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Identification and Molecular Diversity Analysis of *Bacillus subtilis* from Soils of Western Ghats of Karnataka using 16S rRNA Bacterial Universal Primers

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ABSTRACT

Bacillus subtilis (B. Subtilis) is a model organism and to study the chromosomal replication and cell differentiation in bacteria. B.subtilis is the gram positive bacterium and the strains of this bacterium were isolated from soils of Western Ghats of Karnataka and characterized by molecular and morphological methods as a result 13 new B.subtilis strains were identified with reference strain MTCC-2422 (Microbial Type Culture Collection and Gene Bank). The morphology study was carried out and isolated strains were further characterized by 16S rRNA bacterial Universal primers by PCR. Sequence analyses was carried out using NCBI BLASTn tool, sequences were submitted to the NCBI.The sequencing results were showed the variations in the nucleotide sequences. GC content of all the strains between 52 to 55.5% it shows that they are closely correlated to each other. In the phylogenetic tree two major groups were seen KU936344 and KU936333 formed one group and rest of the accessions formed another group which indicates that there is great degree of diversity. The reference strain MTCC-2422 is formed a group with KU936341. Isolates from Western Ghats of Karnataka are high degree of sequence similarity with different NCBI accession numbers. Present findings tell that the genetic diversity among newly isolated strains are closely related to each other.

Key words: *Bacillus subtilis, 16S rRNA, Western Ghats of Karnataka, Molecular diversity, phylogenetic tree, NCBI accession numbers.*

INTRODUCTION

The Western Ghats of Karnataka natural ecosystem and in the world this is one of the eight hottest hotspot of biological diversity, which covers western part of India through four states including Karnataka[3]. The rhizobacterium *B. Subtilis*[13] is an aerobic, endospore-forming, rod-shaped, Gram-positive bacterium[6]. It is a remarkably diverse bacterial species that is capable of growth in different environmental conditions.

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Earlier reports showed that collection of *B. subtilis* isolates obtained from desert soils in a small sample of loci (rpoB, polC, and gyrA) with restriction fragment length polymorphisms method showed that there was considerable diversity[9]. *B.subtilis* is commonly used in laboratory studies directed at discovering the fundamental properties and characteristics of Gram-positive spore-forming bacteria[1]. In particular, the basic principles and mechanisms underlying formation of the durable endospore have been deduced from studies of spore formation in *B.subtilis.*

B. subtilis is one of the best understood prokaryotes, in terms of molecular biology and cell biology. Its super genetic amenability and relatively large size have provided the powerful tools required to investigate a bacterium from all possible aspects. Recent improvements in fluorescence microscopy techniques have provided a novel and amazing insight into the dynamic structure of a single cell organism. Research on *B.subtilis* has been at the forefront of bacterial molecular biology and cytology, and the organism is a model for differentiation, gene or protein regulation, and cell cycle events in bacteria. And it is used as soil inoculant in horticulture and agriculture. It may provide some benefit to saffron growers by speeding corm growth and increasing stigma biomass yield[12]. Monsanto has isolated a gene from *B. subtilis* that expresses cold shock protein B and spliced it into their drought-tolerant corn hybrid MON 87460, which was approved for sale in the US in November 2011[4]. Novel strains of *B. subtilis* that could use 4-fluorotryptophan (4FTrp) but not canonical tryptophan (Trp) for propagation were isolated. As Trp is only coded by a single codon, there is evidence that Trp can be displaced by 4FTrp in the genetic code. The experiments showed that the canonical genetic code can be mutable[15].

Some aerobic spore forming bacteria possess several advantages that make them good candidates for use as biological control agents. Firstly, some of these bacteria produce several different types of insecticidal and antimicrobial compounds. Secondly, they induce growth and defence responses in the host plant. Furthermore, Bacillus species are able to produce spores that allow them to resist adverse environmental conditions and permit easy formulation and storage of the commercial products[11, 2]. Previous reports showed that there is sequence variation in different *B. subtilis* strains isolated from different regions[16].

