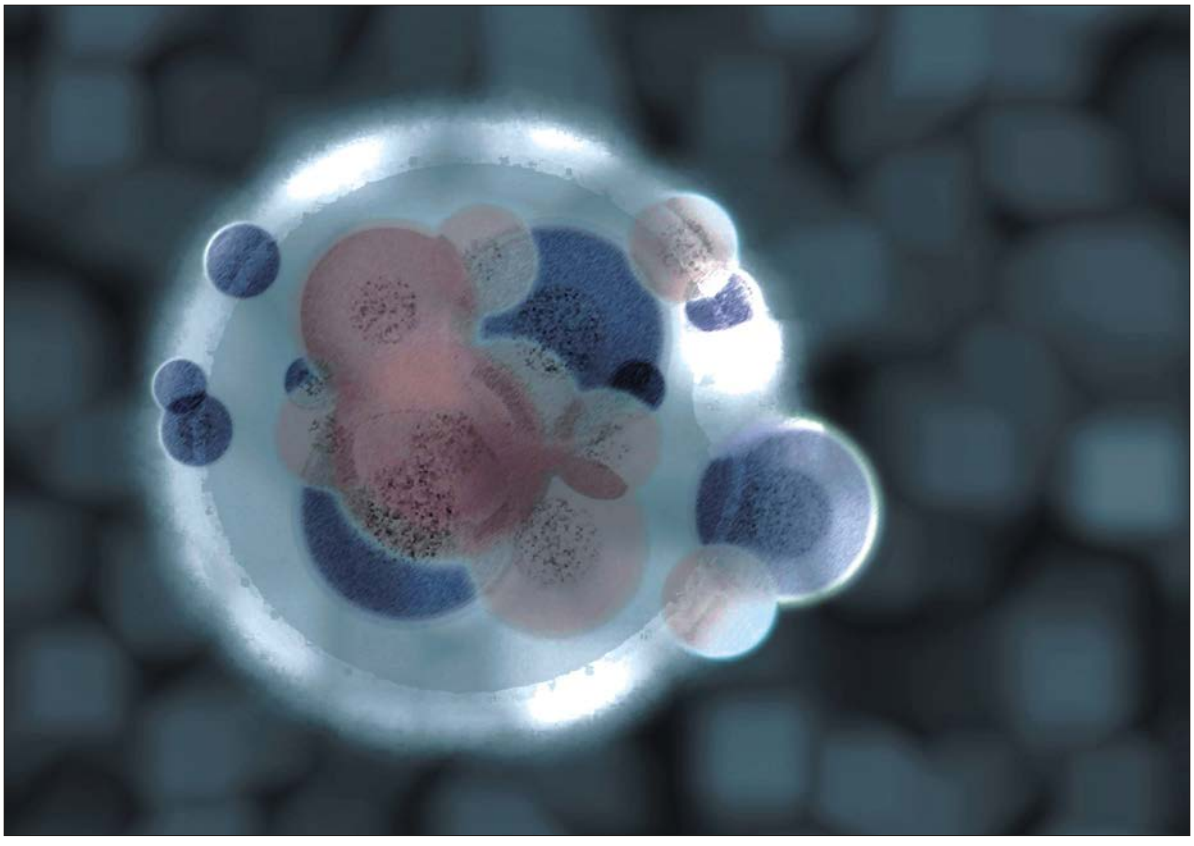

Journal of Biological Sciences

ISSN 1727-3048

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Journal of Biological Sciences 11 (1): 58-63, 2011
ISSN 1727-3048 / DOI: 10.3923/jbs.2011.58.63

Molecular Phylogeny of a Novel Trichloroethylene Degrading Gene of *Bacillus cereus* 2479

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

Abstract: Trichloroethylene (TCE) is one of the important volatile chlorinated organic compounds frequently used as solvents and metal degreasers in various industrial applications. It is a suspected carcinogen and potent mutagen in human. Bacteria that grow on hydrocarbons and able to degrade TCE typically initiate oxidation by incorporating oxygen from the atmosphere into organic compounds by the action of enzymes known as Oxygenases. As per information available on the biochemistry and genetic regulation of toluene dioxygenase in *Pseudomonas putida* Fl, making this gene (*todC1*) the best choice for bacterial TCE degradation. The phylogenetic relationships and divergence among Trichloroethylene (TCE) degrading gene sequences of different bacteria are studied. As the toluene dioxygenase C1 (*todC1*) is crucial gene for TCE degradation, *todC1* extracted from NCBI GenBank database for phylogenetic analysis. Phylogenetic analysis of a new gene *tce1* isolated from *Bacillus cereus* strain 2479 was done to investigate the evolutionary relationship among the toluene dioxygenase genes of different bacteria. 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. A similar Phylogenetic analysis using deduced amino acids sequence (*Tce1*) was also carried out to confirm the gene family of *tce1*. On the basis of Phylogenetic data, it is revealed that *tce1* gene belongs to dioxygenase family.

