GenBank New holotype for Enterobacter cloacae subsp. cloacae strain PR-4 isolated and identified by 16S rDNA gene sequence with Phylogenetic tree view, from explosive laden soil

Dr P Ravi kumar
Associate Professor
Post-Graduate and Research Department of Botany
Government Arts College (Autonomous)
Coimbatore 641018 Tamilnadu INDIA

ABSTRACT

The discovery of novel bacteria with their 16S rDNA and fungi with their 18S rDNA sequencing in explosive laden soil, ribosomal deoxyribonucleic acid sequencing, polymerase chain reaction and deoxyribonucleic acid sequencing has played a pivotal role in the accurate identification of bacterial/fungal isolates. Ten different Bacterial isolates and three different Actinomycetes belongs to the genera Acinetobacter, Bacillus, Enterobacter, Enterococcus, Staphylococcus, klebsiella, Aspergillus, Coriolopsis were isolated and identified with their 16S and 18S rDNA sequences and deposited in the The GenBank Maryland USA and MycoBank Utrecht Netherlands. All the isolates were named after the discoverer P Ravikumar, will be preserved in MTCC, India. Sanger dideoxy sequencing technology was employed and the number of base pairs, the base count of A, T, G and C was also studied. To fully utilise 16S/18S rDNA sequencing of bacteria and fungi in explosive laden soils and their bioremediation, the presence of cat, ben, xplA, xplB and other biodegrading gene/s, catabolic genes and their gene cassettes are being investigated. 16S ribosomal RNA gene of Enterobacter cloacae subsp. cloacae strain PR-4, with the base count 208 a 187 c 245 g 162 t partial sequence with Accession KP261383, Version KP261383.1 GI:758375281, bases 1-802, a novel strain present in the explosive laden soil of cracker industry was deposited in the The GenBank Maryland USA is discussed here.

Keywords: Explosive laden soil, Enterobacter cloacae subsp. cloacae strain PR-4, 16S rDNA sequence, Specific PCR, Novel strain, Discovery, The GenBank deposition.

INTRODUCTION

Explosives are materials with high nitrogen and oxygen contents which on detonation expand to create a shock wave which exerts high pressures on the surroundings, causing an explosion and leaving toxic waste in the environment. The manufacturing, testing and use of explosive have resulted in severe contamination of both soils and groundwater (Brannon et al., 2005; Eisentraeger et al., 2007) thus necessitating their safe removal from the environment. The chemical properties and quantity of explosives waste determine their toxicity and persistence in the environment. The net result has been bioaccumulation and bio magnifications of these explosives waste in aquatic and terrestrial organisms. The incredible versatility inherited in microbes has rendered these explosives as a part of the biogeochemical cycle.

Several microbes catalyse mineralization and/or nonspecific transformation of explosive waste either by aerobic or anaerobic processes. It is likely that on-going genetic adaptation, with the recruitment of silent sequences into functional catabolic routes and evolution of substrate range
by mutations in structural genes, will further enhance the catabolic potential of bacteria toward explosives and ultimately contribute to cleansing the environment of these toxic and recalcitrant chemicals (Singh et al., 2012; Makkar and Rockne, 2003; Mukherji and Vijay, 2002). Over the years, many new biological methods of bioremediation for explosive contaminated soil have been developed (Lewis et al., 2004). Numerous factors can affect the biodegradation processes and depends on the nature of molecules to be degraded (e.g., molecule size, charge, number and position of functional groups, solubility and toxicity) as well as the environmental conditions.

*Enterobacter cloacae* is a clinically significant Gram-negative, facultatively-anaerobic, rod-shaped bacterium. frequently grown at 30 °C on nutrient agar or broth. It is a rod-shaped, Gram-negative bacterium, is facultatively anaerobic, and bears peritrichous flagella. It is oxidase-negative and catalase-positive (Dalban et al., 2008)

**MATERIALS AND METHODS:**

**Study site and collection of sample:**

Valangaiman of Thiruvarur District of Tamilndu India (10°46′17.76″N 79°38′12.48″E) was selected as the sampling site for this study. Approximately 100 gm of explosive laden soil from the cracker manufacturing unit and the grinding unit were collected from five different places and immediately placed into 500 ml sterile air tight container, sealed to avoid contamination and transported to the laboratory for further processing.