For the isolation of these bacteria like *B. subtilis* we randomly collected the soil sample from Western Ghats of Karnataka. In order to study the molecular diversity of *B. subtilis* at nucleotide level PCR was carried out with 16S rRNA bacterial Universal primers. The PCR fragments were cloned and sequenced further the sequences were submitted to NCBI data base and Accession numbers were obtained. Based on the nucleotide sequences phylogenetic tree was constructed by using MEGA 4.0 software. All the results compared with the reference strain MTCC-2422.

MATERIALS AND METHODS

Collection of samples

Total 50 numbers of soil samples were collected from Western Ghats of Karnataka randomly for isolating *B. subtilis*. 5-10 gram of soil sample was collected from 5 cm depth, after gently removing the debris in the top soil, using a sterile spatula and placed immediately inside the sterile polythene covers[14]. Labels containing the details on date of collection, place of collection, collector's name, description of the place of collection, and the agro climatic zone in which the sampling will be carried out, labels were placed inside the bag. The soil samples were stored at 4^0C for further work. Before collection of soil samples it was ensured that there no previous history of use of *B. subtilis* in all the sampling places. And we have confirmed that there is no history of other samples collected from this Western Ghats of Karnataka.

Isolation of *Bacillus subtilis*

In a microfuge tube, suspend a small amount (a loop full) of soil in 1 or 2 drops of distilled

water. Mixed well & incubated in at 80°C heating block for 10 minutes, to eliminate most of the gram positive and gram negative bacteria. After cooling this heat-treated soil samples were streaked on to nutrient agar plates using inoculation loop. Incubated at 30°C for 1-2 days and after incubation different colonies were observed onto nutrient agar media. And different colonies were re-streaked on to Bacillus differentiation Agar media and incubated at 30°C for 1-2 days. White and dry or pasty looking colonies were taken for Gram and Spore staining. And gram positive and spore forming colonies were purified by re-streaking every single colony on Bacillus differentiation agar.

DNA isolation

Confirmed single colonies were inoculated into 10ml of Nutrient broth and overnight incubated at 30°**C** withshaking (200rpm). From this overnight grown culture DNA was isolated using instruction given by HiPurA Bacterial Genomic DNA purification kit (HiMedia Laboratories Pvt ltd). DNA samples were confirmed by 1% agarose gel electrophoresis.

PCR analysis for 16s ribosomal genes

Bacterial universal 16s rRNA oligonucleotides, Forward primer 27F-AGAGTTTGATCMTGGCTCAG and Reverse primer 1492R-CGGTTACCTTGTTACGACTT[17] were synthesized at Sigma Aldrich Pvt Ltd. 200ng of Bacterial DNA were used for PCR analysis with 1.25 U of *Taq DNA polymerase* (Thermo Scientific, 5U/µl), 0.2mM dNTPs each, 1µM both primers and 3mM of MgCl2 to the final volume 50µl. And amplification was carried out with Applied Biosystems thermal cycler. Following parameters are used for amplification 2 min of denaturation at 94°C followed by 30 cycles of amplification with a 40sec denaturation at 94°C, 45 s of annealing at 54°C, and 1 min of extension at 72°C. An extra one extension step of 10 min at 72°C was added after completion of the 30 cycles. Products were analysed by 1% agarose gel electrophoresis.

Purification and Molecular Cloning

PCR products were purified with NucleoSpin Gel & PCR Clean-up (MACHEREY-NAGEL) and ligated to the TA cloning vector pTZ57R/T, which was further used for transforming *Escherichia coli* DH5α by standard protocols[10] using Thermo Fisher Scientific DNA ligation kit. The transformed 50µl of cells spread on LB agar plates containing X-gal (20 mg/ml), IPTG (100mM) and ampicillin (100mg/ml). The plates will be then incubated overnight at 37^0C to screen blue and white colonies for recombinant colony selection. Single white colonies were picked and inoculated into 5ml of LB broth containing 100mg/ml of ampicillin, incubated with shaking at 37°C overnight. Plasmids were isolated using 2ml of overnight grown bacterial culture with Thermo Fisher Scientific GeneJET Plasmid Miniprep Kit. Finally, cloned fragments were sequenced. BLASTn tool was used for DNA sequence analysis. Each gene was sequenced at least three times, and a consensus sequence was obtained.

