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Assessment of Genetic and Functional Diversity of *acdS* Gene Encoding 1-aminocyclopropane 1-Carboxylate Deaminase in Bacteria Isolated from Rhizospheric Soil of Plants Growing under Stressed Climatic Conditions

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Abstract—*Many soil bacteria contain ACC deaminase, a pyridoxal 5′ phosphate, which degrades ethylene precursor ACC to ammonia and α-ketobutyrate. However, the distribution and diversity of acdS gene in the soil bacteria from rhizosphere of plants growing under abiotic stress is poorly documented. In the present study, a set of microorganisms were isolated from rhizosphere of plants growing under saline and alkaline soils and screened for their ability to utilize ACC. A 750 bp long partial fragment of acdS gene was amplified from nine bacterial strains isolated from five different stressed soils. Phylogenetic analysis of acdS nucleotide and deduced amino acid sequences showed partial congruency to the 16S rDNA tree. In silico analysis revealed variability between Gammaproteobacteria and Alphaproteobacteria. Amino acid sequence analysis confirmed the presence of conserved amino acids.*

1. INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) include a diverse group of free-living soil bacteria that can stimulate the growth of plants by different direct or indirect mechanisms [11]. Certain plant growth-promoting bacteria contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase [EC 4.1.99.4] which is a pyridoxal 5'-phosphate (PLP)-dependent enzyme [32] that catalyses the conversion of ethylene precursor ACC to ammonia and α-ketobutyrate. This lowers the level of ethylene in developing or stressed plants [11, 12, 13, 15, 17, 20]. In PGPRs closely associated with roots, this enzyme is thought to lead to a reduction in the amount of ACC available to plant for conversion into ethylene [12]. The ACC deaminase-containing plant growth-promoting bacteria, in part, alleviate the need for the plant to actively defend itself against various environmental stresses [16, 29]. PGPR are known to use the reaction products of ACC hydrolysis as a source of nitrogen. It is an established fact that inoculation of plants with ACC deaminase-positive PGPR reduced ethylene levels in the root zone resulting in enhanced root proliferation [23]. As ACC deaminase activity in bacteria can benefit the plant, it would be of interest to improve our understanding of the range of bacteria displaying this activity. ACC deaminase activity has been documented in microbes belonging to the genera *Pseudomonas*, *Enterobacter*, *Kluyvera*, and in the symbiont *R. leguminosarum* [3, 10, 21, 23, 30]. The biochemical and thermodynamic properties of the ACC deaminase from *Pseudomonas putida* UW4 was analysed by Hontzeas et al. [16]. The presence of *acdS* geen encoding for enzyme ACC deaminase has been shown in *Rhizobium* sp., *Pseudomonas* sp., *Enterobacter* sp., *Mesorhizobium loti*, *M. ciceri* [18, 21, 28]. Putative ACC deaminase sequences derived from the annotation of whole-genome sequences are also available, but these sequences have not been validated by phylogenetic means.

In the present study, ACC deaminase gene was successfully amplified from a range of rhizospheric bacterial species isolated from different stressed environments of North West India. Phylogenetic methods were used to analyze these ACC deaminase-related sequences, with the objectives of confirming their identity and gaining insight into the evolutionary history of *acdS*. *In silico* analysis of predicted gene products was performed using the databases NCBI-BLASTX, CDD v 2.6, AmiGo, and UniProt.

2. MATERIALS AND METHODS

2.1 Soil sample collection and bacterial isolation

Bacterial strains were isolated from the rhizosphere of different crop plants growing in saline and alkaline conditions in different region of North West India (Haryana and Rajasthan) (Table 1). Rhizospheric soil samples were carefully collected by uprooting the root system (15-20 cm deep) by destructive sampling method and placed in a cool box at (4°C) for transport and storage. Soil analysis for determination of

soil texture, pH, electric conductivity, organic carbon, available N, P and K was done according to the methods of Singh et al. [26]. ACC utilizing bacteria were isolated by method of Penrose and Glick [23] using DF salt minimal medium [8] supplemented with ACC. One set of media was spread with ACC (300 µmol per plate) just prior to use and other set of media without any nitrogen source was kept as negative control. Once the ACC was completely absorbed on the plates, bacterial culture was spread. The inoculated plates were incubated at 28˚C for 3 days. Bacterial strains showing better growth on DF medium supplemented with ACC as compared to DF medium without ACC were selected for further characterization. Only morphological distinct colonies were picked up from each soil sample for further screening in order to avoid isolating multiple copies of the same bacterium.

