the rapid and reliable identification of *Leuconostoc s*pecies and to distinguish them from the other phylogenetically related lactic acid bacteria in food samples (Jang *et al* 2003).

2.0 MATERIALS AND METHODS

Chemicals used for the study were of analytical grade purchased from E. Mark Tulip Diagnostics Ltd, Goa. India. Microbiological media components were purchased from Hi-media laboratories Pvt. Limited, Mumbai. Tulip Diagnostics private limited. Goa India, PCR components used in this study were purchased from Geni, Bangalore. The research was carried out in Salem city, Tamil Nadu state in India at Acme Progen Biotechnology (India) Private Limited.

2.1 Soil Sample Collection:

Soil samples were collected from a homestead located near a fertile farm yard. One gram soil was collected using a sterile spatula into a sterile Petri dish. The soil was brought to the laboratory and processed immediately. The bacterial organisms present in the soil were isolated using serial dilution method (Booth, 1971) and the cultures were maintained on a Petri dish of nutrient agar at 25°C over night.

2.2 Isolation of Soil Bacteria by Serial Dilution

Weighed 1 g of soil sample and serially diluted with 10 ml of distilled water. From the dilution, 0.1ml of the sample was taken with sterile pipette and transferred onto the surface of a sterile nutrient agar medium in a Petri dish and the sample was spread over the entire surface with the sterile L-rod. Inoculated plates were incubated at 37°C for 24-48 hours in aerobic condition. Single white colonies were selected from plate labeled 10^{-3} and used for subculture at 37°C for 24-48 hours. A single colony was selected and incubated in the broth and later used in the biochemical tests **(Table 3)**.

2.3 IDENTIFICATION OF BACTERIA:

Bacteria identification was carried out using conventional bacteriological tests that included Gram Stain, Motility Test, Oxidase Test, Methyl Red Test, Voges-Proskauer Test, Indole Test, Catalase Test, Citrate Utilization Test, Urease Test, Test for H_2S Production and Glucose Utilization (Benson, 2001)

2.3.1 Extraction of Genomic DNA

The genomic DNA was extracted from 2ml of overnight culture by centrifugation at 16099xg for 5minutes. The supernatant was decanted and add 2ml of overnight culture was added in the same tube. Centrifugation and decantation of the supernatant were done as stated above and this resulted into a pellet of 4ml culture. Five hundred µl of Tris-EDTA buffer (100 mM Tris,10 mM EDTA) was added to the pellet and completely suspend the cell by taping or repeated pipeting (20mg/ml) and incubate for one hour at 37ºC. One hundred µl of SDS (20%) buffer was added. Lysozyme was added to a tube with gram positive bacteria, and mixed by gentle inversion and later incubated at room temperature for 5minutes. Two hundred µl of 5M NaCl buffer was added and the contents were mixed inverting the tube. Tubes were incubated on ice for 10 minutes,then centrifuged at 11180xg for 5 minutes. The supernatant was transferred to new 2ml tube and 500 µl of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added to the contents, thoroughly mixed by inversion and later centrifuged at 20000xg for 5 minutes. The upper aqueous phase was transferred to new 2ml tube without disturbing the middle white layer. Equal volume of chloroform isoamyl alcohol (25:24:1) was added and centrifugation again at 11180xg for 5 minutes. The upper aqueous phase was transferred to a new 2ml tube without disturbing the middle white layer. Equal volumes of isopropanol was added and mixed by inversion. This was left to stand at room temperature before centrifuged at 11180xg for 5minutes. The supernatant was decanted without disturbing the small white pellet. Five hundred µl of 70% ethanol was added and centrifuged the tube at 11180xg for 5 minutes and decanted the supernatant without disturbing the small white pellet. The eppendorf tubes were opened and kept in upside down position on a tissue paper to drain out and evaporate the remaining fluid for 5-10 minutes. Pellets were dissolved in 50-100µl of TE buffer depending on the pellet obtained. Five µl of RNase A was added and tube tubes were left at room temperature or 37ºC for 30 minutes. The Genomic DNA was confirmed by running the sample on the 0.8% agarose gel electrophoresis and examined under UV-Trans illuminator.

