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BIOINFORMATICAL DETECTION OF RESTIRICTION ENDONUCLEASE IN *BACILLUS MYCOIDES* STRAIN BMI USING ANALYTICAL AGAROSE ETHIDIUM BROMIDE ELECTROPHORESIS

Marimuthu Bakyalakshmi*

N.Mohan*

Abstract: *Bioinformatical method of blasting technology paved way quick to identification of bacterial genome. Such an identified bacterial species traced for Restiriction 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.*

*Centre for advanced study in Botany, Madras University, Chennai, Tamilnadu, India.

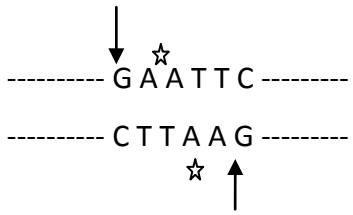
INTRODUCTION:

Restriction Endonucleases are enzymes which posses restriction enzyme and modification methylase. (Yuan 1981; Smith and Nathans, 1973). The restiction 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 recongize 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 invitro (Hayes,1978).

Werner Arber of Basle University, Switzerland (1969) dicovered 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'– $p^{Pu}_{p}{}^{A}_{p}{}^{C}$ and $_{p}{}^{G}_{p}{}^{T}_{P}{}^{Py}$ – 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 methlytransferases 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 $MgCl_2$) 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^{o}C for 1.15 min and the reaction was ceased by kepping the reaction mixture at 65^{o}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^{o}C to 70 ^{o}C. To this level of 10 X TBE pH 8.0 (Tris Borate EDTA-gel and electrophoretic buffer) prepared by adding 1g of sodium hydroxide (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 $MgCl_2$ and 100mM Tris-HCl - in 9ml of NaCl and $MgCl_2$

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^{o}C for 1hr 15min. Afterwards the reaction was ceased by keeping the reaction mixture at 65 ^{o}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 Loyrmat 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.

ProteinEstimation:

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 temperauture 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^{o}C in nutrient broth pH 7.2 and harvested at 7000 g centrifugation (Beckmann, USA) for 30min at 4^{o}C. The cells were washed twice by suspending in 10ml of phosphate buffer (0.01 M, pH 7.2) (0.01 M of K_2HPO_4 –KH_2PO_4 , 1mM EDTA , 7mM β-mercaptoethanol dissolved in 1L double distilled water). 0.05 M NaCl was added to phosphate buffer and stored at 4^{o}C.

Sonication:

The cell suspension in 10ml phosphate buffer was sonicated at 4^{o}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 supernant 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 (ENase) 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 $4^{o}C$ 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)

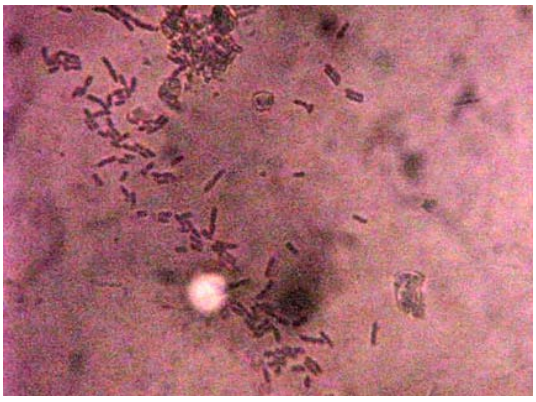


Fig. 1 *Bacillus mycoides* strain BM1

(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^{o}C was studied and harvested at 18 hr (Fig. 5).The cells of bacteria harvested by centrifugation (4^{o}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.

LOCUS : GU812441

DEFINITION : *Bacillus mycoides* strain BM1 (16S ribosomal RNA gene, partial sequence)

ACCESSION : GU812441

VERSION : GU812441

SOURCE : *Bacillus mycoides*

ISOLATION SOURCE : Guindy national park, Adyar, Chennai, Tamil Nadu, India.

ORGANISM : *Bacillus mycoides*, Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus; *Bacillus cereus* group.

AUTHORS : Bakyalakshmi, M. and Mohan, N.

