STUDIES ON MOLECULAR CHARACTERIZATION OF *Azospirillum* spp. ISOLATED FROM THANJAVUR DISTRICT

Kanimozhi.K. and Panneerselvam,A*.,

Dept of Botany and Microbiology, A.V.V.M.Sri Pushpam college(Autonomous),
Poondi- 613 503, Thanjavur Dt , Tamilnadu, India.

Email: Panneer_1959@yahoo.com

ABSTRACT : Altogether 30 *Azospirillum* strains isolated from paddy rhizosphere soil were identified as nitrogen fixers. A preliminary screening of all the species isolated from soils were made for nitrogen fixing activity.Among 30 strains three species of *Azospirillum* showed promising activity. These strains were classified as a unique grass by RAPD fingerprinting restriction enzyme analysis and 16S rRNA gene sequencing. Phylogenetic analysis of the 16S rRNA gene indicated that these diazotrophic strains belonged to the genus *Azospirillum* and were closely related to *A. brasilense* (with 84% similarity), *A.amazonense* (78% similarity) and *A. halopraferens* (93% similarity).

Key words: *Azospirillum*, 16S rRNA sequencing, RAPD fingerprinting.

INTRODUCTION

Soil microorganisms play an important role in soil processes that determine plant productivity. For successful functioning of introduced microbial bioinoculants and their influence on soil health, exhaustive efforts have been made to explore soil microbial diversity of indigenous community and their distribution and behavior in soil habitats. The era of molecular microbial ecology has uncovered only a part of novel microbiota, most of which is based on rRNA and rDNA analysis.

Azospirillum includes five species. *A. brasilense, A. lipoferum, A. halopraferens, A. amazonense* and *A. irakense*. The differentiation between these species has been supported by physiological, morphological and biochemical characterization (Krieg and Dobereiner, 1986), and analysis of DNA restriction profile (Fani *et al.,* 1993). DNA homology (Fallik *et al.,* 1994), 16S ribosomal DNA sequences (Xie and Yokota, 2005).

The purpose of this study was to clarify the taxonomic position of this strain based on phylogenetic analysis of its 16s rRNA gene sequence together with these of other representatives of the genus *Azospirillum*.

MATERIALS AND METHODS

Description of the study area

The present study focused on the area in and around Thanjavur district (Fig.1). The study area is situated in Tamilnadu state (Lat. 11°10′– 11° 30′ N and Long. 78°15′ – 78°30′ E) with the significant features of evergreen forests and also it was a less explored ecosystem for the investigation of *Azospirillum* population.

Collection of soil samples (Bashan and Wolowelsky, 1987)

For the enumeration of *Azospirillum*, soil samples were collected by aseptic manner at a depth of 5-10 cm according to the V - shaped method, at thirty different locations in and around the Thanjavur district. From each site, five samples were collected and pooled together and considered as one sample. The soil samples were brought to the laboratory and kept in the refrigerator for further process.

Isolation of *Azospirillum*

From the collected soil samples, 1 g was taken and serially diluted using sterile distilled water upto 10^{-8} dilutions. One ml of diluted sample from 10^{-6} to 10^{-8} dilutions was taken, and 0.1ml of aliquot was inoculated in test tube containing Nfb (Nitrogen free bromothymol) semisolid media. All the tubes were incubated at 32°C for 48 h and observed the growth by the formation of pellicles. The pellicles were streaked on Nfb solid media and incubated at 32°C for 24 h.

Molecular characterization of *Azospirillum*

DNA Isolation

Total genomic DNA was extracted by standard methods of Boudjella *et al*. (2006). All the isolates were grown at 30°C for 5 days in shake flasks containing 100 ml of Nfb liquid medium. Pellet was obtained by centrifugation and washed twice with distilled water. Approximately 200 mg of pellet were used for genomic DNA extraction. The pellet was suspended in 500 µl of the lysis solution [100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 250 mM NaCl and 2% SDS]. Lysozyme was added to obtain a final concentration of 1 mg/ml, and the solution was incubated at 37°C for 60 min. After the addition of 10 µl of proteinase K (10 mg/ml), the mixture was incubated at 65°C for 30 min. The solution was chilled on ice and extracted with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1). The organic extraction was repeated, and the supernatant was taken and 4M Ammonium acetate and two volumes of isopropanol. Total genomic DNA was precipitated by centrifugation for 10 min at room temperature. The pellet was washed with 70% ethanol, dissolved in Tris EDTA buffer (pH 8.0) and stored at -20°C.

