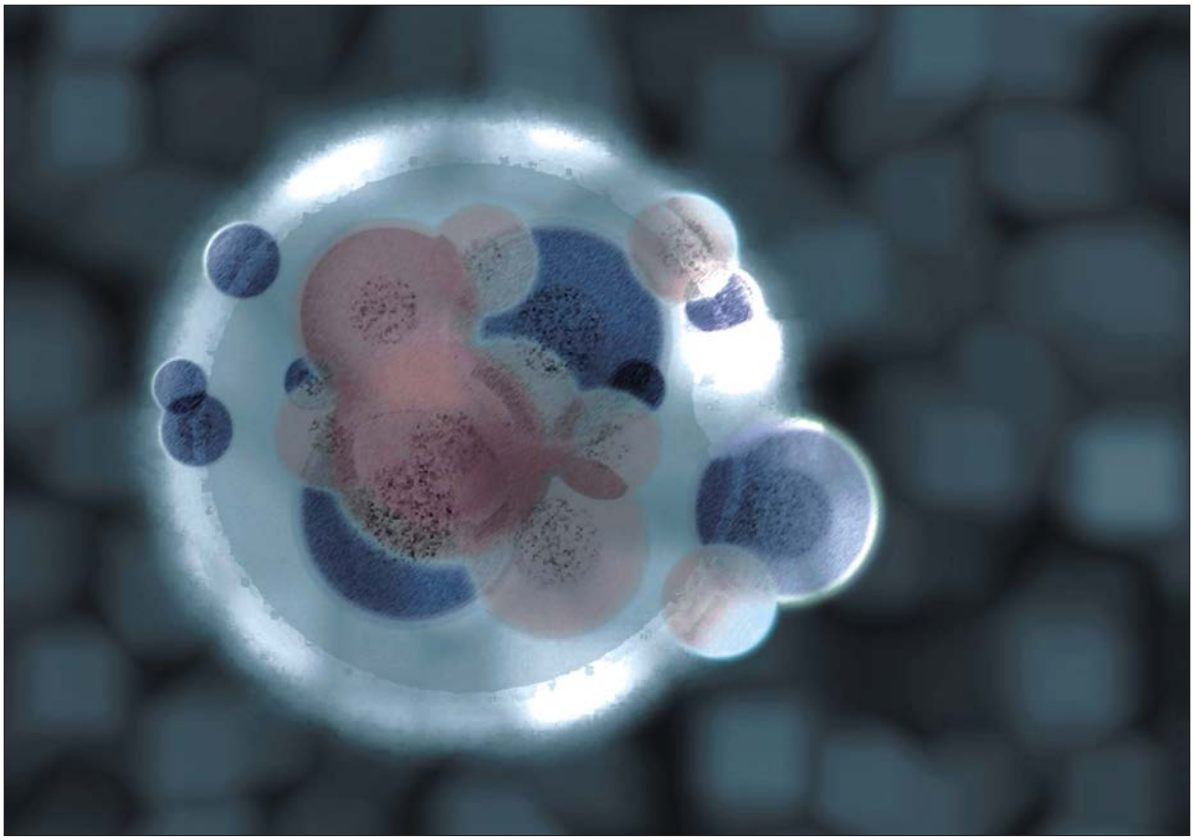


Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 10 (7): 637-642, 2010
ISSN 1727-3048

Molecular Identification by 16S rDNA Sequence of a Novel Bacterium Capable of Degrading Trichloroethylene

Srijata Mitra and Pranab Roy
Department of Biotechnology, The University of Burdwan,
Golapbag, Burdwan, West Bengal, India