**Soil processing and isolation:**

3 gm of the soil samples were vigorously mixed with 3 ml of sterile distilled water and left for overnight.100 microliter of the upper surface soil liquid was then transferred into 5 ml Nutrient broth 2 (Hi-Media M1362) with a sterile micro pipette and incubated at 36° C for 48 hrs. The plates incubated for two days were visually inspected daily until typical colonies formed. The colonies were purified by further subculture on Nutrient Agar 2 M1269 to confirm the purity and preserved at -20°C until further use. The suspected isolate was further screened for large green colonies in Nutrient Agar 2 M1269 medium.

**Molecular confirmation:**

Identification with specific PCR

The colony morphologically identified *Enterobacter cloacae* subsp. *cloacae* strain PR-4 was further identified by PCR procedures based on amplification of 16S rDNA gene. PCR was standardised with forward and reverse PCR primers and performed in a volume of 25 microliter, the reaction mixture containing 200 mM of each dNTP, 1.5μm MgCl2, 1xPCR buffer, 10 pmol of each primer, 1U of taq DNA polymerase and 10 ng DNA. The PCR cycle protocol consists of initial denaturation at 95°C for 6 min and 30 cycles of denaturation at 95°C for 1 min, primer specific annealing for 1 min and extension at 72°C for 2 min with the final extension at 72°C for 10 min. PCR products were electrophoresed on 1% agarose gel and visualised under UV.
16S rDNA ANALYSIS:

16S rDNA sequencing was used to confirm PCR identified isolate and 16S rDNA sequence of the isolate was BLAST analysed (Gee et al., 2003). The PCR reaction mixture for the amplification of the 16S rDNA gene consisted of 200mM of each dNTP, 1.5 mM MgCl2, 1x PCR buffer, 10pmol of each primer, 1 U of Taq DNA polymerase and 10ng DNA. The reaction was made up to 25 microliter with sterile distilled water and the cycle consisted of initial denaturation at 95°C for 6 min and 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min with the final extension at 72°C for 10 min. PCR products were electrophoresed on 1.0% agarose gel and visualised under UV in a gel documentation system as above. Amplified 18S rDNA PCR products were sequenced by the dideoxy chain termination method (Sanger and Coulson, 1975 & Sanger, Nicklen and Coulson, 1977) using Big dye Terminator v 3.1 sequencing kit and Big dye x Terminator Purification kit in an ABI 10 sequencer. The derived sequences were aligned using DNASTAR lasergene 9 Core Suit and BLAST analysis was with NCBI database (Zhang, 2000 and Morgulis, 2008).

RESULTS AND DISCUSSION

From the soil sample 10 different strains were isolated (Figure-1) and sub cultured successfully. One isolate was suspected and selected as possible Enterobacter cloacae subsp. cloacae strain PR-4, based on characteristic colonial morphology. Interestingly all the environmental isolates were able grow on Nutrient broth 2 M1362 and Nutrient agar 2 M1269 including Enterobacter cloacae subsp. cloacae strain PR-4, Figure-2.

FIGURE 1. SHOWING 10 DIFFERENT BACTERIAL ISOLATES IN NUTRIENT AGAR 2 MEDIUM
The one isolate confirmed by 16S rDNA sequencing was subjected to Sanger dideoxy sequencing and the FASTA of *Enterobacter cloacae* subsp. *cloacae* strain PR-4 16S ribosomal RNA gene with 802 base pair is depicted below:

The FASTA Sequence

>gi|758375281|gb|KP261833.1| Enterobacter cloacae subsp. cloacae strain PR-4 16S ribosomal RNA gene, partial sequence

GGGGAAACCCGCGGCGAGGGCGGCCCTGGACAAAAACTGACGCTCAGGTCGGAAAGCGGAGCGAAGCGTGGGGAGCA
AA
CAGGATTAGATACCCCTGGTCAGCCAGCCGTAACGATGTCCATTTGAGGTTGAGGGTGTGGCTTTGGAGGCTGG
CTTTCCGAGCTAACGCGTCGCTGGGG
AGTACCGCCGCAAGGTTAAAACTCAATAGAT
TG
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CT
GCACGCTGCTTGTGAAATGGGTGTTAAGTGGGCTCTCCGCAACGAGCGCAACCCTTTATCCTTGGCCA
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CAAGCGGACCTCATAAAGTGGTCGGGTGAGTCTGCAACTGCTCCATGAAACGCGAAGGAG
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ATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTTGAGCGGCCTACCACCTTTGTGATTGAT
CTGGGGTGATAAAAAAAGAGAGAAATA