Oligonucleotide primers and PCR conditions

The sequences of the oligonucleotide primers used for PCR are listed.

Primer	Sequence	Reference
16S rRNA:		
F243	5'- GGATGAGCCCGCGGCCTA -3'	Heuer *et al.* (1997)
R1378	5'- CGTGTGTACAAGGCCCGGGAACG-3'	
RAPD:		
OP 07	5'- CCGCAGCCAA -3'	Neilan, 1995

The design and synthesis of the 16S rRNA oligonucleotide primers have already been described by Heuer *et al*. (1997) was used in the present study. The random 10-mer oligonucleotide primers (OP-07) used for RAPD-PCR fingerprinting. All the primers were obtained from XDT Technologies (Germany).

All the PCR reactions were carried out in a 50 µl volume containing 1 µl (50 pmol) of each primer, 1 µl of 1.25 mM of dNTPs, 1 µl (50 mg) of template DNA and 1U of DyNAzyme DNA polymerase (Finnzymes, Finland). The buffer supplied with the enzyme was used according to the manufacturer's directions. The amplifications were performed with DNA thermal cycler (Eppendorf Mastercycler Gradient, Germany). The cycling conditions for 16S rRNA and RAPD-PCR differ in annealing temperature. The PCR cycles as follows: one cycle at 95°C for 7 min, 30 cycles at 94°C for 1 min, 62°C for 1 min (16S rRNA) and 42°C for 1 min (RAPD), followed by a single final extension step at 72°C for 7 min. After the reaction completed, 10 µl of amplified DNA was separated on 1.2% low melting agarose (Sigma, USA). Then the agarose gel was stained with ethidium bromide and recorded using a CCD camera in UVP gel documentation system (UVP, England). Standard molecular marker and supplied with DyNAzyme™ II DNA polymerase kit (Finnzymes, Finland) included into all the gels.

DNA fingerprint analysis

The DNA fingerprints of isolates obtained by PCR fingerprinting methods detected by visual examination of the banding pattern. The presence or absence of a band at any position on the gel was used to construct a two-dimensional binary matrix for *Azospirillum* isolates tested. Genetic distances between isolates were calculated using the algorithm of Nei and Li (1979) as provided in the RAP Distance Software package (Armstrong *et al.,* 1994). A pair wise comparison of genetic distances for all *Azospirillum* patterns was used to create a phenogram based on the neighbour-joining method.

Sequencing and phylogenetic analysis of 16S rRNA gene

The amplified PCR products were purified using a QIAquick PCR purification kit (Qiagcn, GmBh, Germany) as recommended by the manufacturer. The sequences of the PCR products were determined by using the BigDye Terminator Cycle Sequencing v2.0 kit on an ABI 310 automatic DNA sequencer (Applied Biosystems, CA, USA) according to the manufacturer's instructions. The 16S rRNA gene sequences determined for the selected *Azospirillum* strains were deposited in the GenBank database and got their accession. The 16S rDNA sequences reported in this study was multiple-aligned using CLUSTAL W, version 1.7 (Thompson *et al.,* 1994) with a selection of *Azospirillum* reference sequences obtained from GenBank. The alignment was corrected manually and converted to a distance matrix. The distance matrix was converted to a phylogenetic tree using the neighbor-joining (NJ) algorithm (Saitou and Nei, 1987) using MEGA 3.1 (Kumar *et al.,* 2004) with multiple substitutions corrected and positions with gaps excluded. The statistical significance of the tree branches was assessed by bootstrap analysis involving the construction of 1,000 trees from the resampled data (Felsenstein, 1985).

