Journal of Applied Biology and Biotechnology Vol. 2 (06), pp. 007-009, Nov-Dec, 2014
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2014.2602

Amplification and cloning of *mcrA*gene from enriched consortia of Methanogens

Rachana Chandragupta Acharya, Usha Mukundan

Department of Biotechnology, Ramniranjan Jhunjhunwala College, Ghatkopar(W), Mumbai-400086, India.

ARTICLE INFO

Article history:
Received on: 10/10/2014
Revised on: 21/10/2014
Accepted on: 12/11/2014
Available online: 27/12/2014

Key words: Anaerobic culture, temperature of annealing, PCR amplification, marker assisted identification.

ABSTRACT

Methanogens were enriched using anaerobic culture conditions. The gDNA extracted from the enriched consortia was used for amplification of *mcrA (methyl coenzyme reductase A)* gene. The temperature of annealing was standardized for amplification. The amplified gene was cloned into cloning vector and transformed into *E.coli* DH5α cells. Screening of recombinant transformants was done using Blue-White selection. The vector containing amplified gene was sequenced. The sequencing results showed that the gene had 97% homology with *mcrA* gene from *Methanoculleus bourgensis*. Thus procedure for *mcrA* gene amplification from enriched *Methanogen* consortia was standardized and its marker assisted identification using bioinformatics tools was carried out.

1. INTRODUCTION

Methanogens are members of the domain Archaea and they fall within the kingdom Euryarchaeota. They are obligate anaerobes and can be differentiated from other organisms since they all produce methane as a major catabolic end product [1]. Methane is a gas with potential for environmental harm, but also an invaluable non-fossil fuel. Functional marker genes encoding key enzymes of characteristic metabolic pathways (denitrification, nitrogen fixation, ammonia oxidation, methane oxidation, methanogenesis and sulfate reduction) have been found to be useful in the detection of microorganisms belonging to different ecophysiological classes in complex microbial assemblages from diverse habitats [2,3]. All known methanogens express the methyl coenzyme M reductase A (*mcrA*) that catalyses the terminal step in methane production during the anaerobic fermentation of biomass [4]. Several researches have analysed the diversity of archaea in laboratory scale and commercial biogas plants fed with different substrates such as wastewaters, maize/beet silages and cattle dung by molecular detection of microbial community using mcrA analysis [5, 6, 7].

The aim of this study was to amplify the *mcrA* gene from an enriched culture of methanogens found in cow dung and confirm their presence using this marker assisted identification technique.

2. MATERIAL AND METHODS

2.1.Enrichment of Methanogenes

Cattle dung was obtained from a local Temple in Ghatkopar, Mumbai. The sample was collected in a plastic container and brought to the laboratory 1g of the sample was suspended in 10 ml of O_2 free sterile saline under aseptic condition.

The suspension was filtered through muslin cloth and centrifuged at 2000 rpm for 5 minutes to separate the particulate matter. 0.5 ml of the supernatant was aseptically transferred to serum bottle containing anaerobic medium using anaerobic culture technique [8]. After 30 days of incubation the culture was analyzed microscopically using Gram's staining and fluorescent as well as confocal microscopy to confirm the presence of Methanogens.

2.2 gDNA Extraction from Enriched Culture

5ml of enriched culture was centrifuged at 12000 g for 5 min, the cell pellet was used for the gDNA isolation. gDNA was extracted using HiPure^{TM} Stool DNA Kit (Hi Media), according to the protocol provided by the Kit. A total of 1mg gDNA was obtained as per the analysis done using agarose gel electrophoresis.

* *Corresponding Author*
Rachana C Acharya, Ramniranjan Jhunjhunwala College, Ghatkopar(W), Mumbai-400086, India.
Email: rachana.acharya@gmail.com,
Residence: 1303/29 Vijay Annex, Waghbil road, Off Ghodbader road, Thane-W Thane-400615

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2.3 Pcr Primers and Amplification

