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1-Aminocyclopropane-1-Carboxylate Deaminase from *Pseudomonas fluorescens* Promoting the Growth of Chinese Cabbage and Its Polyclonal Antibody

Byoung Yul Soh[1,2], Gun Woong Lee[1], Eun Byeul Go[1], Byeo-Ri Kim[1], Kui-Jae Lee[1,3]*, and Jong-Chan Chae[1,3]*

[1]*Division of Biotechnology, Chonbuk National University, Iksan 570-752, Republic of Korea*
[2]*Department of Biochemistry, College of Medicine, Seonam University, Namwon 590-711, Republic of Korea*
[3]*Advanced Institute of Environment and Bioscience, Chonbuk National University, Iksan 570-752, Republic of Korea*

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**Corresponding authors*
J.-C.C.
Phone: +82-63-850-0840;
Fax: +82-63-850-0834;
E-mail: chae@jbnu.ac.kr
K.-J.L.
Phone: +82-63-850-0836;
Fax: +82-63-850-0834;
E-mail: leekj@jbnu.ac.kr

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Bacterial 1-aminocyclopropane-1-carboxlyate (ACC) deaminase (AcdS) is an enzyme that cleaves ACC, a precursor of the plant hormone ethylene, into α-ketobutyrate and ammonia. The *acdS* gene was cloned from *Pseudomonas fluorescens*, which was capable of improving the seedling of Chinese cabbage under salinity condition. The recombinant AcdS (rAcdS) exhibited optimal activity at pH 8.5 and 30°C. Strong activity was sustained at up to 100 mM NaCl. The polyclonal anti-*P. fluorescens* AcdS antibody was produced in a rabbit that had been immunized with the purified rAcdS. This antibody successfully recognized the homologous antigens derived from the total proteins of isolated plant growth-promoting microorganisms. A statistically significant correlation was observed between the intensity of hybridization signal and AcdS activity measured by a biochemical method, suggesting its application as a useful indicator for active deaminases.

Keywords: *Pseudomonas fluorescens*, antibody, deaminase

Introduction

Ethylene, a plant hormone, plays an important role in plant growth and development, and is also known as being a stress hormone [4, 19]. It regulates a wide variety of developmental processes, including germination, root hair initiation, root and shoot primordial formation and elongation, leaf and flower senescence and abscission, and fruit ripening [2]. The production of ethylene in plants is associated with certain environmental and biological factors such as temperature, light, gravity, nutrient, salt, and the other hormones [19]. In spite of its advantageous effect on plants, the overproduction of ethylene in plant tissues retards root growth and causes plant senescence [14]. Thus, increased ethylene level in plants corresponds not only to induction of defense responses that help to enhance the survival of the plant under adverse conditions, but it also causes stress symptoms [7].

1-Aminocyclopropane-1-carboxlyate (ACC), a precursor in the biosynthetic pathway of ethylene in plants [4], is converted to ethylene by ACC oxidase. A variety of different stresses stimulate ACC synthesis, resulting in an increased production of ethylene in plant tissue [7, 15]. ACC deaminase (AcdS) is an enzyme that converts ACC to α-ketobutyrate and ammonia [21]. AcdS-containing plant growth-promoting bacteria (PGPB) bind to plant roots and reduce the endogenous ACC level of root tissue, resulting in alleviated production of ethylene in plants [20]. This mechanism enhances the growth of plants under various stress conditions [7]. Many PGPB strains, such as *Pseudomonas* [5, 22], *Methylobacterium* [16, 17], *Rhizobium* [14], *Enterobacter* [23], and *Bacillus* [6], have been reported to protect plants from the injurious effects of ethylene.

The degree of salinity is one of the major environmental stresses that hamper plant growth. Salt stress causes epinasty, shorter roots, and premature senescence in plant

[9]. Plants under high salt conditions produce high levels of ethylene in tissue *via* elevated levels of ACC [18]. In order to facilitate plant growth under these conditions, the application of PGPB could be a protective strategy [18]. *Pseudomonas* strains are well known to have beneficial effects on not only plant growth, but also plant vitality under abiotic stress including high salinity [5, 11, 22, 24] although the mechanism of PGPB interaction with the plant under salt stress is not clearly known [22].

Herein, we identified the AcdS originated from the strain *P. fluorescens* (KACC10070) that promoted the growth of Chinese cabbage under salt stress. In addition, the purified recombinant AcdS was used to immunize a rabbit in order to produce polyclonal anti-*P. fluorescens* AcdS antibody (anti-*Pf* AcdS Ab), which showed potential as an indicator for PGPB containing active AcdS.