In silico restriction endonuclease digestions

Restriction endonucleases, the commercially available online software used for this study for *in silico* digestion. For each 16S rRNA gene sequence, *in silico* digestions were performed using NEB cutter v2.0. The *in silico* analysis allowed for the selection of specific restriction endonucleases for distinguish of *Azospirillum* genera.

Secondary structure determination

The 16S rRNA gene sequences of *Azospirillum* isolates were used for the construction of secondary structure models and were folded using Genbee-NET Cutter (Brodsky *et al.,* 1995). These secondary structures were used to assess the significance of observed differences in 16S rRNA gene sequence data.

RESULT AND DISCUSSION

Diversity of *Azospirillum*

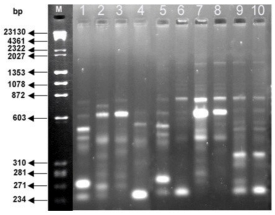
For the isolation of *Azospirillum* spp., Nfb semisolid medium was used. After 24 h incubation, the Nfb semi-solid medium showed white colored pellicle. Appearance of pellicle formation on Nfb semi-solid medium indicated successful isolation of *Azospirillum*. The pellicles were transferred into Nfb plates. After 48 h white, merged colonies were observed on the medium. Typical white or pink, often wrinkled colonies were picked out and transferred into Nfb semi-solid medium. A total number of 30 morphologically distinct *Azospirillum* isolates were isolated. Among the 30 isolates three isolates were highly dominant and selected for molecular characterization.

The genetic diversity among the isolates was assessed by RAPD analysis. All the isolates showed reproducible DNA banding pattern. Diversity among the isolates was assessed on the basis of variation of size number and intensity of bands (Saleena *et al*., 2001). Amplified DNA products were observed for *Rhizobium* and *Azospirillum* isolates. All the *Rhizobium* and *Azospirillum* isolates shared different DNA banding pattern. The strains with identical DNA fingerprints were placed in one group *(*Ilyas *et al*., 2008).

RAPD pattern

RAPD fingerprinting analysis was made using the primer OP07 (Fig.1). All the ten isolates was shared different DNA banding pattern in RAPD finger printing analysis. Most of the amplified fragments were between 234 and 2027 bp. RAPD profiles of *Azospirillum* isolates were greatly diversified based on their banding pattern. It revealed strong reproducible fragments of approximately 615 bp was present in the profile of the isolates *A. lipoferum* TA01, *A. lipoferum* TA05, *A. halopraferens* PA04 and *A. amazonense* PA05, whereas these fragments were absent in other strains. Similarly, another fragments approximately 250 bp was present only in the isolates such as *A. brasilense* PA03, *A. amazonense* PTA03 and *A. amazonense* PTA04. The greatest variations of the banding patterns of *Azospirillum* isolates strongly stated that the strain level variations of the bacterial isolates could be directly reflected in RAPD banding pattern (Plate I).

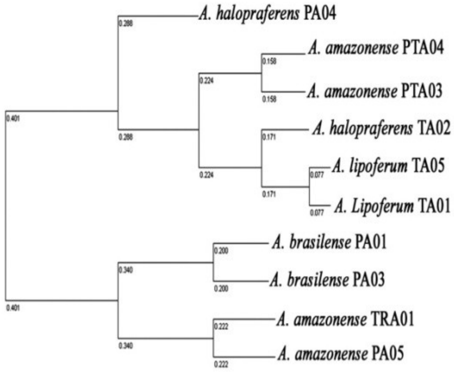
Plate - I



RAPD - Fingerprinting analysis of selected *Azospirillum* isolates

1- *A.halopraferens* TA02; **2-*A.lipoferum* TA01;**
3- *A.lipoferum* TA05; **4- *A.amazonense* TRA01;**
5- *A.brasilense* PA01; **6- *A.brasilense* PA03;**
7- *A.halopraferens* PA04; **8- *A.amazonense* PA05;**
9- *A.amazonense* PTA03; **10- *A.amazonense* PTA04;**
M - Molecular Marker

Figure. 1. Phylogenetic tree constructed based on RAPD – fingerprinting



The figure 1 showed the phylogenetic tree constructed based on RAPD finger printing. Totally 10 *Azospirillum* isolates, they clustered into 2 major distinct groups. In group I contained only 6 isolates, whereas group II contained 4 isolates. These 2 distinct groups comprised 4 major clusters such as cluster (a) A. *halopraferens* PA04, cluster (b) *A. amazonense* PTA04 and PTA03, *A. halopraferens* TA02, A. *lipoferum* TA05 and TA01, cluster (c) *A. brasilense* PA01 and PA03 and cluster (d), *A. amazonense* TRA01 and PA05.