To find regions of similarity between this sequence and other sequences using BLAST, NCBI graphical overview of pairwise alignments for gb|KP261833.1| (802 letters) was found and depicted below:

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**KP261833 Enterobacter cloaceae subsp. cloacae strain PR-4 16S rib...S= 1482 E=0**

**Color key for alignment scores**

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BLAST computes a pair wise alignment between a query and the database sequences searched.
Sequences producing significant alignments and the BLAST hit and E-value are

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Alignment

Enterobacter cloacae subsp. cloacae strain PR-4 16S ribosomal RNA gene, partial sequence
Sequence ID: gb|KP261833.1|Length: 802Number of Matches: 1

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300

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Query 361 TGTTTGGAATGTGGGTCTTGGCCTTCTATCTCTTGCTGAGC 420
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Query 721 AAGTAAGGCTACGTTACCTCGGGAGGGCGCTTACCTTGTGATTTATGCACACTGGAAGTGGGGTGA 780
Sbjct 721 AAGTAGG TAGCTTTAACCTCGGGA GGCGCTTA CCACTTTGTGATTCATGACTGGGTGA 780

Query 781 Taaaaa aaaa aGAGAGAATAAA 802

Sbjct 781 TAAAAAAA AAAAGAGAGAATAAA 802

Tax BLAST report

Results for:

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 Molecule type: nucleic acid
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 Database Name: nr
 Description Nucleotide collection: (nt)
 Program BLASTN 2.3.1

Lineage Report

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Organism Report
Enterobacter cloacae subsp. cloacae [enterobacteria]
Enterobacter cloacae subsp. cloacae strain PR-4 16S ribosomal RNA gene, partial sequence 1482 0.0 KP261833

**Taxonomy Report**

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Phylogenetic Tree was produced using BLAST pairwise alignments (BLAST computes a pairwise alignment between a query and the database sequence searched).

The Blast Tree View (BLAST RI : VVGPCRC201R, Query ID : KP261833.1, Database : nr)
The GenBank Deposition

Enterobacter cloacae subsp. cloacae strain PR-4 16S ribosomal RNA gene, partial sequence

GenBank: KP261833.1
LOCUS KP261833  802 bp  DNA  linear  BCT 25-FEB-2015
DEFINITION Enterobacter cloacae subsp. cloacae strain PR-4 16S ribosomal RNA gene, partial sequence.
ACCESSION KP261833
VERSION KP261833.1  GI:758375281
KEYWORDS .
SOURCE Enterobacter cloacae subsp. cloacae
ORGANISM Enterobacter cloacae subsp. cloacae
  Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Enterobacter; Enterobacter cloacae complex.
REFERENCE 1 (bases 1 to 802)
AUTHORS Ravikumar,P.
TITLE Direct Submission
JOURNAL Submitted (30-NOV-2014) Associate Professor of Botany, Government Arts College (Autonomous), Coimbatore, Tamilnadu 641018, India
COMMENT ##Assembly-Data-START##
  Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
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gtg
121 gttgtgccct tgaggcgtgg cttccggagc taacgcgtta aatcgaccgc ctggggagta
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gtg
241 ggtttaattc gatgcaacgc gaagaacctt acctgtcttt gacatccaca gaacttcca
Conclusion

This research piece of work is the first report of the isolation and molecular confirmation of the Enterobacter cloacae subsp. cloacae strain PR-4 explosive laden soil. This isolate was initially identified by conventional morphological methods and further confirmed by advanced molecular based methods of 16S rDNA sequencing and Enterobacter cloacae subsp. cloacae strain PR-4 specific PCR. The isolation of this important Bacterial strain from this part of India may with their xplA, xplB, other biodegrading gene/s and catabolic gene cassette sequences for the explosives have already been initiated for the further studies on the extent of environmental bioremediation.

ACKNOWLEDGEMENT:

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REFERENCES:


