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ORIGINAL PAPER

Metagenomic analysis of the 1-aminocyclopropane-1-carboxylate deaminase gene (*acdS*) operon of an uncultured bacterial endophyte colonizing *Solanum tuberosum* L

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Abstract Deamination of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) is a key plant-beneficial trait found in many plant growth-promoting bacteria. In this study, we analysed ACC deaminase genes (*acdS*) of bacterial endophytes colonizing field-grown potato plants. PCR analysis revealed the presence of two types of *acdS* genes, the dominant one showing high homology to an *acdS* gene derived from *Pseudomonas fluorescens*. Construction, functional screening and sequence analysis of metagenomic libraries revealed clones containing the *acdS* gene identified in the PCR library. Sequence analysis of one metagenomic clone identified the entire *acdS* operon of an uncultivated endophyte and revealed that the *acdS* gene is coupled upstream with an *acdR* transcriptional regulator gene as previously found in *P. putida* strain UW4 (Grichko and Glick 2000). However, in-silico analysis of 195 fully sequenced, *acdS*-containing bacterial genomes revealed that the majority of strains, including numerous strains belonging to the genus *Pseudomonas*, do not contain an *acdR* regulatory gene in the vicinity of the *acdS* gene or elsewhere in the genome. The $acdR^{+}$–$acdS^{+}$ operon was exclusively found in several *Alpha-* and *Betaproteobacteria* most prominently in the genus *Burkholderia*.

Keywords Endophytes · Metagenomics · 1-aminocyclopropane-1-carboxylate deaminase · *acdS* · *acdR* · *Pseudomonas* · *Burkholderia*

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Introduction

Bacterial endophytes mostly derive from the soil environment and are able to penetrate plants and colonize intercellular spaces and vascular tissues, where they reside at least part of their lives without causing any immediate negative effects or forming any organized symbiotic structures (Wilson 1995; Compant et al. 2010). Plants are generally colonized by a range of different species and strains belonging to various phylogenetic groups (Chelius and Triplett 2001; Reiter and Sessitsch 2006; Berg et al. 2005). The structural composition of endophytic bacterial communities depends on the genotype of the host plant (Sessitsch et al. 2002; Rasche et al. 2006a), the kind of tissue (Sturz et al. 1997) as well as on the vegetation stage (Rasche et al. 2006a) and may be significantly influenced by plant stress (Sessitsch et al. 2002; Reiter et al. 2002; Rasche et al. 2006a, b), indicating that the physiology of the plant has a major impact on endophyte populations (Rasche et al. 2009).

It has been frequently reported that many endophytes have beneficial effects on their host plant such as plant growth promotion or biocontrol of pathogens (Rosenblueth and Martínez-Romero 2006). Many plant growth-promoting bacteria produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Bacteria showing this enzymatic activity can reduce the level of ethylene produced in developing or stressed plants by cleaving ACC, which is the precursor of

ethylene, to α-ketobutyrate and ammonium (reviewed by Glick 2005 and by Hontzeas et al. 2006). Glick (1995) proposed that ACC deaminase-producing plant-associated bacteria act as a sink for ACC, ensuring that plant ethylene levels are not raised to the point where root growth is impaired. This activity has been considered to be important for the promotion of plant growth and is particularly known for protecting plants from biotic and abiotic stresses such as heavy metals (Burd et al. 1998; Belimov et al. 2001, 2005), drought (Mayak et al. 2004a), salt (Mayak et al. 2004b), flooding (Grichko and Glick 2001) and plant pathogens (Wang et al. 2000). Many endophytes have been shown to contain ACC deaminase, and the activity has been detected in a range of gram-negative and gram-positive bacterial strains (Rasche et al. 2006a; Onofre-Lemus et al. 2009; Blaha et al. 2006).

The diversity of bacterial endophytes has been mostly analysed by cultivation, however, as the majority of bacteria cannot be easily cultivated, cultivation-independent methods have revealed an unexpected high diversity of microbes living in the plant apoplast (e.g. Idris et al. 2004). Functional abilities of endophytes such as plant growth-promoting traits have been analysed mostly in isolates. Although degenerate PCR primers targeting ACC deaminase (*acdS*) genes based on published sequences have been designed (Hontzeas et al. 2005; Blaha et al. 2006), they amplify only gene fragments. Consequently, PCR-based analysis may only detect the presence of a (known) ACC deaminase encoding gene, but does not elucidate information on the entire gene or gene operon. Furthermore, so far only *acdS* genes from cultivated bacteria are known; however, the majority of environmental bacteria are not accessible by cultivation. Metagenomic approaches which rely on the isolation of the entire genome of a habitat of interest combined with subsequent cloning and analysis are gaining importance in resolving the ecology and functions of yet uncultured or hardly cultivable bacteria (for reviews see Handelsman 2004; Daniel 2005; Kowalchuk et al. 2007). Whereas PCR analysis usually identifies only gene fragments based on known sequences, metagenomic analysis allows the identification of novel genes and/or of full operons of already known genes irrespective of their culturability. The objective of this study was to study ACC deaminase encoding genes of a potato-associated, bacterial endophytic community including culturable and non-culturable community members by applying a metagenomic approach.

Materials and methods

Dislodging of endophytic bacteria and DNA isolation

Field-grown potato plants (*Solanum tuberosum* L.) of the late maturing cultivar Ditta were collected at flowering stage in August 2005 from a field cultivated under conventional farming management in Stotzing (Burgenland, Austria). Stems and roots of several plants were pooled together, thoroughly washed with tap water and surface sterilized by submerging into 5% bleach for 5 min, in 70% ethanol for 5 min and final flaming. Stems were aseptically peeled to remove DNA of lysed epiphytic bacteria. Roots were vigorously shaken in 0.9% NaCl solution containing 0.3 g acid-washed glass beads (Sigma–Aldrich St. Louis, USA; 0.1 mm) for 20 min to remove surface-associated bacterial debris containing DNA. Stems and roots were rinsed five times with sterile H_2O and tested for their sterility on TSA plates. No growth was observed. To be able to isolate sufficiently high DNA quantities for further metagenomic analysis, the following approach was applied. Sterilized plant material was cut into small pieces, and endophytic bacteria were dislodged by overnight shaking at room temperature in 0.9% NaCl. Bacteria were separated from the plant material by filtration through MN616 filters (Macherey–Nagel, Düren, Germany) and collected by centrifugation. Bacterial pellets were stored at −20°C until used for DNA isolation.

