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1-Aminocyclopropane-1-Carboxylate Deaminase from *Pseudomonas stutzeri* A1501 Facilitates the Growth of Rice in the Presence of Salt or Heavy Metals

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1-Aminocyclopropane-1-carboxylate (ACC) deaminase, which is encoded by some bacteria, can reduce the amount of ethylene, a root elongation inhibitor, and stimulate the growth of plants under various environmental stresses. The presence of ACC deaminase activity and the regulation of ACC in several rhizospheric bacteria have been reported. The nitrogen-fixing *Pseudomonas stutzeri* A1501 is capable of endophytic association with rice plants and promotes the growth of rice. However, the functional identification of ACC deaminase has not been performed. In this study, the proposed effect of ACC deaminase in *P. stutzeri* A1501 was investigated. Genome mining showed that *P. stutzeri* A1501 carries a single gene encoding ACC deaminase, designated *acdS*. The *acdS* mutant was devoid of ACC deaminase activity and was less resistant to NaCl and $NiCl_2$ compared with the wild-type. Furthermore, inactivation of *acdS* greatly impaired its nitrogenase activity under salt stress conditions. It was also observed that mutation of the *acdS* gene led to loss of the ability to promote the growth of rice under salt or heavy metal stress. Taken together, this study illustrates the essential role of ACC deaminase, not only in enhancing the salt or heavy metal tolerance of bacteria but also in improving the growth of plants, and provides a theoretical basis for studying the interaction between plant growth-promoting rhizobacteria and plants.

Keywords: *Pseudomonas stutzeri* A1501, ACC deaminase, ethylene, biology nitrogen fixation, stress

Introduction

Plant growth-promoting rhizobacteria (PGPR) can facilitate the growth and development of their plant host both directly and indirectly by involving growth stimulation or disease prevention/suppression [38]. Certain PGPR carry the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which can promote plant growth and development by cleaving ACC, the intermediate precursor of ethylene biosynthesis in plants, into α-ketobutyrate and ammonia, thereby decreasing ethylene levels in plants [17, 18, 21]. Ethylene is a gaseous plant growth hormone that is produced endogenously by almost all plants. Apart from being an essential plant growth regulator, it was also reported to be a stress hormone. Its overproduction can be induced by biotic and abiotic stresses such as extreme temperature, pathogenicity, flooding, drought, salt, and heavy metals [4]. As a result, high-level ethylene can retard root growth and consequently the growth of the plant as a whole. Research has shown that the inoculation of plants with PGPR producing ACC deaminase lowered the ethylene level in plants and eliminated or at least alleviated the

ethylene-mediated negative impact on plants under a variety of environmental stresses [6, 13, 16, 18, 21, 28, 37].

Given the important role of ACC deaminase in the promotion of plant growth, this enzyme, encoded by the *acdS* gene, has been studied extensively in numerous species of PGPR isolated from various geographical regions and ecosystems [29, 31]. However, most *acdS* genes were identified based on only partial sequences, and some of the strains have been poorly characterized taxonomically. Moreover, the lack of ACC deaminase-inactivated mutant negative controls shadows the importance of ACC deaminase, although its positive effect on plant growth and development has been demonstrated using wild-type isolates with ACC deaminase activity [25, 26, 45].

Pseudomonas stutzeri A1501 (China General Microbiological Culture Collection Center Accession No. 0351) is a highly effective PGP rhizobacterium. It was first isolated from rice paddy soils in South China and was originally designated as *Alcaligenes faecalis* A15 [11]. *P. stutzeri* A1501 is also a versatile soil bacterium that has the ability to fix nitrogen and perform nitrification, denitrification and endophytic association with rice plants [34, 48]. Moreover, inoculation with *P. stutzeri* A1501 can promote the growth of rice [11]. The association of *P. stutzeri* A1501 with rice plants is a promising model system for the study of plant-microbe-soil interactions in the rhizosphere.

In this work, we used bioinformatics, mutation, and complementation experiments followed by a series of physiological assays to analyze the function of ACC deaminase of *P. stutzeri* A1501. We then discuss the role of ACC deaminase in promoting rice growth under salt and heavy metal stresses. Our study has provided solid proof of the importance of ACC deaminase in plant growth promotion, as well as a theoretical basis to study the interaction between PGPR and plants.

