4. Molecular characterization of the mosquitocidal Bacillus subtilis strain (VCRC B-471)
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*Bacillus subtilis* strain (VCRC B-471)

4.1. Introduction

The genus *Bacillus* encompasses a variety of phenotypically heterogeneous species exhibiting a wide range of nutritional requirements, physiological and metabolic diversity and DNA base composition (Claus & Berkeley, 1986; A$^2$h et al., 1991). Due to this, the biochemical approach for species identification can be tedious, expensive, and inaccurate. Hence, a rapid, definitive method is greatly needed. In recent years, nucleic acid fingerprinting, especially the PCR based methods, has been widely applied in bacterial systematics. Use of 16S rRNA genes in molecular systematics provides two primary advantages over phenotypic identification: rapid turn-around time and improved accuracy. The information content of the 5' end of the 16S rRNA gene is sufficient for identification of most bacterial species. The 16S rRNA sequences contain two types of region: highly conserved region that define relationship among distant taxa and variable region which differentiates the genera and species (Yamamoto & Harayama, 1995). It has been reported that in *Bacillus*, the 5' end region (approx. 275 bp) is the hyper variable region (HV region) in the 16S rRNA gene and is highly specific for each type strain of this genus (Goto et al., 2000). Analysis of 16S rRNA sequences is a simple, commonly used method for the identification of microorganisms (Aman et al., 1991; 1995). Hence, sequencing of the 16S rRNA gene was taken up for identifying the mosquitocidal strain VCRC B-471.

Though 16S rRNA gene is used as a framework for modern bacterial classification, often it shows limited variation for members of closely related taxa (Fox et al., 1992). On the other hand, protein-coding genes exhibit much higher genetic variation, which can be used for classification and identification of closely related taxa (Mollet et al., 1997; Yamamota and Harayama, 1995). Chun and Bae (2000) demonstrated the use of *gyrA* sequences (coding for DNA gyrase subunit A) for accurate classification of *Bacillus subtilis* and related taxa, including *Bacillus amyloliquefaciens*, *Bacillus*
vallismortis, Bacillus mojavensis, Bacillus atrophaeus and Bacillus licheniformis. Apart from gyrA gene, rpoB gene, approximately 1,100-amino acid B-subunit of RNA polymerase, is described as possessing the same key attributes as 16S rRNA, in that it is common to all bacteria and is a mosaic of conserved as well as variable sequence domains (Dahllof et al., 2000). Most importantly, the rpoB gene exists as a single copy in bacterial genomes (Mollet et al., 1997). The RpoB protein has been used to infer relationships between archaeal orders (Matte-Tailliez et al., 2002) and Gram-positive and Gram negative bacteria (Morse et al., 2002). Therefore, gyrA and rpoB gene sequencing also was performed for identifying the strain VCRC B-471.

4.2. Objective

Molecular identification of VCRC B-471 using 16S rRNA, gyr A and rpo B gene sequencing.

4.3. Materials and methods

4.3.1. Polymerase chain reaction (PCR) assays

4.3.1.1. 16S rRNA gene

The 16S rRNA gene, corresponding to an internal portion of the B. subtilis group, was PCR-amplified using the primers, Bsub 5F (5’ - AAG TCG AGC GGA CAG ATG G -3’) and Bsub 3R (5’ – CCA GTT CCA ATG ACC CTC CCC -3’), reported by Wattiau et al. (2001). Genomic DNA was extracted from VCRC B-471 using Gen Elute Bacterial Genomic DNA kit (Sigma, St. Louis, USA) according to the manufacturers instructions. The PCR mixture comprised 3.5 mM MgCl₂, 200 μM of each dNTP’s (Promega, Madison, USA), 0.4 μM of each primer (Metabion, Bangalore, India), 2.5 μl of 10X Taq buffer and 1.25 units of Taq polymerase (AmpliTaq Gold, Applied Biosystems, New Jersey (NJ), USA). To this mixture, 1 μl of the DNA template was added. The control tube was added with 1 μl deionised water in place of DNA sample and the reaction mixture in the tubes were made up to 25 μl volume using deionized water. The reaction mixture was amplified in a Thermal Cycler (Biorad, USA). The PCR conditions were: denaturation at 94°C for 12
min., 30 cycles of denaturation at 95°C for 0.5 min, annealing at 65°C for 2 min, extension at 72°C for 2 min, and a final extension step at 72°C for 7 min. The amplified product was checked by electrophoresis of 8 μl on 1.5% agarose (Sigma, St. Louis, USA) gel containing ethidium bromide. To verify the size and purity of the product, a 100 bp ladder (Promega, Madison, USA) was run along with the sample. After confirmation of the size of the amplicon, the amplified PCR product was purified using Qiagen QIA quick gel elution kit (Qiagen Corp., Hilden, Germany) and sequenced (Microsynth AG, Switzerland).

