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Novel Uncultured Bacteria Showing Out Group in Phylogeny Analysis Indicating Failure of NCBI 16S rRNA reference sequence database for its Genus and Species Level Identification can Inhibit *Bacillus safensis* strain MRTV10

Rajesh Dhakane[1], Aarti Deshpande[2], Prajakta Mohite[3], Bipinraj N.K[4], Kshama Murarkar[5], Arati Kamble[6], Shilpa Lokhande[7], Akanksha Dambare[8], Amruta Shinde[9], Arya Kokate[10], Snehal Deshpande[11], Ashwin Haribhakt[12], Amol Kayande[13], Krutadnya Gore[14], Yogita Kasbe[15], Neha Devkate[16], Pratiksha Kusal[17], Rutika Ramgude[18], Janhavi Khadke[19], Sujit Chavan[20], Mahendra Dombale[21], Devaki Salve[22], Vaishnavi Ingale[23], Chaitali Autade[24], Sakshi Sutar[25], Sakshi Nanwate[26], Prathmesh Chalukya[27], Vaibhavi Gate[28], Sneha Sherkar[29], Shital Randive[30], Sakshi Shelar[31], Varsha Thorat[32], Mahek Patel[33], Zara Shaikh[34], Mayuri Takale[35], Janhavi Daundkar[36], Nilam Chobe[37], Vaishnavi Ughade[38], Prerna Kalbhor[39], Hitesh Mehta[40], Pankaj Singh Onkarsingh Baishthakur[41], Ashwini Jadhav[42], Prathmesh Kunjir[43], Saee Chavan[44], Rutuja Rupnawar[45]

[1,13-39,43]Department of Microbiology, Jayawantrao Sawant Commerce and Science College, Hadapsar, Pune, Maharashtra, India.
[1,2]Department of Microbiology, Shankarlal Khandelwal Arts, Science and Commerce College, Akola, Maharashtra, India.
[3,4,10,12]Department of Biotechnology, Rajiv Gandhi Institute of Information Technology and Biotechnology, Katraj, Pune, Maharashtra, India.
[5]Department of Microbiology, Dr. R.G. Bhoyar Arts, Commerce and Science College, Seloo, Nagpur-Wardha Highway, Maharashtra, India.
[6]Department of Microbiology, Sadguru Gadge Maharaj College, Karad, Maharashtra, India.
[7]Department of Microbiology, Sant Gadge Baba Amravati University, Amravati, Maharashtra, India.
[8]Department of Microbiology, Asian College of Science and Commerce, Dhayari, Maharashtra, India.
[9,11]Department of Microbiology, S.M Joshi College, Hadapsar, Pune, Maharashtra, India.
[40]Department of Biotechnology, Smt. S.S. Patel Nootan Science and Commerce College, Sankalchand Patel University, Visnagar, India.
[41]Department of Microbiology, Late Baburao Patil Arts and Science College, Hingoli, Maharashtra, India.
[42]Department of Microbiology, Krishna Institute of Allied Sciences, Krishna Vishwa Vidyapeeth Deemed to be University, Karad, Maharashtra, India.
[44,45]Department of Biotechnology, Vidya Pratishthan's Arts, Science and Commerce College, Baramati, Maharashtra, India.

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ABSTRACT

The uncultured bacteria was isolated from the *Ocimum tenuiflorum* based rhizospheric soil sample of Karad, Maharashtra, India on Glycerol Aspargine Agar. It inhibited the growth of *Bacillus safensis* MRTV 10 and it utilized starch as a source of nutrition. The 16S rRNA gene sequence of the uncultured bacteria showed only 93.52% identity with the reference sequences of NCBI database with failure in genus and species level identification by the database indicating its novelty. The phylogeny tree of the uncultured bacteria constructed using Maximum Parsimony Method by

comparing with 20 NCBI reference sequences showed its placement as out group indicating its recent evolution from its ancestor.

Keywords: *Ocimum tenuiflorum*, Glycerol Aspargine Agar, 16S rRNA gene sequence, *Bacillus safensis* MRTV 10, Maximum Parsimony Method.

INTRODUCTION

The Earth has a magnificently rich microbial diversity, out of which more than 99% of the microbial species are still yet to be discovered and cultured[1,2]. Studies of microbial 16s rRNA gene sequences demonstrated the presence of 4×10^6 diverse microbial taxa per ton of soil and 10^9 cells per gram of soil[3,4,5,6]. Amongst all the microbiota, the uncultured bacteria remain of great interest. 'Uncultured' bacteria are metabolically active in their native environments but they are unable to proliferate in the laboratory media due to lack of appropriate knowledge on their habitats, abiotic-biotic interactions and their ecological roles in soil[7].