In the present study, all the 30 isolates of *Azospirillum* were fell into four groups on the basis of morphological, physiological and biochemical characteristics. Whereas, the results of RAPD finger printing of the maximum 10 nitrogen fixers (TAO1, TA01, TA05, TRA01, PA01, PA03, PA04, PA05, PTA03 and PTA04) were classified into two groups on the basis of banding pattern and phylogeny. Out of 10 *Azospirillum* strains, they clustered into 2 major district groups. Group I contained 6 strains, whereas group II contained 4 isolates. These 2 distinct groups comprised under 4 major cultures, they defined by similarities in phylogenetic patterns were comprised by cluster a) *A. halopraferens* PA04, cluster b) *A. amazonense* PTA04 and PTA03, *A. halopraferens* TA02, *A. lipoferum* TA05 and TA01, cluster c) *A. brasilense* PA01 and PA03, and cluster d) *A. amazonense* TRA01 and PA05.

RAPD analyses are useful for differentiating bacteria at the strain level (Tiedje, 1996). RAPD technique has been frequently used for identification and differentiation of bacterial strains such as *Rhizobium* (Hebb *et al.,* 1998) and *Azospirillum* (Fani *et al*., 1993). According to Young and Cheng (1998) RAPD is a potential tool for the identification of the genetics and systematic of different populations, possible reason for genetic diversity might be low soil moisture may have resulted in genetic adaptations of the strains. However variation among different strains of diverse origin suggested that there is genetic potential to improve N_2 fixation and soil nutrients.

Molecular systematics, which includes both classification and identification, has its origin in the early nucleic acid hybridization studies but has achieved a new status following the identification of nucleic acid sequences through sequencing techniques (O' Donnell *et al*., 1993). The sequence analysis of the genes coding for the ribosomal subunits 16S, 23S and 53S rRNA) in particular the 16S rRNA gene has become an important tool in bacterial identification. Since it provides an information about the phylogenetic placement of the species (Woese, 1987; Brenner *et al*., 2001).

DNA based molecular methods have been used for species differentiation and identification of bacteria. Significance of phylogenetic studies based on 16s rDNA sequences is increasing in the systematics of bacteria (Yokota, 1997).

Tarrand *et al*., (1978) reported that 16S rRNA gene sequence analyses indicated that the close relativeness to strain COC8^T were *A. lipoferum* and *A. largimobile* (96.0% similarity); values of not more than 95.0% were obtained against other recognized species of *Azospirillum*. When alignment gaps and ambiguous bases in the 16S rRNA gene sequences were excluded from the calculations, similarity values for strain COC8^T against *A. lipoferum* and *A. largimobile* were 96.3and 96.8% respectively. *A. lipoferum* was the first species assigned to *Azospirillum* and *A. largimobile* was subsequently reclassified from the genus *Conglomeromonas* (Skerman *et al*., 1983).

16S rRNA sequencing analysis

Among the 10 maximal nitrogen fixing *Azospirillum* isolates, the higher nitrogen fixer's namely *A. brasilense* PA03, *A. amazonense* PTA03 and *A. halopraferens* PA04 were selected for the 16S rDNA sequencing and phylogenetic relatedness studies.

The genomic DNA was isolated from the selected *Azospirillum* isolates and they were partially sequenced using specific 16S rRNA sequencing primer. The length of 16S rRNA fragment of the potential nitrogen fixer's namely *A. halopraferens* PA04, *A. amazonense* PTA03 and *A. brasilense* PA03 were 913, 811, and 894bp respectively. The sequences deposited in Genbank (NCBI, EMBL and DDBJ) with the following accession number such as *A. brasilense* PA03 – HM217758, *A. amazonense* PTA03- HM217759 and *A. halopraferens* PA04 – HM217760.