4.3.1.2. gyrA gene

A gyrA fragment corresponding to B. subtilis gyrA positions 43 to 1070 was PCR amplified by using primers gyrA-f (5'-CAGTCAGGAAATGCGTACCTT-3') and gyrA-r (5'-CAAGGTAATGCTCCAGGCATTG-3') reported by De Clerck et al. (2004). The reactions were carried out with a 50-μl reaction mixture containing 20 pmol of each primer, 10 nmol of each deoxynucleoside triphosphate, 5 μl of 10x PCR-buffer (Applied BioSystems, NJ, USA), 1 U of Taq polymerase (Applied BioSystems, NJ, USA) and 50 ng of template DNA. The PCR profile consisted of denaturation at 94°C for 2 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 45 s, and extension at 68°C for 60 s; and a final extension at 68°C for 10 min. The resultant amplicons were purified with the Qiagen QIA quick gel elution kit (Qiagen Corp., Hilden, Germany) and sequenced in both directions by using the same primers (Microsynth AG, Switzerland).

4.3.1.3. rpo B gene

An rpoB fragment corresponding to Bacillus subtilis rpoB positions 6 to 585 was PCR amplified by using primers rpoB-f (5'-AGGTCAACTAGTACGTATGGGC-3') and rpoB-r (5'-AAGACCGTAACGACGCAACTT-3') reported by De Clerck et al. (2004). The reactions were carried out with a 50-μl reaction mixture containing 20 pmol of each primer, 10 nmol of each deoxynucleoside triphosphate, 5 μl of 10x PCR-buffer (Applied BioSystems), 1 U of Taq polymerase (Applied BioSystems), and 50 ng of template DNA. The PCR profile consisted of denaturation at 94°C for 2 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 45 s, and extension at 68°C for 50 s; and a final extension at 68°C for 90 s. The resultant amplicons were purified with the
Qiagen QIA quick gel elution kit (Qiagen Corp., Hilden, Germany) and sequenced in both directions by using the same primers (Microsynth AG, Switzerland).

4.4. Result and Discussion

Bsub 5F and Bsub 3R primers designed specifically for amplifying the 16S rRNA genes of *B. subtilis* group were used for the identification of VCRC B471 16S rRNA. The primers respectively encompassed nucleotides 59-79 (variable domain I) and 625-646 (variable domain II) relative to the *Escherichia coli* 16S rRNA numbering. PCR amplification of the hyper variable region of 16S rRNA gene of VCRC B-471 yielded an amplicon of size ~600 bp which was sequenced (Fig. 11). When, the nucleotide sequence (577 bp) was blasted against the NCBI database using BLASTN (Altschul et al., 1997), it showed the highest identity score to *B. subtilis* (99%). The sequence has been submitted to the GenBank (Accession number: DQ133461).

PCR amplification of *gyrA* and *rpoB* genes of VCRC B-471 yielded amplicons of size ~1 Kb and ~570 bp respectively (Figs 12 & 13). The sequences of *gyr A* (902 bp) and *rpo B* (552 bp) obtained from VCRC B-471 were blasted against the NCBI database using BLASTN, it showed the highest identity score to *B. subtilis* subsp. *subtilis* (98%). The sequences have been submitted to the GenBank (Accession number: EF687841 & EU057603).

*B. subtilis*, the type species of the genus, encompasses aerobic, endospore forming, rod shaped bacteria which are commonly found in soil, water sources and in association with plants (Claus & Berkeley, 1986). Modern bacterial taxonomy has become increasingly objective with the application of methods based on genomic information. DNA-DNA hybridization is the standard method for the delineation of bacterial species (Hood et al., 1987) but it carries disadvantages, notably it is expensive, labor intensive and time consuming. 16S rRNA sequencing is often used as an alternative method to define species, but this also cannot be applied to those with high pair wise 16S rRNA similarity values. Numerous *Bacillus* species described so far have been observed to
Figure. 11. 16S rRNA gene (~ 595 bp) amplified from VCRC B-471

M  B471

1.5 Kb

500 bp

M -100 bp ladder
Figure 12. *gyrA* gene (~1 Kb) amplicon from VCRC B-471

M  B471

1 Kb

500 bp

M - 100 bp ladder
Figure 13. *rpoB* gene (~570 bp) amplified from VCRC B-471
display rather conserved 16S rRNA sequences compared to other genera, making the use of this taxonomic marker sometimes inadequate for species definition according to generally accepted criteria (Goebel & Stackebrandt, 1994). Such unusual similarity exist for members of the Bacillus 16S rRNA group I, including B. subtilis, which displays 99.3% similarity to B. licheniformis and B. amyloliquefaciens (Ash et al., 1991). In contrast, protein coding genes like gyr A and rpo B with high mutation rates have been successfully used to differentiate taxa that cannot be identified solely using 16S rRNA sequences (Chun and Bae, 2000).

4.5. Conclusion

The mosquitocidal strain isolated from a soil sample collected from Andaman & Nicobar Islands was identified by molecular taxonomic methods based on the DNA sequences of 16S rRNA, gyrA and rpoB as Bacillus subtilis subsp. subtilis.