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Research Paper

Isolation, Phylogenetic Identificationand Proteolytic Activity of *Bacillus Subtilis Jskrsb1*

Biomedical

KEYWORDS : *Bacillus subtilis* , 16S rRna, Phylogenetic analysis

Jeyasree Jayaraju — Research and Development Centre, Bharathiyar University, Coimbatore. Tamilnadu, Department of Biotechnology, Thanthai Hans Roever college, perambalur.

Dr. V. SUKUMARAN — Department of Biotechnology, Periyar Maniammai University, Thanjavur, Tamilnadu, India

ABSTRACT

The present investigation to isolate and identification of Bacillus subtilis JSKRSB1 bacteria from soil sample were collected from mangrove forest region at Muthupettai Thiruvarur District, Tamil Nadu, India. .Partial sequencing of 16SrRNA gene is a good alternative for problematic phenotypic identifications and placing isolates in their right taxonomic position. The 16S rRNA gene had 961 nucleotide base pairs and it was closely related (99%) to the type strain of Bacillus (GenBank Accession Number KC631644). The use of isolates with enzymatic activities of potential advantage and biotechnological applications is considered important now that environmentalists are concerned with the safety which might be endangered by the release of genetically engineered microorganisms (GEMs) in the environment.

INTRODUCTION

Soil contains a variety of microorganisms included bacteria that can be found in any natural ecosystem. Microorganisms play an important role on nutritional chains that are an important part of the biological balance in the life in our planet. Where, bacteria are essential for the closing of nutrient and geochemical cycles such as the carbon, nitrogen, sulfur and phosphorous cycle (Kummerer, 2004). Microorganisms can be used to determine the bioavailability of a given chemical compound in soil. Specifically, measurement of plasmid containing bacteria, using either an endogenous or exogenous approach, serves as a general indicator of environmental contaminants (Arias *et al*)The structural and functional composition of soil bacterial community inhabiting anoxic rice paddy soil was revealed accurately using the same technique of 16S rRNA and comparison with numerical abundant culturable bacteria (Hengstmann, *et al*., 1999). 16S ribosomal DNA (rDNA)-based molecular identification could achieve identification, for reasons including its universal distribution among bacteria and the presence of species-specific variable regions. This molecular approach has been extensively used for bacterial phylogeny, leading to the establishment of large publicdomain databases and its application to bacterial identification, including that of environmental and clinical uncultured microorganisms, unique or unusual isolates and collections of phenotypically identified isolates (Drancourt *et al*., 2000). In the endogenous approach, plasmids are extracted from soil bacteria isolated on agar plates followed by a visualization of the plasmids on agarose gels (Campbell *et al*., 1995).

MATERIALS AND METHODS

Sample Collection

The marine soil sample was collected from Muthupettai Mangrove forest at Thiruvarur District, Tamil Nadu.

Isolation and identification of bacteria from soil sample

The specific bacteria isolated from soil by serial dilution method by using sterile water and forming pure culture of bacteria and due to the dilution concentration of microorganisms in soil is decreased. The identification was made according to the key of Bergey's manual of determinative bacteriology

Molecular characterization of bacteria

Genomic DNA was isolated by Marmur's method (Marmur, 1961). The quality and concentration of the extracted DNA was checked by 0.8% (wt/vol) agarose gel electrophoresis and measured by UV-VIS Spectrophotometer (UV-1700 Pharma Spec, Shimadzu) at 260 and 280 nm, respectively. Approximately 10 ml of pure cultures of the log growth phase were harvested by centrifugation at 10.000 rpm. Samples required: 10 ml or more of an active culture. Yield: depending on the amount of culture and the organisms Size of recovered DNA: native and of high molecular weight. Quality: Good, checked by spectrophotometry, nanodrop, and gel electrophoresis. The 16S rRNA gene fragments were amplified by using PCR Kit (GENEI Pvt.Ltd, India).

Primer used

Forward: AGAGTTTGATCCTGGCTCAG

Reverse: ACGGCTACCTTGTTACGACTT

Gene sequencing

The purified PCR products were sequenced using ABI 3100 sequencer according to manufacturers' instructions (ABI PRISM 3100 Genetic Analyzer User's Man

Phylogenetic analysis

The sequences of 16S rRNA isolated bacteria were compared against the sequences available from GenBank using the BLASTN program and were aligned using CLUSTAL W software developed by Higgins *et al.*, 1992. Phylogenetic analysis was constructed using the Neighbour-joining method Saitou and Nei (1987). Bootstrap analysis was done based on 1000 replications (Felsenstein, 1985). All these analysis were performed by MEGA4 package (Tamura *et al.*, 2007).

Secondary structure prediction

The secondary structure of selected fungal strains was predicted by using Genebee structure prediction software available in online (www.genebee.msu.su/service/ma2-reduced.html).The restriction sites in 16S rRNA were analyzed by using NEB cutter program version 2.0 tools in online (www.neb.com.NEBCutter2/index.php).

