Ann Microbiol (2015) 65:1679–1687
DOI 10.1007/s13213-014-1007-8

ORIGINAL ARTICLE

Effect of mustard rhizobacteria on wheat growth promotion under cadmium stress: characterization of *acd*S gene coding ACC deaminase

Venkadasamy Govindasamy · Murugesan Senthilkumar · Kannepalli Annapurna

Received: 18 April 2014 / Accepted: 21 November 2014 / Published online: 10 December 2014
© Springer-Verlag Berlin Heidelberg and the University of Milan 2014

Abstract Ten mustard rhizobacterial isolates that utilize 1-aminocyclopropane-1-carboxylate (ACC) as sole nitrogen source were screened for plant growth-promoting traits. These isolates enhanced root elongation significantly and minimized ethylene synthesis in wheat seedlings under induced cadmium stress condition. The presence of *acdS* gene coding for ACC deaminase was tested through gene-specific PCR amplification and further confirmed by nucleic acid hybridization. *acdS* gene from isolates Ps 2-3 and Ps 7-12 selected on the basis of wheat growth promotion was cloned and sequenced. Sequence analysis showed an open reading frame of 1017 bp encoding a complete polypeptide with an identity of 86.4 % between each other and sharing 84–95 % similarity with the earlier reported *acdS* genes. The deduced protein sequences were highly conserved and shared a 95.2 % homology at the amino acid level. Eight and thirteen new amino acid residues were found in the protein sequence of Ps 2-3 and Ps 7-12 with variation in numbers of different peptidase acting sites and protein motifs. Based on 16S rDNA sequencing, the isolates Ps 2-3 and Ps 7-12 were identified as *Pseudomonas* sp. and *Pseudomonas fluorescens*, respectively.

Keywords PGPR · Rhizobacteria · ACC deaminase · Abiotic stress · Mustard · Wheat

V. Govindasamy · M. Senthilkumar · K. Annapurna
Division of Microbiology, Indian Agricultural Research Institute, New Delhi 110012, India

V. Govindasamy (✉)
National Institute of Abiotic Stress Management, Indian Council of Agricultural Research, Malegaon, Baramati (Pune) 413115, Maharashtra, India
e-mail: vgs@niam.res.in

Introduction

The growth of plants in the natural environment is influenced by large numbers of biotic and abiotic stresses. Synthesis of high levels of ethylene under different stress conditions triggers onset of plant defense mechanisms followed by exaggerate symptoms leading to senescence in crop plants (Abeles et al. 1992; Pierik et al. 2006). Although a burst of ethylene is necessary to break seed dormancy in many plants, sustained higher levels of ethylene synthesis effectively inhibit root elongation and consequent plant growth. Interestingly, certain plant growth-promoting rhizobacteria (PGPR) are known for their ability to lower the ethylene levels through enzymatic control exerted by 1-aminocyclopropane-1-carboxylate (ACC) deaminase (EC 4.1.99.4). This enzyme hydrolyses ACC, the immediate precursor of ethylene into α-ketobutyrate and ammonia (Honma and Shimomura 1978; Sheehy et al. 1991), thereby reduces the endogenous ethylene level to enhance root elongation in plants (Hall et al. 1996; Glick et al. 1998). In addition, plants treated with ACC deaminase-containing PGPR are highly resistant to the deleterious effects of stress ethylene synthesized as a consequence of various biotic and abiotic stresses (Wang et al. 2000; Grichko and Glick 2001; Mayak et al. 2004; Saravanakumar and Samiyappan 2007).

The ACC deaminase has been isolated from few *Pseudomonas* species, yeast *Hansenula saturnus* and the fungus *Penicillium citrinum* (Sheehy et al. 1991; Jia et al. 1999; Yao et al. 2000). According to Jia et al. (1999), ACC deaminase of *Penicillium citrinum* was quite distinct from all other known ACC deaminases at the biochemical level. Several partial *acdS* gene fragments have been cloned from different bacterial species gave supportive evidence to the existence of different ACC deaminase genes in bacteria isolated from various ecosystems (Belimov et al. 2001; Hontzeas et al. 2005; Blaha et al. 2006; Govindasamy et al. 2008a).

There have been reports on soybean–bacterial endophytes-promoted plant growth through the reduction of biotic stress caused by fungal and bacterial pathogens (Senthilkumar et al. 2009; Mageshwaran et al. 2011). Similarly, rhizobacteria from different crops for their plant growth-promoting traits including the detection of ACC deaminase activity have also been reported (Govindasamy et al. 2008a, b; 2009). Here, we report the ACC deaminase-containing rhizobacteria isolated from Indian mustard, which affects wheat growth by lowering stress ethylene synthesis in the presence of cadmium (Cd)-induced stress and also the initial characterization of their *acdS* genes encoding the enzyme.

Materials and methods

Bacterial isolates, media and culture conditions

A total of 128 bacterial isolates purified from rhizosphere soils supporting eight varieties of Indian mustard. *Pseudomonas fluorescens* 913, having ACC deaminase activity, was a kind gift of Prof. B N Johri, GB Pant University of Agriculture and Technology, Pantnagar, India. The isolates were grown at 28± 2 °C in tryptic soy broth (TSB) or Luria Bertani (LB) medium. DF minimal medium was used for screening bacterial isolates for ACC deaminase activity (Penrose and Glick 2003). *Escherichia coli* DH5α and its transformants carrying plasmids were grown at 37 °C in LB medium with appropriate antibiotics.

Screening for ACC deaminase activity

The bacterial isolates were screened for potential utilization of ACC as described previously by Govindasamy et al. (2009). The ACC positive isolates were further screened for their ability to produce indole-3-acetic acid (IAA) (Bric et al. 1991), siderophore (Schwyn and Neilands 1987) and HCN (Bakker and Schippers 1987) and to solubilize mineral phosphate (Pikovskya 1948). The quantitative estimation of IAA was performed by the spectrophotometric method using Salkowski reagent (Patten and Glick 2002). For the ACC deaminase enzyme assay, the bacterial isolates were initially induced for ACC deaminase activity by growing them in DF minimal medium supplemented with 3 mmol/L ACC (Penrose and Glick 2003). The enzyme activity of the bacterial cell extracts was further determined by measuring the amount of α-ketobutyrate production (Honma and Shimomura 1978).

Root elongation and growth promotion of wheat seedlings

The effect of bacterial isolates on root elongation and plant growth of wheat cv. HD 2687 seedlings on soft agar under gnotobiotic conditions was assessed. Surface-sterilized seeds were imbibed in bacterial suspension (A600 nm=1.0) along with 0.1 % (w/v) Carboxy Methyl Cellulose (CMC) for 4 h and air-dried. The bacterized seeds were placed on soft agar plates (six seeds per plate) and initially incubated at 20 °C in the dark for 12 h, followed by intermittent light in a growth chamber. Root length, shoot length and fresh weight of the seedlings were recorded on 8 days incubation. The dry weight of seedlings was also recorded by keeping them in hot air oven at 60 °C for 7 days for complete desiccation.

Ethylene production assay in wheat seedlings under induced Cd stress conditions

Wheat cv. HD 2687 seeds were treated with bacterial suspension (A600 nm=1.0) and six seeds were placed in each 25-mL Erlenmeyer flask containing a piece of filter paper with the addition of 2 mL of sterile deionized water. Each treatment was replicated five times. subsequent to incubation for 8 days in a growth chamber at 20 °C with a 12-h light/dark cycle of photoperiod, the excess liquid was drained and 2 mL of 150-ppm (w/v) of cadmium chloride ($CdCl_2$) solution was added to induce stress. The flasks were maintained under airtight condition using subaseal. After 36 h of incubation, the gas samples in the headspace were analyzed by gas chromatography (Shimadzu, Tokyo, Japan) equipped with a hydrogen flame ionization detector and an AT-1 column.

Isolation and hybridization of ACC deaminase genes

Genomic DNA of the bacterial isolates was extracted described by Masterson et al. (1985). PCR was performed in a MJ research thermal cycler PTC-200 using the degenerate primers 5'- ATG AAY CTG MAN CGN TTY SMN CGB TAY CC -3' and 5'-TYA GCC RTY NCG RAA NAD RAA RCT RTA NSC -3' to amplify complete ACC deaminase structural gene (*acdS*). Reaction mixture of 100 µL volume was prepared by mixing genomic DNA with 10× polymerase buffer, 1.5 mmol/L $MgCl_2$, 0.5 µmol/L of each primer, 200 µmol/L dNTPs and 1 U of Taq DNA polymerase followed by 30 cycles of amplification.

DNA dot blot and Southern hybridization were performed using the techniques described by Sambrook and Russell (2001). An ~800-bp length of *acdS* from *P. fluorescens* strain 913 was amplified (Blaha et al. 2006), radio-labeled with α-^{32}P dCTP (BARC, India) and used as a hybridization probe (Govindasamy et al. 2009).

Cloning, sequencing and phylogenetic analysis

PCR product of two isolates Ps 2-3 and Ps 7-12 was gel-purified, ligated and transformed into *E. coli* DH5α following standard protocols (Sambrook and Russell 2001). Sequencing was carried out using modified *Tag*-FS enzyme with

fluorescently labeled dideoxy-terminator chemistry. Sequences were subjected to BLAST analysis (Altschul et al. 1997) and their deduced protein sequences were analyzed using software *GeneRunner* v.3.05 (Hastings software) and *BioEdit* version 7.0 (Ibis Biosciences, Carlsbad, CA, USA).

Phylogenetic analysis of *acdS* and deduced AcdS sequences were conducted using software *Treecon* v.1.3b (Yves van de Peer, University of Antwerp). Distances between sequence pairs, the deduced phylogenetic tree, and bootstrap values were all computed using the global gap removal option. Phylogenetic tree for *acdS* sequences was generated by the neighbor-joining method (NJ), using both the Kimura 2 Parameter (K2P) and Jukes-Cantor (JC; with gamma parameter), and maximum parsimony method (MP). For deduced AcdS sequences, a tree was generated by the NJ method using both the Poisson corrections and observed divergence and by the MP method. Nodal robustness of the trees was assessed using 1000 bootstrap replicates.

Identification of bacterial isolates by 16S rDNA sequencing

PCR amplification of 16S rDNA from isolates Ps 2-3 and Ps 7-12 was carried out with universal primers PA and PH as per Blaha et al. (2006). The cloning and sequencing of 16S rDNA was similar to that explained for ACC deaminase gene. The sequences of 16S rDNA obtained were subjected to BLAST analysis.

Statistical analysis and nucleotide sequences accession numbers

The data on root elongation, plant growth measurements and ethylene assay were analyzed by analysis of variance (ANOVA) and the treatment means were compared by Duncan's multiple-range test (DMRT). All hypotheses were tested at the 95 % confidence interval (α=0.05). The full length sequences of 16S rDNA and *acdS* gene of these two isolates were submitted to GenBank of NCBI with accession numbers EU520398, EU520401, EU854429 and EU854430, respectively.

Results

Screening rhizobacteria for ACC deaminase and other PGP traits

Ten of 128 bacterial isolates from mustard rhizosphere grew well in DF minimal medium containing ACC as the sole N source. Rhizobacteria strain A48 exhibited the lowest ACC deaminase activity (2.76 μmol/h α-ketobutyrate/mg protein) while isolates Ps 2-3 and Ps 7-12 showed high level of enzyme activity, viz., 5.15 and 4.98 μmol/h α-ketobutyrate/mg protein, respectively (Table 1).

Rhizobacterial isolates positive for ACC deaminase activity were also positive for IAA production (concentrations ranged from 8.32 to 46.80 μg/mL, the maximum being produced by isolate Ps 2-3 and Ps 7–12) and P-solubilization. Six of the 10 isolates (Ps 2-3, Ps 2-12, Ps 3-7, Ps 4-17, Ps 7-9, Ps 7-12) produced siderophore and seven isolates were found positive in the plate assay for HCN production by change in the color of picric acid-treated filter paper into brownish red (Table 1).