Accession Numbers

The nucleotide sequences of the 16s rRNA genes obtained from Western Ghats of Karnataka were submitted to NCBI GenBank database.

RESULT AND DISCUSSION

Sample collection and Bacillus species identification

50 soil samples were collected from Western ghats of Karnataka, From these samples different bacterial colonies were isolated and identified as *Bacillus*. And for the identification of the bacteria Morphological features like shape, colour and size of the colonies were compared with reference strain MTCC-2422. As per observation out of 50 samples 13 samples confirmed as bacilli and the observations were listed in Table 1.

PCR analysis for 16s ribosomal genes

After the morphological confirmation the colonies used for genomic DNA isolation and successfully 1.5kb product was amplified with the 16s rRNA bacterial universal oligonucleotides Forward primer 27F-

AGAGTTTGATCMTGGCTCAG and Reverse primer 1492R-CGGTTACCTTGTTACGACTT. Amplified products were cloned and sequenced using Sanger dideoxy sequencing at Eurofins genomic India Pvt Limited. And sequence analyses was carried out using NCBI BLASTn tool, all partial sequences were submitted to the NCBI (Table 2) and accession numbers were obtained.

Phyologenetic analysis and GC content analysis

The sequences were aligned using clustalw and a phylogenetic tree was constructed using MEGA 4. The tree was generated by using TREEVIEW. Rooted Neighbour-Joining (NJ) tree showing phylogenetic relationship among different *B. subtilis* isolates based on nucleotide region of ribosomal 16S rRNA sequences genes. In the phylogenetic tree two groups were seen KU936344, KU936333 formed one group and rest of the accessions formed another group which indicates that there is great degree of diversity (Fig 1) among the Bacillus subtilis isolated from soils of Western Ghats of Karnataka. Simultaneously the GC content percentage was calculated for all the isolated strains including reference strain MTCC-2422 shown in the graph (Fig 2).

The GC contents of 16s rRNA for the 13 *B. subtilis* isolates used in the phylogenetic analyses are listed in Table 2. All the *B. subtilis* have GC content more than 52 %.

At the intra- and interspecies levels among bacteria 16S rDNA sequences are good indicators of phylogenetic relationships. In our study the 16S rRNA gene sequence analyses provided very good identification of the isolates at the genus level. Other studies also been reported that analysis of 16S rRNA gene sequences alone was not sufficient to identify *Bacillus* species[5, 8]. Partial sequencing of 16SrRNA gene is a good alternative for problematic phenotypic identifications and placing isolates in their right taxonomic position; it seems necessary in the case of environmental samples and is indispensable in ecological studies, to match relatedness of different species, and gain insight to their diversity in characteristics[7]. According to our study and comparison with the reference strain MTCC-2422 (Table 3) it is confirmed that all the 13 samples are belongs to the *B. subtilis* isolates.

Table 1: Showing the colony morphology and colour of the colony in Bacillus differentiation agar

Sample Name	Shape	Colour	Gram staining	Spore staining	Colony colour in differentiation agar media
HSN_04	Bacilli	Purple	+	+	Yellow
HSN_05	Bacilli	Purple	+	+	Yellow
HSN_06	Bacilli	Purple	+	+	Yellow
HSN_07	Bacilli	Purple	+	+	Yellow
HSN_08	Bacilli	Purple	+	+	Yellow
HSN_09	Bacilli	Purple	+	+	Yellow
HSN_10	Bacilli	Purple	+	+	Yellow
BJP_01	Bacilli	Purple	+	+	Yellow
BJP_03	Bacilli	Purple	+	+	Yellow
HSN_22	Bacilli	Purple	+	+	Yellow
HSN_24	Bacilli	Purple	+	+	Yellow
HSN_33	Bacilli	Purple	+	+	Yellow
HSN_48	Bacilli	Purple	+	+	Yellow
MTCC-2422	Bacilli	Purple	+	+	Yellow

