BIOINFORMATICAL DETECTION OF RESTRICTION ENDONUCLEASE IN

BACILLUS MYCOIDES STRAIN BMI USING ANALYTICAL AGAROSE ETHIDIUM BROMIDE ELECTROPHORESIS

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Abstract: Bioinformatical method of blasting technology paved way quick to identification of bacterial genome. Such an identified bacterial species traced for Restriction Endonuclease using various vectors such as λ DNA (non methylated) pBR322 - Msp digested, pBR322 and pTriEx-1.1 Neo-vector. The cleaved palindrome sites Bmy of Bacillus mycoides strain BM1 is bioinformatically analysed using agarose ethidium bromide electrophoresis.

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INTRODUCTION:

Restriction Endonucleases are enzymes which posses restriction enzyme and modification methylase. (Yuan 1981; Smith and Nathans, 1973). The restriction enzyme cleaves the foreign λ DNA but modification methylase protects the host DNA.

Bacteria have confronted the invasion of foreign DNAs for million of years and have evolved these Restriction – modification system to preserve their own DNA and destroy the invading DNA.

Restriction enzymes recognize short nucleotide sequence on foreign DNA. Different restriction enzymes recognize different but specific sequences of 4 to 8 base pairs length. The specific nucleotide sequences are called recognition site or target site. The target sequences are called palindrome. So restriction enzyme is a powerful tool to cleave predictable fragments of any DNA molecule in vitro (Hayes, 1978).

Werner Arber of Basle University, Switzerland (1969) discovered that certain bacteria contain restriction enzymes which cleave DNA into small fragments. Smith and Nathan (1973) isolated restriction enzyme from *Haemophilus influenzae* and named it Hind II which cleaves viral DNA at specific sites producing fragments with 5’ and 3’ terminals (5’– p^Pu A p^C and p^G T p^Py – 3’). The EcoR1 Restriction endonuclease (Hedgepeth *et al.*, 1972; Dugaiczyk *et al.*, 1975; Gupta *et al.*, 1968) cleaves the host DNA at specific site.

At present many different kinds of restriction endonucleases are discovered. The nomenclature of restriction endonuclease is published by a group of scientists (Richard J. Robert *et al.*, 2003).

There are three main group of restriction endonuclease (RENases) called Type I, II and III (Boyer, Yuan R 1971 and 1981). A new type IV is added to accommodate a class of methyl-dependent restriction enzymes (Belfort and Roberts, 1997).

Isoschizomers are RENases that recognizes the same sequence. The first example discovered is called a prototype and all subsequent enzymes that recognize the same sequence are isoschizomers of the prototype.
Neo schizomers that subret of isoschizomers that recognize the same sequence but cleave at different position from the prototype. Thus Aat II (recognition sequence: G A C G T↓C) and Zra I (Recognition sequence: G A C↓G T C) are neoschizomers of another. But Hpa II (Recognition sequence: C↓C G G) and Msp I (Recognition sequence: C↓C G G) are isoschizomers but not neoschizomers.

REBASE (Roberts, R.J. et al., 2006) database is repository of information about restriction enzyme, DNA methyltransferases and related proteins. It contains referenced information about recognition and cleavage sites, isoschizomers, neoschizomers commercial availability, methylation sentivity and crystal and sequential Data. The contents of REBASE may be browsed from the web (http://rebase.neb.com/rebase.ftp.html) and selected compilations can be downloaded by ftp (ftp.neb.com). RefSeq database of GenBank is much useful for bioinformatical analysis of DNA sequences.

MATERIALS AND METHODS:

BACTERIAL STRAIN ISOLATION AND CULTURE CONDITIONS FOR RESTRICTION ENDONUCLEASE EXTRACTION, BACTERIAL STRAIN ISOLATION:

Soil sample from Guindy National Park (Chennai, Tamilnadu, India) was collected at the depth upto 10 to 15 cm and stored in sterile bag. Then 1 g of soil sample was serially diluted to $10^{-5}$ in sterile distilled water. Bacterial pure line selection was done by pour plate technique and quadrant streak pattern for pure culture was made on nutrient agar culture plates and maintained in culture tubes. Bacterial culture medium was nutrient broth medium (Mahadevan and Sridhar, 1982). 10 g of peptone, 5 g of Beef Extract, 5 g of sodium chloride dissolved in 1L distilled water. The pH 7.2 adjusted with 1M NaOH. The medium with glasswares were sterilized in an autoclave at 15 lb pressure for 20 min. To the nutrient broth, 20% of agar was added. The preparation was heated to dissolve and distributed to the culture tubes. The culture tubes were sterilized by autoclaving and slants were prepared.

Bacterial strain identification:

The bacterial strain was screened for the activity of restriction endonucleases and identified by 16 S ribosomal RNA partial nucleotide sequence Blasted Technique was issued to identify the bacterial strain (Synergy Scientific services, Chennai). The bacterial strain was identified and nomenclatured as *Bacillus mycoides* strain BM1. The nucleotide sequence data of the
identified bacterium was submitted to GenBank (NCBI) and accession number was obtained for publication (GU812441).