Materials and Methods

Microorganisms and Cloning of the ACC Deaminase Gene

The *Pseudomonas fluorescens* strain KACC10070 was obtained from the Korean Agricultural Culture Collection (KACC, South Korea). This bacterium was incubated on nutrient agar (NA) plates at 30°C.

The soil microorganisms were isolated from the rhizosphere of halophytes in Sunchon Bay (Jeollanamdo, South Korea). A sole nitrogen source of 3 mM ACC (Tokyo Chemical Industry, Japan) was used in the DF salt minimal medium. In order to identify the strains, the 16S rRNA gene was obtained from each genomic DNA by polymerase chain reaction (PCR) with the following reaction mixture: 0.2 μM of each primer, 100 ng of genomic DNA as the template, and the EmeraldAmp MAX PCR Master Mix (Takara, Japan). The reactions were initiated at 95°C (2 min), followed by 30 cycles of denaturing at 95°C (30 sec), annealing at 55°C (30 sec), and elongation at 72°C (1.5 min), and ended with incubation at 72°C for 10 min. The PCR amplification and sequencing of the 16S rRNA gene were conducted using universal primers (27F, 5′-AGAGTTTGATCATGGCTCAG-3′; 1492R, 5′-GGCTACCTTGTTACGACTT-3′).

The *acdS* gene was amplified from the genomic DNA of strain KACC10070. PCR was performed to amplify the *acdS* structural gene without the stop codon using the following primers: forward 5′-C*GAGCTC*GATGAACCTGAATCGTTTTGAACGTTATCCG-3′ and reverse 5′-C*AAGCTT*GGCCGTTGCGAAACAAGAAGCTGTAG-3′. The forward and reverse primers were designed on the basis of the *acdS* gene of *Pseudomonas* sp. AT14 (GenBank Accession No. EF011161) and contained *Sac*I and *Hin*dIII sites (in italics) at each 5′-end, respectively. The PCR conditions were the same as described above. The reactions were initiated at 95°C (2 min); followed by 30 cycles of denaturing at 95°C (30 sec), annealing at 55°C (30 sec), and elongation at 72°C (1.2 min); and ended with incubation at 72°C for 10 min. The PCR product was cloned into the pGEM-T easy vector (Promega, USA). The recombinant plasmid was digested with *Sac*I and *Hin*dIII (Takara, Japan), and the fragment containing the *acdS* gene was ligated into pQE31 (Qiagen, USA) that was digested with the same endonucleases. The resultant was transformed into *E. coli* M15 strain (Qiagen, USA).

The nucleotide sequences were determined by SolGent Co., Ltd. (South Korea). The sequence data were assembled and analyzed using the Lasergene software (DNASTAR, Inc., USA) and then compared with existing sequences in the GenBank database by performing a BLAST search [3]. The nucleotide sequences of the *acdS* gene obtained in this study have been deposited under GenBank Accession No. JQ646055.

Purification of Active Recombinant AcdS

The transformant *E. coli* harboring the recombinant plasmid was cultivated in 500 ml of LB medium containing 50 μg ampicillin/ml and 25 μg kanamycin/ml at 37°C with shaking (250 rpm) until reaching an OD_{600} of 0.6. Expression was induced by adding IPTG to a final concentration of 1 mM. The cultures were incubated for an additional 4 h and harvested by centrifugation at 4,000 ×*g* at 4°C for 20 min.

The IPTG-induced cells were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) at 5 ml per gram wet weight and treated with 1 mg lysozyme ml^{-1} for 30 min on ice. The cells were ruptured by sonication on ice using a sonicator (Vibra Cell, Sonics & Materials Inc., USA) equipped with a microtip that was programmed for six 10-sec bursts at 200–300 W, with a 10-sec cooling period between each burst. The cell lysate was centrifuged at 12,000 ×*g* for 30 min at 4°C. The supernatant was transferred to a new tube, and 1 ml of the 50% (w/v) Ni-NTA slurry (Novagen, USA) was added. The solution was gently mixed for 60 min at 4°C. The protein-bound resin was loaded onto a column and washed two times with 4 ml of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0). The bound proteins were eluted four times with 0.5 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The eluted proteins were quantified using the Bradford protein assay solution (Bio-Rad, USA).