Effect of acdS$^+$ isolates on root elongation, plant biomass and ethylene production

The wheat seedlings treated with rhizobacterial isolates Ps 2-3, Ps 7-12 and Fb600 had an increased root length of 20.70, 19.48 and 19.42 cm, respectively, which was on a par with Pf 913 (Table 2; Fig. 1). Shoot length was insignificantly different among treatments. However, bacterization with Ps 2-3, Ps 7-9, Ps 7-12 and Fb600 resulted in significantly higher shoot

Table 1 ACC deaminase enzyme assay, IAA production assay and other PGP traits of the selected mustard rhizobacterial isolates

Bacterial isolate	ACC deaminase activity (μmol/h α-ketobutyrate/mg protein)*	Indole-3-acetic acid (IAA) production (μg/mL)*	Selected PGPR activities†		
			MPS	HCN	Siderophore
Pf 913	4.98 ± 0.11^{ab}	47.34 ± 1.52^{a}	+	−	+
Ps 1-12	3.99 ± 0.20^{bc}	13.12 ± 1.34^{de}	+	+	−
Ps 2-3	5.15 ± 0.13^{a}	46.80 ± 2.11^{a}	+	+	+
Ps 2-12	4.87 ± 0.15^{abc}	24.53 ± 2.54^{c}	+	+	+
Ps 3-7	4.39 ± 0.06^{abc}	09.53 ± 1.62^{ef}	+	+	+
Ps 4-17	3.96 ± 0.12^{c}	12.48 ± 2.45^{de}	+	+	+
Ps 5-1	3.95 ± 0.20^{c}	08.32 ± 2.23^{ef}	+	+	−
Ps 7-9	4.59 ± 0.14^{abc}	18.74 ± 1.12^{d}	+	+	+
Ps 7-12	4.97 ± 0.11^{ab}	38.51 ± 2.01^{b}	+	−	+
A48	2.76 ± 0.21^{d}	15.12 ± 1.51^{de}	+	−	−
Fb600	4.85 ± 0.19^{abc}	09.19 ± 2.25^{ef}	+	−	−

*Values are the means of three-replication±standard error. Values with the same superscripts within column indicate no significant difference with $P \geq 0.05$

†+Positive for that trait; − negative for that trait

Table 2 Effect of ACC deaminase containing mustard rhizobacterial isolates on plant growth and in vitro plant ethylene production in wheat (cv. HD 2687) seedlings under gnotobiotic conditions

S.No.	Treatment	Root length (cm)*	Shoot length (cm)*	Fresh weight (g per plant)*	Dry weight (g per plant)*	Ethylene production (pmol/g fresh weight)†
1	Control	14.12±0.21^{d}	07.00±0.15^{e}	1.06±0.03^{f}	0.10±0.01^{d}	1.43±0.15^{d}
2	Pf 913 (+ve)	19.51±0.18^{ab}	10.29±0.06^{ab}	1.79±0.13^{a}	0.18±0.02^{a}	0.41±0.01^{a}
3	DH5α (−ve)	14.28±0.37^{d}	07.04±0.22^{e}	1.08±0.04^{f}	0.11±0.01^{cd}	1.35±0.14^{d}
4	Ps 1-12	17.05±0.23^{c}	08.62±0.12^{d}	1.38±0.13^{cdef}	0.16±0.02^{ab}	0.90±0.10^{bc}
5	Ps 2-3	20.70±0.09^{a}	10.64±0.03^{a}	1.80±0.13^{a}	0.19±0.02^{a}	0.39±0.01^{a}
6	Ps 2-12	17.69±0.24^{c}	09.25±0.14^{bcd}	1.31±0.09^{ef}	0.16±0.01^{ab}	0.60±0.02^{abc}
7	Ps 3-7	18.30±0.10^{bc}	09.88±0.04^{abc}	1.48±0.15^{abcde}	0.17±0.01^{ab}	0.58±0.07^{ab}
8	Ps 4-17	18.21±0.11^{bc}	09.81±0.05^{abc}	1.33±0.09^{ef}	0.13±0.01^{bcd}	0.68±0.03^{abc}
9	Ps 5-1	17.15±0.18^{c}	08.87±0.16^{cd}	1.35±0.16^{def}	0.14±0.02^{abc}	0.93±0.12^{c}
10	Ps 7-9	18.23±0.14^{bc}	10.05±0.03^{ab}	1.60±0.14^{abcde}	0.17±0.02^{a}	0.42±0.11^{a}
11	Ps 7-12	19.47±0.17^{ab}	10.27±0.12^{ab}	1.78±0.12^{abc}	0.18±0.01^{a}	0.40±0.02^{a}
12	A48	17.09±0.12^{c}	08.82±0.09^{d}	1.65±0.03^{bcdef}	0.15±0.01^{ab}	0.96±0.09^{c}
13	Fb600	19.42±0.16^{ab}	09.94±0.11^{ab}	1.74±0.14^{abcd}	0.18±0.02 ^{a}	0.51±0.06^{ab}

*Values are the means of three-replication±standard error

† Values are the means of five-replication±standard error. Values with the same superscripts within column indicate no significant difference with $P \geq 0.05$

growth, fresh and dry weights when compared with the controls (Table 2). Under Cd stress, these treatments showed 0.39, 0.40 and 0.51 pmol of ethylene production per gram fresh weight, respectively. Stress ethylene production in the wheat seedlings was reduced to the tune of ~72 % when treated with Ps 2-3 and Ps 7-12 (Table 2).