Abstract: Trichloroethylene (TCE) is a widely used organic solvent and metal degreasing agent, one of the most frequently detected groundwater contaminants and a potential health hazard. A novel gram positive, rod shaped bacterial strain 2479 was isolated from soil near the oil depot site at Rajbandh, West Bengal (India) and its exact taxonomic position was investigated on the basis of 16S rRNA gene sequencing and Fatty acid methyl ester analysis (FAME). This novel strain was capable of degrading Trichloroethylene (TCE) efficiently. The major fatty acids detected in the strain 2479 were iso $C_{15:0}$ (24.49%) and iso $C_{16:0}$ (12.12%). The 16S rRNA gene of strain 2479 was amplified by using *Bacillus* specific primers and obtained 1465bp amplified product. Comparison of 16S rDNA region (1465bp) of the isolate 2479 by Ribosomal Database Project II – Sequence match showed greatest similarity with genus *Bacillus* sp. JDM-2-1(Accession No. EF584539). Phylogenetic analysis, involved the identification of homologous sequences, their multiple alignment, phylogenetic reconstruction and the graphical representation of the inferred tree was done in Phylogeny fr. package. Phylogenetic tree showed strain 2479 had 100% similarity with *Bacillus cereus* group. On the basis of phylogenetic data and Fatty Acid Methyl Ester Analysis, strain 2479 should be placed within the genus *Bacillus* and species *cereus*. This is the first instance, *Bacillus cereus* group being used in biodegradation of Trichloroethylene.

Key words: 16S rRNA gene, trichloroethylene, strain 2479, phylogenetics, *cereus*

INTRODUCTION

Trichloroethylene (TCE) is a common pollutant of ground water throughout the United States (Ranya Amer *et al*., 2008). The US Environmental Protection Agency has classified TCE as a priority pollutant on the basis of its ubiquity, suspected carcinogenicity and propensity to be anaerobically degraded to vinyl chloride in ground water (US Environmental Protection Agency, 1980). Vinyl chloride is known to be tumorigenic (Infante and Tsongas, 1982). TCE also affects soil respiration and microbial biomass (Kiyota *et al*., 2006). Aerobic consortia have been shown to degrade TCE in the presence methane, toluene and phenol (Oldenhuis *et al*., 1989). Although no microbial growth on TCE as the sole carbon source has been reported yet, we were the pioneer in reporting microorganism capable of growing on TCE as the sole carbon source (Dey and Roy, 2009).

The identification of Bacterial species has been performed with morphological and physiological criteria and this method is widely employed in various fields. However, the process used requires skillful techniques and is very complex and time consuming. With the advance of genetic engineering, the Randomly Amplified Polymorphic DNA (RAPD) method (Yamazaki *et al*., 1997), the hybridization method (Giffel *et al*., 1997; Timothy *et al*., 1994) or restriction mapping (Henderson *et al*., 1995) were adapted for the identification of species. These methods are effective for identification or detection among a small number of species, but they are not suitable for identification among a large number of species. Over the years, a sizable database of 16S rRNA gene (rDNA) has been built and this has been successfully applied in determining phylogenetic relationship or in identifying bacteria. Because, most 16S rRNA genes are approximately 1500 base pairs in length and certain regions of these genes are characteristics of broad phylogenetic group such as domains (Archea, Eucarya and bacteria), while other regions are characteristics of more narrow groups such as phyla, families and genera. 16S rDNA sequencing provides a cost effective alternative in the identification of unknown bacteria (Wolfe *et al*., 2002). A similarity of 97% in 16S rRNA gene analysis gives information about genus and 99% and above, species (Reddy *et al*., 2009).

Corresponding Author: Pranab Roy, Department of Biotechnology, The University of Burdwan, Golapbag, Burdwan, West Bengal, India Pin: 713104 Tel: +91-342-2657231, +91- 9933037099 Fax: +91-342-2530452

Using this phylogenetic frame work, 305 out of 1025, 16S rDNA sequences presently classified as *Bacillus* sp. could be identified up to a species level. Phylogenetics is the Science of estimating the evolutionary pasts based on the comparison of DNA or protein sequence. Reconstructing the evolutionary history of molecular sequences through phylogenetic analysis is at the heart of many biological research areas such as comparative genomics, functional prediction and detection of lateral gene transfer or the identification of new microorganisms. Starting from a sequence of interest, a typical phylogenetic analysis goes through successive steps that include the identification of homologous sequences, multiple alignment, phylogenetic reconstruction and graphical representation of the inferred tree (Dereeper *et al*., 2008).