The following Degenerate primers were designed and obtained from GeNei™ Merck Specialties forward primer *mcrA*1 5’ -GGTGGTGTMGGATTCACACARTAYGCWACAGC-3’ and reverse primer *mcrA*2 5’ – TTCATTGCRTAGTTWGGRTAGTT-3’. 10 ng of template gDNA was used in a reaction mixture of 50µl containing 10pmol/µl of each primer, 10mM of dNTPs and 5 units of Taq DNA polymerase. The PCR amplification was performed by Thermal Cycler (Techne TC 312). Amplification was carried out at 4 different annealing temperatures. The conditions used were- Initial denaturation for 1 cycle at 95°C for 3 min, 35 cycles of 95°C for 30 s, 50°C/ 55°C/ 60°C for 1 min, 72°C for 1 min and a final elongation at 72°for 10 min. Cloning and sequencing: The PCR product was cloned using INSTA cloning kit (Merck) as per the instructions of the manufacturer. The recombinant TA plasmids then were transformed into *E. coli* DH5 α. The clones containing recombinant plasmids were screened using Blue-White selection. Recombinant plasmids were extracted from white colonies using Hi-Pure mini-prep plasmids extraction kit (Hi Media). The plasmids were again screened for the presence of insert by amplifying the gene with *mcrA*1 and *mcrA*2 primers using PCR program- Initial denaturation for 1 cycle at 95°C for 3 min, 35 cycles of 95°C for 30 s, 60°C for 1 min, 72°C for 1 min and a final elongation at 72°for 10 min. Two of the recombinant plasmids showing the presence of insert were sent for sequencing to Bangalore genei, Merck. The similarity searches for sequences were carried out by BLAST [9] and alignment was done using CLUSTAL W [10].

3. RESULTS AND DISCUSSION

The enriched culture analysed using confocal microscopy showed presence of coccoidal and rod shaped cells with bluish-green fluorescence (Fig. 1) confirming the presence of methanogens [11]. As expected approximately 700bp DNA was obtained when the mcrA gene amplified from the gDNA (Fig. 2) [12]. The amplification was seen best at 60°C annealing temperature as compared to that at 50°C and 55°C.

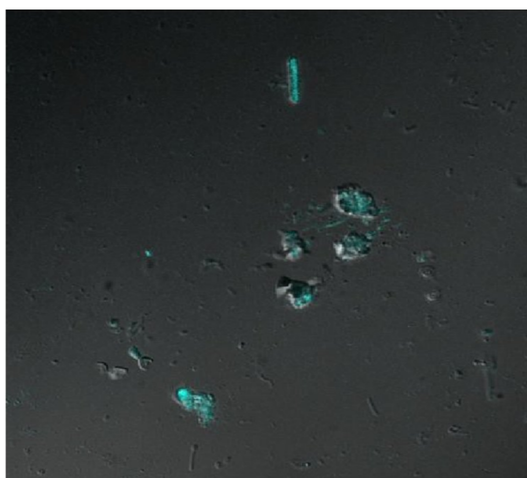

Fig. 1: Enriched Methanogen Consortia as seen using Confocal Microscopy.

The amplified *mcrA* gene which was cloned into the TA-cloning vector and transformed into competent DH5α cells showed presence of both white (cells containing vector plus plasmid) and blue colonies (cells having only vector) when screened using blue white selection. 33 white colonies and 6 blue colonies were obtained. Transformation efficiency was calculated and was found to be 3.9 x 10^2 cfu/µg of DNA.

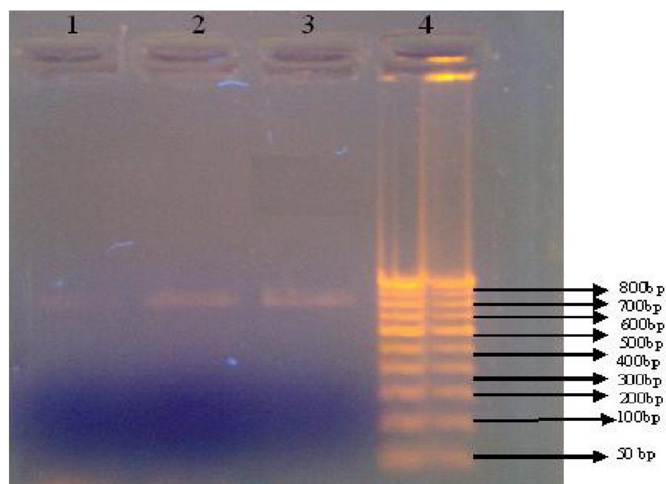

Fig. 2: PCR amplified mcrA gene from gDNA.
Lane 1 annealing at 50°C, lane 2 annealing at 55°C and lane 3 annealing at 60°C.