Key words: MUSCLE, *Tce1 gene*, toluene dioxygenase, trichloroethylene, oxygenase

INTRODUCTION

Volatile organic compounds, especially chlorinated aliphatic hydrocarbons such as 1,1,2-trichloroethylene (TCE) and tetrachloroethylene (PCE) are of major environmental concern (Kiyota *et al*., 2006). The result of improper disposal, TCE enters into the biosphere and contaminates the soil and ground water (Yeh and Kastenberg, 1991). It is a common ground water pollutant throughout the United States (Amer *et al*., 2008). TCE is a potent mutagen and carcinogen in humans. For these reasons, there is great interest in implementing processes to remove TCE from drinking-water supplies (Ensley, 1991). A number of bacterial species can degrade these compounds among those members of the genus *Pseudomonas* are prominent. TCE was found to be co-metabolized by some toluene oxidizing bacterial species (Hubert *et al*., 2005). The uses of *Pseudomonas putida* are capable of degrading TCE in presence of phenol (Ferhan, 2003).The aerobic co-metabolic biodegraders are dependent upon oxygenases, e.g., toluene dioxygenase, methane monooxygenase, toluene monooxygenase and ammonia monooxygense, phenol hydroxylase (Futamata *et al*., 2001). Oxygenases are promising biocatalysts for performing selective hydroxylations not accessible by chemical methods. The inducible enzymatic component of the aromatic degradative pathway is involved in the gratuitous degradation of TCE. The toluene dioxygenase (*todC1C2BA*) in *P. putida* F1 was identified as class IIB multicomponent dioxygenase system. The organization of the TDO (Toluene dioxygenase) system is shown in Fig.1 (Zylstra and Gibson, 1989). Electrons are transferred from NADH through a flavoprotein reductase ($Reductase_{TOL}$) (Subramanian *et al*., 1981) to a Rieske (2Fe-2S) protein ($Ferredoxin_{TOL}$) (Subramanian *et al*., 1985). The latter reduces the oxygenase component, an iron-sulfur protein (ISP_{TOL}) which in the presence of exogenous ferrous iron, catalyzes the stereo specific addition of dioxygen to the aromatic nucleus.

It can be suggested that *todC1* gene is crucial for TCE as well as trichloroethylene co-oxidation on the basis of previous studies (Zylstra and Gibson, 1991).

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

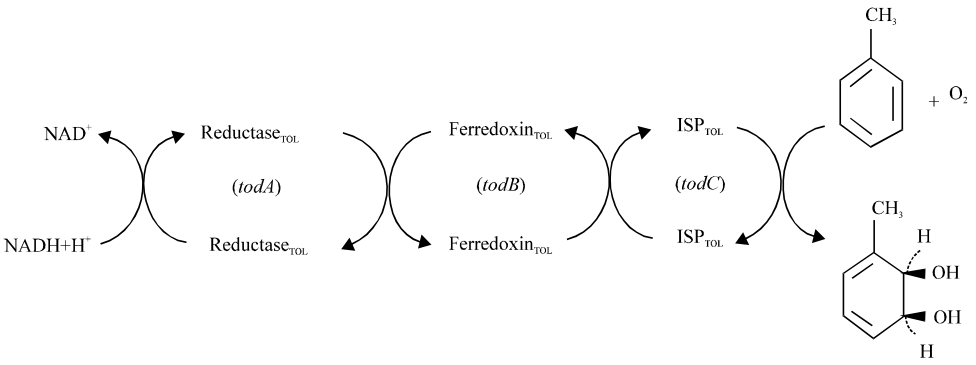

Fig. 1: Oxidation of toluene to cis-toluene dihydrodiol by toluene dioxygenase. Structural genes for each component are given the prefix tod

Previously it was reported the strain 2479 was isolated from the soil near the industrial belt situated at Rajbandh, West Bengal, India (Dey and Roy, 2009) and identified as *Bacillus cereus* 2479 using 16S rDNA (Mitra and Roy, 2010). The present study was focused on exact phylogenetic relationship of a novel gene, *tce1*, isolated from *Bacillus cereus* 2479 that can degrade trichloroethylene efficiently as a sole carbon source. Toluene dioxygenase C1 gene sequences were collected from NCBI GenBank Database. To perform the task, multiple sequence alignment was carried out on toluene dioxygenase, as sequence analysis forms crucial step in molecular evolutionary studies.