Measurement of AcdS Activity

The AcdS activity was estimated by measuring the amount of α-ketobutyrate generated from the cleavage of ACC, according to the method of Penrose and Glick [21] with slight modification. The produced α-ketobutyrate was determined spectrophotometrically and compared with a standard curve of α-ketobutyrate ranging from 0.5 to 5 μmol.

The bacterial cell pellet was resuspended in 1 ml of 0.1 M Tris-HCl (pH 8.5). Thirty microliters of toluene was added to the cell suspension and vortexed. Two hundred microliters of the toluene-treated cells was placed in new microcentrifuge tube. The suspension was treated with 20 μl of 0.5 M ACC dissolved in 0.1 M Tris-HCl (pH 8.5), briefly vortexed, and then incubated at

30°C for 15 min. Following the addition of 1 ml of 0.56 M HCl, the mixture was vortexed and centrifuged for 5 min at 15,000 ×*g* at room temperature. One milliliter of the supernatant was treated with 800 µl of 0.56 M HCl.

The prepared samples were transferred to a new tube, and 300 µl of 0.2% (w/v) 2,4-dinitrophenylhydrazine (Sigma, USA) diluted in 2 M HCl was added. The mixtures were vortexed vigorously and incubated at 30°C for 30 min. During this time, the α-ketobutyrate was derivatized to phenylhydrazone. The color of the phenylhydrazone was developed by adding 2 ml of 2 N NaOH. The absorbance of the mixture was measured at 540 nm with a spectrophotometer (UV-1800; Shimadzu, Japan).

In order to characterize the AcdS, 10 µg of enzymes was prepared in 150 µl of corresponding buffers, and the reaction was initiated by adding 20 µl of 0.5 M ACC to the enzyme mixture after incubation for 15 min. For pH-dependence studies, 0.1 M sodium acetate buffer (pH 4.5–7.0), 0.1M Tris-HCl buffer (pH 6.0–9.0), and 0.2 M glycine buffer (pH 8.5–11.5) were used. For temperature-dependence studies, 10 µg of recombinant AcdS was incubated in reaction buffer (0.1 M Tris-HCl, pH 8.5) from 5°C to 45°C for 10 min just before the activity measurement. Following the addition of 1.5 ml of 0.56 M HCl, the mixture was vortexed. For salinity-dependence studies, 10 µg of recombinant AcdS was incubated in the reaction buffer containing NaCl (0–2 M) at 30°C for 10 min.

SDS-PAGE and Western Blot Analysis

The protein samples were subjected to SDS-PAGE on a 10% (w/v) gel with a Mini-PROTEAN III electrophoresis apparatus (Bio-Rad, USA) and stained with Coomassie brilliant R-250 blue. To confirm the presence of the recombinant AcdS, the separated proteins were transferred onto a nitrocellulose membrane (Hybond-ECL; Amersham Bioscience, Germany). The membrane was blocked with 5% (w/v) skim milk for 2 h at room temperature, incubated with mouse anti-RGS-His antibody (Qiagen, USA) at 1:5,000 dilution or polyclonal AcdS antiserum from rabbit at 1:2,500 in 5% (w/v) skim milk for 2 h at room temperature, and treated with the goat anti-mouse conjugated-HRP antibodies (GE Healthcare Life Science, USA) at 1:5,000 dilution or the goat anti-rabbit conjugated-HRP antibodies (Promega, USA) at 1:5,000 dilution in 1% (w/v) skim milk for 1 h at room temperature.

The hybridization band intensity was determined by image analysis software (Multi-Gauge var. 3.0; Fujifilm, Japan). The correlation coefficient was determined by Pearson's correlation coefficient.

Production of Polyclonal *P. fluorescens* AcdS Antiserum

The polyclonal anti-*Pf* AcdS Ab was generated by immunization with 500 µg of purified recombinant AcdS in complete Freund's adjuvant, using multiple subcutaneous inoculations. The rabbit was boosted two times with 2 mg of the same antigen in incomplete Freund's adjuvant in the same manner. The animal was bled by protocols approved by the Institutional Animal Care and Use Committee (IACUC), and the serum was obtained using standard protocols [12].