Several frozen bacterial pellets originating from different potato plants and different extractions were pooled, and DNA was prepared using the 'UltraClean Bacterial DNA isolation kit' (MO BIO Laboratories, Carlsbad, USA). DNA was analysed by agarose gel electrophoresis and appeared on an agarose gel as a band of approximately 20 kbp.

PCR amplification

16S rRNA gene

Nearly full-length 16S rRNA genes were amplified by PCR using the universal bacterial primer 8f (AGAGTTTGATCCTGGCTCAG) (Edwards et al. 1989) and 1520r (CGGTGTGTACAAGGCCCGGG) (Massol-Deya et al. 1995) using DNA extracted from endophytic bacteria dislodged from potato plants. Reaction mixtures of 50 µl contained 50 ng DNA, 1× PCR buffer (Invitrogen, Carlsbad, USA), 2 mM $MgCl_2$ (Invitrogen), 0.1 µM of each primer, 0.2 mM of each deoxynucleoside triphosphate (Fermentas, St. Leon-Rot, Deutschland), 1 µg BSA (Promega, Madison, USA) and 1 U Taq DNA polymerase (Invitrogen). The PCR parameters were as follows: initial denaturation for 2 min at 95°C followed by 25 cycles consisting of denaturation for 30 s at 95°C, primer annealing for 30 s at 55°C and polymerization for 2 min at 72°C followed by a final extension for 5 min at 72°C. Quality of PCR products was inspected by agarose gel.

acdS gene

For the PCR amplification of *acdS* genes, two primer pairs were used. The forward primer DegACCf (5′-GGBGGV AAYAARMYVMGSAAGCTYGA-3′) and the reverse primer DegACCr (5′-TTDCCHKYRTANACBGGRTC-3′) were reported to be universal (Glick 2005), whereas the forward primer F1937f (5′-MGVAAGCTCGAATAYMT BRT-3′) and the reverse primer F1939r (5′-GARGCR TCGAYVCCRATCAC-3′) were reported to amplify *acdS* genes from *Proteobacteria* (Blaha et al. 2006). PCR were performed under the following conditions: 50 µl PCR volumes contained 50–200 ng DNA, 1× PCR buffer (Invitrogen), 4 mM $MgCl_2$ (Invitrogen), 0.5% DMSO, 1 µM of each primer, 0.2 mM of each deoxynucleoside triphosphate (Fermentas), 1 µg BSA (Promega) and 1 U Taq DNA polymerase (Invitrogen); initial denaturation was performed for 3 min at 95°C followed by 30–35 cycles consisting of denaturation for 30 s at 95°C, primer annealing for 1 min at 46°C (DegACCf/DegACCr) or 53°C (F1937f/F1939r) and elongation for 1 min at 72°C followed by a final extension for 5 min at 72°C.

When *acdS* gene amplicons were directly sequenced, tailed versions of above primers were used, (GCTCC TACTCTGTCACC) and (CTGTCGCTCTGGCTGTC) nucleotide sequences were at the 5′ ends of forward and reverse primers, respectively. Primers with identical nucleotide sequence to added tails were used for sequencing.

Construction of 16S rRNA and *acdS* gene libraries

16S rRNA gene and *acdS* gene libraries using PCR amplicons (see above) were constructed to analyse the diversity of endophytes and the diversity of *acdS* genes detectable by PCR amplification. The TOPO TA cloning kit (Invitrogen) was used following the manufacturer instructions. Genes were first amplified by PCR with gene-specific primers, amplicons were separated on agarose gels and DNA bands of proper size were extracted from agarose gels and cloned by topoisomerase reaction into pCR4 vector (Invitrogen). Positive recombinants, appearing as white colonies on indicator plates containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) (Sigma–Aldrich) and isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Sigma–Aldrich), were picked, their plasmid DNA was isolated using the alkaline lysis method and sequenced.

Construction of metagenomic entry libraries

Metagenomic entry libraries were constructed, which contain metagenomic DNA fragments without additional sequences that facilitate the expression of the cloned genes. Isolated DNA was partially digested with 0.2 U *Sau*3AI (Promega) per µg of DNA in Promega buffer H at ice for 1 h, and fragments between 5 to 15 kbp were isolated from an agarose gel. Four-hundred ng of partially digested DNA was ligated using Mighty mix ligase (Takara) at 16°C for 30 min with 100 ng of dephosphorylated vector pENTR1A (Gateway cloning system, Invitrogen), which was linearized with *BamH*I and previously modified by excision of the *ccdB* selection gene by *Eco*RI digestion. Ligation mixes were electroporated into *TransforMax* EPI300 *E. coli* (Epicentre, Madison, USA), and transformants were selected on LB plates containing 35 mg L^{-1} kanamycin (Sigma–Aldrich). All transformants from one type of library were pooled together. Two libraries were created, one from potato stem endophytes and another one from root endophytes. In total, we obtained approximately 6×10^5 clones for each library. The quality of the entry libraries was estimated by partial sequencing of insert DNA of twenty randomly chosen clones and by double digestion with restriction endonucleases *Dra*I and *Eco*RI. All analysed clones had insert DNA that was different in content and size. Insert sizes ranged between 5 and 15 kbp.

Construction of metagenomic expression libraries

Metagenomic expression libraries were constructed to further facilitate expression of the cloned metagenomic genes. For the construction of these libraries, DNA inserts from entry libraries were transferred into the expression vector pDEST14 (Invitrogen), which is based on bacteriophage T7 RNA polymerase expression system (Studier et al. 1990), by applying the LR reaction enzymatic mixture (Gateway cloning system, Invitrogen) following the manufacturers' instructions. To increase the number of clones, several LR clonase reactions were carried out. Plasmid DNA was electroporated into *E. coli* BL21gold (DE3) (Stratagene, La Jolla, USA), and transformants were selected on LB plates containing 100 mg L^{-1} ampicillin (Sigma–Aldrich). Expression libraries contained 5×10^4 (root endophytes) and 3×10^6 clones (stem endophytes).