MATERIALS AND METHODS

TCE degrading genes: The TCE degrading gene was isolated from *Bacillus cereus* strain 2479, designated as *tce1*, (Mitra and Roy, 2010) by using *todC1* specific primers from *Pseudomonas pudita* F1 (Romine and Brockman, 1996). This gene and deduced amino acid sequences were deposited at NCBI/DDBJ/EMBL (Accession no. GU183105 and ACZ57347). The known toluene dioxygenase C1 (*todC1*) genes and deduced protein sequences were extracted from NCBI to construct the Phylogenetic tree using Phylogeny fr. package (Dereeper *et al*., 2008).

Molecular phylogeny: Toluene dioxygenase C1 gene sequences (>400 bp) of different bacterial species (*Pseudoxanthomonas* sp. strain BD-a59, uncultured soil bacteria, *Acinetobacter* sp. strain B113, *Klebsiella pneumoniae*, *Raoultella ornithinolytica*, *Thauera* sp. strain DNT-1, *Pseudomonas putida*, *Bacillus* sp. strain 2479) were obtained from DDBJ-EMBL-Gene Bank Database (Table 1). The *tce1* gene sequence 454 bp

Table 1: The TCE degrading genes (*todC1* genes and *tce1* gene) of different microorganisms with accession nos

Organism	Accession No.	Gene
Pseudoxanthomonas sp. BD-a59	EU734590	*todC1* type D iron-sulfur aromatic dioxygenase alpha subunit
Uncultured soil bacterium	AJ512674	*todC1*pseudogene
Uncultured soil bacterium	AJ512672	*todC1*pseudogene
Acinetobacter sp. B113	EU883930	Type D iron-sulfur multicomponent aromatic dioxygenase alpha-subunit gene
Uncultured soil bacterium	AJ512673	*todC1*
Klebsiella pneumoniae	AY918492	*todC1*
Raoultella ornithinolytica	AY918491	*todC1*
Thauera sp. DNT-1	AB066264	*todC1*
Pseudomonas putida	J04996	*todC1*
Bacillus sp. 2479	GU183105	*tce1*

(Accession No. Gu183105) of strain 2479 was aligned with known toluene dioxygenase gene sequences. MUSCLE (Edgar, 2004) algorithm was used to process the multiple alignment of the sequences. Subsequently, the alignment was refined by GBlock (Castresana, 2000) algorithm in the Phlogeny fr. Package (Dereeper *et al*., 2008).

Parameters in the the processes of MUSCLE and Gblock: The parameters in the the processes MUSCLE and GBlock were set as follows: Maximum No. of iterations: 16; Minimum No. of sequences for a conserved position: Half the number of sequences + 1; Minimum No. of the sequences for a flank position; 85% of the number of the sequences; Maximum No. of contiguous non conserverd position: 8; Minimum length of a block; 10. Gaps at the 5 and 3 ends of the alignment were omitted.

Computer analysis and construction of phylogenetic tree: Phylogentic tree was inferred using the tree making algorithm the Phylogeny using maximum likelihood in the PhyML (version 3.0) (Anisimova and Gascuel, 2006) with

aLRT program ((sequence size)×(No. of taxa) ^{2}<10 000 000). This test is based on an approximation of the standard likelihood ratio. The parameters were 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 entire process was done in phylogeny.fr package by using Advanced Mode.Partial cds of deduced protein sequences (*TodC1*) of different bacteria and deduced amino acid sequence (*Tce1*) were assembled into a multiple sequence alignment using the MUSCLE in Phylogeny fr. software and tree was constructed by the method applied earlier (Table 2).