Screening for proteolytic activity

Proteolytic activity was detected by casein hydrolysis on agar plates containing YNB (DIFCO) medium supplemented with 0.5% of casein, 0.5% of glucose, and 2% of agar (w v-1), pH 7.0 (LARSEN et al., 1998). .

RESULTS AND DISCUSSION

Isolation and Identification of *Bacillus* sp from marine soil

In the present study, isolation and identification of specific bacteria from soil. The specific bacteria (*Bacillus subtilis*) isolated from soil by serial dilution method by using sterile water and forming pure culture of bacteria and due to the dilution concentration of microorganisms in soil is decreased. According to gram staining the isolated bacteria was rod shaped, violet in colour and Gram positive in nature. The identification was made according to the key of Bergey's manual of determinative bacteriologyand its Proteolytic activity. Various methods using a range of media or pretreated soil are utilized to isolate microorganisms

Molecular characterization of bacteria

DNA isolation

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The molecular characteristic of *B.subtilis* JSKRSB1 was evaluated by PCR amplification of 16S rRNA. The amplified product were separated by agarose gel and shown in Plate-6

Nucleotide sequence accession numbers

The 16S rRNA gene sequences *B.subtilis* JSKRSB1 obtained in this study were deposited in GenBank under the accession number KC631644 . Comparative sequence analysis of the 16S rRNA (~550 bp) in the GenBank database revealed that the six bacterial isolates were most closely related each to other and to the genus *Bacillus*. Members of this genus, e.g. *Bacillus stearothermophilus* BR219 (Subramanian, 1992), *Bacillus thermoglucosidasius* A7 (Duffner *et al.,* 2000), and *Bacillus thermoleovorans* strain A2 (Duffner and Muller, 1998; Feitkenhauer *et al.,* 2001, 2003), were described to degrade phenol, cresols, nitrophenols, bromophenols. *Bacillus* strains also degrade 4-chlorobiphenyl (Saagua *et al.,* 1998) and polychlorinated biphenyls (Kim *et al.,* 2004). The DNase treatment of the PCR mixtures successfully eliminated all unwanted DNA. 16S rRNA PCR using universal primers can otherwise amplify the conserved regions of the eubacteria 16S rDNA genes giving unclear results. Our data suggest that this simple DNase treatment represents an effective step to eliminate unwanted DNA prior to the PCR amplification.

Evolutionary relationships

The evolutionary history was inferred using the Neighbor-Joining method. . The 16S rRNA gene had 961 nucleotide base pairs and it was closely related (99%) to the type strain of . (GenBank Accession Number KC631644) by Blast analysis. The topology of the NJ tree inferred from the whole dataset clearly illustrates the very strong signal of 16S rDNA of the species level in genus *Bacillus*. There were a total of positions in the final dataset. The overall tree topology suggests that the tree is divided into 2 main clades namely A, B. The clade A with three taxa showed higher bootstrap value . The clade B had totally 7 taxa including the test strain. The test strain *B.subtilis* JSKRSB1 shared with in great bootstrap value 100%.

Secondary structure prediction.

Restriction sites analysis

The restriction sites of isolated bacteria were shown . The total restriction enzyme sites of *B.subtilis* JSKRSB1 is 68. However, the cleavage sites and the nature of restriction enzymes differed from one another. The GC content of *B.subtilis* JSKRSB1 was found to be 57% respectively. Similarly, the AT content of *B.subtilis* JSKRSB1 was found to be 43% respectively using NEB Cutter Programme V 2.0 in www.neb.com/nebcutter2/index.php .

Screening for proteolytic activity

In the study, smear prepared from the suspension of isolated bacteria which is violet coloured rod shaped bacteria that are Gram positive bacteria. The isolated bacteria were gram positive rod shaped according to its appearance it is *B.subtilis.* Holt *et al.,* (1994) investigated protease enzymes are important in industry and most of the members of the *Bacillus species* have proteolytic activity. In the present investigation, proteolytic activity was detected by casein hydrolysis on agar plates containing YNB medium. Enzyme activity was indicated by the formation of a clear zone around colonies after precipitation with 1 M HCl solution

CONCLUSION

The present investigation to isolate and identification of specific bacteria from marine soil. The isolated pure culture shows the proteolytic activity. These isolated Proteolytic activity was detected by casein hydrolysis on agar plates containing yeast nitrogen base (YNB) medium. The proteolytic activity is observed by zone of inhibition by plate method. The present work concludes that the isolated bacteria show proteolytic activity. Partial sequencing of 16SrRNA gene is a good alternative for problematic phenotypic identifications and placing isolates in their right taxonomic position; The 16S rRNA gene had 961 nucleotide base pairs and it was closely related (99%) to the type strain of *Bacillus* (GenBank Accession Number KC631644).

Table: 1 Biochemical characterization of *Bacillus* sp.