Screening for ACC deaminase activity [EC 3.5.99.7]

Screening for the ACC deaminase activity was performed on solid minimal medium plates containing 1-amino-cyclopropane-1-carboxylate (ACC) (Calbiochem-Merck, Darmstadt, Germany) as sole nitrogen source prepared according to Brown and Dilworth (1975) (B&D plates). Shortly, 12 g L^{-1} agarose (SeKam LE Cambrex, Biozym, Germany) was sterilized by boiling for 30 min and mixed with filter-sterilized solutions to obtain final concentrations: 0.36 g L^{-1} KH_2PO4, 1.4 g L^{-1} K_2HPO_4, 0.25 g L^{-1} $MgSO_4.7H_2O$, 0.02 g L^{-1} $CaCl_2.2H_2O$, 0.2 g L^{-1} NaCl, 0.011 g L^{-1} $FeCl_3.6\ H_2O$, 150 µg L^{-1} EDTA, 1 mg L^{-1}

Table 1 Phylogenetic analysis of 16S rRNA gene sequences of potato endophytes

Clone	16S rRNA gene identity (NCBI accession number/% identity)	Phylogenetic group
Root-associated endophytes		
3A2	*Enterobacter aerogenes* strain T2 (GU265554/99%)	*Gammaproteobacteria*
3B4	*Enterobacter cloacae* strain Ag02 (EU554428/99%)	*Gammaproteobacteria*
3B3	*Enterobacter ludwigii* strain EN-119T (AJ853891/99%)	*Gammaproteobacteria*
3C4	*Enterobacter* sp. JM1-2 (FJ472852/99%)	*Gammaproteobacteria*
3D3	*Enterobacter* sp. PIC15 (GQ359959/100%)	*Gammaproteobacteria*
3B1	*Enterobacter* sp. PIC15 (GQ359959/98%)	*Gammaproteobacteria*
3C1	*Enterobacter* sp. Px6-4 (EF175731/99%)	*Gammaproteobacteria*
3G4	*Klebsiella* sp. Tam (GQ416399/96%)	*Gammaproteobacteria*
3E3	*Klebsiella terrigena* strain ATCC 33257T (Y17658/99%)	*Gammaproteobacteria*
3H4	Uncultured *Enterobacter* sp. clone TCCC 11071 (EU567038/99%)	*Gammaproteobacteria*
3F4	Uncultured *Enterobacteriaceae* bacterium clone Ko' (AY635978/100%)	*Gammaproteobacteria*
3B2	Uncultured *Enterobacteriaceae* bacterium clone Ko' (AY635978/99%)	*Gammaproteobacteria*
3C3	Uncultured *Kluyvera sp*. clone F3feb.36 (GQ417620/99%)	*Gammaproteobacteria*
Stem-associated endophytes		
1E4	*Enterobacter hormaechei* (FM883661/99%)	*Gammaproteobacteria*
1C4	*Enterobacter* sp. 638 (CP000653/99%)	*Gammaproteobacteria*
1C3	*Enterobacter* sp. AN2 (GQ451698/100%)	*Gammaproteobacteria*
1D4	*Enterobacter* sp. pptphilum (AY596467/99%)	*Gammaproteobacteria*
1C1	*Enterobacter* sp. SPi (FJ405368/99%)	*Gammaproteobacteria*
1A2	*Enterobacter* sp. strain WAB1938 (AM184277/99%)	*Gammaproteobacteria*
1F3	*Enterobacter* strain 3.1.1.C (DQ916133/99%)	*Gammaproteobacteria*
1G2	*Enterobacteriaceae* bacterium HH31 (AB304398/98%)	*Gammaproteobacteria*
1G3	*Enterobacteriaceae* bacterium HH31 (AB304398/98%)	*Gammaproteobacteria*
1F2	*Klebsiella oxytoca* strain PYR-1 (GU253335/99%)	*Gammaproteobacteria*
1B3	*Lactococcus* sp. F51 (FJ405283/100%)	*Firmicutes*
1A3	Uncultured *Acinetobacter* sp. clone GI5-004-C08 (FJ192625/99%)	*Gammaproteobacteria*
1E1	Uncultured *Acinetobacter* sp. clone GI5-007-C11 (FJ192785/99%)	*Gammaproteobacteria*
1C2	Uncultured *Klebsiella* sp. clone F4jan.33 (GQ417933/100%)	*Gammaproteobacteria*
Root- and stem-associated endophytes		
1D3	*Acinetobacter calcoaceticus* strain LUH 5820 (FJ867364/98%)	*Gammaproteobacteria*
3A1	*Enterobacter* sp. AN2 (GQ451698/99%)	*Gammaproteobacteria*
3E1	*Enterobacter cloacae* strain Ag02 (EU554428/99%)	*Gammaproteobacteria*
3F4	Uncultured *Enterobacteriaceae* bacterium clone Ko' (AY635978/99%)	*Gammaproteobacteria*

Thiamine-HCl, 2 mg L^{-1} Ca-pantothenate, 20 μg L^{-1} biotin, 2 g L^{-1} glucose and 5 mM ACC. The ACC solution was always prepared freshly before use. Small aliquots of entry and expression library were plated onto B&D plates and incubated at 30° for couple of days until colonies appeared. About 1 mM IPTG was added to B&D plates before screening of the expression library. ACC deaminase activity of positive clones was confirmed by re-inoculation onto solid B&D medium with and without ACC, supplemented with 35 mg L^{-1} kanamycin in the case of entry clones and 100 mg L^{-1} ampicillin and 1 mM IPTG in the case of expression clones. DNA of positive clones was analysed by *acdS* gene-specific PCRs (see above) and sequencing.

Sequence analysis

Sequencing was performed by the dideoxy chain termination method (Sanger et al. 1977) using an ABI 3100 automated DNA sequencer and Big-Dye Terminator v1.1 Cycle kit (Applied Biosystems). In some cases, DNA was sequenced by AGOWA GmbH, Germany. 16S rRNA and *acdS* gene sequences were obtained by sequencing of corresponding plasmid DNA using M13 forward and reverse primers (Invitrogen). *acdS* gene sequences of isolated entry and expression library clones were obtained by direct sequencing of ACC deaminase gene-specific PCR amplicons (see *acdS* gene PCR) and by sequencing of isolated plasmid DNA using vector-specific primers

(Invitrogen). Insert DNA of entry clone mp16 was sequenced by primer walking starting from vector arms using primers ForAttL1 (TTGTACAAAAAAGCAGGC) and attL2rev (GACCCAGCTTTCTTGTACAAAG) and from inside the *acdS* gene in both directions using ACC deaminase-specific primers. Nucleotide sequences were subjected to BLAST analysis (Altschul et al. 1997) with the National Centre for Biotechnology Information (NCBI) database.