Table 2: Protein sequences of various microorganisms

Organism	Protein	Accession No.
Thauera sp. DNT-1	*TodC1*	BAC05504
Pseudomonas putida	*TodC1*	ADI95397
Novosphingobium aromaticivorans	Large subunit aromatic oxygenase	NP-049186
Pseudomonas putida	Toluene dioxygenase large subunit	ABA10809
Uncultured soil bacterium	Toluene dioxygenase	CAD56197
Uncultured soil bacterium	Toluene dioxygenase	CAD56195
Klebsiella pneumoniae	Toluene dioxygenase	AAX14500
Raoultella ornithinolytica	Toluene dioxygenase	AAX14499
Pseudomonas putida	*TodC1*	AAA26005
Pseudoxanthomonas sp. BD-a59	Type D iron-sulfur subunit aromatic dioxygenase alpha	ACE73692
Novosphingobium aromaticivorans	Large subunit aromatic oxygenase	AAD03982
Acinetobacter sp. B113	*TodC1*type D iron-sulfur multicomponent aromatic dioxygenase alpha-subunit	ACG69433
Bacillus cereus 2479	*Tce1*	ACZ57347

RESULTS

A Phylogenetic tree was constructed based on toluene dioxygenase C1 gene sequence retrieved from Genbank to investigate the evolutionary relationship of *tce 1* gene with the known toluene dioxygenase gene of different bacteria. Phylogenetic tree showed *todC1* gene sequences were clustered into two groups: one containing *Thauera* sp. strain DNT-1 and four uncultured soil bacteria another group containing *Pseudoxanthomonas* sp. strain BD-a59, *Acinetobacter* sp. strain B113, *Klebsiella pneumoniae*, *Raoultella ornithinolytica, Pseudomonas putida.* The *tce1* gene of *Bacillus* sp. strain 2479 is closely related to *todC1* type D iron-sulfur aromatic dioxygenase alpha subunit of *Pseudoxanthomonas* sp. (Fig. 2). Protein phylogeny was also done to confirm the gene family of *tce1* gene, constructed the. Figure 3 showed that the deduced protein sequence, *Tce1* (Accession No. ACZ57347) was clustered with large subunit aromatic oxygenase (*todC1*) of *Novosphingobium aromaticivorans* (*NP-049186*) *and Novosphingobium aromaticivorans* (AAD03982). This branching is confirmed by Bootstrap values.

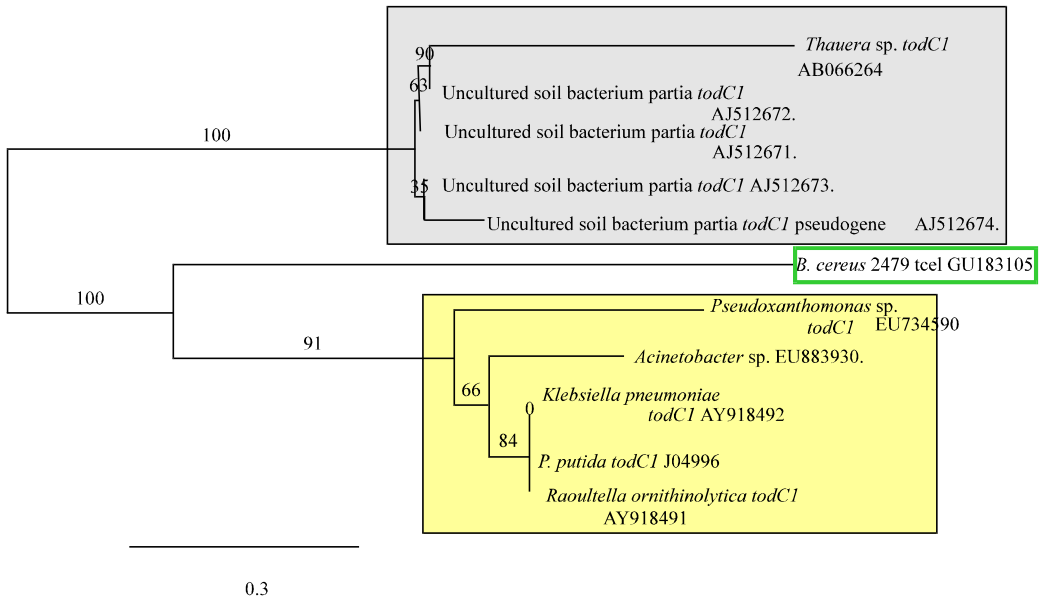

Fig. 2: Phylogenetic analysis based on *todC1* gene sequence with *tce1* gene isolated from strain 2479 using the Phylogeny fr. package. Bar, 0.3 substitution per nucleotide position. Both the clusters are shown in two different colors