2.3.2 Polymer chain reaction (PCR-16S rDNA analysis of sample)

The primer selected and used for the 16S rDNA analysis were,

16S F 5'AGAGTTTGATCCTGGCTCAG 3' and
16SR 5'GTACGCTACCTTGTTACGAC 3'
The master mix was prepared as shown on the table one.
The master mix was mixed thoroughly by vortex and aliquot of 24.5 µl were transferred into each of the five reaction tubes. In each of the PCR tubes 0.5 µl of each DNA sample was added. The negative control did not receive DNA sample. The contents were mixed by flicking by a finger, and then centrifuged briefly for about 10 seconds at 112xg to concentrate the reaction mixture at the bottom of the tube. Tubes were placed in the thermocycler, then ran using the following program; initial denaturation at 94^{o}C for 5 minutes, cycle denaturation at 94^{o}C or 45 seconds, annealing at 50^{o}C for 45 seconds and extension at 72^{o}C for 60 seconds. From cycle denaturation to extend, the cycle was repeated 34 times. This was followed by a final extension at 72^{o}C for 5 minutes and holding stage at 22^{o}C until when used for further steps. The PCR products were resolved on to 1 % Agarose gel and observed using trains illuminator.

2.3.3 Isolation, Cloning, Transformation and DNA Analysis.

The isolated genomic DNA of dichloromethane degrading bacteria (1) was confirmed by running the sample in 0.8% v/v Agarose gel Electrophoresis along with DNA marker and approximately 10000 base pairs were estimated (**Figure 1**). Polymerase chain reaction using 16SrDNA Primers [16S Forward Primer 5'AGAGTTTGATCCTGGCTCAG 3' and 16S Reverse Primer 5'GTACGCTACCTTGTTACGAC 3'] with the help of a thermocycler. The amplicon was confirmed by running the product in 1% v/v Agarose gel electrophoresis along with DNA marker. Approximately 1500 base pairs were estimated on visualization using U.V trains illuminator or measured using a UV spectrophotometer at 260 nm, and 280nm. The DNA bands **(Figure 2)** were purified by the Gene Jet™ extraction Kit. Hybrid DNA was prepared by cloning/ ligating genomic with plasmid DNA (PTZ57R/T cloning vector) transformed in *E. coli* DH5 cells and grown in selective media X-gal IPTG ampicillin **(Figure 3)** hybrid DNA was isolated from the colonies and ran in 1% v/v agarose Gel Electrophoresis

2.3.4 Gel Elution

Heated block or water bath, gel slice containing DNA fragment was excised using a scalpel or razor blade. The gel was cut as close to the DNA as possible to minimize its volume. The gel slice was placed into a pie-weighed 1.5 ml tube, weighed and recorded. Ratio 1:1 (volume: weight) volume of binding buffer was added to the gel slice .100 µl of the binding buffer for every 100 mg of Agarose gel. The gel mixture was incubated at 50ºC - 60ºC for 10 minutes until the gel slice was completely dissolved. The tubes were mixed by inversion every few minutes to facilitate the complete melting process. Eight hundred µl of the solubilized gel solutions was transferred into the GenJet™ purification column and centrifuged at 252xg for 1minute. Discarded the flow thoroughly and placed the column back into the same collection tube. Seven hundred µl of wash buffer (diluted with ethanol) was added into the GenJet™ purification column and centrifuged at 252 xg for 1 minute. The flow was discarded through and placed the column back into the same collection tube. The empty GenJet™ purification column was centrifuged for an additional 1minute to completely remove residual wash buffer. Transferred the GenJet™ purification column into a clean 1.5ml Microcentrifuge tube and fifty µl of Elution buffer was added to the center of the purification column membrane. This was followed by centrifugation at 252xg for 1minute. The GenJet™ purification column was discarded and the purified DNA was stored at –20ºC.

2.3.5 Ligation

To a clean 0.5 ml Microcentrifuge tube 4µl of sterile water, 2 µl of ligation buffer, 1 µl of Λ DNA (insert), 2 µl of plasmid (PTZ57R/T cloning vector) and 1 µl DNA ligase were added.. The content in the tubes was mixed and briefly spun to bring down the reaction mixture and the tube were incubated at room temperature for 1-2 hours at 4ºC for overnight.