Isolation, sequencing and characterization of ACC deaminase gene

PCR yielded an amplicon of ~1000 bp size in all the bacterial isolates except A48. However, positive signal was obtained in A48 in dot blot and Southern hybridization (Fig. 2). In BLASTN analysis, sequences of Ps 2-3 and Ps 7-12 showed 87 and 89 % identity, respectively, with transcriptional regulator (*acdR*) gene and ACC deaminase (*acdS*) gene of *Pseudomonas fluorescens* strain 2P24. The *acdS* gene of Ps 2-3 and Ps 7-12 contained an open reading frame of 1017 bp nucleotides encoding a protein of 338 amino acids length. The G+C content was 59.6 and 61.3 % for Ps 2-3 and Ps 7-12, respectively, and 86.4 % identical to each other. About 14 % of the ORF (137 nucleotides) were dissimilar especially in the wobble position constituting of 111 codons (Fig. 3). Multiple alignments of nucleotide

Fig. 1 Effect of ACC deaminase containing mustard rhizobacterial isolates on root elongation of wheat (cv. HD 2687) seedlings under gnotobiotic conditions

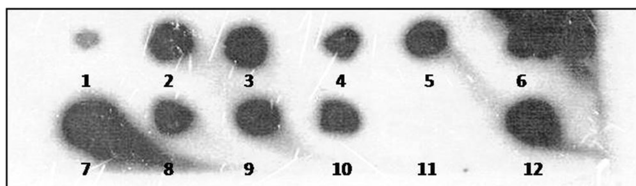

Fig. 2 Nucleic acid dot blot hybridization showing the presence of *acdS* gene in mustard rhizobacterial isolates. *1* Ps 1-12; *2* Ps 2-3; *3* Ps 2-12; *4* Ps 3-7; *5* Ps 4-17; *6* Ps 5-1; *7* Ps 7-12; *8* Ps 7-9; *9* A48; *10* Fb600; *11* DH5α; *12* Pf 913

sequences of Ps 2-3 and Ps 7-12 with sequences of complete ACC deaminase genes available in the GenBank databases showed sequence homology with *Pseudomonas putida* strain UW4 (PpUW4) as ~84 and ~85 %, respectively. Sequence identity of Ps 2-3 and Ps 7-12 was 74.5 and 76.2 %, respectively, when compared to ACC deaminase gene sequence of *Pseudomonas* sp. strain ACP (PspACP). However, the deduced amino acid sequences of Ps 2-3 and Ps 7-12 showed 96 and 95 % similarity respectively, with the ACC deaminase protein of *P. putida* UW4.

A high level of conservation (95.2 %) was observed in Ps 2-3 and Ps 7-12 and only 16 amino acids of 338 were found to be different between them. Additionally, 8 and 13 new amino acids were observed in the AcdS protein sequence of Ps 2-3 (positions K^{4}, A^{7}, Q^{33}, E^{125}, I^{222}, D^{273}, F^{303} and D^{304}) and Ps 7-12 (positions H^{4}, E^{7}, Q^{33}, E^{125}, D^{146}, S^{150}, E^{181}, Q^{185}, T^{189}, Q^{218}, I^{222}, K^{230} and D^{304}), respectively (Fig. 4). Ten different types of peptidase sites were present in the deduced protein sequences of both isolates. Hydroxylamine (NH_2OH) acting site was absent in Ps 2-3 compared to Ps 7-12 AcdS protein. However, an additional number of peptidase acting sites for *S. aureus* V_8 protease (Endoproteinase Glu-C) and Chymotrypsin in Ps 7-12, and Carboxypeptidase B site and Modified trypsin (Trypsin) site in Ps 2-3 AcdS proteins, were observed (Table 3). Three types of motifs with varying patterns, viz., protein kinase phosphorylation site, N-glycosylation site and N-

Fig. 3 Alignment of the nucleotide sequences of *acdS* genes of mustard rhizobacteria Ps 2-3 and Ps 7-12. Identical nucleotides are shown as *dots*

```
Ps2-3   1    TATGAATCTGAAACGATTTGCTCGTTATCCGCTGACCTTTGGTCCCTCTCCCATCACCCCCTTGAAGCGT
Ps7-12  1    ......C...C.T..G....AG..G......T.......C.....A...........G...........C

Ps2-3   71   CTCAGCGAACATTTGGGCGGCAAGGTGCAGTTGTATGCCAAGCGTGAAGACTGCAACAGTGGCCTGGCTT
Ps7-12  71   ..G..T......C....G........C...C...................T.................C.

Ps2-3   141  TCGGCGGGAACAAAACCCGCAAGCTTGAATACCTGATTCCCGAAGCACTTGAGCAAGGCTGCGATACCCT
Ps7-12  141  .............G...........C....................GA.C....................

Ps2-3   211  GGTTTCCATCGGCGGAATCCAGTCGAACCAGACCCGCCAGGTAGCCGCCGTCGCTGCTCACCTGGGCATG
Ps7-12  211  ...C........T..C.........................G..G..G..G..C..G............

Ps2-3   281  AAGTGCGTACTGGTGCAGGAAAACTGGGTGAACTACTCCGATGCGGTGTATGACCGGGTAGGCAATATCG
Ps7-12  281  ........G...................C..............C..............G.....C....

Ps2-3   351  AGATGTCCCGGATCATGGGTGCAGAGGTACGTCTGGACGCCGCCGGGTTTGACATCGGCATTCGTCCCAG
Ps7-12  351  .......T..C........G..G........A...........G.....C........T..C..G..A..

Ps2-3   421  CTGGGAGAAGGCCATGAGCGACGTGGTCGAGCGAGGCGGCAAGCCATTCCCGATCCCGGCGGGGTGTTCC
Ps7-12  421  ................GA.........GTC...C...........G.................T......

Ps2-3   491  GAGCATCCCTACGGCGGCCTCGGGTTCGTGGGTTTCGCCGAAGAGGTACGGCAGCAGGAAAAAGAACTGG
Ps7-12  491  ...........T.................C..C..T........A..G..AG...........C.G....

Ps2-3   561  GCTTCAAGTTTGATTACATTGTGGTCTGCTCGGTGACCGGTAGTACCCAGGCCGGCATGGTGGTCGGTTT
Ps7-12  561  ......C...C..C.....C........T..C........C.............................

Ps2-3   631  CGCTGCCGACGGCCGCTCGAAGAACGTCATCGGTATCGACGCCTCGGCCAAACCGGAGCAGACCAAGGCA
Ps7-12  631  ...C..G.........T......C.A..G.....C.....T............G..A...A.A.........

Ps2-3   701  CAGATTCTGCGCATTGCCCGGCACACCGCTGAGCTGGTGGAGCTGGGTCGGGAGATCACTGAAGAGGACG
Ps7-12  701  .....C.....T..C.........T.....G..A...............C..C..A.....C..G.......

Ps2-3   771  TGGTCCTCGATACGCGCTTCGCCTACCCGGAATATGGCTTGCCCAACGACGGCACGCTGGAAGCTATCCG
Ps7-12  771  ....G.....C..T..G.........T.........C.............................C..T..

Ps2-3   841  ACTGTGCGCGAGCCTTGAGGGCGTACTGACAGACCCGGTGTATGAAGGTAAATCCATGCACGGGATGTTT
Ps7-12  841  C.......GC.....G..A..T..G.....C..T.........C.........................A..

Ps2-3   911  GATATGGTGCGTCGTGGTGAGTTCCCTGAAGGCTCGAAAGTGCTTTATGCGCATTTGGGCGGGGTTCCCG
Ps7-12  911  ..A.....C..C.....C.........C.................C..C.....CC.......A.CG..T.

Ps2-3   981  CGCTGAACGCATATAGCTTTCTGTTTCGAGACGGCTAA
Ps7-12  981  .C........C..C..T...A.A..C...A........
```