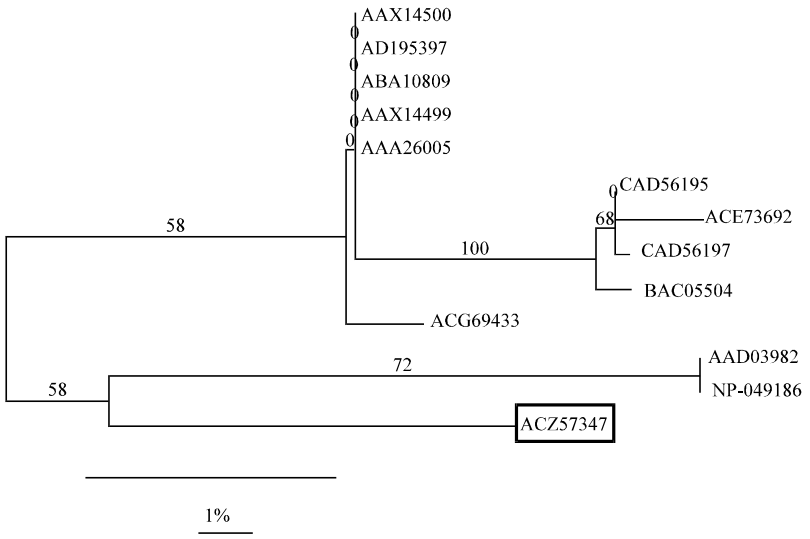

Fig. 3: Phylogenetic analysis of *Tce1* from *B. cereus* 2479 protein and *TodC1* protein sequence of other bacteria using the Maximum Likelihood (ML) method. The ML tree is constructed by Phylogeny fr. package. Accession numbers are shown on the branches. Bootstrap values (expressed as percentage of 1000 replications) >50% are shown at branch points as per 1000 replications). The Bar represent 1% sequence divergence, as determined by measuring the length of the horizontal lines any two species

DISCUSSION

A number of bacterial species can degrade the toluene and trichloroethylene, with member of the genus *Pseudomonas* prominent among them. *Pseudomonas putida* F1, *Pseudomonas* sp. strain JS150, *Pseudomonas fluorescens* CFS215 and *Pseudomonas* sp. strain W31, these stains are expressing *todC1* for TCE degradation under aerobic condition (Leathy *et al*., 1996). *Pseudomonas putida* CEMB 10124 was best degrader of phenol as well as TCE (Ferhan, 2003). *P. putida* F1 expressing *todC1* gene were detected in sea water contaminated with toluene but same did not happen in the case of *Pseudomonas putida* AC108 not expressed the todC1 (Chen *et al*., 1999). Besides, TCE degraders microorganism generally belong to the *Proteobacteria* such as *Methylococcus capsulatus* (Stainthorpe *et al*., 1990), *Methylosinus trichosporium* OB3b, *Methylocystis* sp. strain M (McDonald *et al*., 1997), *Pseudomonas putida* strain H (Herrmann *et al*., 1995), *Pseudomonas mendocina*, *Burkholderia cepacia* G4 (Shields *et al*., 1989), *Ralstonia pickettii* (objective synonym of *Pseudomonas pickettii*) (Byrne *et al*., 1995) and *Ralstonia eutropha* (objective synonym of *Alcaligenes eutrophus*) (Kim *et al*., 1996). In these orgamisms, TCE degradation is catalysed by dioxygenases or monoxygenases which are induced by specific substrates relevant to the enzymes. Previously it was reported that pulsing of methane was found to significantly improve biodegradation of TCE rates by methanotrophs (Hazen *et al*., 2009). In present study, a new gene designated as *tce1* found to degrade TCE isolated from *Bacillus cereus* 2479. Hence, the objective of this study is to find out exact family of new gene through phylogenetic analysis.

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, detection of lateral gene transfer or the identification of new micro-orgamisms. 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. The result of the phylogenetic tree analysis identified that *tce1* gene was clearly clustered with aromatic dioxygenase gene specially toluene dioxygenase (*todC1*). It is concluded that *tce1* gene belongs to toluene dioxygenase C1 (*todC1*) gene family.

CONCLUSION

From previous report, it can be established that no other *Bacillus cereus* having *todC1* gene involved in TCE degradation. This is the first instance when *Bacillus cereus* containing *todC1* gene can degrade TCE efficiently.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. Ashis Kumar Mandal for generously supporting the program. The authors are indebted to Dr. Amal Kumar Bandyopadhyay for providing his valuable suggestions in Phylogenetic analysis. Also, the authors would like to thank Sri Sushil Kumar Sinha to provide technical help in the study.

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