**Assay for restriction activity:**

Nucleic acid used for restriction enzyme digestion were λ DNA (non methylated) (Sanger *et al.*, 1982) Msp (*Moraxella species*) (GeNei Cat. No.105650) restriction enzyme digested pBR322 fragments and pBR322. The λ DNA, Msp digested pBR322 and pBR322 were purchased from GeNei- Bangalore, India. pTrix – 1.1 Neo Vector, donated by Prof M. Parthiban, Madras Veterinary College, Chennai.

10 μl of DNA (0.2 μg of DNA in 20 μl) of the above mentioned λ DNA, pBR322 / Msp I digest and pBR322 were taken separately in a sterilized appendorf tubes. 3 μl of supermix DNA ladder was used as control. To each DNA 20 μl of RE buffer pH 7.5 (sterilized) was added (100M Tris, pH 7.5, 50mM NaCl and 5mM MgCl₂) and then 100μl of purified restriction endonuclease Bmy was added to each DNA and tapped well to get uniform mixture. The digestion was completed at 37°C for 1.15 min and the reaction was ceased by keeping the reaction mixture at 65°C for 5min

**Horizontal Agarose Gel Electrophoresis:**

**Template preparation:**

The template was cleaned and air dried. The two open ends of the template was sealed with autoclave tape and an eight teeth comb was clamped at one of the template.

**Agarose preparation:**

0.8% agarose (Sima electrophoresis grade) gel was prepared by adding 32mg of agarose in 36ml of double distilled water and boiled for 10min. Then it is cooled to 65°C to 70 °C. To this level of 10 X TBE pH 8.0 (Tris Borate EDTA-gel and electrophoretic buffer) prepared by adding 1g of sodium hydrooxide (MW. 40); 108g of Tris base (MW. 121.10), 55g ofBoric acid (MW.61.83), 7.4g of EDTA in 1L lass distilled water. 1 vol of TBE concentrate was distilled with 9 volumes of distilled water before use.

The agarose solution was poured on the template and the gel was allowed to set for 1 to 2 hrs.

**Enzyme Assay:** (Roberts. J, 1978)

To 10μl of DNA (0.5 μg of λ DNA or pBR322 or pBR322/Msp1 digest) 20 μl of REbuffer pH 7.5 was added (50mM of NaCl, 5mM of MgCl₂ and 100mM Tris-HCl - in 9ml of NaCl and MgCl₂
1ml of Tris-HCl buffer is mixed and autoclaved. To this 0.2 nM of ATP and 10 μl and 20 μl was added). Then 100 μl of purified restriction endonuclease was added and tapped well to get uniform mixture and incubated at 37°C for 1hr 15min. Afterwards the reaction was ceased by keeping the reaction mixture at 65 °C for 5 min.

**Electrophoresis:**

The restriction enzyme digested DNA (λ DNA, pBR322 and pBR322 Msp 1 digest ) were analysed by a horizontal agarose gel electrophoresis (0.8%). To the reaction mixture (sample) 10μl loading dye bromophenol blue (8% sucrose – 0.025% bromo phenol blue and 1M Tris ) was added and loaded into the wells of the casted gel which was immersed in Tris Borate Ethylene diammine Tetra Acetic Acid (TBE) electrophoretic buffer. Electrophoresis was done at 50V for 2 hrs and stained with Ethidium Bromide (5 μg /ml) for 15 min and destained in glass distilled water for 15 min. The gel was observed under UV light (260 nm) and photographed with red filter.

**Bioinformatic Analysis of Digested DNA Fragment Length:**

The agarose gels were scanned by Vilber Loyrat Gel documentation system with Bioimage Software. The mobility of cleaved fragments of nucleotides were scanned and the mode value of fragmented nucleotide base pairs (length) were screened by comparing with supermix DNA ladder was calculated.

The genome nucleotide sequence of λ DNA (Sanger et. al., 1982) and pBR322 (Sutcliffe 1979) and pTriEx 1.1 Neo Vector (Trautner, 1974) were obtained from database. This enabled bioinformatical statistical analysis of fragments length and the restriction site of the isolated restriction enzyme.

**Protein Estimation:**

Beckman (USA) spectrophotometer was used to determine the quantity of protein present in the eluted fractions from the column chromatography. Coomassie Brilliant Blue (G-250) (Sigma,USA) dye was used as protein reagent, according to the method of Bradford (1976). The absorbance (595nm) values were plotted against the standard graph constructed with Bovine Serine Albumin (Sigma, USA) and the protein content of each fractionated sample was determined.
Analysis of Protein Profile by SDS – PAGE:

Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS – PAGE) was carried out by modified method of Laemmli (1970). 15% resolving gel was prepared (30% acrylamide mix, 1.5 M Tris pH 8.8, 3ml of distilled water, 10% SDS, Ammonium Per Sulphate and 10 μl of TEMED). 5% of stacking gel was prepared by mixing the following components. 30% acrylamide mix, 1M Tris pH 6.8, 2ml distilled water, 10% SDS, 10% APS and 5μl TEMED. The purified and dialysed protein fractions were directly used for gel electrophoresis. Approximately 50 μg protein was loaded in each well. An equal volume of sample buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 10% Mecaptoethanol, 20% glycerol, 0.02% Bromophenol and 30 μl distilled water) was added to protein sample, mixed well and heated in a boiling water bath for 5 min and cooled before loading onto the gel.