The 16S rRNA gene sequences of *Azospirillum* isolates were BLAST to already submitted sequences. The results revealed that the closes relativeness to the isolates PA03 with *Azospirillum* sp. TSH51 at 84% of similarity level. Similarly, both the isolates PTA03 and PA04 were closely related to an already existing but uncultured *Azospirillum* sp. DQ 312-25 at 78% and 93% similarity level respectively. Phylogenetic relatedness of the *Azospirillum* sp. were analyzed by neighbor joining method and showed in Figure.2.

The isolate *A. brasilense* PA03 showed 33 stems and 23 loops in their RNA secondary structure (Fig. 3), where as *A. amazonense* PTA03 showed 33 stems and 22 loops (Fig. 4) and 35 stems and 24 loops were found in *A. halopraferens* PA04 (Fig. 5). The free energy structure of 16S rRNA secondary structure of *A. brasilense* PA03, *A. amazonense* PTA03 and *A. halopraferens* PA04, were -193.0 kkal/mol, -192.3 kkal/mol and -215.5 kkal/mol respectively (Figure. 3- 5).

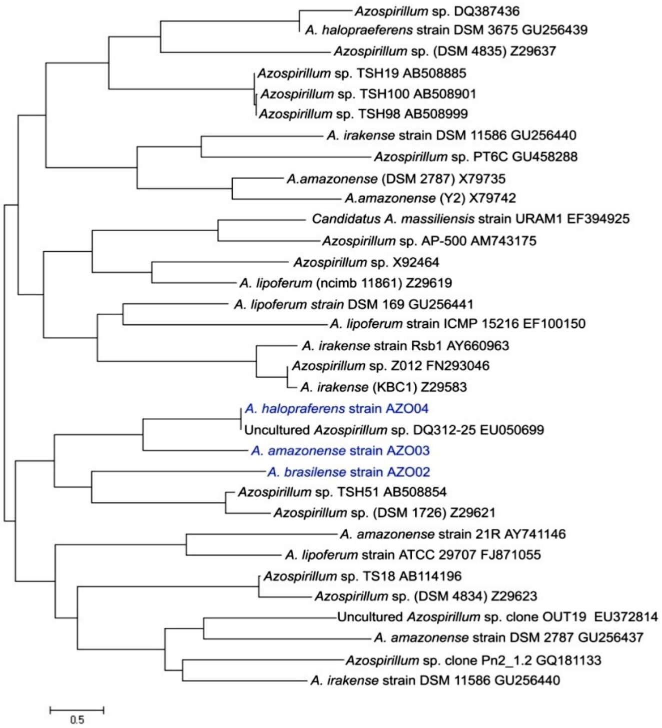


Figure. 2. Phylogenetic tree constructed based on 16s rRNA gene sequences

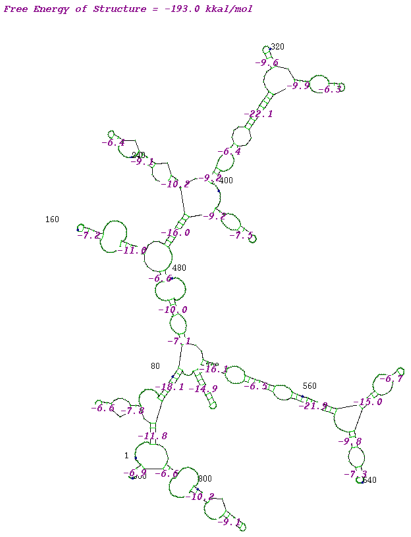


Figure.3. Secondary structure of 16s rRNA of *Azospirillum brasilense* PA03 (ACC.NO.HM217758)