2.2 Acds gene and 16S rDNA gene amplification

Colony PCR method was used for rapid screening of the bacterial isolates for the presence of the ACC deaminase (*acdS*) gene. Degenerate primers DegACC5 (5'-GGBGGVAAYAARMYVMGSAAGCTYGA 3') and DegACC3 (5'-TTDCCHKYRT ANACBGGRTC 3') were used (14) to amplify 750 bp long segment of *acdS* gene. The reaction conditions for PCR involved 3 min. initial denaturation; 30 cycles of denaturation (94˚C for 30 secs), annealing (55˚C for 30 secs), and extension (72˚C for 30 secs); followed by a final extension of 10 minutes at 72˚C. The isolates were identified on the basis of 16S rDNA gene sequences. The PCR was performed in a thermocycler (Applied biosystems, USA). Universal prokaryotic primers, FD1 (5′AGAGTTTGATCCTGGCTCAG3′) and RD1 (5′AAGGAGGTGATCCAGCC3′), were used to amplify a 1.5 kb segment from the 16S rDNA gene [31]. The PCR products were run on 1.2% and 0.8% agarose gel respectively and extracted using a Qiagen Min Elute Gel Extraction Kit (Qiagen, USA). The amplicon were ligated into pGEMT easy cloning vector using TA Cloning Kit (Promega) and ligated product was transformed in *E. coli* strain DH5α using electroporation. The transformants were screened on the basis of blue or white colony. The restriction analysis of randomly selected clones was performed with fast digest restriction enzymes (Fermentas, USA) according to manufacturer's instructions. DNA sequences of the PCR products were determined using Big Dye Terminatior Cycle Sequence Kit (PE Applied Biosystems, CA, USA). The products of the sequencing reaction were analyzed using a DNA sequencer (ABI PRISM^{tm} 310 Genetic Analyzer, PE Applied Biosystems).

2.3 Analysis of *acdS* gene and deduced amino acid sequences

Gene sequences of the *acdS* and 16S rDNA were subjected for BLAST analysis. BLASTN and BLASTP [1,6] were performed to detect similarity in DNA and deduced amino acid sequences respectively. Potential coding regions were obtained by ORF finder ((http://www.ncbi.nlm.nih.gov/projects/gorf/)) which gave an entire open reading frame with a potential start and stop codon for each sequence. DNA sequences and deduced amino acid sequences were aligned using the multiple sequence alignment software CLUSTAL X and CLUSTAL W respectively and compared with other four well studied reference strains (*Enterobacter cloacae*, *E. aerogenes*, *Bradyrhizobium* and *Mesorhizobium*). In order to avoid misleading phylogenetic conclusions, different reference organisms were selected to be used as outgroups from those for which *acdS* gene sequences have already been determined [27]. The aligned sequence data were subjected to unweighted pair group method with arithmetic mean (UPGMA) [23] using Kimura 2 parameter (K2P) and Poisson for *acdS* gene and its deduced amino acid sequences respectively. Phylogenetic trees were made for *acdS* and deduced amino acid sequences of the isolates with reference strains [5].

2.4 In silico analysis

Functional domains were detected from BLAST analysis by utilizing the Conserved Domain Database (CDD v 2.6) (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).The conserved patterns of amino acid from all sequences were predicted by multiple sequence alignments using BioEdit version 7.0.5 programmes (Ibis Biosciences, Carlsbad, CA) (http://www.mbio.ncsu.edu/bioedit/). The protein function analysis was done using AmiGo (http://amigo.geneontology.org/cgi-bin/amigo/blast.cgi) and Uniprot (http://www.uniprot.org/uniprot/) databases. The nucleotide sequences of *acdS* gene obtained during this study were submitted to GenBank under the accession numbers presented in Table 2.

3. RESULTS AND DISCUSSION

Soil samples were collected from five different regions of north west India exposed to harsh climatic conditions. On the basis of texture of the soil samples, three samples (soil 2,3 and 5) were found to be loam whereas rest two were sandy loam (soil 4) and sandy clay loam (soil 1). Soil 1 and soil 3 were saline. Soil 2 was saline as well as alkaline whereas soil 4 and 5 was found to be alkaline (Table 1). After enrichment and selection, fifty five isolates were selected from a total of ninety four isolates which were showing bigger colonies on DF medium supplemented with ACC as compared to the growth on DF medium without ACC**.**

3.1 PCR amplification and sequencing of *acdS* gene

After screening of the isolates by plate assay method, PCR based screening for the presence of *acdS* gene was done. Out of fifty five isolates, amplification of *acdS* gene was obtained only in nine (17%) of the isolates (Fig. 2). There are reports that ACC utilizing bacteria may not be authentically identified

based on phenotypic characterization by traditional methods used for screening germplasm collections [30]. Degenerate oligonucleotide primers based on conserved amino acid sequences of *acdS* gene were used to amplify 750 bp long fragments. The BLAST search showed high sequence similarity with *Enterobacter cloacae*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Bradyrhizobium* and *Mesorhizobium*. The *acdS* gene, encoding enzyme ACC deaminase, has been isolated from different species and strains of genera belonging to *Alphaproteobacteria*, *Betaproteobacteria, Gammaproteobacteria*, *Firmicutes* and *Actinobacteria* [2,4,7,14, 18, 25].

