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Molecular Characterization of Endosulfan Tolerant Rhizospheric Microbes from Tea Gardens of Silchar Assam, India

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ABSTRACT: 16s rDNA gene analysis is one of the most reliable techniques for identification of unknown and uncultured bacterial strains as sequence of rRNA molecule is highly conserved between organisms. Following growth on selective media, identification of the endosulfan metabolizing rhizospheric microbes inhabiting roots of shade trees (*Albizia* sp.) from selected tea garden soils of South Assam, was done by performing PCR amplification of 16S rRNA gene followed by cloning in to pGEMT® easy vector. Molecular identification and phylogenetic analysis of 16S rRNA gene sequences revealed that tea garden soils harbored bacterial species belonging to *Rhizobium*, *Burkholderia* and *Enterobacter*. Identification of bacterial strains growing under heavy input of chemicals in the form of fertilizers and pesticides from tea garden soils, have spurred continued interest in exploring prokaryotic diversity and its relationship to ecosystem processes that can be exploited for developing effective bioremediation strategies so as to remediate pollutants from the environment.

Key words: 16s rRNA, *Albizia, Rhizobium, Burkholderia, Enterobacter*

Introduction

Tea (*Camellia sinensis*) belonging to family *Theaceae*, is one of the largest consumed beverages in the world. Among the processed tea types, black tea represents ~78% of total consumed tea in the world, followed by green tea accounting for ~20% of tea consumed (Siddiqui *et al.*, 2004). Tea contains several naturally occurring dietary polyphenols such as catechins possessing anticarcinogenic activity that act as effective chemopreventive agents against the initiation, promotion and progression stages of multistage carcinogenesis (Katiyar and Mukhtar, 1997; Khan and Mukhtar, 2007). To meet the needs of consumers, tea industry largely rely on use of chemicals in the form of fertilizers and pesticides for better production. Tea cultivation is one of the major cash crops of Assam (North East India). In Cachar district of south Assam, area under tea cultivation is about 32124 Ha, as cultivation of tea being the most important foreign exchange earner, acts as sole source of economy of the state. As its production intensified over past few decades, producers became dependent on agrochemicals such as endosulfan (recommended dose 2ml/lt) as a relatively reliable method of crop protection. Keeping in view growing concerns regarding continued use of agrochemicals, one of the major challenges for 21th century is ecologically sound, compatible strategies in agriculture for sustainable crop production (Botelho and Mendonca-Hagler, 2006).
Increased use of agrochemicals for better production has resulted in pollution of garden tea soil, besides rendering native microbiota resistant to these chemicals through development of series of mechanisms that play a major role in the biological transformation of chemical pesticides (Burd *et al.*, 2000; DellAmico *et al.*, 2008). Shade trees of tea gardens like *Albizia, Dalbergia,* etc being leguminous, their root nodules form an ideal niche for nitrogen fixing organisms like *Rhizobium.* Isolation and identification of soil microbes thriving in such polluted soil would be helpful in screening for tolerant bacteria that can be exploited for pesticide degradation; we designed our study for bacteria inhabiting rhizospheric soils of shade trees from five tea gardens of south Assam for identification of rhizosphere inhabiting bacteria for studying their role in endosulfan degradation.

Materials and Methods

Study site

The study was conducted at five tea estates in Cachar district (Barak Valley) of south Assam (92.51°E longitude and 24.5°N latitude). The Barak Valley located at an altitude of 22 meters above sea level, covers an area of 6922 km^2. Average annual rainfall of the place is 280 cms. The maximum temperature ranges from 25.4°C (Jan) - 32.6°C (Aug) while as minimum temperature ranges from 11°C (Jan) - 25°C (Aug). The average humidity of the place varies from 97.5% (max) to 47.5 % (min).