SDS–PAGE Electrophoresis was carried at room temperature at 50V until the bromophenol dye reached 1-2 cm above the bottom of the resolving gel. Then the gel was separated from glass plate and stained CBB (R-250) (1.25 g of CBB R-250 dissolved in 500ml of methanol and stirred for 30min. 100ml of Acetic acid is added and final volume made upto 1L with deionised water and filtered through Whatman No-1 filterpaper and stored in amber colour bottle. Later the gel was transferred to destaining solution. 600ml of methanol mixed with 140 ml of acetic acid and made upto 2L with deionised water. It was continued until the bands were clear. The gel was scanned by Vilber Lourmat Gel documentation with Bioimage software. The gel was stored in 7% (V/V) acetic acid solution.

Isolation of Restriction Endonuclease from Bacillus mycoides strain BM1: (Greene et al, 1975 - modified procedure)

Harvesting the bacteria from culture:

Bacteria were grown at 37°C in nutrient broth pH 7.2 and harvested at 7000 g centrifugation (Beckmann, USA) for 30min at 4°C. The cells were washed twice by suspending in 10ml of phosphate buffer (0.01 M, pH 7.2) (0.01 M of K₂HPO₄ – KH₂PO₄, 1mM EDTA, 7mM β-mercaptoethanol dissolved in 1L double distilled water). 0.05 M NaCl was added to phosphate buffer and stored at 4°C.

Sonication:

The cell suspension in 10ml phosphate buffer was sonicated at 4°C for 30 min at amplitude 50% (ultrasonic UP2005 Leilscher).
Restriction Enzyme Freed From the Bacterial Nucleic Acid:
The cell free extract after sonication was centrifuged at 10000 g for 20 min at 4°C. The supernatant was treated with 2% Streptomycin sulphate (to remove nucleic acids) stirred for 10 min and centrifuged at 10000 g for 20 min at 4°C.

The supernatant was saturated with 50% ammonium sulphate for 12 hr, at 4°C and centrifuged at 10000 g for 20 min at 4°C. The protein pellet - I was obtained. To the supernatant 70% ammonium sulphate was saturated 12 hr, at 4°C and centrifuged at 10000 g for 20 min at 4°C. The protein pellet – II was obtained. The supernatant with 80% ammonium sulphate saturation, no pellet was formed after centrifugation.

The two protein pellets I and II dissolved in 5ml of phosphate buffer (0.01 M, pH7.2) and dialysed against 50% glycerol with phosphate buffer for 12 hrs and concentrated. The crude protein stored in sterilized vial and kept at -20°C for further purification and restriction analysis with λDNA pBR322 and p Tri-Ex 1.1 Neo- Vector.

Purification of Restriction Endonuclease from Bacillus mycoides strain BM1:
10 ml of crude and dialysed enzymatic proteins (ENa se) were loaded onto the activated DEAE-cellulose ion exchanger (GeNei, Bangalore) column-1 (12 x 2cm) previously equilibrated with 0.2M NaCl in 0.01M phosphate buffer (pH7.2) in cold room at 4°C. Sample was eluted eight times to bind the proteins with ion exchanger.

The unbound elutants through the column were collected in 5ml fraction (5ml/10 min) of 31 test tubes. The fractions were eluted with 0.2M NaCl-1.0M NaCl gradient in 0.01M phosphate buffer, 1mM mercaptoethanol pH -7.2. The optical density of the fractions was observed at A595 and plotted in graph. The peaks of protein fractions eluted at 0.3M NaCl (30ml) and 0.4M NaCl (20ml) in 1 x SB, 1 mM Mercaptoethanol pH 7.2 were pooled together and dialyzed against 0.2M NaCl in 1 x SB, 1mM Mercaptoethanol pH 7.2 for 12 hrs at 4°C.

The dialyzed protein fractions of 0.3M to 0.4M NaCl gradient of bacterial strain was loaded on activated phosphocellulose IE-03 cation exchanger (GeNei, Bangalore) column-2 (15 x 2cm) to bind the protein at 4°C kept in a cold room. The columns were equilibrated with column II buffer 0.2M NaCl in 1x SB, 1mM Mercaptoethanol pH 7.2 at 4°C.

Bacterial strain RENase:
The bound bacterial strain RENase was eluted with 180 ml linear gradient of 0.2M to 1.0 M NaCl in 1x SB, pH 7.2 (5ml/10min) 5ml fractions were collected. The high protein peaks at
595nm were eluted at 0.2M NaCl (40 ml), 0.3M NaCl (20 ml) and 0.4M NaCl (20 ml) were pooled separately and dialyzed against 0.2M NaCl in 1 x SB buffer pH 7.2. Protein Fractions of *Bacillus mycoides* strain BM1 RENase were pooled individually and dialyzed against 0.2M NaCl in 1 x SB, 1mM Mercaptoethanol with pH- 7.2 with 50% glycerol for 12 hrs at 4°C separately.

The RENase fractions from DEAE cellulose column-I elution and phosphocellulose column-II elution were assayed with pBR322/Msp1digest, pBR322, λ DNA and assayed with pTriEx-1.1 Neo vector.