ORIGIN 1 taaatctcgt ggggtggagg gcggtgtgga caaggaccgg gaacgtattc accgcggctg

61 ctgatccgcg attactagcg attccagctt catgtaggcg agttgcagcc tgcatccgaa

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181 tggtactagt gtggtaccca gggcataagg ggcatgatga tttgacgtca tccccacctt

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541 cggagtgctt aatgcgttaa cttcagcact aaagggcgga aaccctctaa cacttagcac

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661 gcctcagtgt cagttacaga ccagaaagtc gccttcgcca ctggtgttcc tccatatctc

721 tacgcatttc accgctacac atggaattcc actttcctct tctgcactca agtctaccgc

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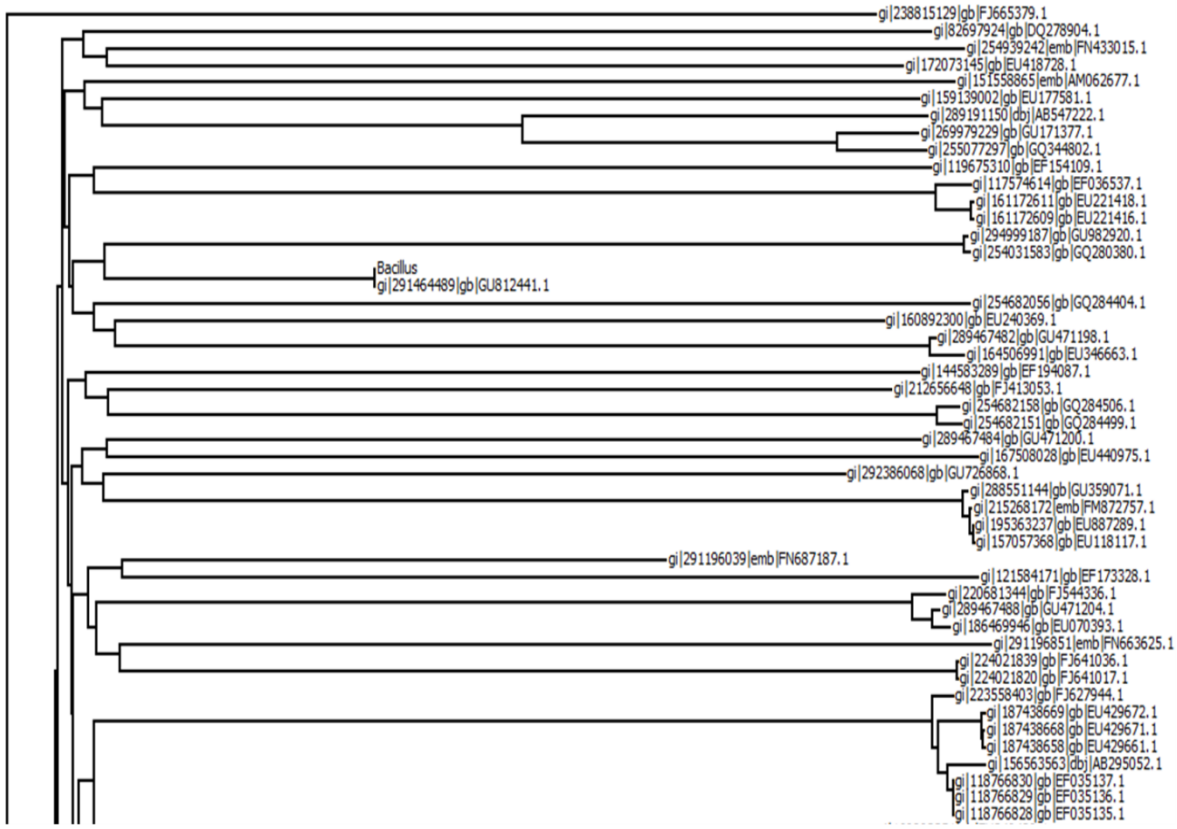
Fig. 2 GenBank flat file for *mycoides* strain BM1