We have isolated (Dey and Roy, 2009) and identified one TCE degrading bacterium from TCE contaminated soil. In this study, we report the exact taxonomic position of strain 2479, which can degrade Trichloroethylene (TCE) efficiently by using the phylogenetic analysis based on partial sequencing of 16S rRNA gene and Fatty Acid Methyl Ester Analysis (FAME). This is the first report of any *Bacillus cereus* strain carrying out degradation of Trichloroethylene.

MATERIALS AND METHODS

Bacterial strain: The strain 2479 was isolated (Dey and Roy, 2009) from the soil of industrial belt, situated at Rajbandh, West Bengal (India) where the use of polychlorinated hydrocarbons (including TCE) is abundant.

Growth of organism: The isolate was grown in rich medium (Bacto tryptone- 10 g; yeast extract -5 g; NaCl -10 g; Distilled water -1000 mL) at 31°C and pH 7.2.

DNA isolation and PCR amplification: 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. The concentration of DNA was 1.5 mg mL^{-1}. Part of the 16S rRNA gene was amplified by *Bacillus* specific primers (database bank) synthesized by Metabion International AG (Germany). Amplification reaction was performed with reagents supplied by Bangalore Genei, India, as follows: Taq DNA polymerase 2 units; Magnesium ion concentration of 1.5 mM; 10X buffer (containing 100 mM Tris-HCl, pH-9.0; 500 mM KCl; 0.1% Gelatin); 0.2 mM each of the four dNTPs and 100 ng template DNA of strain 2479, 25 pmol each of the primers. The reaction was carried out in a DNA Thermocycler for 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. Primer sequences were as follows:

- Oligo1 Forward sequence - 5'-AGAGTTTGATCC TGGCTCAG-3'
- Oligo2 Reverse sequence - 5'-TACGGCTACCTT GTTACGACTT-3'

Amplified product was then checked for its purity and size by 1.2% (wt/vol) agarose gel electrophoresis in Tris Acetate EDTA buffer (pH 8.3).

Nucleotide sequencing: Nucleotide sequencing was carried out by automated DNA sequencer from MWGAG BIOTECH. Primers used for this purpose were the same as for the PCR.

Computer analysis and construction of phylogenetic tree: Partial 16S rRNA gene sequences of genus *Bacillus* sp. (>1200 bp; isolates) were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmBH, Braunschweig, Germany), ATCC (American Type Culture Collection, Manassas, VA, USA.), RDP (Ribosomal Database Project), DDBJ-EMBL-GeneBank Database. The 16S rRNA sequence was aligned manually to 16S rRNA sequences of type strain of other *Bacillus* sp. obtained from GenBank database and the common overlapping region of forward and reverse sequence was deleted and continuous sequence stretch was constructed by using Gene Runner Software (Version 3.0). The sequence was subjected to BLAST (Basic Local Alignment Search Tool) (version 2.2.18) (Altschul *et al*., 1990) for searching the neighbours of the query sequence (1465 bp). MUSCLE (version 3.7) (Edgar, 2004) algorithm was used to process the multiple alignment of the sequences. Subsequently, the alignment was refined by GBlocks (0.91b) (Castresana, 2000) algorithm in the Phylogeny fr. package (Dereeper *et al*., 2008). The parameters in both the processes were set as follows: - Maximum number of iterations: 16; Minimum number of sequences for a conserved position: Half the number of sequences +1; Minimum number of the sequences for a flank position: 85% of the number of the sequences; Maximum number of contiguous non conserved position: 8; Minimum length of a block: 10. Gaps at the 5' and 3' ends of the alignment were omitted (http://www.phylogeny.fr). Phylogentic tree was inferred using the tree making algorithm the Phylogeny using

maximum likelihood in the PhyML (version 3.0) (Guindon and Gascuel, 2003) with aLRT program (Anisimova and Gascuel, 2006) [(sequence size) × (no. of taxa)^2 < 80000000]. This test is based on an approximation of the standard likelihood ratio. The parameters in this process were set as follows: Number of substitutions rate catergories: 4; gamma parameter: estimated; Transition / Transversion ratio: 4. The phylogenetic tree was analyzed by Tree Dyn Software (version 198.3) (Chevenet *et al.*, 2006). The input data was in Newick Format. The stability of relationship was assessed by a Bootstrap analysis (>100 replications). The entire process was done in Phylogeny.fr package by using 'Advanced' Mode.