3.2 Phylogeny of *acdS* gene and deduced amino acid sequences

The phylogenetic tree derived from UPGMA analysis of *acdS* sequences and deduced amino acid sequences gave the same overall topology. Two main groups that were robust and contained significant number of sequences (Fig. 2, 3) were arbitrarily defined for clusters. Out of nine sequences, six sequences of isolates KTM, KTZ3, KTO, KTZ1, KT18A and KT15S were clustered in group I of *Gammaproteobacteria* whereas group II contained the remaining three KT7113, KTMG24 and KTM8 of *Alphaproteobacteria*.

Only minor topological differences were observed when the phylogenetic trees of *acdS* nucleotide sequences and deduced amino acid sequences were compared. Phylogenies were highly congruent when considering the *Proteobacteria*, regardless of the method used and it appeared that the *acdS* based group I and group II were also relevant with deduced amino acid sequences. The same classification was obtained with nucleotide and amino acid sequences, but phylogenetic distances for the amino acid tree were shorter possibly due to the masking of synonymous nucleotide substitution after translation.

The two gram positive *Bacillus* strains KTZ1 and KTMG24 clustered in group I and group II of *Proteobacteria*, rather than in a separate group. Therefore, the phylogenetic relationship derived from *acdS* analysis was only partly congruent with the one based on 16S rDNA gene sequence analysis (Table 2). Our results are in consonance with observations made by Honteaz et al. [14] that ACC deaminase genes have not evolved in the same manner as 16S rDNA genes, but have evolved through horizontal transfer.

3.3 In silico analysis of deduced amino acid sequence

The functional domains were identified and found to belong to the Pyridoxal Phosphate (PLP) functional domain, which represent the tryptophan synthase beta superfamily (TRPSβ, fold type II). Amigo and uniprot databases exploration showed that ACC deaminase (EC 4.1.99.4) is a pyridoxal 5'-phosphate (PLP)- dependent enzyme [32] that catalyses the conversion of ACC to α-ketobutyrate and ammonia. The conserved patterns of the ACC deaminase amino acid sequences, predicted by multiple sequence alignments illustrate that most of the important amino acid residues (valine, aspartic acid, alanine, glycine, proline, tyrosine, serine, leucine, phenylalanine, glycine, methoinine, glutamic acid and arginine) which are were conserved during evolution as they share one or more functional domains (Fig. 4). The two groups– *Alphaproteobacteria* and *Gammaproteobacteria* exhibited differences at certain positions. Interestingly, there were deletions in *Gammaproteobacteria* group of four amino acids namely arginine, glycine, isoleucine and serine. On the other hand one deletion of alanine was observed in *Alphaproteobacteria* as compared to *Gammaproteobacteria* group. Only isolate KTZ1 and KT15S contained cysteine. Lysine and tryptophan were present in all the bacteria belonging to *Alphaproteobacteria*, whereas they did not code for threonine. The *Gamaproteobacteria* on the other hand, contained a high frequency of threonine which was absent in *Alphaproteobacteria* except isolate KTMG24. In addition, true ACC deaminases are expected to have a leucine residue in close proximity to the glutamic acid residue which was found true in our study.

The present study revealed the diversity of ACC utilizing bacteria in the rhizospheric soil of plants growing under abiotic stress (salinity, drought etc). A comparison of 16S rDNA phylogeny of the isolates and their *acdS* gene sequence (both nucleotide and amino acid sequence) suggests that ACC deaminase gene did not evolve exclusively vertically but instead some of these genes have undergone horizontal gene transfer. Based on comparative *acdS* gene sequence analysis of the isolates, it was found that *acdS* gene showed differences in amino acid constitution in *Alphaproteobacteria* and *Gammaproteobacteria*. Our study provided novel information in this regard. The DNA approaches used in the current work were useful in proper identification of *acdS* gene in soil bacteria and improving our understanding on distribution of *acdS* gene in bacterial kingdom. A systematic study involving a larger collection of bacterial strains can go a long way in improving and understanding of the distribution and functional aspects of ACC deaminase gene and enzyme in different subdivision of bacterial kingdom as a prelude to developing *acdS* producing microbes as bioinoculants for mitigating the effect of abiotic stress effects on crop plants.