Collection of soil sample and isolation of bacteria

Rhizosphere soil around root nodules of shade trees (*Albizia sp.*) was collected aseptically from a depth of 0-10cm for isolation of bacteria. Soil samples were maintained at -20°C prior to analysis. The collected samples were screened for bacterial strains initially by allowing growth on nutrient agar media followed by growth on YEMA (Yeast Extract Mannitol Agar) medium containing 0.0025 % Congo red dye. After incubation at 28±1°C for 48-72 hrs, isolated colonies were picked up and streaked on fresh YEMA plates. Pure cultures were obtained by sub-culturing. Microorganisms obtained as pure cultures by streak-plate technique, were inoculated in luria broth and incubated aerobically at 28±1°C for 3-5 days on a rotator platform shaker (Scigenics) operating at 150rpm.

Growth inhibition assay

After inoculation, all the isolates (AULS-B3, AULS-R2, AULS-B3a, AULS-B3b, AULS-E4 and AULS-R5) were screened for resistance to pesticide endosulfan by subjecting them to growth inhibition study against varied concentration of endosulfan (25 µM, 50 µM, 100 µM and 150 µM). Growth was monitored by measuring optical density (OD_{595}) of the aliquots taken after every 24 hr interval by means of spectrophotometer 169 (SYSTRONICS).

Chromosomal DNA extraction and PCR amplification

High-molecular weight chromosomal DNA isolation was carried out by following the method of Felnagle et al. (2007) with a little modification (Felnagle *et al.*, 2007). The 16S rRNA gene sequence data obtained for primer designing was first analysed using advanced BLAST search program at the NCBI website: http://www.ncbi.nlm.nih.gov. PCR amplification for 16S rRNA gene from different isolates was achieved using two degenerate primers in a 50µl reaction volume in an automated thermocycler (Techne Tc-312) using 1U Taq DNA polymerase (Fermentas, USA) and the recommended buffer system with following amplification profile: Initial denaturation at 94°C for 5 min, 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 2 min followed by final extension at 72°C for 5 min. Amplified product was analysed by agarose gel electrophoresis and photographed using Gel doc system (BioRad, USA).

Cloning and Sequencing of 16S rDNA gene

PCR amplified product of expected size for 16S rDNA gene was purified using silica gel DNA extraction kit (Fermentas). Purified product for 16S rDNA gene was subjected to cloning by ligation into the pGEMT® easy vector (Promega, USA) following manufacturer's instructions followed by transformation in *E. coli* DH5α competent cells. Positive transformants were checked for the desired gene

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using same set of primers by colony PCR and sent for sequence analysis at Xcelris Labs Limited, Ahmedabad. BLAST search of GenBank was used to identify known bacterial species with 16S rRNA gene sequences similar to isolates under study.

Nucleotide sequence accession numbers

Partial 16S rRNA gene sequence of all 6 isolates has been deposited in GenBank database under accession numbers GU391260 (AULS-R2), GU569895 (AULS-B3), GU479029 (AULS-B3a), GU569896 (AULS-B3b), GU569897 (AULS-E4) and GU569898 (AULS-R5).

Results and Discussion

Screening of bacteria for tolerance to endosulfan and Growth inhibition study

Following collection of rhizospheric soil from the roots of shade trees (*Albizia sp.*), initial screening for bacterial strains was carried out on nutrient agar media followed by growth on YEMA (Yeast Extract Mannitol Agar) medium containing 0.0025 % Congo red dye. Isolates showing resistance towards endosulfan were selected for study of tolerance towards varied concentration of endosulfan (Table 1). Microbial tolerance of endosulfan was observed by allowing bacteria to grow under endosulfan stress for 5 days. Significant and pronounced effect of 150µM concentration of endosulfan was observed for all isolates except AULS-B3. Fig 1a-1f shows effect of varied concentration of endosulfan on the growth pattern of tolerant isolate of bacteria (AULS-B3) under study.