**Horizontal Agarose gel electrophoresis:**

The assayed pBR322/Msp1digest, λ DNA, pBR322 and pTriEx-1.1 Neo vector were analyzed by a horizontal agarose gel electrophoresis (0.8%)

**Analysis of restriction sequence:**

The restriction endonuclease *Bmy* isolated and purified form *Bacillus mycoides* strain BM1 cleaved specific site on λDNA, pBR322 and pTriEx-1.1 Neo vector were recognized from the data collected from gel doc with Bioimage software using Bioinformatics.

**RESULTS:**

**Bacterial strain identification (biochemical test):**

(i) **Gram stain test:**

*Bacillus mycoides* strain BM1 belonging to Bacillaceae .It is identified as Gram negative bacteria and rod shaped which exhibited Diplobacilli (Fig.1)

![Fig. 1 Bacillus mycoides strain BM1](image)

(ii) **16S Ribosomal RNA gene, partial sequence:**

The genomes of the *Bacillus mycoides* strain BM1 was partially sequenced with 16S ribosomal RNA gene and submitted to Gen Bank on March 29, 2010. Accession number was
provided and the GenBank flat file of this organism was produced (Fig 2). The Phylogenetic
tree of the identified organism was charted (Fig.2).

(iii) Phylogenetic tree

Phylogenetic tree of the identified bacterial strains were constructed through the blasting
technique based on 16S rRNA ribotyping (Fig 3).

(iv) Isolation and purification of Restriction endonuclease from *Bacillus mycoides*
strain BM1 (Greene et.al., 1975 modified procedure):

*Bacillus mycoides* strain BM1 pure line selection was made from quadrant streak (Fig. 4). The
growth rate of the cells in nutrient broth (pH7.2) at 37°C was studied and harvested at 18 hr
(Fig. 5). The cells of bacteria harvested by centrifugation (4°C) and the cell paste weighed to 12g
and washed with sonication buffer (SB). Cells suspended in 20ml (SB) was sonicated and
centrifuged to remove cell debris. Exonucleases were removed from the cell free extract by
the addition of Streptomycin sulphate after centrifugation. The cell free extract with 50%
(314 g/l) and 70% (135 g/l) ammonium sulphate saturation, Proteinaceous restriction
endonuclease pelleted. Protein pellet dissolved in 5ml of phosphate buffer (pH 7.2) and the
quantity of protein extraction was read at 595nm. The optical density was 1.636 (50 µl) of the
extract. It was quantified to 160 µg/ml.

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<th>LOCUS</th>
<th>GU812441</th>
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<td>DEFINITION</td>
<td><em>Bacillus mycoides</em> strain BM1 (16S ribosomal RNA gene, partial sequence)</td>
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<tr>
<td>VERSION</td>
<td>GU812441</td>
</tr>
<tr>
<td>SOURCE</td>
<td><em>Bacillus mycoides</em></td>
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<td>ISOLATION SOURCE</td>
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<td>ORGANISM</td>
<td><em>Bacillus mycoides</em>, Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus; <em>Bacillus cereus</em> group.</td>
</tr>
<tr>
<td>AUTHORS</td>
<td>Bakyalakshmi, M. and Mohan, N.</td>
</tr>
</tbody>
</table>
| ORIGIN      | 1 taatctctgt ggggtgaggg cgggttgtga caaggacgg gaacgtattc accgcgggtg  
61 ctgatccgcg attactagcg attccagctt catgtaggcg agttgcagcc tgcatccgaa  
121 ctgagatggt tttatgaggt tagctccccc tcgcggtctt gcagctcttt tgtccgtcct  
181 tggtactagt gtgtaccca gggcataagg ggcatgatga ttgacgtcga tcccccacctt |
Fig. 2 GenBank flat file for *mycoides* strain BM1

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241 cctcgcgttt tgtcaccggg cagtcacctc tagagtgccc aacttaatga tggcaactaa
301 gatcaagggg ttgcgctcgt tgcgggactt aacccaacat ctcacgcacac gagttgacgaa
361 caaccatgca ccacctgtca ctctgccccc gaaggggaag ccctatctct agggtgtca
421 gaggatgtca agaccttggta aggtttctcg cgttgccttcg aatataacca catgcctccac
481 cgctttgtcg gggccccgccctc aatcccttttg agtttacgcct tgccggccgct acttccccagg
541 cggagtgtctt atagcgttta aacctgacct aaaggccgag aacccctttaa cacttagacac
601 tcatcgttta ccgcctggac taccaggta tctaatcttg ttgctcccc acgcctttgcctc
661 gcctcagtgt cagttacaga ccagaaagtc gccttgcctca ctggtgttcc tccatatcttc
721 tacgcatttc accgctacac atgggaatccc aatccaactct tctgcaactca agtcattgcgc
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901 tacgcgcggct cgtctcaggt gttactcgtg cgtttctgct cagttacgcgc