Nucleotide sequence accession No.: The partial sequence of 16S rRNA was deposited in DDBJ- EMBL-GenBank database under the accession no. EU754844 (1465bp).

RESULTS AND DISCUSSION

PCR amplification of 16S rRNA gene sequence and FAME test: In (0.8%) agarose gel electrophoresis~1500 bp band was obtained by PCR amplification (Fig. 1). Partial sequencing of 16S rRNA gene and Fatty acid methyl ester test were performed to identify the novel isolate. BLAST analysis of the sequence data revealed that the strain 2479 shows closest similarity (99%) with *Bacillus* sp. JDM-2-1 (EF584539). The organism was submitted to Microbial Type Culture Collection (MTCC) at IMTECH, Chandigarh and was provided an accession no. MTCC-8159. Strain 2479 belongs to the genus *Bacillus* by 16S rRNA gene sequencing.

Fatty Acid Methyl Ester (FAME) analysis was done at Institute of Microbial Technology (IMTECH), Chandigarh, India. FAME test showed that the major cellular fatty acids were iso $C_{15:0}$ (24.49%) and iso $C_{16:0}$ (12.12%) (Table 1). The Fatty acid profile of Strain 2479 was similar to those of recognized genus *Bacillus* species *cereus*. Strain 2479 belongs to the genus *Bacillus* by 16S rRNA gene sequencing and species *cereus* by FAME analysis.

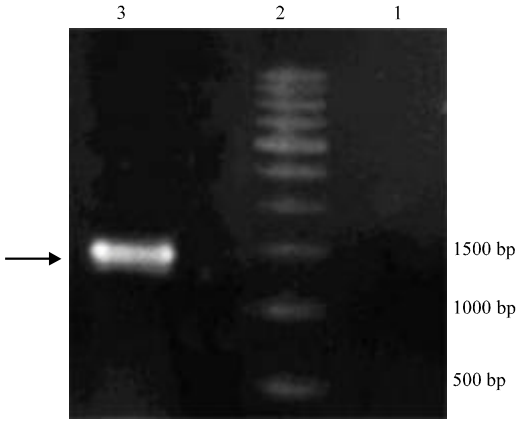


Fig. 1: 0.8% Agarose gel electrophoresis of Polymerase Chain Reaction of strain 2479 genomic DNA using *Bacillus* specific primers of 16S rRNA gene. Lane 1: Negative control without any template; Lane 2: Molecular weight marker, 500 bp ladder; Lane 3: PCR amplified product of 16S rRNA gene of strain 2479 (Size of band approximately 1500 bp)