Table 2: Percentage (%) match of Western Ghats of Karnataka samples with NCBI 16S rRNA Gene libraries

Deposited Accession numbers	% match	% match with existing Accession Numbers
KU936333	(99)	KF010353
KU936334	(97)	AM396493
KU936335	(98)	JN872500
KU936336	(99)	KF956558
KU936337	(98)	AB627012
KU936338	(98)	GU056809
KU936339	(98)	JX406823
KU936340	(99)	FJ435215
KU936341	(99)	KR140184
KU936342	(99)	KJ580578
KU936343	(99)	JX406823
KU936344	(98)	KF010353
KU936345	(99)	EU257453

Table 3: Sequence confirmed each strain with NCBI accession numbers and GC content(%) with reference strain MTCC-2422

Species selected for GC content	Accession numbers	GC content
Bacillus subtilis HSN-04	KU936333	53.66%
Bacillus subtilis HSN-05	KU936334	53.93%
Bacillus subtilis HSN-06	KU936335	53.40%
Bacillus subtilis HSN-07	KU936336	54.26%
Bacillus subtilis HSN-08	KU936337	53.26%
Bacillus subtilis HSN-09	KU936338	52.60%
Bacillus subtilis HSN-10	KU936339	54.33%
Bacillus subtilis BJP-01	KU936340	53.26%
Bacillus subtilis BJP-03	KU936341	54.93%
Bacillus subtilis HSN-22	KU936342	55.33%
Bacillus subtilis HSN-24	KU936343	54.13%
Bacillus subtilis HSN-33	KU936344	53.86%
Bacillus subtilis HSN-48	KU936345	53.60%
Bacillus subtilis reference strain MTCC-2422	---------	55.2%

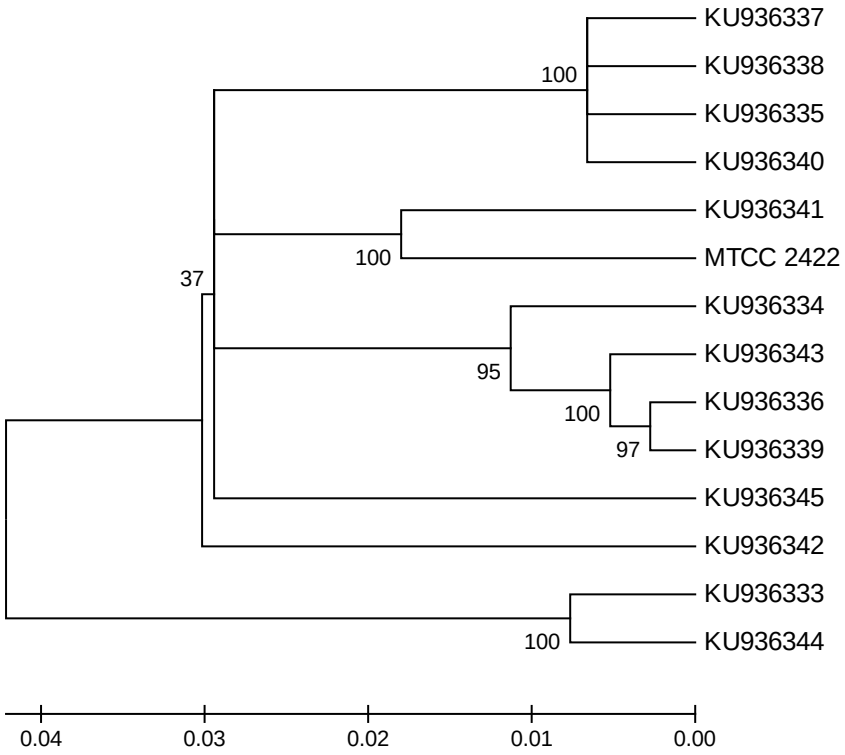

Fig. 1: Rooted Neighbour-Joining (NJ) tree showing phylogenetic relationship among different *B. subtilis* isolates with reference strain MTCC-2422. The gene sequences were responsible for geographic separation for divergence within *B. subtilis* isolates.