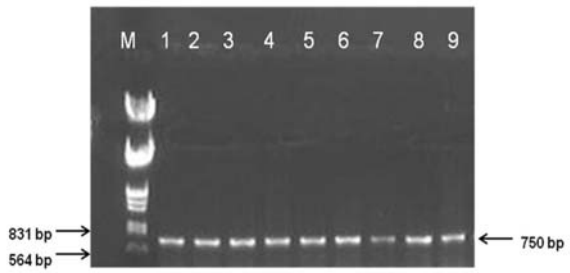

Fig. 1: PCR amplification of *acdS* gene in isolated bacterial strains; Lane M- λ/ EcoRI+ HindIII digest; Lane 1-9- 750 bp amplicon of *acdS* gene

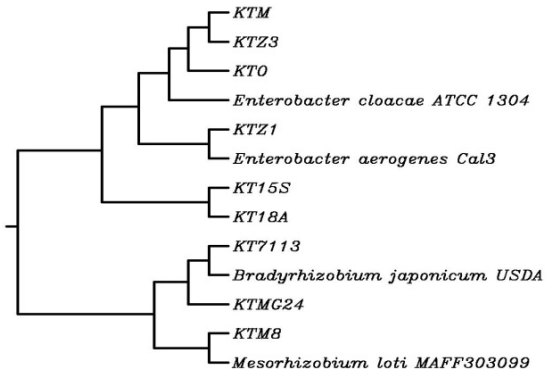

Fig. 2: Dendrogram generating using UPGMA algorithm with the aligned partial *acdS* gene nucleotide sequence of the isolates with reference strains.

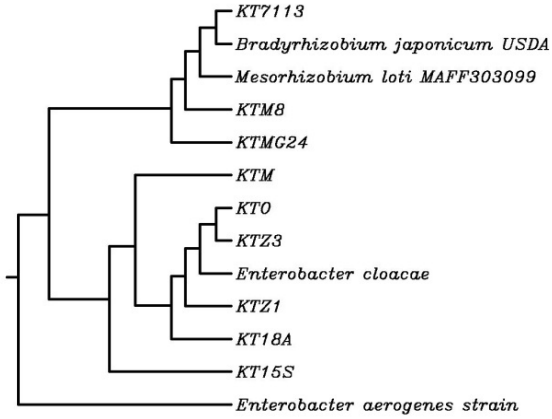

Fig. 3: Dendrogram built by use of the UPGMA algorithm with the aligned partial *acdS* gene amino acid sequence of the isolates with reference strains.

Table 1: Characteristics of different soils

Soil	Site	Geographical location	Texture	pH (soil reaction 1:2)	E.C (dS/m) *	Organic carbon (%)	Available P (kg ha^{-1})	Available K (kg ha^{-1})	Available N (kg ha^{-1})
1	Bhagotipur	28° 59' 3" N, 76° 30' 58" E	Sandy clay loam	7.89	3.63	0.39	22.5	139	313.5
2	Samargopalpur	28° 57' 5" N, 76° 31' 40" E	Loam	8.87	2.23	0.81	21.2	323	334.5
3	Singhpura	29° 27' 3" N, 76° 54' 35" E	Loam	8.18	2.83	0.42	18.3	116	355.4
4	Tataulai	28° 58' 23" N, 76° 33' 38" E	Sandy loam	8.70	0.70	0.30	21.2	150	376.3
5	Mahaveer ji	26°27'19"N 85°58'32" E	Loam	8.36	0.89	0.55	24.6	134	418.7

* Measure of total soluble salts

Table 2: Identification of ACC deaminase utilizing bacteria by 16S rDNA sequencing

S. No	Isolate	Accession number	Homology match on the basis 16S rDNA sequence	soil
1	KT15S	JF304555	Klebsiella sp.	Soil 1
2	KT7113	JF304554	Mesorhizobium ciceri	Soil 1
3	KTZ1	JF304552	Bacillus licheniformis	Soil 2
4	KT18A	JF304549	Klebsiella sp.	Soil 2
5	KTO	JF304551	Enterobacter cloacae	Soil 3
6	KTMG24	JF304547	Bacillus sp.	Soil 3
7	KTM	JF304550	Klebsiella oxytoca	Soil 4
8	KTZ3	JF304553	Pantoaea stewarti	Soil 4
9	KTM8	JF304548	Mesorhizobiumm ciceri	Soil 5

Fig. 4: Primary structure analysis of *acdS* gene homolog indicating conserved functional domain

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