2.4 Bacterial transformation

Bacterial transformation was carried out by using *Escherichia coli* competent cells. Transformation of *E.coli* with plasmid DNA, competent cell was prepared by the calcium chloride method (Sambrook *et al*, 1989). *Escherichia. coli* was streaked on a LB agar plate from a glycerol stock maintained in -70ºC. The cells were grown at 37ºC overnight to obtain single colonies. A single colony was inoculated into five ml LB broth in a fifty ml culture tube and was allowed to glow overnight at 37 ^{0}C under vigorous shaking at 352xg in an orbital shaker incubator. One ml of this overnight culture was added to fifty ml of YT broth dud placed at 37ºC in an orbital shaker incubator at 1000xg for 2-3 hours. The optical density was checked every one hour at 600nm in a spectrophotometer. The culture was removed when the optical density at 600nm reached 0.4-0.5 and chilled at 4ºC. Thirty ml of the cell was transferred to fifty ml centrifuge tubes and harvested by centrifugation at 1006xg

at 4ºC. Four ml of fresh calcium chloride with 15% glycerol were added to the tubes and the pellets were very gently re-suspended and 100µl of cells were transferred into 1.5ml Microcentrifuge tube. The tubes were frozen in liquid nitrogen and stored at -70ºC until further use. The transformation efficiency was measured as Number of colonies log of DNA 1ml of the competent cells. *Escherichia coli* DH5αF were grown in LB solid or liquid culture. The bacteria were streaked out on a LB Agar plate and allowed to grow overnight on a single colony isolation for plasmid extraction. The bacteria were grown with appropriate selective antibiotics in 5ml liquid medium for mini-prep and fifty ml of maxi prep. They were grown since 12-14 hrs at 37ºC in an incubator shaker at a speed of 168xg. The transformed cells grew as colonies in plates and were used for further analysis. Basing on the previous study carried out in order to detect *Rhizobium* species directly in the environment (De Oliveira *et al.,* 1999), specific PCR primers were designed on the basis of sequence analysis of 16S rDNA spacer regions of strains. Primer specificity was checked by comparing with available rDNA spacer sequences in databases, and also PCR using DNA from the target and reference strains. Denaturing gradient gel electrophoresis (DGGE) were used to detect the sequence polymorphisms of RDNA spacer fragments among strains of the same species. The PCR products were purified and characterized by single digestions with restriction endonuclease, Taq I polymerase was found to discriminate the respective reference strains. In the previous studies, twenty-three isolates, out of 33, were assigned to one of the reference species, on the basis of their restriction profiles by digestion with HpaII (Sato *et al,* 1998).

3.0 RESULTS

The isolated microorganisms from the soil samples were described and characterized using both conventional, morphological and biochemical methods (Table 3) as well as by DNA based techniques.

3.1 Isolation, Cloning, Transformation and Analysis of Genomic DNA

Approximately 12216 OC- form hybrid plasmid DNA of pPG017 (1) was estimated **(Figure 4)**. Sequencing was carried out to confirm the gene of the bacteria alignment with BLAST (Basic Local Alignment Search Tool) and a partial sequence of 16S rDNA of *Burkholderia Cenocepacia* was located with the accession No. AM747721 with 99% similarity. Confirmation was done using RDP classifier. The analysis showed 100 % similarity to the genus *Burkholderia. Burkholderia cenocepacia* specific oligonucleotide primer sequence described by 16 S rDNA primer was found in the oligonucleotide. The partial sequence of 16SrDNA sequence of *Burkholderia cenocepacia* showed 99% of the class beta *Proteobacteria* and family *Burkholderiaceae* **(Figure 5)**