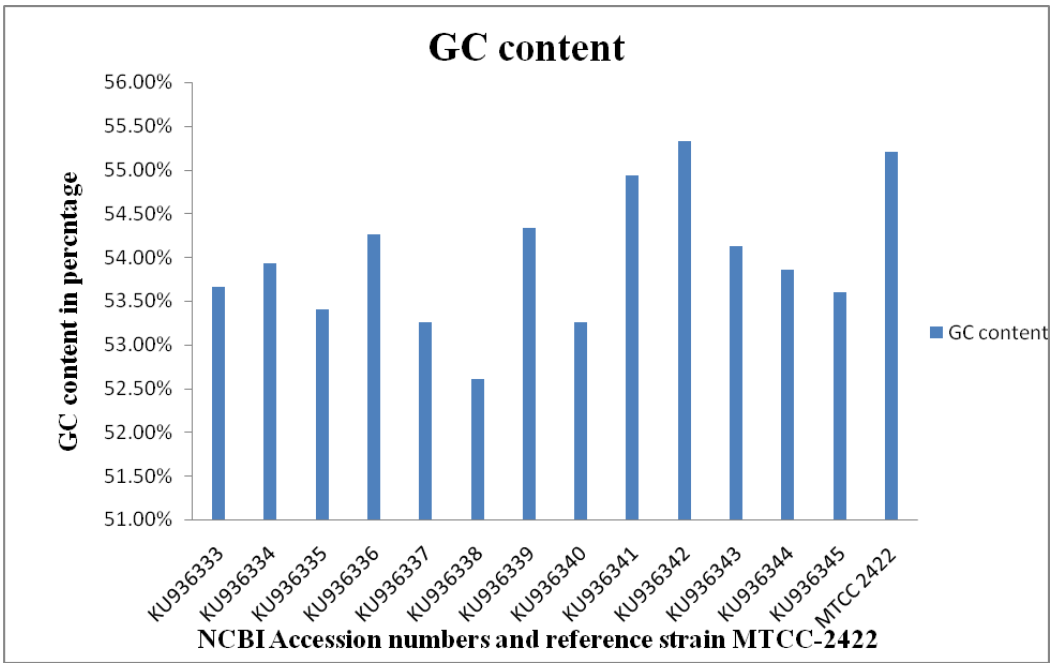

Fig. 2: Bar chart of GC (%) contents for 13 sequences deposited to the NCBI with reference strain MTCC-2422

CONCLUSION

Analysis of the 16S rRNA nucleotide sequences with reference strain MTCC-2422 using the BLAST algorithm revealed that Western ghats of Karnataka isolates have a high degree of sequence similarity with different NCBI accession numbers (Table 3). This study shows that the *B. subtilis* isolates are highest similarity between the NCBI deposited isolates i.e. 97-99%. 16S rRNA nucleotide sequence will be informative and useful in detecting the genetic diversity of populations of *B. subtilis*. It suggests that it is an efficient method for describing genetic relationships and estimating genetic diversity. To determine the molecular diversity of the environmental ecosystem the PCR mediated analysis of 16S rRNA is a very power full tool.