Table 1: Percentage of cellular fatty acid composition of *Bacillus cereus* 2479

RT	Response	Ar/Ht	Rfact	ECL	Peak name	Percent	Comment1	Comment2
1.448	2.743E+8	0.019	-	7.142	Solvent peak	-	<min rt	
1.527	22146	0.020	-	7.309		-	<min rt	
1.600	4973	0.029	-	7.463		-	<min rt	
1.673	2389	0.020	-	7.619		-	<min rt	
2.246	737	0.019	-	8.833		-	<min rt	
4.356	6446	0.025	1.037	12.002	12:0	6.40	ECL deviates 0.002	Reference -0.001
5.048	4779	0.027	1.014	12.612	13:0 ISO	4.64	ECL deviates -0.002	Reference -0.005
6.326	3057	0.028	0.984	13.617	14:0 ISO	2.88	ECL deviates -0.002	Reference -0.006
6.850	5410	0.032	0.975	14.002	14:0	5.05	ECL deviates 0.002	Reference -0.002
7.805	26567	0.034	0.963	14.624	15:0 ISO	24.49	ECL deviates 0.001	Reference -0.004
7.943	4363	0.034	0.961	14.714	15:0 ANTEISO	4.01	ECL deviates 0.001	Reference -0.004
9.185	1856	0.033	0.950	15.482	Sum in feature 2	1.69	ECL deviates 0.002	16: ISO I/14:00.3OH
9.425	5588	0.035	0.945	15.626	16:0 ISO	5.07	ECL deviates -0.001	Reference -0.005
9.811	5533	0.036	0.946	15.859	Sum in feature 3	5.01	ECL deviates 0.007	15:0 ISO OH/16:1 w7c
10.048	13409	0.035	0.944	16.0001	16:0	12.12	ECL deviates 0.001	Reference -0.004
10.707	2429	0.038	0.941	16.383	ISO 17:1 w10c	2.19	ECL deviates 0.005	
10.837	5085	0.039	0.940	16.459	ISO 17:1 w5c	4.58	ECL deviates 0.002	
11.131	12709	0.037	0.939	16.629	17:0 ISO	11.43	ECL deviates -0.001	Reference -0.005
11.292	1693	0.038	0.939	16.722	17:0 ANTEISO	1.52	ECL deviates 0.001	Reference -0.005
13.523	9948	0.040	0.935	18.000	18:0	8.90	ECL deviates 0.000	Reference -0.003
-	1856	-	-	-	Summed feature 2	1.69	12:0 ALDE?	Unknown 10.928
-	-	-	-	-		-	16:1 ISO I/14:0 3OH	14:0 3OH/16: 1 ISO I
-	5533	-	-	-	Summed feature 3	5.01	16:1 w7c/15 iso 2OH	15:0 ISO 2OH/16:1w7c

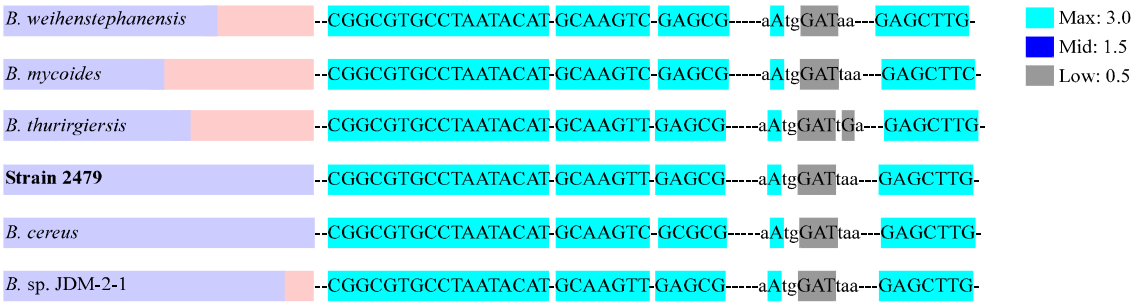


Fig. 2: In MUSCLE Alignment, strain 2479 shows most conserved sequences with other five *Bacillus* species (according to BLOSUM 62).Average BLOSUM 62 score was: Max: 3.0 Mid: 1.5 Low: 0.5