Seed Germination and Seedling Growth Assays

The Chinese cabbage seeds were surface-sterilized for 5 min in 70% ethyl alcohol and then for 5 min in a mixing buffer (2% sodium hypochlorite, 50% (v/v); dH_2O, 49.8% (v/v); Triton X-100, 0.2% (v/v)) with agitation. After immersion, the seeds were rinsed 10 times with sterile water. *P. fluorescens* was grown on LB broth for 48 h at 30°C. The growing bacterial cells were harvested by centrifugation at 4,000 ×*g* for 15 min and resuspended in sterilized distilled water. The suspension was calculated using a UV/VIS spectrophotometer to approximately A_{600} = 1.0. The disinfected seeds were soaked in 5 ml of bacterial suspension (A_{600} = 1.0) for 60 min. Then, they were sown in a soil mix (soil/peat/perlite = 1/1/1) in plastic pots (12 cm diameter). A salinity level was adjusted to 200 mM using NaCl dissolved in distilled water for salt stress condition. After nine Chinese cabbage seeds were sown per pot, each pot was maintained in a growth chamber at 25°C with day/night cycles (light 14 h/dark 10 h) for 6 days. The germination percentage of the seeds was recorded and the vigor index was calculated using the following formula: Vigor index = percentage of germination × seedling length (shoot and root length) [1].

Results and Discussion

Promoted Seedling of Chinese Cabbage Under Salinity Stress

Salinity stress conditions are known to suppress seedling and plant growth [11, 18]. Bacteria exhibiting AcdS activity have been reported to confer resistance to various stresses, including salinity, through the alleviation of endogenous ethylene in plant tissues [8]. Severe inhibition of the Chinese cabbage seedlings was observed with increased concentration of salt (Table 1).

Table 1. Growth parameters of Chinese cabbage as influenced by *P. fluorescens* KACC10070.

Growth condition	Shoot length (mm)	Root length (mm)	Seed germination (%)	Vigor index
Control[a]	11.6 ± 1.3	13.1 ± 2.7	100.0	2,466.7
Non-treatment[b]	5.0 ± 1.4	5.5 ± 0.7	22.2	233.1
Treatment[c]	7.0 ± 1.1	6.0 ± 1.3	88.8	1,154.4

[a]0 mM NaCl; [b]200 mM NaCl; [c]200 mM NaCl + *P. fluorescens* KACC10070.

Values are averages from three experiments.

P. fluorescens KACC10070 was capable of utilizing ACC as a sole nitrogen source and exhibited AcdS activity. In order to elucidate the effects of this strain that conferred promotion of plant growth and tolerance to salt to the Chinese cabbage, the seeds coated with this strain were sown in pots under salinity conditions. The Chinese cabbage seeds that had been treated with this bacterium showed significant improvement in seedling at NaCl concentrations less than 200 mM compared with the non-treated control (Table 1). This result indicates that the strain is one of the candidates as plant growth-promoting bacteria.

Protein Expression and Characterization of Recombinant *P. fluorescens* AcdS

The *acdS* gene was amplified from the genomic DNA of strain KACC10070 and cloned in the pQE31 vector. The recombinant AcdS (rAcdS) with His-Tag was expressed in *E. coli* M15 by induction with IPTG. The expressed rAcdS was approximately 39.1 kDa in size and consisted of 357 amino acid residues including *N*-terminal tagged residues with six consecutive histidines (His-Tag). The actual molecular mass of *P. fluorescens* AcdS was 37 kDa consisting of 338 amino acids, which was similar to other bacterial AcdSs, multimeric enzymes with a monomeric subunit molecular mass of approximately 35 to 42 kDa [7]. When the deduced amino acid sequence of the *acdS* gene was compared with other characterized AcdSs from different strains and species, the highest sequence identity was 95.3% with AcdS of *P. putida* (data not shown). Based on the findings of Karthikeyan *et al*. [10], the active site of the *P. putida* AcdS associated with pyridoxal 5′-phosphate (PLP)-dependent catalysis was composed of Lys51, Lys54, Ser78, Asn79, Thr199, Thr202, Tyr268, Tyr294, and Glu295. The Tyr294 played a critical role as a nucleophile in the opening of the cyclopropane ring of ACC. The nine residues of the active site were all conserved among the compared sequences except those from *Bacillus* spp. and *Enterobacter cloacae* UW4 (data not shown), indicating the possibility of different catalytic mechanisms [13]. The *E. cloacae* AcdS was known to be distinct from the other bacterial AcdS and consisted of 232 amino acids. It belonged to the amidohydrolase superfamily, which hydrolyzes ACC through metal-binding catalysis instead of the pyridoxal phosphate-dependent reaction [13]. Actually, the sequence identity between the AcdSs of *P. fluorescens* and *E. cloacae* was only approximately 11.8% at the amino acid level.