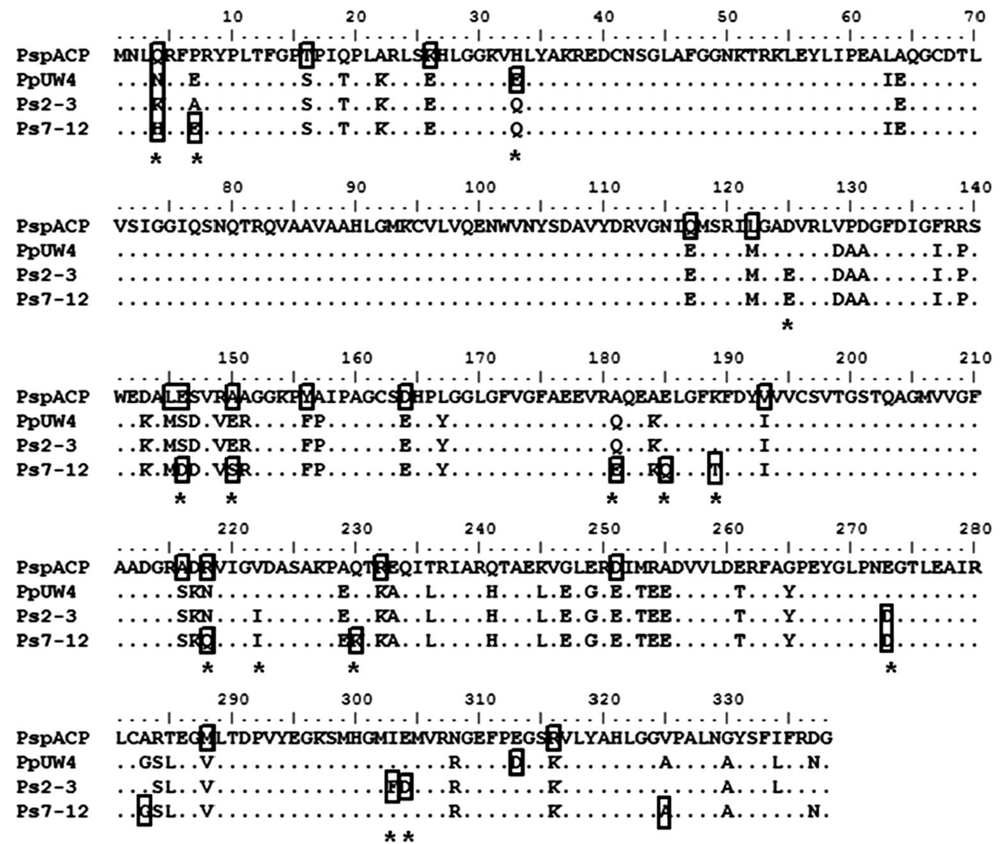

Fig. 4 Alignment of the deduced amino acid sequences from *acdS* genes of mustard rhizobacteria Ps 2-3 and Ps 7-12 with *Pseudomonas* sp. ACP and *P. putida* UW4. The identical amino acid sequences are showed as *dots*. The dissimilar amino acids are showed in *boxes*. The new amino acids are indicated as *stars*

myristoylation site, were present. An additional N-myristoylation motif site was observed in the deduced protein sequence of Ps 7-12 (Table 4).

Table 3 Number of different types of peptidase acting sites present in the deduced amino acid sequences from *acdS* gene of mustard rhizobacteria Ps 2-3 and Ps 7-13

Name of the peptidase site	Number of sites	
	Isolate Ps 2-3	Isolate Ps 7-12
Hydroxylamine (NH_2OH)	_	1NG
pH 2.5	1DP	1DP
Iodosobenzoate (IBzo)	2 W	2 W
NTCB+Ni (NTCB)	6C	6C
Cyanogen bromide (CnBr)	9 M	9 M
Endoproteinase Lys-C	16 K	15 K
Myxobacter protease	16 K	15 K
Proline endopeptidase (Proline)	16P	16P
Clostripain (Endoproteinase Arg-C)	21R	21R
S. aureus V_8 protease (Endoproteinase Glu-C)	27E	28E
Chymotrypsin	28 (WFY)	29 (WFY)
Carboxypeptidase B	37KR	36KR
Modified trypsin (Trypsin)	37KR	36KR
Iso-electric point	pH 5.75	pH 5.72

Phylogenetic analysis of *acdS* and deduced AcdS sequences

A distinct polymorphism was observed between our isolates and others existing in the database. Two distinct clades were observed which were different from *Pseudomonas* sp. ACP, *Rhizobium leguminosarum* 128C53K and *Pseudomonas plecoglossicida* AM10. *acdS* sequence of isolate Ps 7-12 was more similar to strains Ps 2P24, Pf 17, CH-CRS-8 and PSNL (Fig. 5), but the deduced amino acid sequences of Ps 2-3 showed better homology to other *P. fluorescens* AcdS protein sequences (Fig. 6).