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REFERENCES

1. Earl Ashlee, M., Losick, Richard., and Kolter, Roberto., Ecology and genomics of *Bacillus subtilis*. *Trends in Microbiology*, **16 (6):** 269–75 (2008).
2. Francis, I., Holsters, M., and Vereecke, D., The Gram-positive side of plant–microbe interactions. *Environ Microbiol,* **1:** 1-12 **(**2010**)**.
3. H, M, Mahadeva Swamy., R, Asokan., Riaz, Mahmood., and S, N, Nagesha., Molecular Characterization and Genetic Diversity of Insecticidal Crystal Protein Genes in Native *Bacillus thuringiensis* Isolates. *Curr Microbiol,* **66(4):** 323-30 (2013).
4. Harrigan, G.G., Ridley, W.P., Miller, K.D., et al. The forage and grain of MON 87460, a drought-tolerant corn hybrid, are compositionally equivalent to that of conventional corn. *J. Agric. Food Chem* **57 (20):** 9754–63 (2009).
5. Lima - Bittencourt, C. I., Astolfi - Filho, S., Chartone - Souza, E., Santos, F. R., Nascimento, A. M., Analysis of *Chromobacterium* sp. natural isolates from different Brazilian ecosystems. *BMC Microbiol*, **7:** 1-9 (2007).
6. Moszer, I., Jones, L, M., Moreira, S., Fabry, C., and Danchin, A., SubtiList: the reference database for the *Bacillus subtilis* genome. *Nucleic Acids Res*, **30:** 62–65 (2002)
7. Ola, M, Gomaa., and Osama, A, Momtaz., 16S rRNA characterization of a Bacillus isolate and its tolerance profile after subsequent subculturing. *Arab J Biotech* **10 (1):** 107-116 (2007).
8. Pontes, DS., Lima-Bittencourt, CI., Chartone-Souza, E., and Nascimento, AMA., Molecular approaches: advantages and artifacts in assessing bacterial diversity. *J Ind Microbiol Biotechnol*, **34:** 463-473 (2007)
9. Roberts, M.S., and Cohan, F.M., Recombination and Migration Rates in Natural Populations of *Bacillus subtilis* and Bacillus mojavensis. *Evolution*, **49:** 1081-1094 (1995).
10. Sambrook, J., and Russell, D.W., Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, *Cold Spring Harbor*, (2001).
11. Schallmey, M., Singh, A., and Ward, O.P., Developments in the use of *Bacillus species* for industrial production. *Can J Microbiol*, **50:** 1-17 (2004)
12. Sharaf-Eldin, Mahmoud., Elkholy, Shereen., Fernández, José-Antonio., Junge, Helmut., Cheetham, Ronald., Guardiola, José., and Weathers, Pamela., *Bacillus subtilis* FZB24® Affects Flower Quantity and Quality of Saffron (Crocus sativus). *Planta Medica*, **74(10):** 1316–20 (2008).
13. Sonenshein, A, L., Hoch, J.A., and Losick, R., *Bacillus subtilis* and Its Closest Relatives. From Genes to Cells. Washington, DC: *American Society for Microbiology Press*. (2001)

14. Travers, R.S., Martin, P.A.W., and Reichelderfer, C.F., Selective process for efficient isolation of soil *Bacillus spp*. *Appl Environ Microbiol,* **56:** 1263–1266 (1987).
15. Yu, A., Chi-Shing., Yim Aldrin, Kay-Yuen., Mat, Wai-Kin., et al., Mutations Enabling Displacement of Tryptophan by 4-Fluorotryptophan as a Canonical Amino Acid of the Genetic Code. *Genome Biology and Evolution*, **6 (3):** 629–641 (2014)
16. YU, Gang., WANG, Xun Cheng., TIAN, Wang Hong., SHI, Ji Chun., WANG, Bin., YE, Qiang., DONG, Si Guo., ZENG, Ming., and WANG, Jun Zhi., Genomic Diversity and Evolution of *Bacillus subtilis***.** *Biomed Environ Sci*, **28(8):** 620-625 (2015).
17. Weisburg, W.G., Barns, S.M., Pelletier, DA., and Lane, DJ., 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol.* **173 (2):** 697-703 (1991)