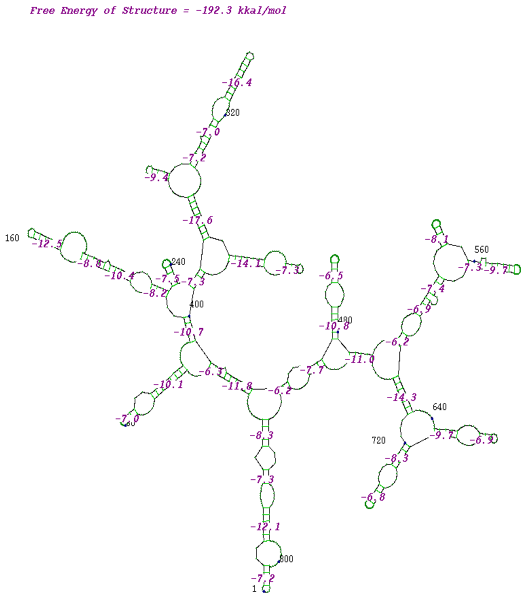


Figure. 4. Secondary structure of 16s rRNA of *Azospirillum amazonense* PTA03 (ACC.NO.HM217759)

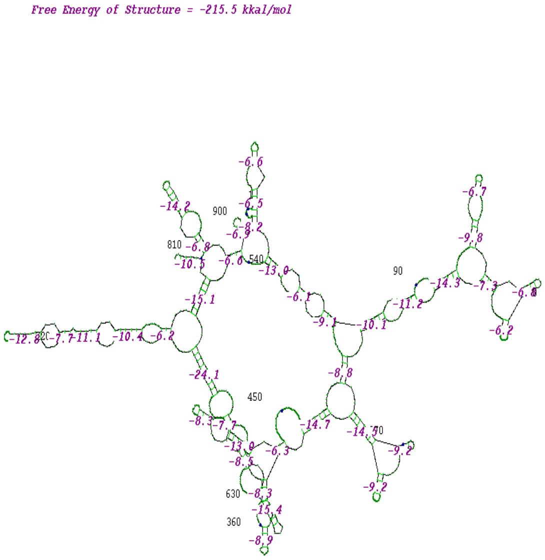


Figure. 5. Secondary structure of 16s rRNA of *Azospirillum halopraferens* PA04 (ACC.NO.HM217760)

A large number of restriction enzyme sites were found in all the *Azospirillum* isolates. As much as, the total restriction enzymes sites of *A. brasilense* PA03 and *A. amazonense* PTA03 were 42 and 46 respectively (Fig.6 and 7). Whereas *A. halopraferens* PA04 was showed 61 restriction enzyme sites (Fig.8). However the cleavage sites and the nature of restriction enzymes of azospirilla differed from one another. The GC content of *A. brasilense* PA03, *A. amazonense* PTA03 and *A. halopraferens* PA04, were found to be 50, 53 and 53% respectively. Similarly, the AT content of *A. brasilense* PA03, *A. amazonense* PTA03 and *A. halopraferens* PA04 were found to be 50, 47 and 47% respectively using NEB Cutter Programme V 2.0 in www.neb.com/nebcutter2/index.php (Figure. 6-8).

In the present study variation in the 16S rDNA sequences of three different *Azospirillum* isolates were observed. The phylogenetic analysis of partial 16s rDNA sequences showed that the *A. brasilense* PA03 – HM217758 was closely similar (84%) to the existing species of *Azospirillum* sp. TSH51 AB508854 whereas *A. amazonense* PTA03 – HM217759 was closely similar (78%) to existing species of uncultured *Azospirillum* sp. DQ312–25 EU050699. *A. halopraferens* PA04 – HM217760 was closely similar (93%) to existing species of uncultured *Azospirillum* species DQ312–25 EU050699. In the present study, distinct variation in the secondary structure, GC composition, presence of restriction enzymes in 16S rRNA sequence of three different *Azospirillum* isolates showed molecular level specificity of each and every individual isolates for the development of universal identification system of not only *Azospirillum* but all microorganisms, a polyphasic taxonomic approach utilizing morphological, biochemical, physiological cultural, ecological and molecular characteristic will only help taxonomists for the developments of meaningful taxonomic identification system.