Identification of rhizobacterial isolates by 16S rDNA sequencing

Isolates Ps 2-3 and Ps 7-12 were closely related to *Pseudomonas* sp. and *Pseudomonas fluorescens*, respectively, based on BLASTN analysis of their full length 16S rDNA sequences.

Discussion

The cyclopropanoid amino acid, 1-aminocyclopropane-1-carboxylic acid (ACC), is known to participate only in the ethylene biosynthesis pathway; it is likely that the only

Table 4 Number and pattern of different types of motifs present in the deduced amino acid sequences from *acdS* gene of mustard rhizobacteria Ps 2-3 and Ps 7-13

Name of the motif	Pattern	Number of sites	
		Isolate Ps 2-3	Isolate Ps 7-12
cAMP & cGMP dependent protein kinase phosphorylation site	[RK] (2)×[ST]	1	1
Casein kinase II phosphorylation site	[ST]×(2) [DE]	1	1
Protein kinase C phosphorylation site	2 [ST]×[RK]	2	2
N-Glycosylation site	N {P} [ST]{P}	3	3
N- Myristoylation site	G {EDRKHPFYW}×(2) [STAGCN] {P}	10	11

biochemical consequence to the plant of ACC degradation is inhibition of ethylene synthesis (Abeles et al. 1992). The ability to hydrolyze ACC with ACC deaminase and utilize the reaction products as nutrients is considered to be phyto-beneficial trait found in some plant growth-promoting rhizobacteria (Glick 1995; Glick et al. 1998). In the present study, we have identified ten ACC deaminase producing rhizobacteria out of a large number of rhizobacteria screened, which were isolated from eight varieties of Indian mustard. Their ability to utilize ACC as the sole nitrogen source, suggesting production of ACC deaminase enzyme, was confirmed by in vitro estimation of α-ketobutyrate. ACC deaminase enzyme activity of these bacterial isolates significantly promoted root elongation in the treated seedlings as compared to unbacterized and *E. coli*-treated control seedlings. On the other hand, the ability of these bacterial isolates to utilize ACC was associated with other properties, such as production of IAA, phosphate solubilization, siderophore and HCN formation, and could probably also affect their interaction with plants (Glick et al. 1999; Dey et al. 2004).

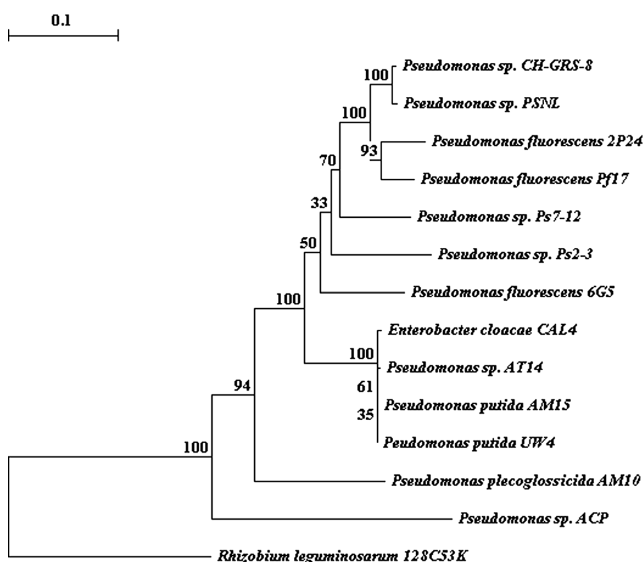

Fig. 5 Phylogenetic relationship of mustard rhizobacteria Ps 2-3 and Ps 7-12 based on nucleotide sequence of complete *acdS* genes. The *numbers at the nodes* indicate the level of bootstrap support, based on neighbour-joining analysis of 1000 replicated datasets

Differential AcdS enzyme activity of the bacterial isolates significantly correlated to their potential to lower stress ethylene synthesis in the inoculated wheat seedlings. Although the endogenous level of ACC and its related compounds was not quantified, the efficiency of these isolates to reduce ethylene level was found to vary from 32 to 72 % as compared to the unbacterized and *E. coli*-treated controls in which ethylene production was maximum under cadmium-induced stress. These results were consistent with the proposed model that $AcdS^+$ bacteria are closely associated with plant roots and act as a sink for a significant amount of ACC present in the root exudates (Glick et al. 1998; Penrose et al. 2001). Reduction in ACC concentration within the plant resulted in reduced amounts of ethylene synthesis, and thus decreased the extent of ethylene inhibition on root elongation and improved the plant survival under stress (Penrose and Glick 2001; Pierik et al. 2006). Even though IAA production was shown by all $AcdS^+$ isolates, and bacterial IAA at low levels is known to have a positive effect on plant root growth (Patten and Glick 2002), the result obtained from the Cd-treated seedlings with low-stress ethylene synthesis clearly implicated the role of ACC deaminase enzyme of the isolates on root elongation and plant growth promotion under abiotic stress conditions. These results are in accordance with others (Grichko and Glick 2001; Mayak et al. 2004; Saravanakumar and Samiyappan 2007).