The inability to culture and study the uncultured bacteria causes a significant loss, as these bacteria hold untapped potential across various fields. For example, in medicine, to find treatment for the diseases caused due to uncultivable bacteria[8]. Studies of these uncultured bacteria can lead to various novel discoveries in fields of Medicine, Microbiology, Agriculture, Ecology leading to advancement in technology. *Bacillus safensis* NBRC 100820 showed toxic effect on the spotted bollworm or the spruce budworm[9] causing an imbalance of the biodiversity. In the present study, the antagonistic potential of novel uncultured bacteria against *Bacillus safensis* strain MRTV 10 was investigated with its identification success and novelty.

MATERIALS AND METHODS

Sampling

For isolation of *Bacillus safensis* strain MRTV 10, *Ocimum tenuiflorum* based rhizospheric soil sample was collected from Karad, Maharashtra, India.

Isolation of uncultured bacterium

Initially, 1 gram of the soil sample was diluted in 100 ml of distilled water, followed by serial dilutions up to 10^{-2}. The diluted sample was then spread on Glycerol Asparagine Agar plates[10] by the spread plate technique. Plates were incubated at 28°C[11] for 4–6 days[12]. Colonies exhibiting morphological characteristics consistent with uncultured bacterium were isolated and purified. Molecular identification of the purified isolates was performed at Progenome Life Science, Sambhajinagar, Maharashtra, India.

Molecular Identification

Genomic DNA was extracted from the isolated culture using the Nucleospin Microbial DNA Kit as per the manufacturer's instructions. The quality was checked by agarose gel electrophoresis (on 1% agarose gel). The gel was visualized using a UV Transilluminator). PCR amplification of the 16S ribosomal RNA gene was performed with primers 27_F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492_R(5′-TACGGYTACCTTGTTACGACTT-3′)[13]. The following conditions were maintained for the PCR: initial denaturation at 95^0C for 3 minutes with 1 cycle, priming at 95^0C for 45 seconds, extension at 52^0C for 50 seconds, termination at 72^0C for 70 seconds (35 cycles), final extension at

72^0C for 10 minutes (1 cycle) and the final storage at 4^0C. The resulting PCR product, a single distinct band, was visualized on a 1.2% agarose gel by UV transilluminator.

PCR products were processed for cleanup to remove unincorporated nucleotide and residual primers using Exonuclease-I and Shrimp Alkaline phosphatase enzyme followed by cycle sequencing reaction using BigDye® Terminator v.3.1 Cycle Sequencing Kit. The cycle sequencing was followed by sequencing cleanup by ethanol precipitation followed by dissolving template in HiDi formamide and bidirectionally sequencing in ABI 3730 Genetic analyzer. PCR products were then processed for direct bi-directionally sequencing using ABI PRISM 3730 × 1 Genetic Analyzer. The resulting DNA sequences were aligned, manually trimmed and edited using CodonCode Aligner to obtain complete sequences. Homology searches were carried out using the BLASTn program[14] against the NCBI GenBank database to identify the obtained 16S rRNA gene sequence. The obtained 16S rRNA gene sequence of *Bacillus safensis* strain MRTV 10 was submitted to the NCBI database with accession number PQ375347.

Biochemical Characterization

Starch Utilization Test

To perform the starch utilization test[15], 40 ml of Starch Agar Medium was prepared and poured on two different Petri plates. In the first plate, considered as experimental, the colony of uncultured bacteria was grown. In the second plate, considered as control, no bacteria was cultivated. The plates were incubated at 28^0C for 8 days.

Phylogney Analysis

The phylogenetic trees were constructed to understand the evolutionary relations of the uncultured bacteria with its counter parts and same for the *Bacillus safensis* MRTV 10. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model[16]. The bootstrap consensus tree inferred from 500 replicates[17] is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test 500 replicates are shown next to the branches[17]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 21 nucleotide sequences. There were a total of 830 positions in the final dataset. Evolutionary analyses were conducted in MEGA11[18].

Antibacterial activity

100 ml of Aspargine Glycerol Agar was prepared, autoclaved at 121^0C at 15 psi (pounds per square inch) and poured into the five sterile Petri plates[19]. The culture on uncultured bacterium was placed at the center of each plate except control one. The plates were placed for incubation at 28^0C for 8 days for growth of the uncultured bacterium. The plates were naturally contaminated by *Bacillus safensis* strain MRTV 10.