DNA sequences were aligned using the Clustal W computer program version 1.4 (Thompson et al. 1994), and phylogenetic trees were constructed using DNAml DNA Maximum Likelihood program version 3.6a2.1 (Copyright 1986–1993 by Joseph Felsenstein and by the University of Washington) included into BioEdit Sequence Alignment Editor software package (Hall 1999). Sequences of *acdS* genes were obtained from DOE Joint Genome Institute (JGI) (http://img.jgi.doe.gov) and the National Center for Biotechnology Information (NCBI) database.

Nucleotide accession numbers

Nucleotide sequences have been deposited in the NCBI database under accession number HQ166711 (metagenomic clone mp16) and numbers HQ189393-HQ189422 (16S rRNA gene sequences).

Results

16S rRNA and ACC deaminase gene diversity in potato endophytes

Metagenomic DNA isolated from potato stem, and root endophytes was analysed by 16S rRNA gene PCR and sequencing. Sixty-four clones were partially sequenced and blasted against NCBI database. One sequence showed highest homology to *Lactococcus*, whereas the remaining sequences showed affiliation to *Gammaproteobacteria*, in particular to the genera *Enterobacter, Klebsiella, Acinetobacter* and *Kluyvera* (Table 1). Roots and stems showed different population structures, and only few sequences were found to be present in both tissues. Plant-derived ribosomal RNA genes were not detected.

The occurrence of ACC deaminase genes in metagenomic DNA was tested by PCR using published protocols (Hontzeas et al. 2005; Blaha et al. 2006) and subsequent cloning and sequencing. Amplicons were obtained with the primer pair F1937f/F1939r (Blaha et al. 2006) from root-derived endophytes but not from stem endophytes. Two distinct *acdS* genes were found. Fifty-nine *acdS* gene sequences showed 95% nucleotide identity to the *acdS* gene of *Pseudomonas fluorescens* strain F113, while five sequences showed 78% nucleotide identity to the *acdS* gene of *Rhizobium sullae* strain 43767.

Construction and screening of metagenomic libraries for ACC deaminase activity

Two types of metagenomic libraries were constructed from potato stem and root endophytes DNA. Entry libraries, in which transcription is possible only from promoters present in insert DNA and expression libraries, in which transcription is driven by the T7 promoter of the vector. Screening for ACC deaminase activity was performed on modified B&D plates (Brown and Dilworth 1975) supplemented with ACC as a sole nitrogen source. In both libraries, we found ACC deaminase-positive clones, 20 in the entry library obtained from root endophytes, 19 and 6 clones in the expression libraries obtained from stem and root endophytes, respectively. No positive clones were detected in the entry library derived from stem endophytes. PCR amplicons were obtained for all ACC deaminase-positive clones using *Proteobacteria*-specific *acdS* gene primers, indicating that potato endophytes hosted no novel, yet unknown type of ACC deaminase. Sequencing of *acdS* genes found in metagenomic libraries revealed that they were all identical to that *acdS* gene amplified by PCR and being related to the *P. fluorescens* strain F113 *acdS* gene.

The *acdS* operon and flanking regions of one of the ACC deaminase-positive entry clones (mp16) were sequenced (Fig. 1). The entire sequence of *acdS* gene showed highest (95%) homology to the *acdS* sequence of *P. fluorescens* strain FY32 (Farajzadeh et al. 2010). The nearest gene upstream of the *acdS* gene was identified as *acdR* gene that encodes the leucine-responsive regulatory-like protein AcdR, shown to regulate expression of *acdS* in *Pseudomonas putida* UW4 (Grichko and Glick 2000; Li and Glick 2001). It is oriented in the opposite direction from *acdS* and

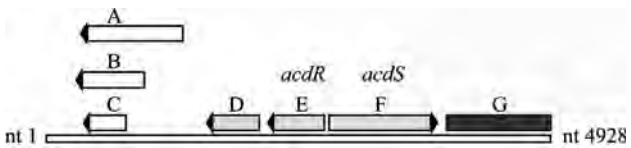

Fig. 1 Nucleotide blast sequence analysis of insert DNA of the ACC deaminase–positive metagenomic clone mp16. *Bars* with *arrowhead* indicate open-reading frames, size of bars corresponds to ORF size. *A–G*: graphic presentation of best NCBI nt Blast hits. *A*: *Rhizobium leguminosarum* bv. viciae, 65% (952 bp), putative transmembrane efflux pump; *B*: *Nematostella vectensis*, 74% (620 bp), predicted hypotetical protein; *C*: *Sinorhizobium medicae* WSM419 plasmid pSMED02, 66% (372 bp), secretion protein, HLyd family; *D*: *Pseudomonas fluorescens* Pf0-1, 67% (467 bp), conserved hypotetical protein; *E*: *Pseudomonas fluorescens* 2P24, 85% (517 bp), Lrp like protein, transcriptional regulator; *F*: *Pseudomonas fluorescens* FY32, 95% (1,015 bp), ACC deaminase; *G*: *Enterobacter cloacae* homologous DNA, 70% (1,071 bp), non-coding region

is 85% identical to *acdR* gene of *P. fluorescens* strain 2P24. Downstream of *acdS* approximately 1.3 kb of non-coding DNA were found, which had 70% identity to a sequence derived from *Enterobacter cloacae*. Upstream of the *acdR–acdS* gene cluster, we found an ORF with 67% homology to a hypothetical gene of *P. fluorescens* Pf0-1. Further upstream, three overlapping open-reading frames were found, which showed homology to a putative transmembrane efflux pump of *R. leguminosarum* (952 bp, 65% identity), a hypothetical protein from *Nematostella vectensis* (620 bp, 74% identity) and a secretion protein of the HlyD family of *Sinorhizobium medicae* WSM419 located on a plasmid (372 bp, 66% identity).

No metagenomic clone was found containing an *acdS* gene related to *R. sullae* strain 43767, although this gene was obtained by PCR amplification. Furthermore, this gene could not be amplified by PCR from plasmid DNA isolated from pooled metagenomic libraries using primers targeting specifically the *R. sullae* related *acdS* gene (not shown). Most likely this gene was not detectable in metagenomic libraries due to its low abundance.