The rAcdS was purified through Ni-NTA affinity chromatography of which the purification yield was over 20 folds and the purity was greater than 80%. When 1 unit (U) of rAcdS was defined as produced 1 nmol α-ketobutyrate per 1 mg of protein from ACC, the specific activity of purified rAcdS was 18,725 U/mg. We determined the optimal pH and temperature for rAcdS activity. Once the enzyme was exposed under each pH condition for 15 min at 30°C, the activity was measured. The enzyme

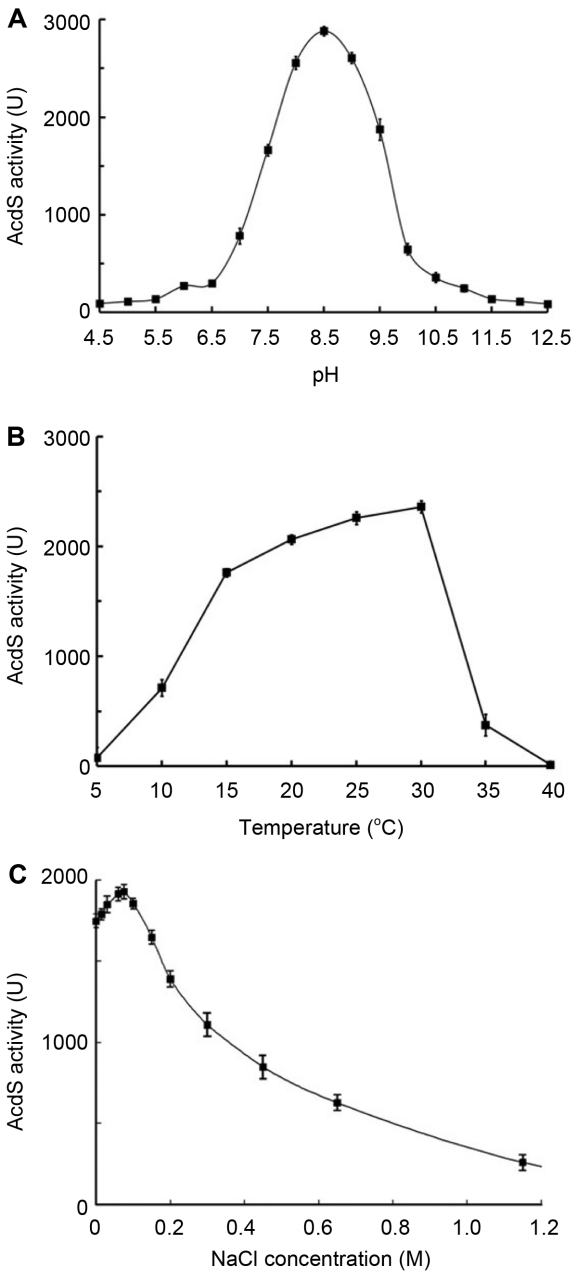

Fig. 1. Effects of pH (**A**), temperature (**B**), and salinity (**C**) on recombinant ACC deaminase activity.
Acetate (pH 4.5–6.5) and Tris (pH 5.5–11.5) buffers were used to examine the effect of pH. Data are presented as the mean ± SD.

exhibited optimal activity at pH 8.5 and sustained significantly stable activity in the pH ranges from 8.0 to 9.0 (Fig. 1A). However, the activity was lost below pH 6.5 and above pH 10.5. The optimal activity for rAcdS was observed at 30°C, and the activity was preserved from 15°C to 25°C, although there was no activity below 4°C or above 35°C (Fig. 1B). In addition, AcdS sustained strong activity at NaCl concentrations less than 100 mM, and the optimal salinity condition for activity was 75 mM NaCl (Fig. 1C). However, the AcdS activity decreased under high salinity concentrations greater than 150 mM NaCl.