Bacillus
```
Fig. 3 Bacillus mycoides strain BM1

Purification of Bmy Restriction endonuclease:
The dialysed enzyme extract was eluted through DEAE cellulose column (I) chromatograph (2 x 12 cm). Fractions of 5ml/10min were collected in 34 test tubes (170 ml). Fractions collected at 0.3M and 0.4M elution buffer had high peaks of protein content (Fig.6). At 0.3M salt gradient elution buffer fractions of 1 to 6 test tubes (30ml) were pooled and dialysed against only phosphate buffer (pH 7.2) without salt for 12 hrs at 4°C. Fractions collected at 0.4M salt gradient elution buffer from 7 to 10 test tubes (20ml) had high peaks of protein were pooled and dialysed against phosphate buffer (pH 7.2) without salt. These two peaks of enzymatic fractions (0.3 M and 0.4 M elutants) were assayed with λDNA. The two enzymatic fractions cleaved λDNA into more than 12 fragments (Fig.7). The above mentioned enzymatic fractions were pooled together. 20 ml of such enzymatic fraction eluted through the phosphocellulose column (II) (2 x 16cm) with phosphate elution buffer with salt gradient (0.2M to 1M). Fractions (5ml/10min) were collected in 45 test tubes.
Fractions with high enzymatic protein peaks were eluted at 0.2M, 0.3M and 0.4M salt gradient (Fig.6).

All of these eluted fractions collected through phosphocellulose at 0.2M, 0.3M and 0.4M with high protein content were pooled together separately and dialyzed against phosphate buffer (pH 7.2) without salt with 50% glycerol. As per the rules of the nomenclature, the restriction endonuclease of Bacillus mycoides strain BM1 was denoted as Bmy. The selected elution profile of Bmy RENase fractionated protein from column I and column II were quantified (Table.1).

Protein Analysis Bmy:

SDS- PAGE separation of protein bands of restriction endonuclease of Bacillus mycoides strain BM1 (Bmy) stained with CBB R-250. The complexity of protein nature of the Bmy restriction endonuclease was characterized with protein marker (Fig. 8).

Analysis of Bmy restriction endonuclease assay:

A. Bmy restriction endonuclease assay with pBR322/ Msp 1 digest:

The newly isolated Bmy restriction endonuclease recognize cleavage site on plasmid DNA pBR322 proved through the agarose gel electrophoresis assay with pBR322/ Msp1 digest restriction enzyme. (Fig. 9). The isolated Bmy crude restriction enzyme methylated the fragments of already digested pBR322/ Msp1 digest. The methylated fragments remained in the well (lane-3) on the agarose gel. The purified Bmy restriction enzyme did not methylate the fragmented pBR322/ Msp1 digest (control). Fragments sizes and number of bands resolved at the lanes from 4 to 8 on the agarose gel were similar to pBR322/ Msp 1 digest (Control).
Fig. 5 Growth Curve of *Bacillus mycoides* strain B

Fig. 6 Elution profiles of *Bmy* eluted from DEAE cellulose column I and Phospho cellulose column II

Fig. 7 *Bmy* Restriction endonuclease assay with Phage *λ* DNA
1. Super mix ladder DNA (100 Kb), 2. λ DNA control, 3. Crude ENase protein (Enzyme) digested λ DNA, 4, 5. DEAE- Cellulose column fractionated protein (1-6, 7-10 (5ml)) digested λ DNA, 6, 7, 8. Phosphocellulose column fractionated protein (1-7, 8-12, 13-16 (5ml)) digested λ DNA fractionated protein (1-7, 8-12, 13-16 (5ml)) digested λ DNA determined the isoschizomeric property of Bmy restriction enzyme with Msp (Fig. 9).

### Table 1 Quantification of fractional protein eluted through column chromatography

<table>
<thead>
<tr>
<th>Protein source</th>
<th>Fractions</th>
<th>O.D value</th>
<th>Protein quantified/BSA (µg/ml)</th>
</tr>
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<tbody>
<tr>
<td><em>Bacillus mycoides</em> strain BM1 sonicated in phosphate buffer (pH 7.2)</td>
<td>Crude extract</td>
<td>0.208</td>
<td>26</td>
</tr>
<tr>
<td>Ammonium sulphate saturation (50% &amp; 70%)</td>
<td>Dialysed protein</td>
<td>1.014</td>
<td>95</td>
</tr>
<tr>
<td><strong>Column I</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elution at 0.2M</td>
<td>Fractions (1-6)</td>
<td>0.127</td>
<td>12</td>
</tr>
<tr>
<td>Elution at 0.3M</td>
<td>Fraction (7-10)</td>
<td>0.095</td>
<td>10</td>
</tr>
<tr>
<td><strong>Column II</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elution at 0.2M</td>
<td>Fractions (20ml)</td>
<td>0.089</td>
<td>9</td>
</tr>
<tr>
<td>Elution at 0.3M</td>
<td>Fractions (25ml)</td>
<td>0.101</td>
<td>10</td>
</tr>
<tr>
<td>Elution at 0.4M</td>
<td>Fractions (20ml)</td>
<td>0.096</td>
<td>10</td>
</tr>
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</table>

**Fig. 8 Characterization of Bmy protein elution profile**  
DEAE column I chromatography  
Lane 1- *Bmy* elution fraction at 0.2, 2- *Bmy* elution fraction at 0.2; Phosphocellulose column II chromatography, 3- *Bmy* elution fraction at 0.2M, 4- *Bmy* elution fraction at 0.3M, 5- *Bmy* elution fraction at 0.4M, 6- Protein marke, 7- Crude RE of *Bmy*. Each lane received 50 µg of protein.