4.0 DISCUSSION

The isolation of bacteria using conventional techniques employed to yield two different isolates. However during the molecular typing and identification by PCR analysis using 16S r DNA spacer gene (forward and reverse primers) yielded a product of about 1500 Bp (**Figure 2**) which was carried further for sequencing. The PCR technology amplifies the spacer regions for specific purpose of detecting heterogeneity between and within species (Barry *et al.,* 1991). The heterogeneity is found in terms of both the number and the length of the spacer and broadly applicable for bacterial identification and typing. The primers used in PCR are designed according to specific segments which should give accurate parameters (Hassan *et al*, 2008) with reduced errors. The bacteria were identified as *Burkholderia cenocepacia* (**Figure 5**). The sequence was submitted and retrieved via the World Wide Web and new sequence compared with those held in the database using the basic local alignment tool (BLAST), http://ncbi.nih.gov/cgi/nph-blast?jform=1 and Ribosomal Database Project (RDP), University of Illinois, Illinois. USA, http://rdpwww.life.uiuc.edu/index2.html which provides both phonetic and phylogenetic analysis of query sequences with those online at the RDP and BLAST has a greater number of sequences held online at the European Molecular Biology Laboratory (EMBL), NCBI, and the DNA Database of Japan (DDBJ). The sequence of 1374 base pairs was obtained and deposited in Genbank with the accession number AM 747721 with higher (99%) similarity with 16SrDNA gene. The focus of this study has been the proposal that a rapid and universal bacterial identification and typing scheme are possible based on amplification of the variable length of 16S RDNA spacer regions which is based on observation that many bacteria carry rDNA operons and a considerable length and sequence heterogeneity is apparent when the 16S rDNA sequences of such strains are aligned. Currently, a segment of 734 out of 736 nucleotides of 16S rDNA gene of *Burholderia Cenocepacia* is the region of choice for primer construction because of proximity that provides a successful discrimination in strains of *Burholderia Cenocepacia* in soil. In relation to other alternative techniques of bacterial identification it is rapid, cost-effective and accurate. Other studies involving other regions in rRNA primer either failed to detect the spacer variation at the species or strain level (Gill S. *et al* 1993) or had limited success (Jensen *et al,* 1993). The 16S rDNA sequence provides unambiguous data even for rare isolates which are reproducible in and between laboratories. This is a genetic characterization procedure involving molecular techniques. It provides solution to drawbacks related to alternative molecular techniques with primers like 23S rDNA ,16S rRNA, denaturing gradient gel electrophoresis (Ahn *et al.,*2009), temperature gradient gel

electrophoresis with anomalous hybridization during the reaction. The complement of the sequence forms the 3' end of the primer sequences at which the Taq polymerase bind to commence amplification in PCR procedure. Matching at the region increases the efficiency of priming hence detecting the spacer variation present. Pertinent to the latter outcome are our findings of significant viability in particular regions of *Burluloderia cenocepacia* rDNA gene used. These 16S rDNA primers are therefore recommended in the detection of all copies of spacer regions ranging more than ten copies. The 16S rDNA gene account to 99% similarity score in molecular typing and identification of bacteria which concerns deposition of sequences into established microbial genomic database and accuracy depend on the extensiveness of bacterial strains from which the bacteria 16S rDNA gene derived has been characterized.

ACKNOWLEDGEMENT
The principal author would like to acknowledge supervisors of the project, Biochemistry, Biotechnology and Microbiology department Periyar University, Kampala International University and Acme Progen Biotechnology (India) Private Limited, on their persistent patience and their valuable assistance towards my research in "molecular identification of soil bacteria by 16S rDNA sequence".

TABLES

Table 1: Preparation of the Amplicon during polymerase chain reaction.

Components	Volume (µl) per reaction
Double distilled water	18.3
Taq DNA polymerase buffer(10x)	2.5
dNTP mix(2mM)	2.5
Forward primers (10µM)	0.5
Reverse primers(10µM)	0.5
Template DNA	0.5
Taq DNA polymerase enzyme (5U/µl)	0.2
Total reaction volume	25.0

Table 2: Gram Stain, Motility Test and Biochemical Tests results for the isolated from soil samples

Biochemical test	Sample 6	Sample 7
Gram stain	-	-
Motility test	+	+
Indole Test	-	-
Methyl red	-	-
Voges-Proskauer-test	-	-
Citrate Utilization Test	+	+
Urease Test	+	+
Catalase Test	-	-
Oxidase Test	-	-
Test H_2S production and glucose utilization	+	+
Triple sugar iron agar test	+	+

FIGURES:

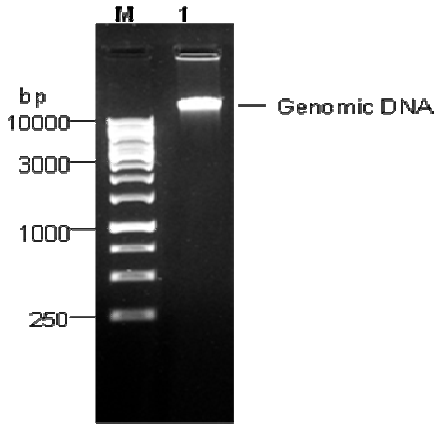

Figure 1

(Agarose gel Electrophoresis (0.8% v/v) of Genomic DNA of Dichloromethane Degrading Bacterium)

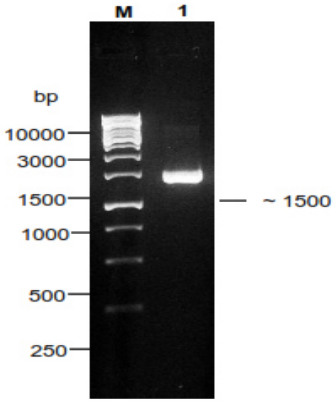

Figure 2

(Agarose Gel electrophoresis (1%v/v) of PCR amplified product)

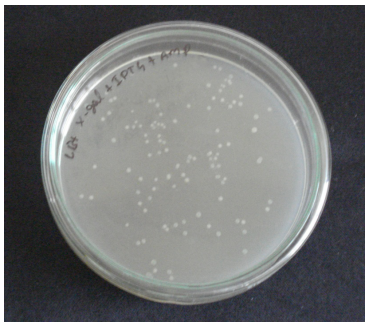

Figure 3

Transformed colonies of Hybrid Plasmid (selection based on X-GAL IPTG ampicilin)

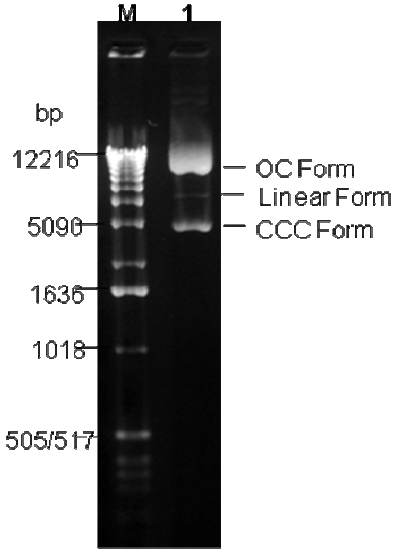

Figure 4

Agarose Gel Electrophoresis (1% v/v) of Hybrid Plasmid pPG017

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1   AGTATTGGCC CAGGGGGCTG CCTTCGCCAT CGGTATTCCT CCACATCTCT  50
51  ACGCATTTCA CTGCTACACG TGGAATTCTA CCCCCCTCTG CCATACTCTA 100
101 GCCTGCCAGT CACCAATGCA GTTCCCAGGT TGAGCCCGGG GATTTCACAT 150
151 CGGTCTTAGC AAACCGCCTG CGCACGCTTT ACGCCCAGTA ATTCCGATTA 200
201 ACGCTTGCAC CCTACGTATT ACCGCGGCTG CTGGCACGTA GTTAGCCGGT 250
251 GCTTATTCTT CCGGTACCGT CATCCCCCGA CTGTATTAGA GCCAAGGATT 300
301 TCTTTCCGGA CAAAAGTGCT TTACAACCCG AAGGCCTTCT TCACACACGC 350
351 GGCATTGCTG GATCAGGCTT TCGCCCATTG TCCAAAATTC CCCACTGCTG 400
401 CCTCCCGTAG GAGTCTGGGC CGTGTCTCAG TCCCAGTGTG GCTGGTCGTC 450
451 CTCTCAGACC AGCTACTGAT CGTCGCCTTG GTAGGCCTTT ACCCCACCAA 500
501 CTAGCTAATC AGCCATCGGC CAACCCTATA GCGCGAGGCC CGAAGGTCCC 550
551 CCGCTTTCAT CCGTGGATCG TATGCGGTAT TAATCCGGCT TTCGCCGGGC 600
601 TATCCCCCAC TACAGGACAT GTTCCGATGT ATTACTCACC CGTTCGCCAC 650
651 TCGCCACCAG GTGCAAGCAC CCGTGCTGCC GTTCGACTTG CATGTGTAAG 700
701 GCATGCCGCC AGCGTTCAAT CTGAGCCAAT CAAAA 735
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Figure 5*: Partial sequence of 16S rDNA of Burkholderia cenocepacia* (Ribosomal Database Project (RDP), University of Illinois, Illinois USA. http://rdpwww.life.uiuc.edu/index2.html)

REFERENCE:

Ahn JH, Kim YJ, Kim T, Song HG, Kang C and Ka JO, 2009. Quantitative improvement of 16S rDNA DGGE analysis for soil bacterial community using real-time PCR. *J Microbiol Methods.* 78(2):216-222.