RESULTS

The uncultured bacteria was isolated, purified and preserved into the slant with Glycerol Aspargine Agar (figure 1). The bacteria were able to use the starch as a source of nutrition in the starch utilization test (figure 2). The comparison of 849 bp 16S rRNA gene sequence of the uncultured

bacteria with reference sequences from NCBI showed only 93.52% identity (figure 3a). In the evolutionary history analysis using Maximum Likelihood method and Tamura-Nei model[16], the uncultured bacterium (SEQ24-0210KJ1) showed an out group when compared with 20 NCBI 16S rRNA reference sequences (figure 3b). Moreover, *Bacillus safensis strain* MRTV 10 with 20 NCBI reference sequences showed clade with OP646631.1:24-717 *Bacillus safensis* strain LYYY117 16S ribosomal RNA gene partial sequence (figure 4). In addition, the uncultured bacteria showed zone of inhibition against *Bacillus safensis* strain MRTV 10 in the experimental 1 plate (figure 5).

DISCUSSION

In the study, the uncultured bacteria, not identified by NCBI 16S rRNA gene sequence database up to genus and species level, was first time cultivated on the Glycerol Aspargine Agar (figure 1). This has provided optional growth medium to the researchers for cultivation of the uncultured bacteria. In addition, the work confirmed the utilization of starch as a source of nutrition by the bacteria under study (figure 2) supporting further study of the bacteria by designing the medium composition. The obtained uncultured bacteria was not identified by the NCBI 16S rRNA gene database (figure 3a) up to genus and specie level suggesting its novelty. It explicitly indicated that the NCBI sequence database is needed to be increased with the sequence quantity. Moreover, the uncultured bacteria under study may be novel since it showed its single out group (figure 3b) when analysed through phylogenetic analysis. In addition, *Bacillus safensis strain* MRTV 10 was properly identified by NCBI reference sequence database and grouped with OP646631.1:24-717 *Bacillus safensis* strain LYYY117 16S ribosomal RNA gene partial sequence (figure 4). Interestingly, the uncultured bacteria under study has potential to inhibit the growth of *Bacillus safensis* strain MRTV 10 (figure 5). This indicated that the bacteria under study may have pharmaceutical applications in the future.

CONCLUSION

The rhizospheric soil of *Ocimum tenuiflorum* belonging to Karad, Maharashtra, India has the presence of *Bacillus safensis* MRTV 10 against which the novel uncultured bacteria has antagonistic property. The Glycerol Aspargine Agar supports the growth of novel uncultured bacteria providing the growth medium for its further study for its pharmaceutical applications by the future researchers. The NCBI 16S rRNA gene sequence database is not able to identify the 16S rRNA gene sequence of the uncultured bacteria. The uncultured bacteria under study shows its recent evolution from the ancestor.

Fig 1: Isolated uncultured bacteria preserved in the slant with Glycerol Aspargine Medium.

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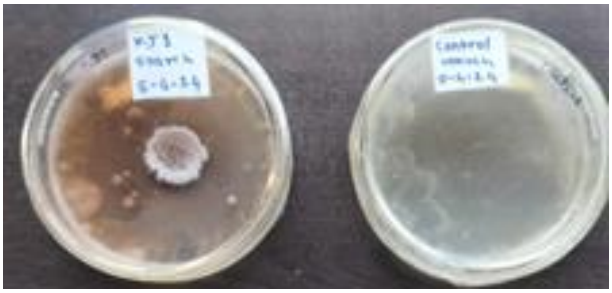

Fig 2: Starch utilization test of the uncultured bacterium showing positive test (left plate) in contrast to the control plate (right) showing negative test.