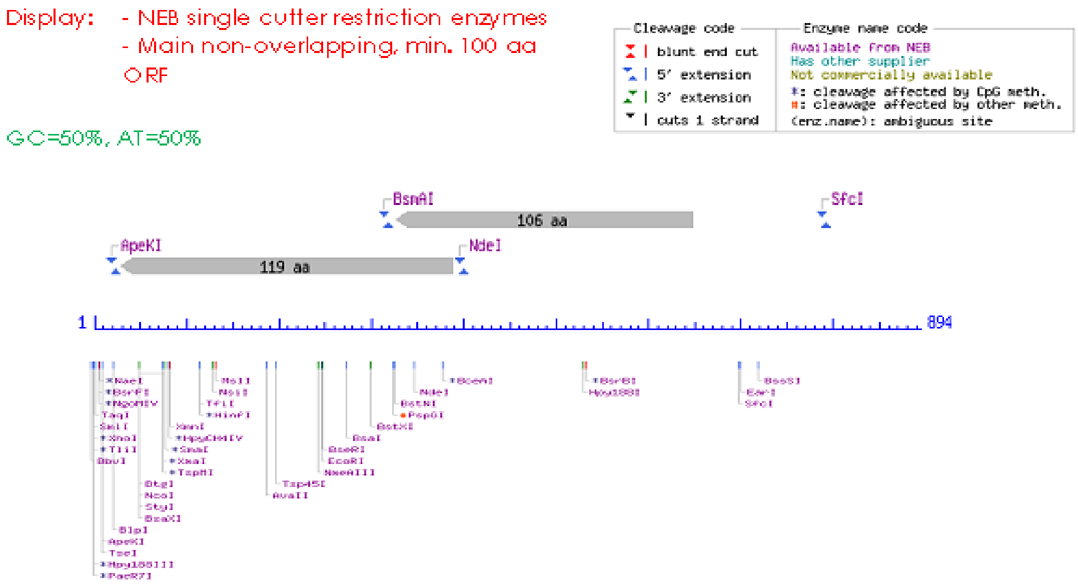


Figure. 6 Restriction site analysis and GC content of 16S rDNA of *Azospirillum brasilense* PA03 (ACC.NO. HM217758)

Figure. 7 Restriction site analysis and GC content of 16S rDNA of *Azospirillum amazonense* PTA03 (ACC.NO. HM217759)

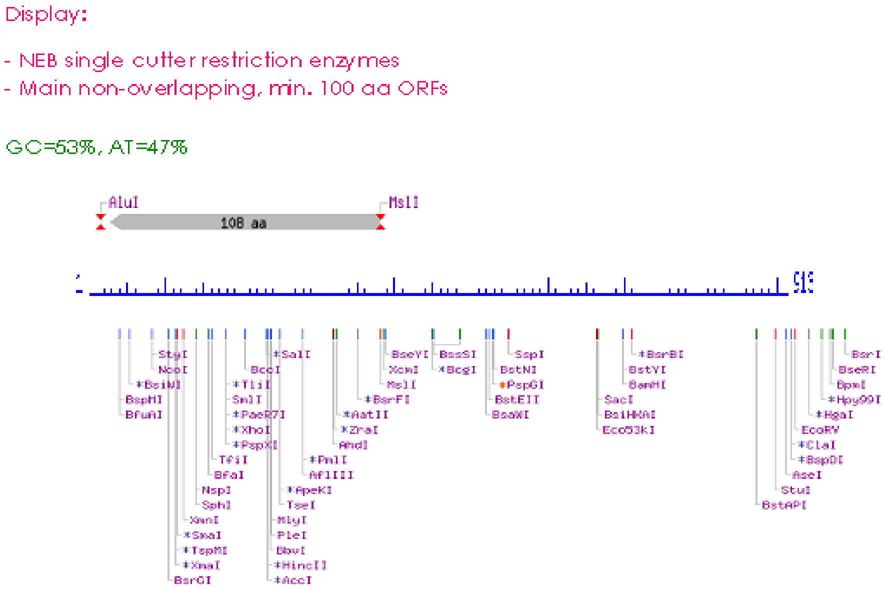


Figure. 8 Restriction site analysis and GC content of 16S rDNA of *Azospirillum halopraferens* PA04 (ACC.NO. HM217760)

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