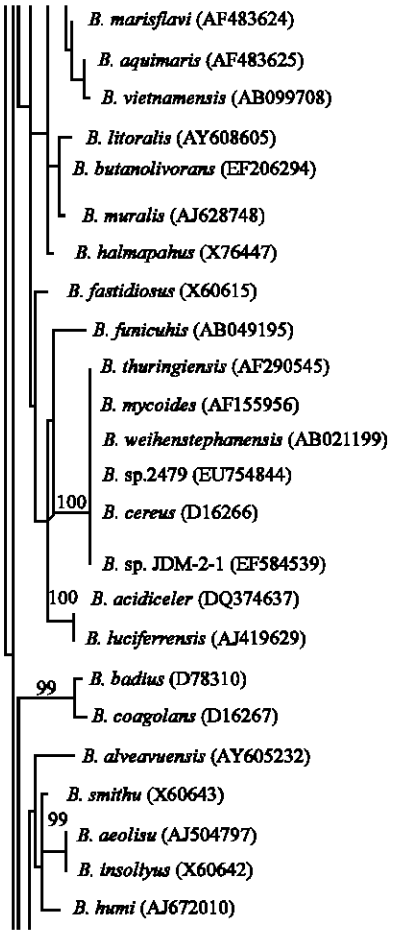
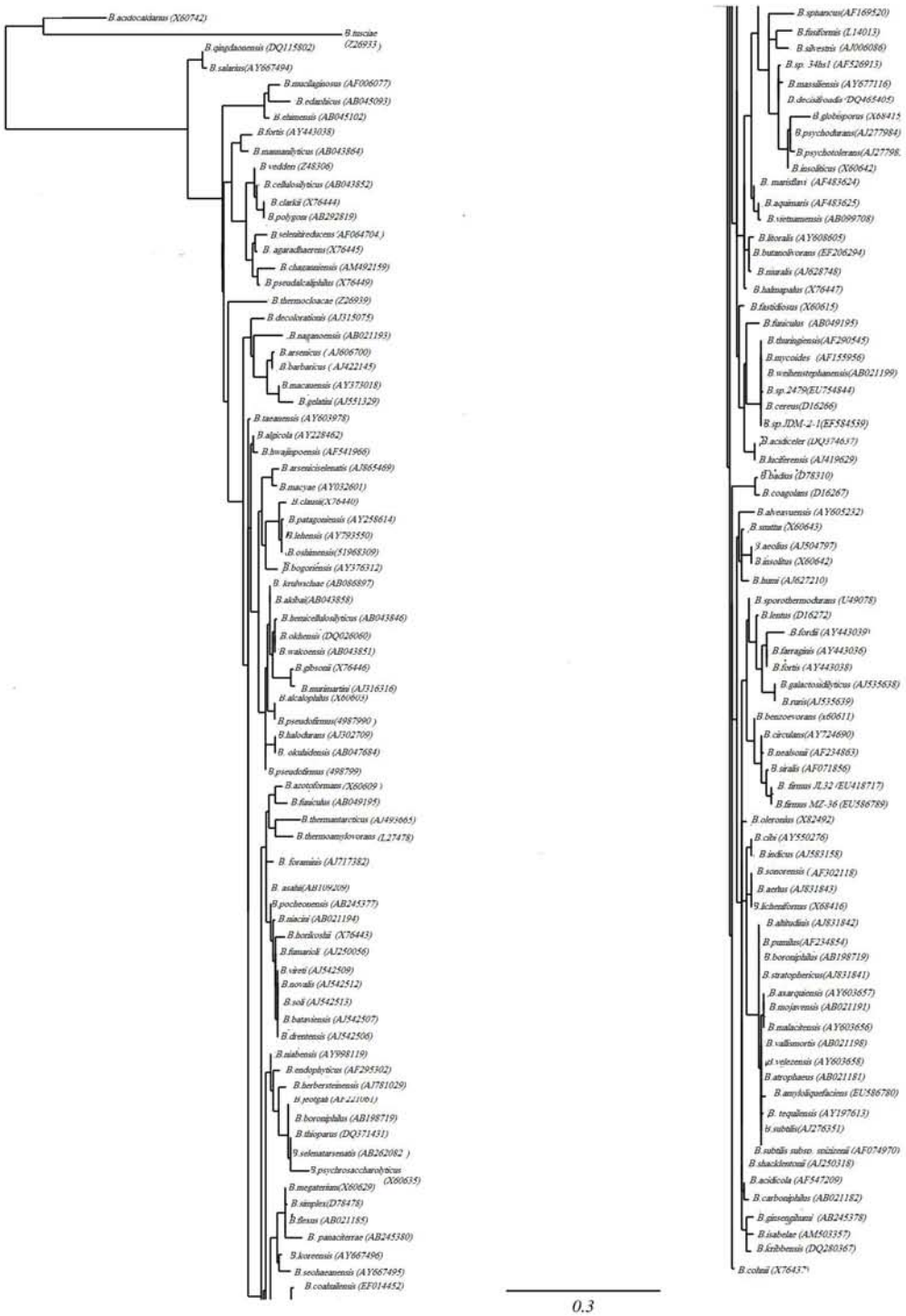