Materials and Methods

Growth Conditions

The plasmids and bacterial strains used in this study are listed in Table 1. *P. stutzeri* A1501 and its mutant derivative were grown at 30°C in Luria-Bertani medium, Tryptic Soybean Broth medium, DF salt minimal medium [12], or in minimal lactate-containing medium (medium K), as described previously [11]. *Escherichia coli* and its mutant strains were grown in LB medium at 37°C. The antibiotic tetracycline (Tc), ampicillin (Amp), or kanamycin (Km) was added to the medium at a concentration of 10, 100, or 50 μg/ml, respectively, as required.

Rice was grown in a growth chamber under a controlled environment and illuminated using cool-white fluorescent lights with a light/dark cycle of 16 h/8 h at 28°C.

Construction of Mutants

An *acdS* nonpolar mutant was constructed by homologous suicide plasmid integration, as described previously [47], using pK18mob as the vector [40]. A 292 bp internal fragment of the *acdS* gene was amplified from the genomic DNA of *P. stutzeri* A1501 using appropriate oligonucleotide primers (*acdS*-M-F (*Eco*RI): 5′-CTGTAGGAATTCCGGAAACAAGTGGT-3′; and *acdS*-M-R (*Bam*HI): 5′-ATATTAGGATCCAGTAACCGCGATGG-3′), which were designed to generate amplicons for the creation of nonpolar mutations without interfering with the transcription of downstream genes. The PCR product was cloned into the multiple cloning sites of the pK18mob vector, and the resulting plasmid, pacdS, was introduced into *P. stutzeri* A1501 from *E. coli* JM109 by triparental conjugation using pRK2013 [14] as the helper plasmid. The nonpolar mutant strain A1815 with the *acdS* gene silenced but

Table 1. Strains and plasmids used in this study.

Strain and plasmid	Genotype or phenotype	Source or reference
Strain		
P. stutzeri A1501	Wild-type *Pseudomonas stutzeri* A1501	Yan *et al.* [48]
A1815	*Pseudomonas stutzeri* A1501 *acdS* insertion mutant, Km^r	This work
Comp *acdS*	A1815 harboring complement plasmid pLacdS	This work
Plasmid		
pK18mob	Km^r; *ori*ColE1 Mob^+ *lacZá*$^+$, used for directed insertional disruption	Schafer *et al.* [40]
pLAFR3	Tcr; Tra^-, Mob^+, *cos*, RK2 replicon	Staskawicz *et al.* [43]
pRK2013	ColE1 replicon with RK2 *tra* genes Helper plasmid used for mobilizing P-and Q-group cloning vector; Tc^r	Figurski & Helinski [14]
pGD926	Tc^r IncP *lacZ* fusion vector	Sambrook & Russell [39]
pacdS	Km^r; 292 bp *Eco*RI-*Bam*HI fragment containing part of *acdS* in pK18mob	This work
pLacdS	Tc^r; 1.5 kb *Eco*RI-*Hin*dIII fragment containing *acdS* in pLAFR3	This work

without blocking the transcription of its downstream genes was thereby generated. Correct recombination was confirmed by PCR analysis using appropriate oligonucleotide primers (PK18conF: GCCGATTCATTAATGCAGCTGGCAC; and Y*acdS*: TCAGGG TGCAGCGGTTTGTTC). For further complementation assays, the complete *acdS* gene was amplified from the genomic DNA of *P. stutzeri* A1501 using appropriate oligonucleotide primers (*acdS*-C-F (*Eco*RI): GCCGAATTCGCCGTTTGTTGATTT; and *acdS*-C-F (*Hin*dIII): GCCCAAGCTTTGTTCATCCTGG). *Eco*RI and *Hin*dIII were used to digest the DNA fragment of the *acdS* gene, which was then ligated into the broad host vector pLAFR3 to construct the complementation plasmid, pLacdS, as described previously [43]. The complementation strain was designated Comp *acdS*.

Sensitivity Assays

For the sensitivity assay, the strains were cultured in medium K at 30°C overnight to reach stationary phase. Bacterial cells from the overnight culture were then subjected to the stress sensitivity assays. The starter cultures were diluted 1:50 in fresh liquid LB medium, and the cells were grown for 3 h until an $OD_{600} < 0.75$ was reached. Then, salt (1 M NaCl in LB) or heavy metal (13.2 μmol in LB) was added to the growth medium, and the cultures were grown for another 3 h. As a negative control, autoclaved distilled water was added to a culture of each treatment. After treatment, 1 ml aliquots of the bacterial cultures were sampled and diluted to 1×10^{-4}, and 10 μl of every dilution was dripped onto LB plates, which were incubated at 30°C overnight before the colonies were enumerated. Each experiment was repeated at least three times.