Bacillus

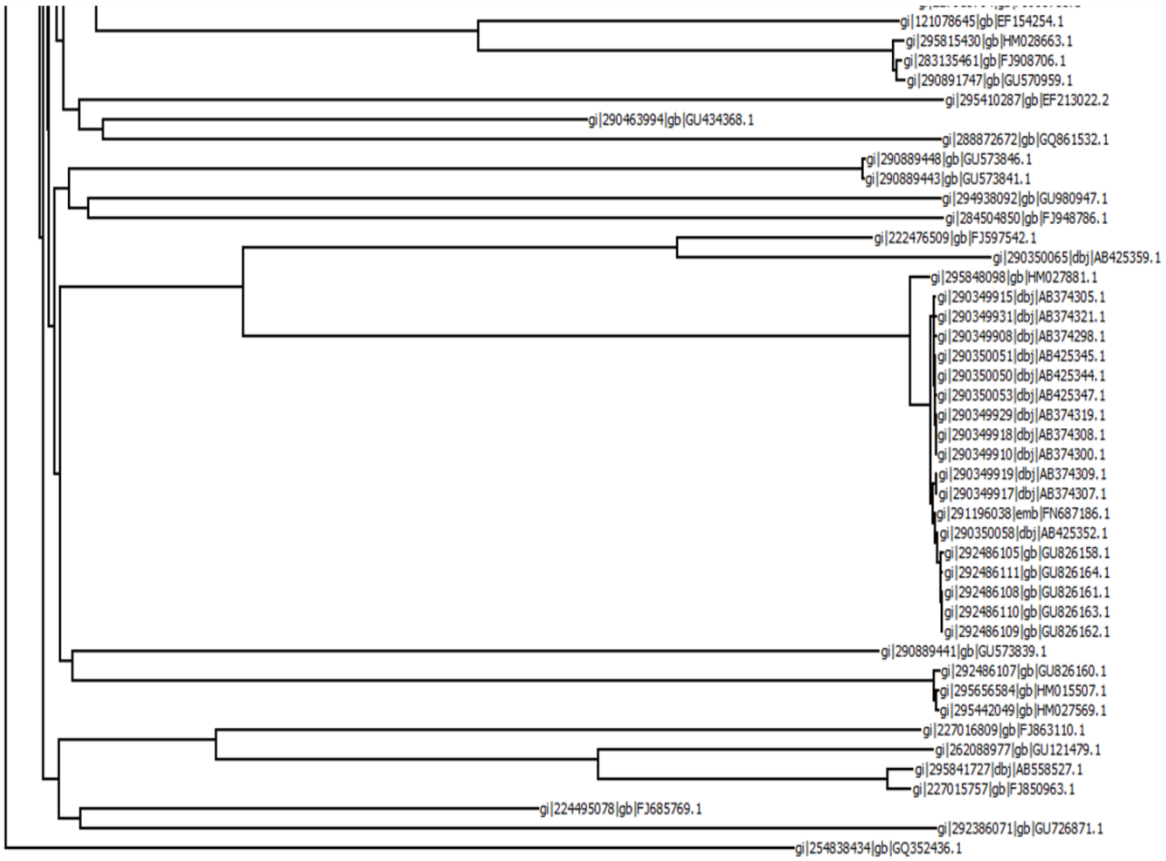


Fig . 3 *Bacillus mycoides* strain BM1

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^{o}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 12fragments (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

(225ml).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/*Msp*1 digest restriction enzyme. (Fig. 9).The isolated *Bmy* crude restriction enzyme methylated the fragments of already digested pBR322/*Msp*1 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/*Msp*1 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. 4 *Bacillus mycoides* strain BM1

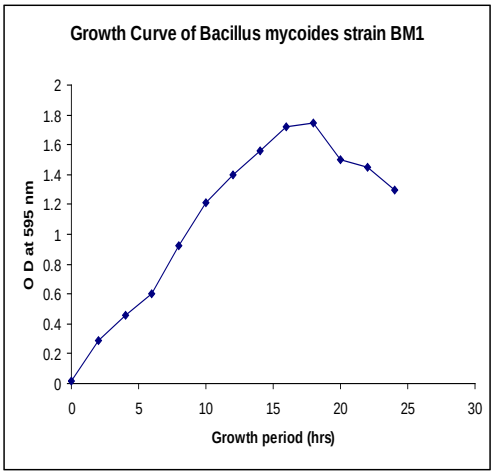


Fig. 5 Growth Curve of *Bacillus mycoides* strain B

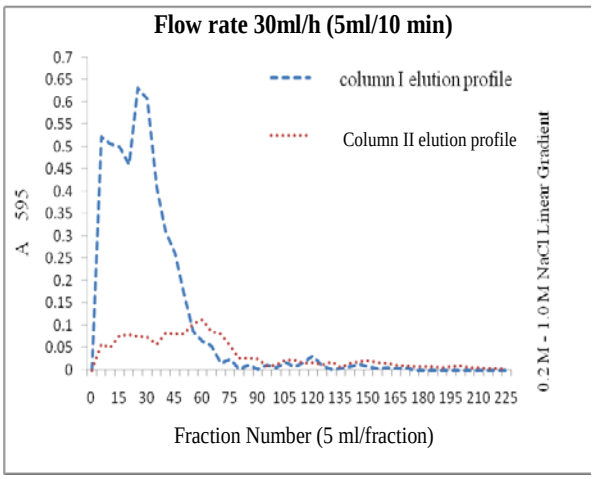


Fig. 6 Elution profiles of *Bmy* eluted form DEAE cellulose column I and Phospho cellulose column II