Barry ,T., Colleran,G., Glennon M., Dunican,L.K. and Cannon,F. 1991. The 16S/23 S ribosomal spacer region as a target for DNA probes to identify *eubacteria*. *PCR Methods Appl* 1: 51-56

Benson: Microbiological Applications Lab Manual , 2001. Eighth Edition. The McGraw-Hill Companies.

Booth C. 1971. Fungal Culture Media. In: Methods in Microbiology, 4th ed. (J. R. Norris and D. W. (eds). Academic Press, London, New York. p. 49-94.

De Oliveira VM, Coutinho HL, Sobral BW, Guimarães CT, van Elsas JD, Manfio GP.Fundação André Tosello, and Campinas, SP, 1999. Discrimination of *Rhizobium tropici* and *R. leguminosarum* strains by PCR-specific amplification of 16S-23S rDNA spacer region fragments and denaturing gradient gel electrophoresis (DGGE) *Lett Appl Microbiol.* 28(2):137-141

Gill,S., Belles-isles,J. Brown,G., Gagne,S., Lemieux,C., Mercier J.P and Dion,P. 1994. Identification of variable of ribosomal DNA spacer from pseudomonas soil isolates. *Can J microbial* 40:541-547

Hassan AA, Vossen A, Lämmler C, Siebert U and Fernández-Garayzábal JF, 2008. PCR amplification of species specific sequences of 16S rDNA and 16S-23S rDNA intergenic spacer region for identification of *Streptococcus phocae*. *Microbiol Res.* 163 (2):132-135.

Jang J, Kim B, Lee J and Han H, 2003. A rapid method for identification of typical *Leuconostoc* species by 16SrDNA PCR-RFLP analysis. *J Clin Microbiol*. 29(12):2774-2778

Jayarao BM, Doré JJ Jr and Oliver SP ,1992. Restriction fragment length polymorphism analysis of 16S ribosomal DNA of *Streptococcus* and *Enterococcus* species of bovine origin. *J Clin Microbiol.* 30(9):2235-40

Jensen, M.A., Webster,I.A. and Straus N.1993. Rapid identification of bacteria on the basis of polymerase chain reaction amplified ribosomal DNA spacer polymorphism. *Appf Envin Microbiof* 59:945-952.

Kur J, Burkiewicz A, Zablocka A and Gospodarek E. 1995. Identification of *Pseudomonas aeruginosa* on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Acta Microbiol Pol.* 44(2):111-117.

Rachman CN, Kabadjova P, Prévost H, Dousset X.Unité de Recherche Qualité Microbiologique et Aromatique des Aliments, 2003 .Identification of Lactobacillus alimentarius and Lactobacillus farciminis with 16S-23S rDNA intergenic spacer region polymorphism and PCR amplification using species-specific oligonucleotide . *J Appl Microbiol.* 95(6):1207-1216

Sambrook, J.; Fritsch, E.F. And Maniatis,T., 1989. Molecular Cloning: A Laboratory Manual, vol. I. 2nd edition. Cold Spring Harbor Laboratory Press. ISBN 0-87969-309-6

Sato T, Sato M, Matsuyama J, Kalfas S, Sundqvist G and Hoshino E, 1998. Restriction fragment-length polymorphism analysis of 16S rDNA from oral *asaccharolytic Eubacterium* species amplified by polymerase chain reaction. *Oral Microbiol Immunol.* 13(1):23-29

Stein A, Kruszewska D, Gouvernet J and Raoult D, 1997. Study of the 16S-23S ribosomal DNA internal spacer of *Coxiella burnetii*. *Eur J Epidemiol.* 13(4):471-475