Characterization of the *acd*S gene in Ps 2-3 and Ps 7-12 isolates (selected for higher in vitro enzymatic activity and better reduction of stress ethylene in plants) showed that it encoded a complete protein of 338 amino acids in length. As most of the nucleotide dissimilarities were observed in the wobble position, the deduced amino acid sequences showed better homology than nucleotide sequences. Isolate Ps 7-12 branched closer with other strains Pf 2P24, Pf 17, CH-CRS-8 and PSNL based on nucleotide sequences; however, Ps 2-3 shared more affinity with them based on amino acid sequences. Variation in numbers of different peptidase acting/cleaving sites and the presence of different types of motif sites

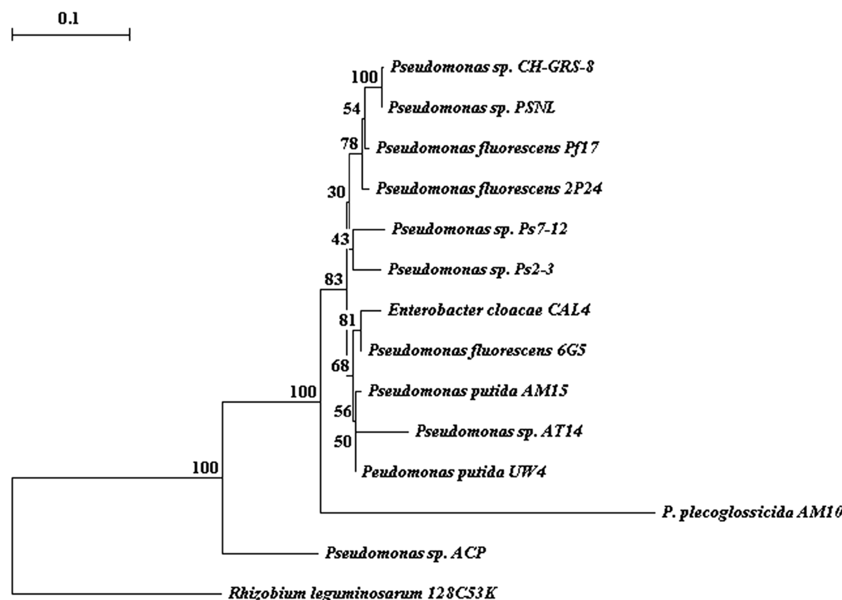

Fig. 6 Phylogenetic relationship of mustard rhizobacteria Ps 2-3 and Ps 7-12 based on complete amino acid sequences deduced from *acdS* gene sequences. The *numbers at the nodes* indicate the level of bootstrap support, based on neighbour-joining analysis of 1000 replicated datasets

were observed between the two AcdS protein sequences with an additional N-myristoylation site in Ps 7-12. This motif site plays an important role in plant responses to environmental stress by membrane targeting and signal transduction during plant–rhizobacterial interactions. Deduced amino acid sequence analysis indicated the conservation of key residues, for example, K^{51} which acts as a PLP binding site, S^{78} which act as nucleophile in the enzymatic attack, C^{162}/A^{162} which is important for enzymatic activity of protein, Y^{294} which is involved in substrate/product transportation, and E^{295} which stabilizes the PLP binding as required for the protein confirmation and enzymatic activity in *P. fluorescens* ACP and *H. saturnus* (Sheehy et al. 1991; Yao et al. 2000). These conserved key residues were also observed in the AcdS sequence of isolates Ps 2-3 and Ps 7-12. In addition, there were 8 (K^4, A^7, Q^{33}, E^{125}, I^{222}, D^{273}, F^{303} and D^{304}) and 13 (H^4, E^7, Q^{33}, E^{125}, D^{146}, S^{150}, E^{181}, Q^{185}, T^{189}, Q^{218}, I^{222}, K^{230} and D^{304}) new amino acid residues observed in Ps 2-3 and Ps 7-12, respectively, as compared to earlier reports. These results are consistent with the possibility that there were a limited number of pre-existing gene containing a few motifs for ACC deaminase genes (Hontzeas et al. 2005; Blaha et al. 2006) that could be mutated (without large numbers of changes) to encode ACC deaminase protein and could be evolved through multiple horizontal gene transfer.

The present study demonstrated the growth-promoting ability of mustard rhizobacteria containing ACC deaminase in wheat seedlings through modulation of stress ethylene synthesis. Nucleotide sequence variations were observed in *acd*S of *Pseudomonas* sp. Ps 2-3 and *P. fluorescens* Ps 7-12, but at the same time key amino acid residues of its AcdS protein were highly conserved. However, overexpression of these cloned genes and the potentiality of these characterized genes in the rhizobacterial isolates Ps 2-3 and Ps 2-17 for growth promotion of wheat should be validated under field experiments with appropriate abiotic stress conditions.

Acknowledgments We are grateful to Prof. Bernard R. Glick, Department of Biology, University of Waterloo, Ontario, Canada, for his valuable suggestions during the course of our investigation. The comments and suggestions of Dr. Tilak R. Sharma, National Research Centre on Plant Biotechnology, New Delhi, India, helped to considerably enhance the manuscript. The first author thanks Indian Agricultural Research Institute, New Delhi, India, for financial assistance in the form of Senior Research Fellowship. A financial grant received from the Department of Science and Technology (DST), India is also gratefully acknowledged.

Conflict of interest The authors of this manuscript declare that they do not have any conflict of interest.

References

Abeles FB, Morgan PW, Saltveit Jr ME (1992) Regulation of ethylene production by internal, environmental and stress factors. In: Ethylene in plant biology (2nd edn). Academic, San Diego pp 56–119

Altschul SF, Madden TL, Schaffer AA, Zhang J, Miller W, Lipman DJ (1997) Gapped BLAST and PSI- BLAST: a new generation of protein database search programme. Nucl Acids Res 25:3389–3402

Bakker AW, Schippers B (1987) Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp. mediated plant growth stimulation. Soil Biol Biochem 19:451–457

Belimov AA, Safronova VI, Sergeyeva TA, Egorova TN, Matveyeva VA, Tsyganov VE, Borisov AY, Tikhonovich IA, Kluge C, Preisfeld A, Dietz KJ, Stepanok VV (2001) Characterization of plant growth promoting rhizobacteria isolated from polluted soils and containing

1-aminocyclopropane- 1-carboxylate deaminase. Can J Microbiol 47:242–252

Blaha D, Prigent-Combaret C, Mirza MS, Moënne-Loccoz Y (2006) Phylogeny of the 1-aminocyclopropane-1-carboxylic acid deaminase-encoding gene *acdS* in phytobeneficial and pathogenic Proteobacteria and relation with strain biogeography. FEMS Microbiol Ecol 56:455–470