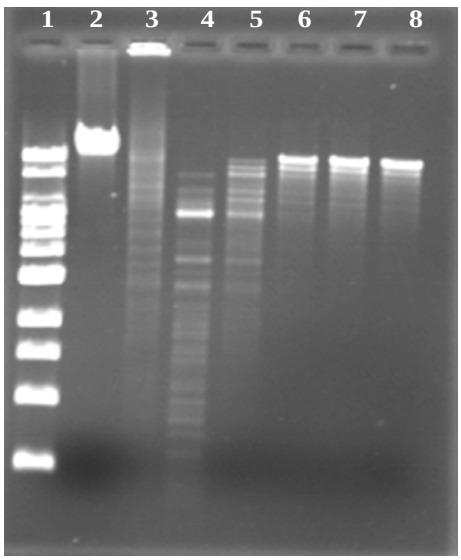


Fig. 7 *Bmy* Restriction endonuclease assay with Phage λ DNA

Lane 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

Protein source	Fractions	O.D value	Protein quantified/BSA (µg/ml)
Bacillus mycoides strain BM1 sonicated in phosphate buffer (pH 7.2)	Crude extract	0.208	26
Ammonium sulphate saturation (50% & 70 %)	Dialysed protein	1.014	95
Column I			
Elution at 0.2M	Fractions (1-6)	0.127	12
Elution at 0.3M	Fraction (7-10)	0.095	10
Column II			
Elution at 0.2M	Fractions 1-7 (20ml)	0.089	9
Elution at 0.3M	Fractions 8-12 (25ml)	0.101	10
Elution at 0.4M	Fractions 13-16 (20ml)	0.096	10

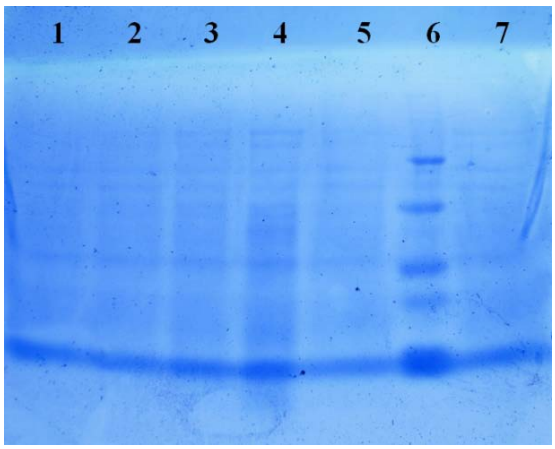


Fig. 8 Characterization of *Bmy* protein elution profile DEAE column I chromatography Lane 1- *Bmy* elution fraction at 0.2, 2- *Bmy* elution fraction at 0.2; Phosphocellulose column II chromatography,3- *Bmy* elution fraction at 0.2M ,4- *Bmy* elution fraction at 0.3M, 5- *Bmy* elution fraction at 0.4M,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

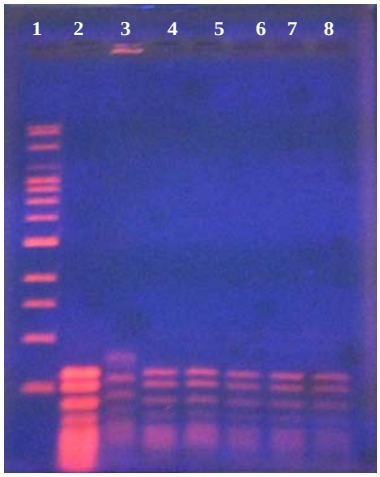


Fig. 9 *Bmy* assy with pBR322/*Msp* 1 digest

Lane 1.Supermix DNA ladder 100 Kb,

2. Control of pBR322/*Msp* 1digest

3. Crude enzyme of *Bmy* with pBR322/*Msp*1digest

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).

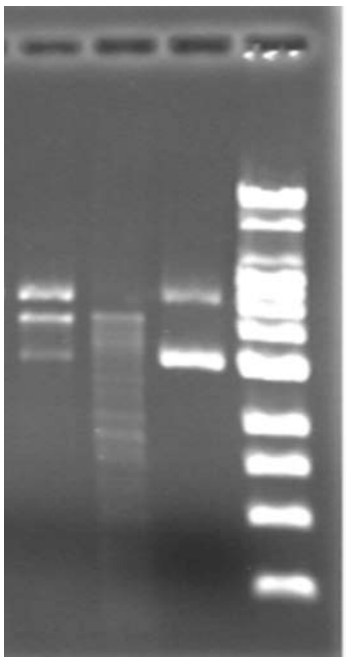


Fig. 10 Bmy restriction endonucleases assay with pBR322

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

C↓CGG
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/*Msp*1 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

C↓CGG
GGC↑C

The number of nucleotide basepairs in between the above mentioned specific nucleotide sites of Pehag 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 *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.10).