Fig 1a-1f: Effect of endosulfan on growth rate of different isolates of bacteria

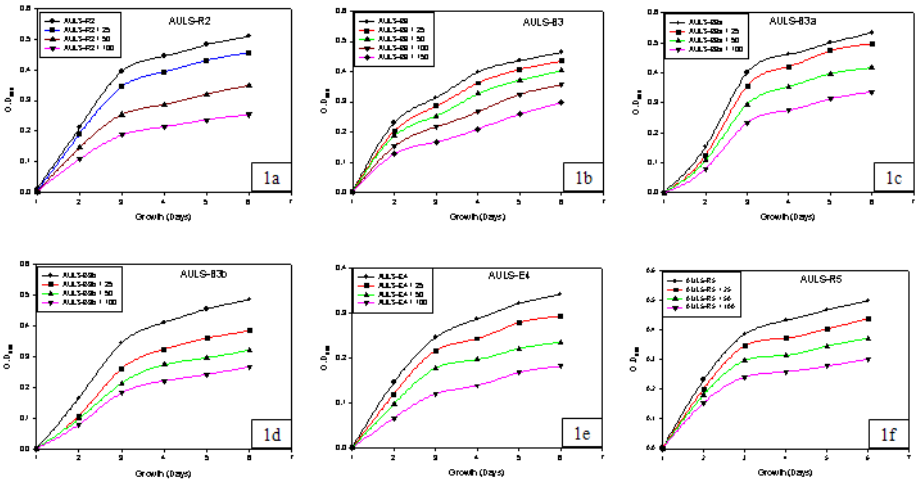


Table 1: Resistance and sensitivity of bacterial isolates at varied concentration of Endosulfan

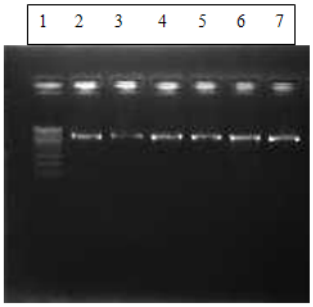
Conc	AULS-R2	AULS-B3	AULS-B3a	AULS-B3b	AULS-E4	AULS-R5
Control	++	++	++	++	++	++
25 µM	++	++	++	++	++	++
50 µM	++	++	++	++	++	++
100 µM	++	++	++	++	++	++
150 µM	--	++	--	--	--	--

++ = Good Growth
+ = less (late) growth
-- = no growth

Chromosomal DNA extraction and PCR amplification

Genomic DNA isolated from all isolates following method of Felnagle et al. (2007) with a little modification, was resuspended in 50µl of TE. The purity of DNA was checked by measuring absorbance at 260 nm ($A_{260/280}$ ratio) in a UV spectrophotometer (Specord 200, Analytik Jena). The isolated DNA concentration was higher (~40 ng/µl) in most of the samples compared with (~30 ng/µl) in some samples. Amplification of 16S rRNA gene was achieved from DNA of sufficient purity using gene specific primers PF-5′ GCAGTGGGGAATATTGGACAATGG3′; PR-5′ ATGAGGACTTGACGTCATCCCCA 3′ (GenexBio, USA). For 16S rRNA gene profiling, consensus primer set resulted in the amplification of approximately ~830 bp of 16S rRNA gene (Fig 2).

Fig 2: Gel photographs showing amplification of 16S rDNA gene



Lane 1: Marker 100 bp

Lane 2 - 7: Amplification product of 16S rDNA gene (~830 bp) from the collected samples.

Cloning and Sequencing of 16S rDNA gene

Amplified product was electrophoresed in 1.5% Agarose gel along with 100 bp ladder (Fermentas) and eluted following standard protocol by silica gel DNA extraction kit (Fermentas). Eluted PCR product was cloned in pGEMT® easy vector (Promega, Madison, WI) following manufacturer's instruction and transformed in *E. coli* DH5α. Good number of white colonies on Luria agar plate supplemented with ampicillin along with X-gal and IPTG signifies successful ligation of desired gene. Positive transformants were checked for the desired gene using same set of primers by colony PCR and sequenced. Unaligned sequence was submitted for sequence match using advanced BLAST search program of NCBI and aligned against retrieved sequences using CLUSTAL W option in the BioEdit 5.0.9 sequence analysis software (Fig 3). Sequence analysis of 16S rRNA gene reveals close relationships to a wide range of bacterial species belonging to *Rhizobium*, *Burkholderia* and *Enterobacter* (Table 2). Bacterial isolate AULS-B3 showed 99.5% similarity with *Burkholderia* sp. JZ4 (EU826644). Likewise, isolate, AULS-R2 showed 99.3% homology with *Rhizobium* sp. (GU433459), isolate AULS-B3a showed 98.8% homology with *Burkholderia* sp. (EU826644), isolate AULS-B3b showed 99.2% homology with *Burkholderia cepacia* (FJ169472), isolate AULS-E4 showed 98.7% homology with *Enterobacter hormaechei* (HM771693) and isolate AULS-R5 showed 99.4% homology with *Rhizobium* sp. (GU433459).