Phylogenetic analysis of the *acdR–acdS* gene cluster

Our knowledge regarding the organization and regulation of the *acdS* gene locus derives from studies performed with *Pseudomonas putida* UW4 (Grichko and Glick 2000; Li and Glick 2001; Ma et al. 2003). In this strain, *acdR* and *acdS* genes are clustered, separated by a short intergenic region of 165 bp that contains promoters and binding sites for AcdR, FNR and CRP transcription regulatory proteins (Online Resource 1). The *acdR–acdS* gene cluster of clone mp16 shows highest homology to the same cluster from *P. putida* UW4.

Table 2 Bacterial strains carrying an *acdR* gene adjacent to the *acdS* gene

Phylogenetic affiliation	Genus[a]
Alphaproteobacteria	*Agrobacterium, Methylobacterium* (3), *Sinorhizobium, Bradyrhizobium* (2)
Betaproteobacteria	*Burkholderia* (42), *Acidovorax* (2), *Diaphorabacter, Polaromonas, Ralstonia* (4), *Variovorax*
Gammaproteobacteria	***Pseudomonas*** (3)

Bold letters indicate the position of the genus *Pseudomonas*

[a] Numbers in parenthesis indicate the number of different strains belonging to same genus

Table 3 Bacterial strains carrying an *acdS* gene but lacking an *acdR* gene

Phylogenetic affiliation	Genus[a]
Alphaproteobacteria	*Jannaschia, Magnetospirillum, Nisaea, Parvibaculum, Rhizobium, Rhodopseudomonas, Roseovarius, Sinorhizobium, Xanthobacter* (2)
Betaproteobacteria	*Burkholderia* (20), *Methylibium*, *Methylobacillus*
Gammaproteobacteria	*Acinetobacter* (3), *Aeromonas, Congregibacter, Escherichia* (10), *Francisella, Hahella, Kangiella, Legionella* (2), *Oceanospirillum,* ***Pseudomonas*** (17), *Reinekea, Saccharophagus* (2), *Shewanella* (3), *Vibrio* (7)
Actinobacteria	*Catenulispora, Clavibacter, Kineococcus, Mycobacterium* (3), *Nocardioides, Nocardiopsis, Rhodococcus, Saccharopolyspora* (2), *Streptomyces* (10), *Brevibacterium* (2), *Actinosynnem* (2)
Bacteroidates	*Parabacteroides*
Bacilli	*Bacillus* (4), *Staphylococcus*
Clostridia	*Blautia* (unclassified), *Clostridium, Natranaerobius*
Cyanobacteria	*Nodularia, Anabaena* (2), *Nostoc*
Deinococci	*Meiothermus*
Deltaproteobacteria	*Anaeromyxobacter, Desulfatibacillum*
Epsilonproteobacteria	*Campylobacter* (4)
Flavobacteria	*Capnocytophaga* (3), *Cellulophaga, Chryseobacterium, Flavobacteria* (4), *Gramella, Kordia*
Sphingobacteria	*Chitinophaga, Cytophaga, Dyadobacter, Microscilla, Pedobacter, Sphingobacterium*
Spirochaetes	*Leptospira* (4)
Thermococci	*Pyrococcus* (3)

Bold letters indicate the position of the genus *Pseudomonas*

[a] Numbers in parenthesis indicate the number of different strains belonging to same genus

Table 4 Analysis of closest open-reading frame upstream of *acdS* genes

Closest ORF upstream of *acdS* gene	Phylum/Class	Species
AcdR	*Alphaproteobacteria*	*Agrobacterium, Azorhizobium, Bradyrhizobium, Methylobacterium, Sinorhizobium*
	Betaproteobacteria	*Burkholderia, Acidovorax, Ralstonia, Variovorax, Polaromonas, Methylibium, Diaphorobacter*
	Gammaproteobacteria	***Pseudomonas***
	Azorhizobium	*Azorhizobium*
AraC	*Alphaproteobacteria*	*Rhizobium*
	Betaproteobacteria	*Acidovorax*
YdhC/GntR	*Actinobacteria*	*Actinosynnema, Brevibacterium (putative), Mycobacterium, Streptomyces*
	Deinococci	*Meiothermus*
Known or hypothetical* protein	*Alphaproteobacteria*	*Jannaschia*, Phenylobacterium*, Roseovarius*, Magnetospirillum, Nisaea, Parvibaculum, Rhizobium, Rhodopseudomonas, Sinorhizobium, Xanthobacter*
	Betaproteobacteria	*Burkholderia, Methylobacillus*
	Gammaproteobacteria	*Congregibacter*, Kangiella*, Legionella*, Marine gamma Proteobact. HTCC2143*, Oceanospirillum*, **Pseudomonas***, Shewanella*, Sodalis*, Vibrio*, Yersinia*, Acinetobacter, Aeromonas, Escherichia, Francisella, Hahella, **Pseudomonas**, Reinekea, Saccharophagus, Shigella, Shewanella*
	Deltaproteobacteria	*Anaeromyxobacter*, Desulfatibacillum*
	Epsilonproteobacteria	*Campylobacter*
	Actinobacteria	*Mycobacterium*, Nocardiopsis*, Streptomyces*, Actinosynnema, Brevibacterium, Catenulispora, Clavibacter, Kineococcus, Mycobacterium, Nocardioides, Rhodococcus, Saccharopolyspora*
	Bacteroidetes	*Capnocytophaga*, Cellulophaga*, Chryseobacterium*, Flavobacteria*, Flavobacterium*, Gramella*, Sphingobacteria*, Capnocytophaga, Parabacteroides*
	Chlamydiales	*Candidatus, Protochlamydia*
	Chloroflexi	*Thermomicrobium*
	Clostridia	*Blautia*
	Cyanobacteria	*Anabaena, Nodularia, Nostoc*
	Euryarchaeota	*Pyrococcus**
	Firmicutes	*Bacillus*, Bacillus, Clostridium, Natranaerobius, Staphylococcus*
	Sphingobacteria	*Cytophaga, Dyadobacter, Leptospira, Microscilla, Pedobacter, Sphingobacterium*
	Spirochaetes	*Leptospira*, Spirochaetes*, Leptospira*
	Thermococci	*Pyrococcus*, Pyrococcus*
	Thermotogae	*Thermotoga*