Production and Reactivity of Polyclonal Anti-*Pf* AcdS Ab

The polyclonal anti-*Pf* AcdS Ab was obtained from a rabbit that had been immunized with purified rAcdS. The specific hybridization was verified by western blotting, in which the 39 kDa rAcdS was reacted with the anti-*Pf* AcdS Ab and also strongly hybridized with anti-His-tag antibody, showing the same size bands on each reaction (data not shown). This finding suggests that both antibodies in the blotting hybridize with the same proteins. The anti-*Pf* AcdS Ab was successively hybridized with both purified rAcdS of 39 kDa and native AcdS of 37 kDa in the cell lysate of strain KACC10070 (data not shown). Since rAcdS has His-Tag on its *N*-terminus, it is 2 kDa greater in size than the native form. In order to identify the homologous proteins to AcdS, isolated rhizobacterial strains were examined by western blotting using the polyclonal antibody along with measuring the AcdS activity through the biochemical method [21]. The used strains were isolated from Sunchon Bay for the capability of utilizing ACC as a sole nitrogen source and identified as *Enterobacter*, *Stenotrophomonas*, and *Pseudomonas* strains by 16S rRNA gene sequences. Hybridization of the total proteins of the bacterial strains showing AcdS activity with anti-*Pf* AcdS Ab successfully generated 37 kDa bands, as shown in Fig. 2A. In addition, the signal intensity was likely to have a correlation with the AcdS activity measured by the colorimetric method [21]. In order to investigate the association between the levels of hybridization and the enzyme activities, we determined the band intensity of each sample using image analysis software. As shown in Fig. 2B, a statistically significant correlation was observed between the band intensity and AcdS activity ($r = 0.907$, $p = 0.002$; Pearson's correlation coefficient by SPSS var. 12.0 program), implying that the Ab might have significant potential for biomonitoring of active AcdS.

The AcdS from genus *Stenotrophomonas* has still not been identified. However, the isolated *Stenotrophomonas* was expected to have a closely homologous AcdS to the

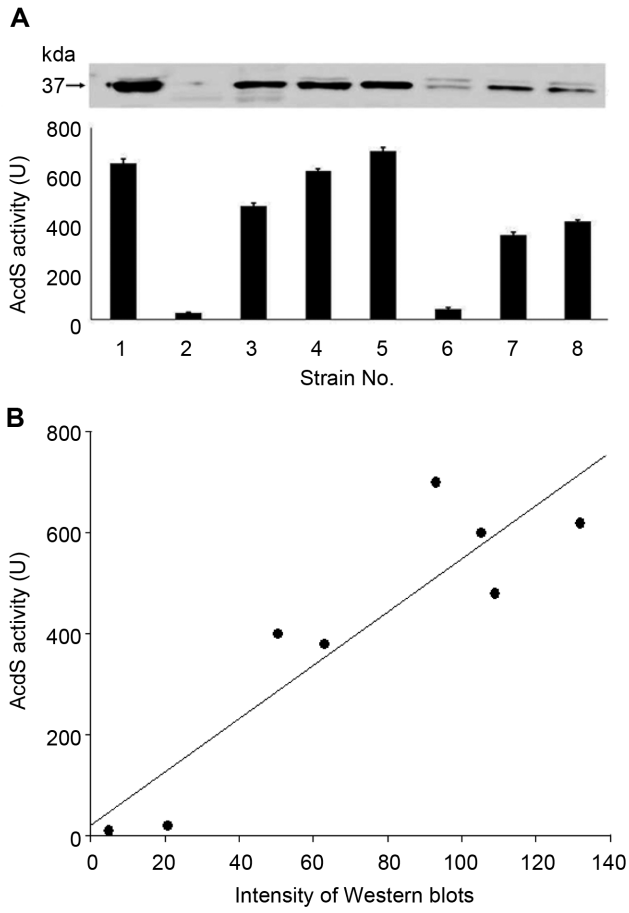

Fig. 2. Detection of AcdS in soil microorganisms isolated from Sunchon Bay by western blotting and the comparison with biochemical analysis.

(**A**) Western blotting of bacterial ACC deaminases using polyclonal anti-AcdS antibody and the corresponding enzyme activities measured by a colorimetric method. Hybridization was performed with anti-AcdS serum (1:2,500) and the hybridization signals show the 37 kDa AcdS band in each sample. Lane 1, *P. fluorescens* KACC10070; lane 2, *Enterobacter*; lane 3, *Stenotrophomonas*; lanes 4–8, *Pseudomonas*. (**B**) Correlation between the intensities of western blotting and the enzyme activities ($r = 0.907$, $p = 0.002$; Pearson's correlation coefficient). The band intensities in the western blots were determined by Multi-Gauge software.

P. fluorescens AcdS with a similar protein structure and size, since the polyclonal antibody recognized the epitope in the extracted total protein and the strain capable of utilizing ACC for growth exhibited AcdS activity (lane 3 in Fig. 2A). On the other hand, another isolate, *Enterobacter*, grew on minimal medium containing ACC as a sole nitrogen source but exhibited no AcdS activity and negligible hybridization signal in the western blot (lane 2 in Fig. 2A).

It suggested that the isolated *Enterobacter* utilized ACC *via* an unknown catabolic reaction rather than using hydrolysis of ACC catalyzed by metal-binding or pyridoxal phosphate-dependent AcdS.

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