PLASMID 1

>H05_40A.RA.JJ.SAMPLE.10.T7U20_2013-08-31_1.ab1

GACGTTGTCGGATTCACCGAGTACGCGACCGCGGCCTACACCGACAACATCCTCGAT
GAGTTCACTACCACGGTAAGGACTACATCAAGGACAAGTACAAGGTCGACTGGAAG
AAGCCGAACGCGCAACGACAAGGTCAAGCCGAGCTGCCACGTCGTAAGCGACATGG
CAACCGAGGTCACCCTCAACGCCATGGAGCAGTACGAGCAGTTCCCGGCCATGATTG
AGGACCACTTCGGCGGGTCCCAGCGTGCCGGTGTCATCGCCGCTGCGTCCGGTCTCA
CGGCCGCCATCGCAACCGGCAACTCGAACGCCGGCCTGAACGCCTGGTACCTCCCGA
TGCTTCTGCACAAGGACGGCTGGTCGGGTCTCGGCTTCTTCGGCTACGACCTCCAGG
ACCAGTGCGGGTCTGCAAACTCGCTCTCCATCCGGGGCGACGAGGGTGCGATCGGCG
AGCTGCGTGGTCCGAACTACCCTAACTACGCAGTGA

PLASMID2

>B06_42A.RA.JJ.SAMPLE.12.T7U20_2013-08-31_1.ab1

GGCGGTGTCGGATTCACCCAGTACGCGACCGCGGCCTACACCGACAACATCCTCGAT
GAGTTCACCTACTACGGTATGGACTACATCAAGGACAAGTACAAGGTCGACTGGAAG
AACCCGAACGCGAACGACAAGGTCAAGCCGACCTGCGACGTCGTCAACGACATGGC
AACCGAGGTCACCCTCAACGCCATGGAGCAGTACGAGCAGTTCCCGACCATGATGGA
GGACCACTTCGGCGGGTCCCAGCGTGCCGGTGTCATCGCCGCTGCGTCCGGTCTCAC
GGCCGCCATCGCAACCGGCAACTCGAACGCCGGCCTGAACGCCTGGTACCTCCCGAT
GCTCCTGCACAAGGACGGCTGGTCGCGTCTCGGCTTCTTCGGCTACGACCTCCAGGA
CCAGTGCGGGTCTGCAAACTCGCTCTCCATCCGGGGCGACGAGGGTGCGATCGGCGA
GCTGCGTGGTCCGAACTACCCTAACTACGCAATGA

Fig. 3: Sequences of the mcrA Gene from two plasmids.

PCR amplification carried out to confirm the presence of the insert in the vectors of transformed colonies showed presence of insert in 2 of the 33 white colony plasmids. These two clones when sequenced showed the sequences as given in Fig. 3.

The sequences obtained when analysed using bioinformatics tool ‘BLASTn’ available on NCBI website, gave 101 blast hits for the query sequences. Three sequences with which the query sequences showed more than 90% homology were of *mcrA* gene from *Methanoculleus bourgensis* (97%), Uncultured *Methanomicrobiaceae* archaeon MRR-mcr57 (97%) and Uncultured *Methanomicrobiaceae* clone LC17. Both the

sequences showed same matches. This could be because the gDNA that was used to amplify the mcrA gene was taken from anaerobically cultivated enriched consortia. Hence same type of organisms may have got enriched.

The amplification of specific *mcrA* gene from enriched consortia and its cloning and identification was successfully carried out. Thus it was proved that *mcrA* genes can be used for marker assisted identification and phylogenetic analysis of *Methanogens.*

4. CONCLUSIONS

Amplification of *mcrA* gene from the gDNA was achieved using *mcrA* specific primers. The amplicon was analysed electrophoretically and was cloned into cloning TA vector. Plasmid from two transformed recombinant clones were sent for sequencing to Merck Biosciences, Bangalore, India. The sequences obtained were matched with the database available online and analysis was done using bioinformatics tool BLASTn, which gave 101 blast hits for the query sequence. The sequences showed 90% homology matches with *mcrA* gene of Methanoculleus and Methanomicrobiaceae spps. both belonging to Methanogen family. Thus methanogen specific gene from enriched culture was successfully amplified, cloned and identified using molecular techniques.

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How to cite this article:

Rachana Acharyaa and Usha Mukundan. Amplification and cloning of *mcrA*gene from enriched consortia of Methanogens. J App Biol Biotech. 2014; 2 (06): 007-009.