Bold letters indicate the position of the genus *Pseudomonas*

To see how common this type of *acdS* operon is among other *acdS* gene-containing bacteria, we analysed DNA sequences of fully sequenced bacterial stains publicly available from the DOE Joint Genome Institute (JGI) database. Among 195 *acdS* gene-containing and fully sequenced strains, the *acdR–acdS* gene cluster was exclusively found in few *Proteobacteria* (Table 2), while in all other strains *acdS* was not flanked by *acdR* but by diverse other genes (Tables 3, 4). Strains containing an *acdR–acdS* gene cluster belonged to the *Beta-* (*Burkholderia, Acidovorax, Diaphorabacter, Polaromonas, Ralstonia, Variovorax*), *Alpha-* (*Agrobacterium, Methylobacterium, Sinorhizobium, Bradyrhizobium*) and *Gammaproteobacteria* (*Pseudomonas*) (Table 2). The *acdR–acdS* gene cluster is much less abundant among *Gammaproteobacteria* than among *Alpha-* and *Betaproteobacteria* (Tables 2, 3). Phylogenetic analysis showed that the *acdS* genes of three $acdR^{+}$–$acdS^{+}$ *Pseudomonas* strains are distinct from those that are found in many more $acdR^{-}$–$acdS^{+}$ *Pseudomonas* strains (Fig. 2). In addition, in the maximum likelihood

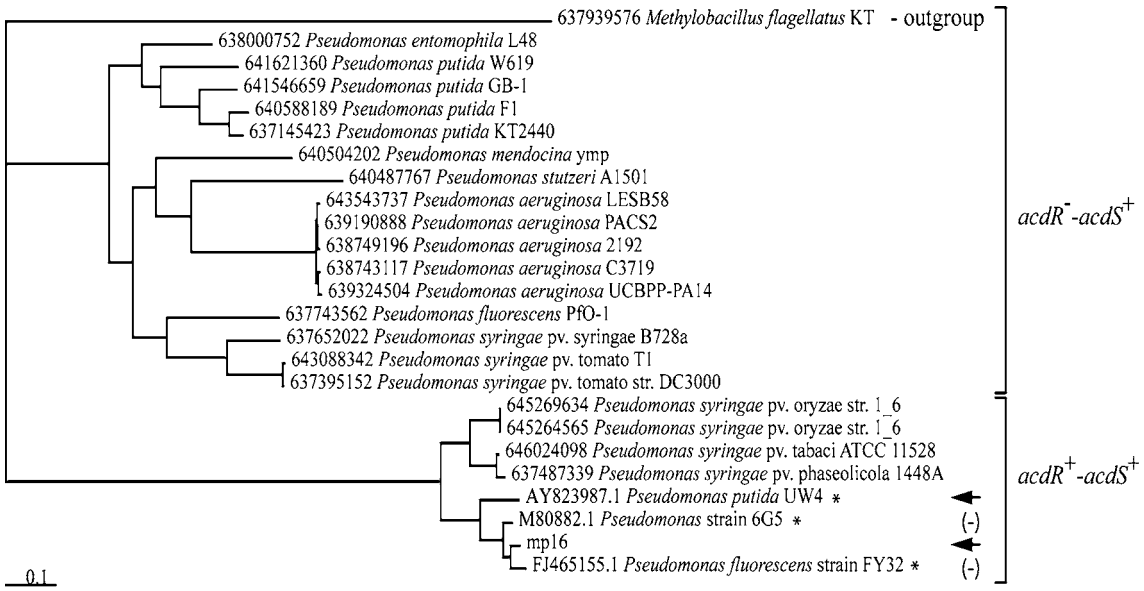

Fig. 2 Maximum likelihood phylogenetic tree of *acdS* genes of *Pseudomonas* strains sequenced over the *acdR–acdS* gene locus. *acdR*$^+$–*acdS*$^+$, strains that comprise both, *acdR* and *acdS* genes in one locus; *acdR*$^-$–*acdS*$^+$, strains that lack the *acdR* gene. (−), strains for which sequence upstream of *acdS* gene is not available. Accession numbers of sequences in JGI or NCBI database (marked with *) are written before strain names. *Arrowheads* indicate the position of the metagenomic clone mp16 and the *Pseudomonas putida* UW 4 *acdS* gene

phylogenetic tree built for *acdS* genes from 195 different bacterial strains affiliating to 72 different genera available from the JGI database, *acdR*$^+$–*acdS*$^+$-type and *acdR*$^-$–*acdS*$^+$-type strains were clearly separately organized (Fig. 3). Interestingly, *acdS* genes of several *acdR*$^-$–*acdS*$^+$-type strains but not of *acdR*$^+$–*acdS*$^+$-type strains showed homology to genes encoding D-cysteine desulfhydrase [EC: 4.4.1.15] (Fig. 3).

Protein sequence comparison of AcdR and other AsnC/Lrp transcription regulator proteins encoded by genes present in genomes of twenty fully sequenced *Pseudomonas* strains showed that AcdR proteins share only moderate homology with other AsnC/Lrp regulators (Fig. 4). Moreover, in *Pseudomonas* and other *acdS* gene-positive strains, *acdR* genes were found only when they are coupled with *acdS* genes and never elsewhere apart from *acdS*. NCBI and JGI database Blast searches for genes that are homologous to the *P. putida* UW4 *acdR* gene resulted only in *acdR* genes that are located adjacent and upstream of *acdS* genes in *acdR*$^+$–*acdS*$^+$ strains (not shown).

Discussion

Metagenomic analysis of microorganisms allows a cultivation-independent approach to study characteristics or genes not easily accessible by PCR or related methods (Riesenfeld et al. 2004). Metagenomics has the potential to identify novel enzymes or genes (Kimura 2006) as well as to analyse larger gene regions, whereas by PCR analysis usually only gene fragments are amplified. Potato endophytes analysed in this study showed limited diversity of putative ACC deaminase encoding genes despite the fact that ACC deaminase is considered an important and frequently encountered trait among endophytes (Glick et al. 2007). Partly, this might be due to the procedure used to isolate DNA derived from endophytes, which was applied to avoid the extraction and analysis of plant-derived DNA and to be able to isolate the quantity of DNA needed for the construction of metagenomic libraries. The majority of endophytes covered in the metagenomic library included mostly *Gammaproteobacteria*; however, a broader range of endophytes is usually encountered within potato plants (Sessitsch et al. 2004; Rasche et al. 2006a; Manter et al. 2010). A high number of endophytes belonging to the *Enterobacteriaceae* were found. This bacterial group is frequently found in association with higher organisms, either as human or plant pathogen but also as plant growth-promoting bacteria (Holden et al. 2009; Bulgari et al. 2009). In addition, endophytes belonging to *Acinetobacter* sp. were detected, and a genus commonly found to colonize plants endophytically (Rasche et al. 2009; Thomas and Soly 2009).