Fig. 3: Phylogenetic analysis of strain 2479 based on partial 16S rRNA gene sequence using the Maximum Likelihood (ML) method. The ML tree is constructed by Phylogeny fr. package. The accession no. is shown in parentheses. Bootstrap values (expressed as percentage of 1000 replications) >50% are shown at branch points as per 1000 replications). Bar, 0.3 substitution per nucleotide position

Phylogenetic analysis: *Bacillus* 2479 showed highest similarity with other five *Bacillus* species *(B. mycoides*, *B. cereus*, *B.* sp. *JDM-2-1*, *B. thuringiensis*, *B. weihenstephanensis*) based on the result of the MUSCLE Alignment (version 0.3) (Fig. 2) (Edgar, 2004). Strain 2479 showed 100% similarity with *Bacillus cereus* group as shown in the phylogeny tree (Fig. 3).

The complete identification of the isolated organism was done by the polyphasic approach (Vandamme *et al*., 1996). First morphological and biochemical tests with the strain 2479 indicated that it is *Bacillus* (Dey and Roy, 2009). Biolog system where different carbon source utilization pattern is examined also established the same. Fatty acid methyl ester analysis of the total lipids of the microorganism showed this to be *Bacillus cereus*. Lastly, unequivocal identification and molecular phylogeny was performed by amplification of 16S rDNA and sequencing 1465 bp using the Phylogeny.fr software (online version). In the phylogenetic analysis, we have tried to establish the relationship between strain 2479 and other related species of *Bacillus*. *B. anthracis*, *B. cereus* and *B. thuringiensis* belonging to the *B.cereus* cluster was not distinguished from each other, but they formed one group with the same sequence of the HV region (Goto *et al*., 2000). Further, it has been reported that the *Bacillus cereus* group contains five species, *B. cereus* sensu stricto, *B. thuringiensis*, *B. anthracis*, *B. mycoides* and *B. weihenstephanensis* (McIntyre *et al*., 2008). We have come to know that strain 2479 showed 100% similarity with *Bacillus cereus* group as reflected in the phylogeny tree (Fig. 3). However, the present isolate is unique and sharply different from the usual *Bacillus* strains in its biodegrading nature. This isolate that belongs to the *Bacillus cereus* was designated as a novel strain and given a name strain 2479. The complete phylogenetic tree is shown as appendix.

APPENDIX



Appendix: Phylogenetic analysis of strain 2479 based on partial 16S rRNA gene sequence using the maximum likelihood (ML) method with aLRT program. The ML tree is constructed by Phylogeny fr. package. The accession no. is shown in parentheses. Bar, 0.3 substitution per nucleotide position

ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. Ashis Kumar Mondal for generously supporting the program and Sri Kaushik Dey of Durgapur College of Commerce and Science for providing the organism 2479. Also, the authors would like to thank Sri Sushil Kumar Sinha to provide technical help in the study. Prof. Pranab Roy and Srijata Mitra occupy the chairs of Sri Mrigendra Nath Mondal endowment Head of the Dept. and Smt. Saralabala Mondal endowment Senior Research Fellow respectively in the Department of Biotechnology, The University of Burdwan to whom the authors express their indebtness.

REFERENCES

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman, 1990. Basic local alignment search tool. J. Mol. Biol., 215: 403-410.

Anisimova, M. and O. Gascuel, 2006. Approximate likelihood ratio test for branches: A fast accurate and powerful alternative. Syst. Biol., 55: 539-552.

Castresana, J., 2000. Selection of conserved blocks for multiple alignments for their use in phylogenetic alignments. Mol. Biol. Evol., 17: 540-552.

Chevenet, F., C. Brun, A.L. Banuls, B. Jacq and R. Christen, 2006. TreeDyn: towards dynamic graphics and annotations for analyses of trees. BMC Bioinformatics, 7: 439-439.

Dereeper, A., V. Guignon, G. Blanc, S. Audic and S. Buffet *et al*., 2008. Phylogeny.fr: Robust phylogenetic analysis for the non-specialist. Nucleic. Acids Res., 36: 465-469.