Fig. 9 Bmy assay with pBR322/Msp 1 digest

Lane 1. Supermix DNA ladder 100 Kb,
2. Control of pBR322/Msp 1 digest
3. Crude enzyme of Bmy with pBR322/Msp1 digest
4,5. Elution fractions of Bmy at 0.2 M and 0.3 M of DEAE cellulose column I with pBR322
6, 7, 8. Elution fractions of Bmy at 0.2 M, 0.3 M and 0.4 M phosphocellulose column II with pBR322

B. Bmy restriction endonuclease assay with pBR322:

Bmy restriction endonuclease was assayed with pBR322 Bmy cleaved pBR322 into four fragments were resolved on the agarose gel. (Fig. 10).

Fig. 10 Bmy restriction endonucleases assay with pBR322
Lane 1,2- Bmy cleaved pBR322 into fragment

3- control - pBR322

4- supermix DNA ladder 100 kb

**Bioinformatical determination of Bmy RENase specific restriction site on the DNA of pBR322**

The completely sequenced the nucleotide of plasmid pBR322/Msp restriction enzyme cleaves the DNA of pBR322 at 24 specific sites of the nucleotide at

\[ \text{C↓CGG} \]
\[ \text{GGC↑C} \]

Restriction map of pBR322/Msp 1 digest has 24 cleavage sites which was referred bioinformatically. The lengths of the cleaved DNA fragments basepairs of pBR322/Msp1 digest were scored and tabulated (Table.2).

The newly isolated Bmy RENase fragmented nucleotide basepairs of pBR322 were scored and tabulated (Table.3).

The newly isolated Bmy RENase fragmented nucleotide basepairs of pBR322 were scored and tabulated (Table.3).

**C. Bmy Restriction endonuclease assay with Phage λ DNA**

The newly isolated Bmy restriction endonuclease cleaved λ DNA into 14 fragments. The fragments mobility was compared with supermix DNA ladder (500bp-33,500bp). The purified elution profile of Bmy from DEAE cellulose column I and phosphocellulose column II had optimal restriction activity on λ DNA (Fig.7).

Sanger et al., (1982) sequenced the genomic nucleotide of Phage λ DNA (48,560bp). It was referred bioinformatically that Phage λ DNA has 311 specific cleavage sites

\[ \text{C↓CGG} \]
\[ \text{GGC↑C} \]

The number of nucleotide basepairs in between the above mentioned specific nucleotide sites of Phage λ DNA were scored and tabulated bioinformatically (Table.4).

The newly isolated Bmy restriction endonuclease cleaved λ DNA. The electrophoretic mobility of fragmented λ DNA by Bmy (Fig.7) was compared with supermix DNA ladder (100 kb). The mode value of the gel doc profile of fragmented λ DNA digest by Bmy restriction
endonuclease was scored (Tab More than 12 fragments resolved on the agarose gel revealed the restriction activity of \textit{Bmy} restriction endonuclease.

The newly isolated \textit{Bmy} also cleaved the plasmid DNA pBR322 into five fragments. But the newly isolated another restriction endonuclease \textit{Bsu} revealed restriction alleviation (RA) of pBR322 DNA. This enzyme condensed the plasmid DNA of pBR322 and increased the molecular weight, which decreased the mobility on the gel (Fig.10).

D. \textit{Bmy} restriction endonucleases assay with pTriEx-1.1 Neo vector

\textit{Bmy} cleaved the pTriEx-1.1 Neo vector DNA into many fragments which were dissolved clearly at lane 4 on agarose gel (Fig.11)

![Fig. 11 Bmy restriction endonucleases assay with pTriEx-1.1 Neo vector](Image)

Lane1-supermix DNA ladder 100 , 2-Control pTriEx-1.1 Neo vector DNA, 3,4 - \textit{Bmy} RE fractions of elution profile from DEAE at 0.2 M and 0.3 M, 5 - \textit{Bmy} RE fractions from phosphocellulose at 0.2 M

<table>
<thead>
<tr>
<th>Fragment length</th>
<th>pBR322/Msp 1 digest</th>
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<td>699</td>
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<td>600-500</td>
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<td>500-400</td>
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<tr>
<td>300-200</td>
<td>213,234</td>
</tr>
<tr>
<td>200-100</td>
<td>160,119,158,156,197,176,143,153,188,173</td>
</tr>
<tr>
<td>Less than 100</td>
<td>5,5,72,86,21,30,22,30</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total 24 fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>8</td>
</tr>
</tbody>
</table>
Table 3  $Bmy$ RE digested fragment length of pBR322.

Derived Gel doc data tabulation

<table>
<thead>
<tr>
<th>Fragment length (Nucleotide bp)</th>
<th>$Bmy$ RE digest of pBR322</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fragment length (Nucleotide bp)</td>
</tr>
<tr>
<td></td>
<td>(Gel doc mode value)</td>
</tr>
<tr>
<td>700-600</td>
<td>700</td>
</tr>
<tr>
<td>600-500</td>
<td>598</td>
</tr>
<tr>
<td>500-400</td>
<td>448</td>
</tr>
<tr>
<td>400-300</td>
<td>328</td>
</tr>
<tr>
<td>300-200</td>
<td>241</td>
</tr>
<tr>
<td>200-100</td>
<td>172</td>
</tr>
<tr>
<td>Less than 100</td>
<td></td>
</tr>
</tbody>
</table>

Total fragmented bands 5.

$Bmy$ restriction endonuclease was scored (Table.5) More than 12 fragments resolved on the agarose gel revealed the restriction activity of $Bmy$ restriction endonuclease.