By PCR analysis two types of *acdS* genes were found, a highly abundant one showing high homology to an *acdS* gene derived from *Pseudomonas* and an additional one showing moderate homology to an *acdS* gene derived from *Rhizobium*. Only the abundant *acdS* type was encountered

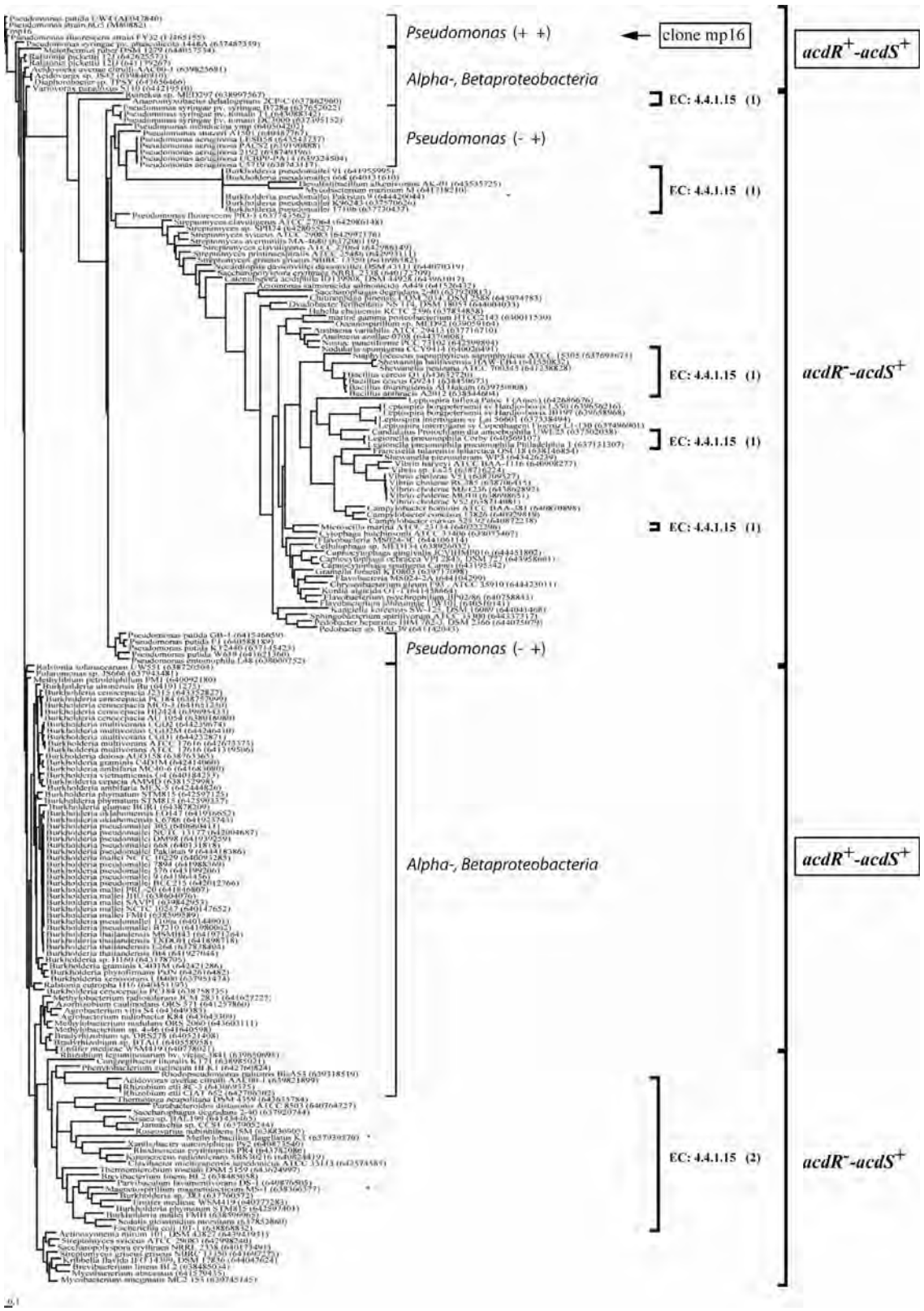

Fig. 3 Maximum likelihood phylogenetic tree of *acdS* genes from bacterial strains, which are fully sequenced over the *acdR–acdS* gene locus by JGI. *acdR*$^+$*–acdS*$^+$, strains with *acdR–acdS* gene cluster; *acdR*$^-$*–acdS*$^+$: strains with *acdS* genes but lacking an *acdR* gene. EC: 4.4.1.15 (1) and (2): genes homologous to *acdS* and D-cystein desulfhydrase gene, except those labelled with * (only *acdS* gene homology). (++, −+), + or − indicates the presence of *acdR* gene adjacent to *acdS* gene. Accession numbers of *acdS* gene sequences in the JGI and NCBI database are written in parenthesis after the strain names. The position of the metagenomic clone mp16 is indicated