Dey, K. and P. Roy, 2009. Degradation of trichloroethylene by *Bacillus sp.*: Isolation strategy, strain characteristics and cell immobilization. Current Microbiol., 59: 256-260.

Edgar, R.C., 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res., 32: 1792-1797.

Giffel, M.C., R. Beumer, N. Kligin, A. Wagendrop and F.M. Rombotus, 1997. Discrimination between *Bacillus cereus* and *Bacillus thuringiensis* using specific DNA probes based on variable regions of 16S rRNA. FEMS Microbiol. Lett., 146: 47-51.

Goto, K., T. Omura, Y. Hara and Y. Sadaie, 2000. Application of the partial 16S rDNA sequence as an index for rapid identification of species in the genus *Bacillus*. J. Gen. Applied Microbiol., 46: 1-8.

Guindon, S. and O. Gascuel, 2003. A simple, fast and accurate algorithm to estimate larges phylogenies by maximum likelihood. Syst. Biol., 52: 696-704.

Henderson, I., D. Yu and P.C. Turnbull, 1995. Differentiation of *Bacillus anthrasis* and other *Bacillus cereus* group Bacteria using IS 231-derive sequences. FEMS Microbiol. Lett., 128: 113-118.

Infante, P.F. and T.A. Tsongas, 1982. Mutagenic and oncogeniceffects of chloromethanes, chloroethanes and halogenated analogues of vinyl chloride. Environ. Sci. Res., 25: 301-327.

Kiyota, H., S. Kanazawan, A. Yokoyama and H. Wada, 2006. Effects of highly volatile organo chlorine solvents on soil respiration and microbial biomass. Int. J. Soil Sci., 3: 235-242.

Marmur, J., 1961. A procedure for isolation of deoxyribonucleic acid from microorganism. J. Mol. Biol., 3: 208-218.

McIntyre, L., K. Bernard, D. Beniac, J.L. Isaac-Renton and D.C. Naseby, 2008. Identification of Bacillus cereus group species associated with food poisoning outbreaks in British Columbia. Applied Enviorn. Microbiol., 23: 7451-7453.

Oldenhuis, R., R.L.J.M. Vink, D.B. Janssen and B. Withelt, 1989. Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus Trichosporium* 0B3b expressing soluble methane monooxygenase. Applied Environ. Microbiol., 55: 2819-2826.

Ranya Amer, A., M. Mahmoud Nasier and R. Ehab El-Helow, 2008. Biodegradation of monocyclic aromatic hydrocarbons by a newly isolated *Pseudomonas* strain. Biotechnology, 7: 630-640.

Reddy, D.M., D. Paul, H.K. Reddy and G. Reddy, 2009. Characterization and identification of *Bacillus cereus* GMHS: An efficient 2-picoline degrading bacteria. Int. J. Integrative Biol., 3: 187-191.

Timothy, C.R., J. Malcolam, D.P. Suresh and C. Mitchell, 1994. Identification of capsule-forming *Bacillus anthrasis* spores with the PCR and a novel dual probe hybridization format. Appl. Environ. Microbiol., 60: 1622-1625.

US. Environmental Protection Agency, 1980. Ambient Water Quality Criteria for Trichloroethylene. Publication 440/5-80-073. National Technical Information Service, Springerfield, Va.

Vandamme, P., B. Pot, M. de Gillis, P. Vos, K. Kersters and J. Swings, 1996. Polyphasic taxonomy a consensus approach to bacterial systematics. Microbiol. Rev., 60: 407-438.

Wolfe, M.D., D.J. Altier, A. Stubna, C.V. Popescu, E. Münck and J.D. Lipscomb, 2002. Benzoate 1, 2-dioxygenase from *Pseudomonas putida*: Single turnover kinetics and regulation of a two-component rieske dioxygenase. Biochemistry, 41: 9611-9626.

Yamazaki, K., T. Okubo, N. Inoue and H. Shinano, 1997. Randomly amplified polymorphic DNA (RAPD) for rapid identification of spoilage bacterium *Alicyclobacillus acidoterrestris*. Biosci. Biotechnol. Biochem., 61: 1016-1018.