Fig 3: Phylogenetic affiliation of bacteria with corresponding group members

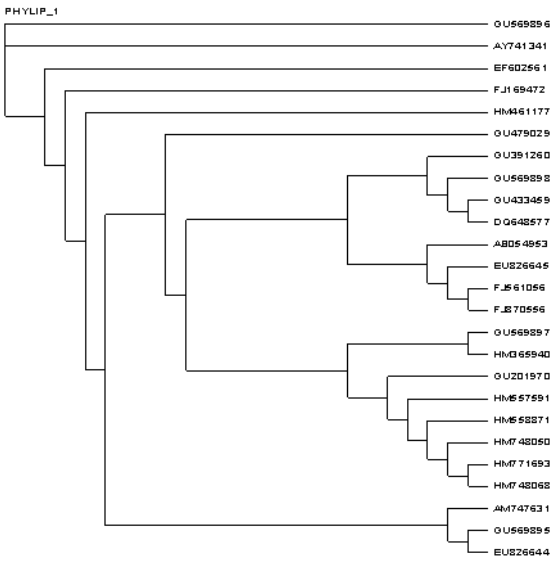


Table 2: Phylogenetic affiliation and Percentage similarity of the isolated bacteria based on 16S rRNA gene

GenBank Acc. no.	Collection site	Best match (GenBank Acc. no.)	Family	Similarity (%)	Microbial group affiliation
AULS-B3	Silchar (South Assam), India	*Burkholderia* sp. (EU826644)	β-proteobacteria	99.5	*Burkholderia* sp.
AULS-R2	Silchar (South Assam), India	*Rhizobium* sp. (GU433459)	α-proteobacteria	99.3	*Rhizobium* sp.
AULS-B3a	Silchar (South Assam), India	*Burkholderia* sp. (EU826644)	β-proteobacteria	98.8	*Burkholderia* sp.
AULS-B3b	Silchar (South Assam), India	*Burkholderia* sp. (HM461177) *Burkholderia cepacia* (FJ169472)	β-proteobacteria	99.2	*Burkholderia cepacia.*
AULS-E4	Silchar (South Assam), India	Uncultured bacteria (GU201970) *Enterobacter hormaechei* (HM771693)	γ-proteobacteria	98.7	*Enterobacter* sp.
AULS-R5	Silchar (South Assam), India	*Rhizobium* sp. (GU433459)	α-proteobacteria	99.4	*Rhizobium* sp.

Conclusion

Producing nutritious foods sufficiently and sustainably is the goal of modern agriculture. Increasing crop yields by enhancing the concentrations of agrochemicals represents a challenging problem that requires concerted efforts from researchers amid their distribution along with potential deleterious effects on human health. Sensing chemicals in the environment and responding to changes in their concentrations is a fundamental property of a living cell. Microorganisms possess simple yet effective systems that allow them to regulate numerous cellular functions in response to changes in their surroundings. Plants enhance degradation of soil contaminants by releasing exudates that nourish microbes in the rhizosphere, besides inducing biochemical pathways within bacteria. Keeping in view that neither every microbe possesses the ability to thrive in the sites where the contamination is present nor have the ability to degrade toxic compound, it would be of great advantageous to search for the one that posses the ability to survive in a given environment, besides possessing the capability to degrade the contaminant present. Diversity and exploitation of beneficial microbes is very less known in the northeastern part of India in spite of its rich biodiversity. Interest in the biodegradation mechanisms

have lead us to carry out study of microbes inhabiting rhizospheric soils for their ability to degrade pesticides mainly endosulfan that can be exploited to genetically engineer microbes for enhancement of degradative capabilities under other selective environmental pressures.

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