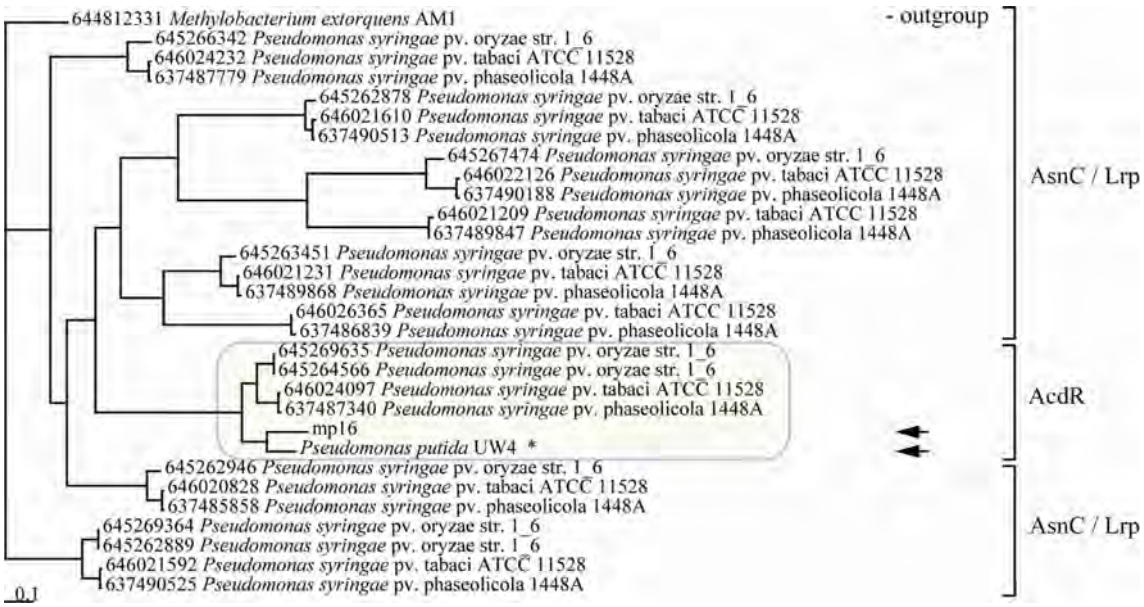

Fig. 4 Maximum likelihood phylogenetic tree of AsnC/Lrp transcriptional regulator protein sequences encoded in genomes of *acdR*$^{+}$–*acdS*$^{+}$ *Pseudomonas* strains. Groups of AsnC/Lrp transcription regulators and AcdR transcription regulators (encoded by *acdR* genes that are coupled with *acdS* genes) are indicated. Accession numbers of sequences in JGI or NCBI database (marked with *) are written before strain names. *Arrowheads* indicate the position of the AcdR protein from the metagenomic clone mp16 and *Pseudomonas putida* UW4

in the metagenomic libraries, despite the fact that the entry libraries contained app. 10^9 bp, which presumably covers more or less the potato endophyte metagenome. Nevertheless, the second type of the *acdS* gene might have been undetected due to its low abundance. Based on our results, we cannot conclude on the taxonomy of strains containing *acdS* genes.

Whereas current PCR protocols only allow the analysis of a rather short fragment of the *acdS* gene, we were able to analyse the entire operon and surrounding gene regions of an uncultured endophyte. Sequencing of one metagenomic clone revealed the presence of a full-length *acdS* gene is 95% identical to that found in *P. fluorescens* strain FY32. The metagenomic clone containing this gene also showed ACC deaminase activity. Furthermore, upstream of *acdS,* the regulatory *acdR* gene was identified. Similarly, this operon was identified in *P. putida* UW4, which has been used as a model to study the regulation of ACC deaminase. In strain UW4, expression of the *acdR* gene is absolutely necessary to induce ACC deaminase production (Grichko and Glick 2000; Li and Glick 2001) (Fig. 2). The Lrp of *E. coli* could not substitute AcdR to induce expression (Li and Glick 2001). Due to the numerous studies performed with strain UW4, our perception is that *acdS* is generally regulated by *acdR*. However, by analysis of available, fully sequenced bacterial genomes, we for the first time found that the *acdR–acdS* gene cluster is rather rare and typically occurs in few alpha- and betaproteobacterial genera. Among 195 genomes containing an *acdS* gene and affiliating to 72 genera, only 58 genomes contained an *acdR–acdS* gene cluster with the majority genomes (42) belonging to *Burkholderia*. The *acdR–acdS* gene cluster was found in four alpha-, six betaproteobacterial genera and in only three *Pseudomonas syringae* strains indicating that this operon is rather uncommon among *Gammaproteobacteria*. Considering the information found in genomes sequenced so far, the presence of the *acdR–acdS* operon is rather rare in *Pseudomonas*, but is commonly found in *Burkholderia* strains.

The AcdR regulator protein belongs to the family of AsnC/Lrp-family regulator proteins (Grichko and Glick 2000; Thaw et al. 2006). We showed that AcdR regulator proteins are evolutionary conserved in *acdR*$^{+}$–*acdS*$^{+}$-containing *Pseudomonas* strains and that they are only moderately homologous to related AsnC/Lrp regulators encoded in genomes of other fully sequenced *Pseudomonas* strains. Strains lacking the *acdR* gene next to the *acdS* gene also do not possess an *acdR* homologue elsewhere in the genome. The regulation of *acdS* genes in strains lacking the AcdR regulator protein is not known. However, ACC deaminase activity is common in many strains not belonging to those genera typically containing the AcdR regulator protein (Rasche et al. 2006a; Idris et al. 2004), we conclude that at least some of these strains *acdS* genes are functional as well.

Several *acdR*$^{-}$–*acdS*$^{+}$-type *acdS* genes showed high homology to the D-cysteine desulfhydrase gene. D-cysteine desulfhydrase is an enzyme that belongs to the same family as ACC deaminase, but utilizes D-cysteine as a substrate.

Todorovic and Glick (2008) found that the alternation of two amino acid residues within the predicted active site of D-cysteine desulfhydrase converts the substrate specificity of the enzyme to ACC deaminase and vice versa. This leads to the assumption that ACC deaminase in *acdR*$^-$–*acdS*$^+$ strains might have originated from D-cysteine desulfhydrase as a result of gene mutations that changed amino acids in the active site of ancestor enzyme resulting in altered substrate specificity. This observation also indicates that PCR-based detection of *acdS* gene fragments does not necessarily indicates ACC deaminase activity but might detect also D-cysteine desulfhydrase.

ACC deaminase activity is typically found in plant-associated bacteria; however, little information is available on the activity level, whether all strains show activity, how these genes are regulated and how they interact with plants. Similarly, the regulation and level of activity has not been analysed so far in *acdR*$^-$–*acdS*$^+$-type *acdS* genes. Anyhow, the majority of strains carry an *acdS* gene that lack an adjacent *acdR* gene and might be consequently differently regulated.

In conclusion, we were able to analyse the whole *acdR*$^+$-*acdS*$^+$ operon and surrounding gene regions of an *acdS* gene of a bacterial endophyte colonizing potato plants without the need of cultivation by applying metagenomics. Analysis of the entire operon yielded information on the type of activity and regulation, and ACC deaminase activity was also confirmed. A major limitation of PCR-based analysis is that usually few genes or gene regions are targeted and therefore cannot elucidate differences in activity or functionality. Metagenomic analysis, however, can complement PCR-based analysis and yield information on whole gene operons.

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