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 dissolved clearly at lane 4 on agarose gel (Fig.11)

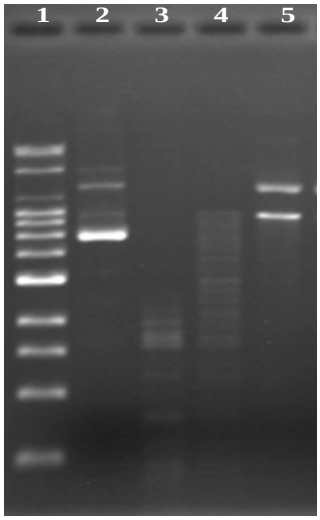


Fig. 11 *Bmy* restriction endonucleases assay with pTriEx-1.1 Neo vector

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

Table 2: pBR322/*Msp* 1 digest has 24 cleavage sites at CCGG. Bioinformatical data of the length of the possible fragments

Fragment length	pBR322/Msp 1 digest	
	Fragment length	Fragment No
700-600	699	1
600-500	542	1
500-400	400	1
400-300	303	1
300-200	213,234	2
200-100	160,119,158,156,197,176,143,153,188,173	10
Less than 100	5,5,72,86,21,30,22,30	8

Total 24 fragment

Table 3 *Bmy* RE digested fragment length of pBR322.

Derived Gel doc data tabulation

Fragment length (Nucleotide bp)	*Bmy* RE digest of pBR322
	Fragment length (Nucleotide bp) (Gel doc mode value)
700-600	700
600-500	598
500-400	448
400-300	328
300-200	241
200-100	172
Less than 100	

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 incRENased the molecular weight, which decRENased 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

λ DNA Fragment length	Cleaved fragments (Bioinformatical data)	
	Fragment length	Fragment No.
3000-2500	2289	1
2500-2000	2164	1
2000-1500	1985,1566	2
1500-1000	1248,1168,1045	3
1000-900	-	
900-800	847	-
800-700	767	1
700-600	665,650,611,617	1
600-500	595	4
500-400	506,484,482,455,433,423,422,413	1
400-350	374,359,379,373	8

350-300	333,343,330,320,354,331,336	4
300-250	301,295,267,263,266,281,283,278,255	7
250-200	Many fragments	9
200-100	Many fragments	21
Less than 100	Many fragments	57
		194

Total 311 fragments

Table 5 The newly isolated Bmy restriction endonuclease digest of λ DNA

Mobility of supermix DNA ladder (bp)	Fragmented length (bp) of λ DNA
33500,24500	
15000	14530
8990	10820
7000	8530
6000	6460
5000	
4000	3640
3000	2720
2000	2200
1500	1850 1500
1000	1100 825
500	

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 Ava* II and *Asu* II from cyanobacteria have also been purified by the

combination of these two column. 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^{o}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^{o}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^{o}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

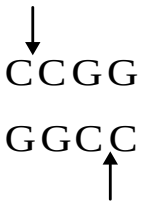
The purified restriction endonuclease *Bmy* from *Bacillus mycoides* strain BM1 cleaved the λ DNA into more than 12 fragments and pBR 322 into four fragments.

The 70% Ammonium sulphate precipitated crude extract of *Bmy* restriction endonuclease methylated some of the fragmented pBR322/*Msp*1 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/ *Msp*1 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



While the crude restriction endonuclease of *Bmy* isolated from *Bacillus mycoides* strain BM1 methylated the fragmented DNA of pBR322 /*Msp*1 digest, the purified endonuclease subunit did not methylate the fragmented pBR322/*Msp*1 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 *Eco*R1 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)

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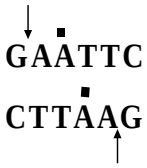


Similarly *Bmy* modification methylase and restriction enzyme should recognize specific sequence on substrate pBR322 DNA. So that the specific cleavage site and methylation site of *Bmy* on pBR322 sequences is

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).

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

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

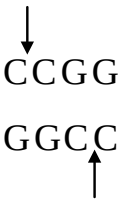
on λDNA and pBR322 determine the nucleotide recognition cleavage site of *Bmy* restriction endonucleases.

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 *Bsu*I of *Bacillus subtilis* cleaved pTriEx-1.1 Neo vector at specific cleavage site at

The *Bmy* restriction endonuclease may be an isoschizomer of *Msp* and *Bsu*I 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



with staggered cohesive ends. It may be an isoschizomer of *Msp* and *Bsu*I restriction endonucleases.

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