S.No	Name of the test	Result
1.	Gram staining	+ve Rod
2.	Motility	Motile
3.	Indole test	-
4.	MR test	-
5.	VP test	+
6.	Catalase test	+
7.	Citrate test	-
8.	Oxidase test	+
9.	TSI test	Gas Production & Acid butt
10.	Urease test	-
11.	Carbohydrate	+

LOCUS KC631645 1006 bp DNA linear BCT 22-MAR-2013
DEFINITION Bacillus licheniformis strain JSKRSB1 16S ribosomal RNA gene,
partial sequence.
ACCESSION KC631645
VERSION KC631645
KEYWORDS .
SOURCE Bacillus subtilis
ORGANISM Bacillus subtilis
Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.
REFERENCE 1 (bases 1 to 1006)
AUTHORS Jeyasree,J., Sukumaran,V., Kalaivani,R., Ramya,V., Subha,K. and
Prabakaran,M.
TITLE Isolation and identification of Bacillus sp. from marine soil
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1006)
AUTHORS Jeyasree,J., Sukumaran,V., Kalaivani,R., Ramya,V., Subha,K. and
Prabakaran,M.
TITLE Direct Submission
JOURNAL Submitted (16-FEB-2013) Department of Biotechnology, Thanthai Hans
Roever College, Perambalur, Tamilnadu 621212, India
COMMENT ##Assembly-Data-START##
Assembly Method :: CAP3 v. 3
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
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121 tgcttgattg aaccgcatgg ttcaattata aaaggtggct tttagctacc acttacagat
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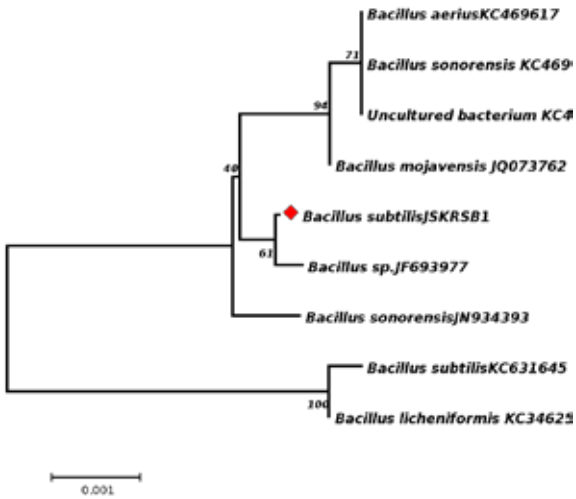

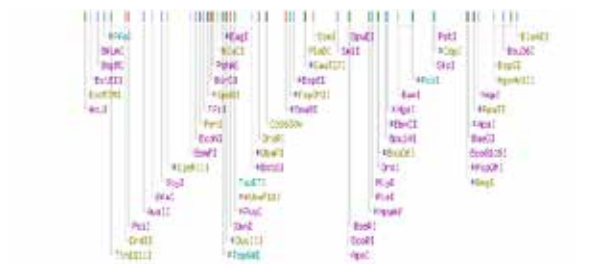

REFERENCE

Adinarayana, K. Bapi Raju, K.V.V.S.N., Ellaiah, P., 2004. Investigations on alkaline protease production with B. subtilis PE-11 immobilized in calcium alginate gel beads. Process BiocheM, 39: 1331–1339. | Campbell, J. I. A., Albrechtsen, M. and Sorensen, J., 1995. Large pseudomonas phages isolated from barley rhizosphere. FEMS Micro Ecol., 18: 63–74. | Drancourt, M., Bollet, C., Carlioz, A., Martelin, R 2000. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J. Clin. Microbiol., 38(10): 3623-3630. | Duffner, F. and Muller, R., 1998. A novel phenol hydroxylase and catechol 2, 3-dioxygenase from the thermophilic Bacillus thermoleovorans strain A2: nucleotide sequence and analysis of the genes. FEMS Microbiol. Lett., 161: 37–45. | Duffner, F., Kirchner, U., Bauer, M. and Muller, R., 2000. Phenol/cresol degradation by the thermophilic Bacillus thermoglucosidasius A7. cloning and sequence analysis of five genes involved in the pathway. Gene. 256: 215–221. | Hengstmann, U., Chin, K., Janssen, P.H. , 1999. Comparative phylogenetic assignment of environmental sequences of genes encoding 16S rRNA and numerically abundant culturable bacteria from anoxic rice paddy soil. Appl. Environ. Microbiol., 5(11): 5050-5058. | Kim, A., Pestsov, G., Yadgarov, K., Dzhumaniyazova, G., Zinov'ev, P., Dzhuraeva. G., Abdukarimov, A. and Gins, V., 2004. Microorganisms degrading polychlorinated biphenyls. Appl. Biochem. Microbiol., 40: 70-73. | Kummerer, K., 2004. Resistance in the environment. J. Antimicrob. Chemoth., 45: 311–320. | Saagua, M., Vieira, G., Paveia, H. and Anselmo, A., 1998. Isolation and preliminary characterization of Bacillus sp. MCS, a gram-positive 4- chlorobiphenyl degrading bacterium. Int. Biodeterior. Biodegradation, 42: 39-43.