The newly isolated $Bmy$ also cleaved the plasmid DNA pBR322 into five fragments. But the newly isolated another restriction endonuclease $Bsu$ revealed restriction alleviation (RA) of pBR322 DNA. This enzyme condensed the plasmid DNA of pBR322 and increased the molecular weight, which decreased the mobility on the gel (Fig.11).

D. $Bmy$ restriction endonucleases assay with pTriEx-1.1 Neo vector

$Bmy$ cleaved the pTriEx-1.1 Neo vector DNA into many fragments which were resolved clearly at lane- 4 on agarose gel (Fig.11).

Table 4  The fragments lengths were found out through bioinformatics

<table>
<thead>
<tr>
<th>$\lambda$ DNA Fragment length</th>
<th>Cleaved fragments (Bioinformatical data)</th>
<th>Fragment length</th>
<th>Fragment No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3000-2500</td>
<td>2289</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>2500-2000</td>
<td>2164</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>2000-1500</td>
<td>1985,1566</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>1500-1000</td>
<td>1248,1168,1045</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>1000-900</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>900-800</td>
<td>847</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>800-700</td>
<td>767</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>700-600</td>
<td>665,650,611,617</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>600-500</td>
<td>595</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>500-400</td>
<td>506,484,482,455,433,423,422,413</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>400-350</td>
<td>374,359,379,373</td>
<td></td>
<td>8</td>
</tr>
</tbody>
</table>
DISCUSSION:

Nomenclature of Isolated Soil Bacteria

*Bacillus mycoides* strain BM1 (Accession no GU812441) belongs to Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group. This is a tropical organism isolated from the soil of Adyar-Guindy National park, Chennai, Tamil Nadu. Greene, *et al.*, (1975) suggested that most of the restriction endonuclease (*Bam* I) activity was in the 50% to 80% ammonium sulphate saturation.

Most of the restriction endonucleases of Type II were highly purified by ion exchanging column chromatography. The DEAE cellulose and phospho cellulose column chromatography were used for purification of *Mal* I (DeWaard and Duyvesteyn, 1980) from *Mastigocladus leminasus Av* II and *Asu* II from cyanobacteria have also been purified by the

<table>
<thead>
<tr>
<th>Mobility of supermix DNA ladder (bp)</th>
<th>Fragmented length (bp) of λ DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>33500-24500</td>
<td></td>
</tr>
<tr>
<td>15000</td>
<td>14530</td>
</tr>
<tr>
<td>8990</td>
<td>10820</td>
</tr>
<tr>
<td>7000</td>
<td>8530</td>
</tr>
<tr>
<td>6000</td>
<td>6460</td>
</tr>
<tr>
<td>5000</td>
<td></td>
</tr>
<tr>
<td>4000</td>
<td>3640</td>
</tr>
<tr>
<td>3000</td>
<td>2720</td>
</tr>
<tr>
<td>2000</td>
<td>2200</td>
</tr>
<tr>
<td>1500</td>
<td>1850</td>
</tr>
<tr>
<td>1000</td>
<td>1100</td>
</tr>
<tr>
<td>500</td>
<td>825</td>
</tr>
</tbody>
</table>
combination of these two columns. The combination of DEAE cellulose and phospho cellulose column chromatography was used successively for the enzyme Bmy.

The elution profile of the restriction enzyme Bmy, a newly isolated enzyme adsorbed on DEAE cellulose column was found between 0.3M to 0.4M NaCl salt gradient which was similar to EcoR1 elution profile.

The elution profile of Bmy adsorbed on phosphocellulose was also similar to EcoR1 (Paniyum, 1985). The enzymatic fractions were eluted from 0.3M to 0.4M salt gradient. The restriction activity of Bmy assayed with λDNA, pBR 322 and pTriEx-1.1 Neo vector. It is essential to use substrate DNA with several recognition sites for all enzymes (Greene et al., 1975).

Most of the restriction endonucleases had a temperature optimum at 37°C and above. Lyra, et al., (2000) suggested that the factor which affects the endonuclease activity was the growth temperature of respective organism. The restriction endonucleases of Bacillus mycoides strain BM1 were grown at 37°C.

The most important cofactor needed by all endonuclease is Mg^{2+} other than ATP and S-AdoMet. Cowan (1988) suggested that the preference of Mg^{2+} by Type II enzymes than other metal ions due to its favourable physical and chemical properties like small ionic reaction with extensive hydration, high charge density, high transport numbers and abundance in the cell. Divalent metal ions like Mn^{2+} and Ca^{2+} may substitute Mg^{2+} for DNA binding (Vipond and Halford, 1995).

To cease the endonuclease activity, the temperature is raised to 65°C or EDTA can also be used. The presence of EDTA, a metal chelating agent reduced the activity. (Solaiman and Somukutti, 1991)

Restriction endonuclease varies with respect to their ability to maintain activity in a reaction over an extended period of time.