Bric JM, Bostock RM, Silverstone SE (1991) Rapid in situ assay for indole acetic acid production by bacteria immobilized on a nitrocellulose membrane. Appl Environ Microbiol 57:535–538

Dey R, Pal KK, Bhatt DM, Chauhan SM (2004) Growth promotion and yield enhancement of peanut (*Arachis hypogaea* L.) by application of plant growth-promoting rhizobacteria. Microbiol Res 159:371–394

Glick BR (1995) The enhancement of plant growth by free-living bacteria. Can J Microbiol 41:109–117

Glick BR, Penrose DM, Li J (1998) A model for the lowering of plant ethylene concentrations by plant growth promoting bacteria. J Theor Biol 190:63–68

Glick BR, Patten CL, Holguin G, Penrose DM (1999) Biochemical and genetic mechanisms used by plant growth promoting bacteria. Imperial College Press, London

Govindasamy V, Senthilkumar M, Gaikwad K, Annapurna K (2008a) Isolation and characterization of ACC deaminase gene from two plant growth promoting rhizobacteria. Curr Microbiol 57:312–317

Govindasamy V, Senthilkumar M, Kumari A, Annapurna K (2008b) Isolation of ACC deaminase containing plant growth promoting bacteria from wheat rhizosphere. Pusa Agric Sci 31:57–63

Govindasamy V, Senthilkumar M, Mageshwaran V, Annapurna K (2009) Detection and characterization of ACC deaminase in plant growth promoting rhizobacteria. J Plant Biochem Biotechnol 18:71–76

Grichko VP, Glick BR (2001) Amelioration of flooding stress by ACC deaminase-containing plant growth-promoting bacteria. Plant Physiol Biochem 39:11–17

Hall JA, Peirson D, Ghosh S, Glick BR (1996) Root elongation in various agronomic crops by the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. Isr J Plant Sci 44:37–42

Honma M, Shimomura T (1978) Metabolism of l-aminocyclopropane-1-carboxylic acid. Agric Biol Chem 42:1825–1831

Hontzeas N, Richardson AO, Belimov AA, Safranova VI, Abu-Omar MM, Glick BR (2005) Evidence for horizontal gene transfer (HGT) of ACC deaminase genes. Appl Environ Microbiol 71:7556–7558

Jia YJ, Kakuta Y, Sugawara M, Igarashi T, Oki N, Kisaki M, Shoji T, Kanetuna Y, Horita T, Matsui H, Honma M (1999) Synthesis and degradation of 1-aminocyclopropane-1-carboxylic acid by *Penicillium citrinum*. Biosci Biotechnol Biochem 63:542–549

Mageshwaran V, Walia S, Govindasamy V, Annapurna K (2011) Antibacterial activity of metabolite produced by *Paenibacillus polymyxa* strain HKA-15 against *Xanthomonas campestris* pv. phaseoli. Ind J Expt Biol 49:299–233

Masterson RV, Prakash RK, Amerly AG (1985) Conservation of symbiotic nitrogen fixation gene sequence in *R. japonicum* and *B. japonicum*. J Bacteriol 163:21–26

Mayak S, Tirosh T, Glick BR (2004) Plant growth promoting bacteria that confer resistance in tomato to salt stress. Plant Physiol Biochem 42:565–572

Patten CL, Glick BR (2002) The role of bacterial indole acetic acid in the development of the host plant root system. Appl Environ Microbiol 68:3795–3801

Penrose DM, Glick BR (2001) Levels of 1-aminocyclopropane- 1-carboxylic acid (ACC) in exudates and extracts of canola seeds treated with plant growth-promoting bacteria. Can J Microbiol 47:368–372

Penrose DM, Glick BR (2003) Methods for isolating and characterizing ACC deaminase-containing plant growth promoting rhizobacteria. Physiol Plant 118:10–15

Penrose DM, Moffatt BA, Glick BR (2001) Determination of 1- aminocyclopropane −1-carboxylic acid (ACC) to assess the effects of ACC deaminase-containing bacteria on roots of canola seedlings. Can J Microbiol 47:77–80

Pierik R, Tholen D, Poorter H, Visser EJW, Voesenek LACJ (2006) The janus face of ethylene: growth inhibition and stimulation. Trends Plant Sci 11:176–183

Pikovskya RI (1948) Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. Mikrobiologiya 17:362–370

Sambrook J, Russell DW (2001) Molecular cloning: A laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Saravanakumar D, Samiyappan R (2007) ACC deaminase from *Pseudomonas fluorescens* mediated saline resistance in groundnut (*Arachis hypogea*) plants. J Appl Microbiol 102:1283–1292

Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophore. Anal Biochem 160:47–56

Senthilkumar M, Swarnalakshmi K, Govindasamy V, Lee YK, Annapurna K (2009) Biocontrol potential of soybean bacterial endophytes against charcoal rot fungus, *Rhizoctonia bataticola*. Curr Microbiol 58:288–293

Sheehy RE, Honma M, Yamada M, Sasaki T, Martineau B, Hiatt WR (1991) Isolation, sequence, and expression in *Escherichia coli* of the *Pseudomonas* sp. strain ACP gene encoding 1-aminocyclopropane-1-carboxylate deaminase. J Bacteriol 173:5260–5265

Wang C, Knill E, Glick BR, Défago G (2000) Effect of transferring 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase genes into *Pseudomonas fluorescens* strain CHA0 and its *gacA* derivative CHA96 on their growth-promoting and disease-suppressive capacities. Can J Microbiol 46:898–907

Yao M, Ose T, Sugimoto H, Horiuchi A, Nakagawa A, Wakatsuki S, Yokoi D, Murakami T, Honma M, Tanaka I (2000) Crystal structure of 1-aminocyclopropane-1-carboxylate deaminase from *Hansenula saturnus*. J Biol Chem 44:34557–34565