Sequences producing significant alignments Download Select columns Show 100

select all *100 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Uncultured bacterium clone B5_21 16S ribosomal RNA gene, partial sequence	uncultured bacterium	1118	1118	86%	0.0	93.52%	1071	EU790073.1
Uncultured bacterium clone nbw790d06c1 16S ribosomal RNA gene, partial sequence	uncultured bacterium	1112	1112	86%	0.0	93.39%	1359	GQ011051.1
Uncultured Ralstonia sp. clone F3Bjun.47 16S ribosomal RNA gene, partial sequence	uncultured Ralstonia sp.	1112	1112	86%	0.0	93.39%	1461	GQ417765.1
Uncultured Burkholderia sp. partial 16S rRNA gene, clone CM14C8	uncultured Burkholderia sp.	1112	1112	86%	0.0	93.39%	1358	AM936871.1
Uncultured Ralstonia sp. clone 1P-2-D24 16S ribosomal RNA gene, partial sequence	uncultured Ralstonia sp.	1112	1112	86%	0.0	93.39%	1265	EU705001.1
Uncultured Ralstonia sp. clone NpFkyB16Ral 16S ribosomal RNA gene, partial sequence	uncultured Ralstonia sp.	1112	1112	86%	0.0	93.39%	1460	JQ726778.1
Uncultured bacterium clone B31_38 16S ribosomal RNA gene, partial sequence	uncultured bacterium	1112	1112	86%	0.0	93.39%	1183	EU790462.1
Uncultured Ralstonia sp. clone 3P-4-1-O23 16S ribosomal RNA gene, partial sequence	uncultured Ralstonia sp.	1112	1112	86%	0.0	93.39%	1239	EU706260.1
Uncultured Ralstonia sp. clone 1P-1-E22 16S ribosomal RNA gene, partial sequence	uncultured Ralstonia sp.	1109	1109	86%	0.0	93.25%	1291	EU704771.1
Uncultured bacterium clone aab26h03 16S ribosomal RNA gene, partial sequence	uncultured bacterium	1109	1109	86%	0.0	93.26%	1466	DQ819188.1
Uncultured bacterium clone nbw316c09c1 16S ribosomal RNA gene, partial sequence	uncultured bacterium	1109	1109	86%	0.0	93.26%	1360	GQ088965.1
Uncultured bacterium clone nbw319d01c1 16S ribosomal RNA gene, partial sequence	uncultured bacterium	1109	1109	86%	0.0	93.25%	1359	GQ089236.1
Uncultured bacterium clone BIGO786 16S ribosomal RNA gene, partial sequence	uncultured bacterium	1109	1109	86%	0.0	93.26%	1371	HM558789.1
Uncultured Ralstonia sp. clone BF64A_B18 16S ribosomal RNA gene, partial sequence	uncultured Ralstonia sp.	1109	1109	86%	0.0	93.26%	1390	HM141115.1
Uncultured bacterium clone nbw905a07c1 16S ribosomal RNA gene, partial sequence	uncultured bacterium	1109	1109	86%	0.0	93.26%	1360	GQ027342.1
Uncultured bacterium clone nbw1119h07c1 16S ribosomal RNA gene, partial sequence	uncultured bacterium	1109	1109	86%	0.0	93.26%	1360	GQ055776.1
Uncultured bacterium clone nbw774e09c1 16S ribosomal RNA gene, partial sequence	uncultured bacterium	1109	1109	86%	0.0	93.26%	1360	GQ016542.1

Fig 3a: The comparison of 849 bp 16S rRNA gene sequence of the uncultured bacteria with reference sequences from NCBI showed only 93.52% identity.

Fig 3b: The uncultured bacterium (SEQ24-0210KJ1) showed an out group when compared with 20 NCBI 16S rRNA reference sequences.

Fig 4: The phylogenetic analysis of *Bacillus safensis strain* MRTV 10 with 20 NCBI reference sequences showed clade with OP646631.1:24-717 *Bacillus safensis* strain LYYY117 16S ribosomal RNA gene partial sequence.

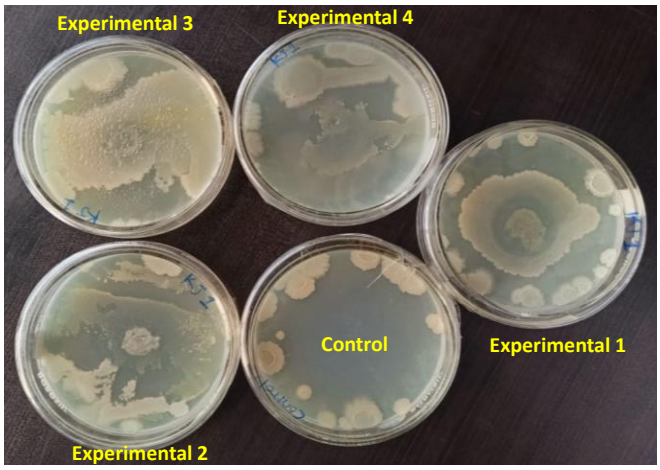

Fig 5: The uncultured bacteria showed antibacterial activity against *Bacillus safensis* strain MRTV 10 in experimental 1 plate.

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Ethical statement

No ethical guidelines were violated during the research.

Author contributions

All authors have considerable contributions for concept development as well as design, data acquisition, analysis, along with the interpretation of the data; involved in the process of drafting and revising of the article; agreed for the submission to the current journal; provided the final approval of the article to be published; and agree for the accountability of all dimensions of the work. All included authors are eligible for authorships as per the International Committee of Medical Journal Editors (ICMJE) guidelines or requirements.

Conflict of interest

Authors declare that no conflict of interest exists among them.

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