Most of the restriction endonucleases needed pH 7-8 for optimum activity (Narsi and Thomas, 1987). Restriction buffer used in the reaction mixture had pH 8.

**Bioinformatical determination of Bmy cleavage site on substrate DNA**

The purified restriction endonuclease Bmy from Bacillus mycoides strain BM1 cleaved the λ DNA into more than 12 fragments and pBR 322 into four fragments.
The 70% Ammonium sulphate precipitated crude extract of Bmy restriction endonuclease methylated some of the fragmented pBR322/Msp1 digest substrates. So that the methylated fragments with increased molecular weight remained in the particular well of the agarose gel.

The other lanes loaded with reaction mixture contained purified Bmy restriction endonuclease elution fractions did not express methylation. The fragmented pBR322/ Msp1 digest substrates were resolved into four clean bands on the agarose gel.

This revealed that Bmy restriction endonuclease is a kind of Type IIA restriction endonuclease. This kind of Type IIA has separate modification and restriction enzymes, (Yuan, 1981). Type IIA (Eg: EcoR1) cleaved the DNA within the recognition site in a staggered pattern generating specific fragments with identical single-stranded cohesive ends.

Based on the above principle it is concluded that Msp restriction endonuclease cleaved the pBR322 DNA at the specific site at

\[
\begin{align*}
\text{CCGG} \\
\text{GGCC}
\end{align*}
\]

While the crude restriction endonuclease of Bmy isolated from Bacillus mycoides strain BM1 methylated the fragmented DNA of pBR322 /Msp1 digest, the purified endonuclease subunit did not methylate the fragmented pBR322/Msp1 digest and the bands of fragments resolved on the agarose gel were identical to the control.

This indicates that Bmy restriction endonuclease (Bacillus mycoides strain BM1) has separate modification and restriction enzymes. Bmy is a Type IIA a restriction endonucleases as this required only Mg^{2+} cofactor for its optimal activity. No other cofactors like ATP and S-AdoMet enhanced the activity.

The ATP and S-AdoMet cofactors are required for the restriction activity of Type I and Type III restriction endonucleases.

The Type IIA EcoR1 restriction endonuclease and modification methylase together with the substrate provide a model system for the study of sequence-specific DNA protein interactions. The sequence of base pairs that serves as substrate for both enzymes has been determined to be (Hedgepeth et al., 1972; Dugaiczyk et al., 1974)
Similarly, Bmy modification methylase and restriction enzyme should recognize specific sequence on substrate pBR322 DNA. So that the specific cleavage site and methylation site of Bmy on pBR322 sequences is

\[
\begin{align*}
\text{GAATTC} \\
\text{CTTAAG}
\end{align*}
\]

The Bmy restriction endonuclease also cleaved the λDNA into more than twelve fragments. The λDNA has 322 specific sequential sites at (Sanger et al., 1982).

\[
\begin{align*}
\text{CCGG} \\
\text{GGCC}
\end{align*}
\]

The number of base pairs of cleaved DNA fragments of λDNA by Bmy restriction endonucleases were scored and tabulated. The restriction map of cleaved fragments of λDNA has more than twelve different lengths of fragments with different number of nucleotide base pairs.

Sutcliffe, (1979) completely sequenced the nucleotide of the Escherichia coli plasmid. It has 24 cleavage specific sequence site at

\[
\begin{align*}
\text{CCGG} \\
\text{GGCC}
\end{align*}
\]

The number of base pairs of cleaved DNA fragments of pBR322 by Bmy RENase were scored and tabulated.

The bioinformatical data about sequence specific cleavage site at
on λDNA and pBR322 determine the nucleotide recognition cleavage site of Bmy restriction endonucleases.

The Bmy restriction endonuclease cleaved the pTriEx-1.1 Neo vector into 5 fragments. It is identical with Bsu (Trautner, 1974). It was reported that BsuI of Bacillus subtilis cleaved pTriEx-1.1 Neo vector at specific cleavage site at

C C G G
G G C C

The Bmy restriction endonuclease may be an isoschizomer of Msp and BsuI restriction endonucleases. The Bmy restriction endonuclease protein was characterized by the SDS-PAGE electrophoreses. The purified Bmy RENase elution profiles were loaded on agarose gel. The restriction activity of Bmy RE was well resolved on the agarose gel. The resolved bands expressed the protein content of the elution profile of the Bmy RENase.

CONCLUSION:

Bmy is a Type IIA restriction endonuclease isolated from Bacillus mycoides strain BM1. The purified restriction endonuclease Bmy is characterized. The protein profile on SDS-PAGE was resolved well. It has a separate modification and restriction enzyme. Bmy cleaves the host DNA (λDNA, pBR322 and pTriEx-1.1 Neo Vector) at the specific recognition site at

C C G G
G G C C

with staggered cohesive ends. It may be an isoschizomer of Msp and BsuI restriction endonucleases.

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REFERENCES