Enzymatic Assays

Nitrogenase activity was determined according to the protocol described by Desnoues *et al*. [11]. In brief, cells from an overnight culture in LB medium were centrifuged and resuspended in a 50 ml flask containing 10 ml of N-free minimal medium K at an OD_{600} of 0.1. The suspension was incubated for 3 h at 30°C with vigorous shaking under an argon atmosphere containing 0.5% oxygen and 10% acetylene. Nitrogenase-specific activity was expressed as nmol ethylene/mg protein/h.

For ACC deaminase activity determination, recombinant *P. stutzeri* A1501 cells were grown as described in Penrose and Glick [32], and ACC deaminase activity was determined by measuring the production of α-ketobutyrate, as described by Honma and Shimomura [23]. ACC deaminase activity was expressed as μmol α-ketobutyrate/mg protein/h.

Protein concentrations were determined using the Bio-Rad protein assay. Each experiment was repeated at least three times.

RT-PCR and Quantitative Real-Time PCR

The procedure used to extract total RNA was as described

Table 2. Primers for RT-PCR and quantitative real-time RT-PCR.

Primer name	Sequence (5′-3′)	Amplified fragment[a]
PST1814F	CGTGCCGCCGTCTTTTT	382 bp internal fragment
PST1814R	TCCCGCTCGTGACTTTCG	
PST1815F	TGCCCCGCTGATTCTGC	280 bp internal fragment
PST1815R	CCGTCGGCGTGTCCTG	
PST1816F	TTCCTCCGCAGAACATACG	320 bp internal fragment
PST1816R	CGACCTCGCTCACCACC	
nifH-F	GAGATGATGGCGATGTATGC	113 bp internal fragment
nifH-R	GGTCGGTGTTGCGGCTGTTG	
nifD-F	ACATGATCCACATTTCCCACG	197 bp internal fragment
nifD-R	GAACAGCGTCTCGATCTCGTC	
nifK-F	TCGAGACCTACCTGGGCAACT	104 bp internal fragment
nifK-R	GGGGTATCGAGCACTTCTTCC	
Rpon-F	CTTCTTCTCCAGCCACGTCAG	137 bp internal fragment
Rpon-R	CCAGTAAACCAGCGATCTTGC	
nifA-F	CGCGAAGACCTCTACTACCG	134 bp internal fragment
nifA-R	CAGCTTGAGTTTGCGACCCT	
glnA-F	CGGAGCCGGAGTTCTTCATC	110 bp internal fragment
glnA-R	GACGTCCTGGTCGGTCATCC	
16S-F	CCTACGGGAGGCAGCAG	150 bp internal fragment
16S-R	ATTACCGCGGCTGCTGG	

[a]PCRs were carried out with the sets of primers indicated on the left.

previously [48]. Specifically, total RNA was isolated with an SV Total RNA Isolation System (Promega, Madison, WI, USA) and treated with RNase-free DNase I (Promega). The integrity of the RNA was analyzed by agarose gel electrophoresis. To check for DNA contamination, the samples were analyzed by PCR using primers for *nifH*. First-strand cDNAs were synthesized from 1 μg of total RNA in a 20 μl reaction volume using the Protoscript First-Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA).

For quantitative real-time PCR (Q-PCR) experiments, primer pairs, as shown in Table 2, were designed based on the genome sequence of *P. stutzeri* A1501 using the Primer Premier 5.0 software. The amplicons (100 to 200 bp) and reaction specificity were confirmed by agarose gel electrophoresis and product dissociation curves. Each sample was examined in triplicate using the ABI7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's recommendation; 16S rDNA was used as an internal control to normalize variation due to different reverse transcription efficiencies.

Evaluation of Rice Growth-Promotion Responses to Inoculation of Bacteria

Rice seeds were surface-sterilized with 75% ethanol for 2 min followed by with 50% sodium hypochlorite solution for 10 min, 99% sodium hypochlorite solution for 20 min, and 75% ethanol for 2 min, and then washed five times with sterile deionized water. Sterilized seeds were transferred to Petri dishes containing filter paper moistened with sterilized water. After 5 days of incubation in the dark at 30°C, the rice seedlings were transferred to tubes containing 10 ml of semisolid medium and a bacterial suspension containing wild-type A1501, A1815 (OD_{600} of 0.1; 8×10^7 CFU), or no bacteria. After 7 days of incubation, the root length, root fresh weight, root dry weight, and plant height were measured.

For the growth-promotion assays of *P. stutzeri* A1501 and A1815 in the presence of stresses, the same protocol as described above was used, except that salt (120 mM or 2 M NaCl) or a heavy metal (0.3 mM Co^{2+}, Cu^{2+}, Zn^{2+}, or Ni^{2+}) was added to the semisolid medium.

Results and Discussion

PGPR expressing ACC deaminase can enhance the growth of a wide range of plants in the presence of various biotic and abiotic stresses [15, 18, 21]. However, only a few studies have focused on the effects of ACC deaminase activity in nitrogen-fixing bacteria [7]. In this work, an *acdS* insertion mutant of the root-associated nitrogen-fixing *P. stutzeri* A1501 was constructed, and the function of the gene was investigated.

Sequence Analysis of the *acdS* Gene

While ACC deaminase has been suggested to play a key role in free-living plant growth-promoting bacteria, in the past few years, a large number of bacterial ACC deaminase genes have been isolated, characterized, and shown to encode active ACC deaminase [16, 18, 21]. The establishment of the complete genome sequence of *P. stutzeri* A1501 (GenBank Accession No. CP000304) revealed the presence of a single copy of an ACC deaminase-encoding gene (*acdS*, PST1815), and a BLAST search of the predicted ACC deaminase sequence returned many ACC deaminase homologs from other *Pseudomonas* species that function as predominant PGPR [33, 42]. High-identity homologs were found in the well-documented *P. putida* FCA-8 [2, 49] (66%), *P. fluorescens* Pf-5 (62%), and *P. aeruginosa* PF23 [1, 46] (62%), implying that the *acdS* gene in *P. stutzeri* A1501 may favor the growth promotion of its host rice.

To construct an *acdS* mutant without blocking the transcription of downstream genes, transcriptional units of *acdS*, with its adjacent genes, were identified by RT-PCR. As shown in Fig. 1A, *acdS* (PST1815) is adjacent to PST1814 (encoding a chromosome segregation ATPase) and PST1816 (encoding a cAMP-binding protein), with the same transcription

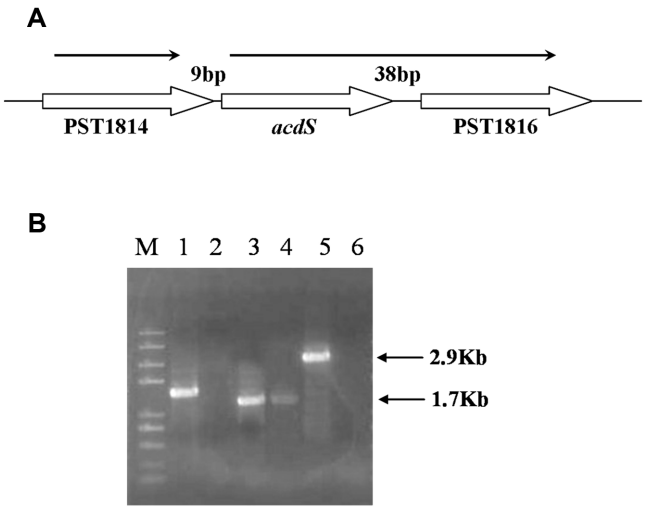

Fig. 1. Transcriptional organization of the three consecutive genes, PST1814–16, in the genome of *P. stutzeri* A1501.
(**A**) The number of nucleotides in noncoding regions is shown above the respective regions. Transcriptional units and directions are denoted by horizontal arrows in the upper panel. (**B**) RT-PCR analysis of mRNA transcripts using gel electrophoresis of the amplified cDNA fragments. The first lane was loaded with *Trans*2K PlusII DNA Marker; lanes 1, 3, and 5 were loaded with the PCR products of PST_1814/1815, PST_1815/1816, and PST_1814/1816 when using A1501 DNA; and lanes 2, 4, and 6 were loaded with the PCR products of PST_1814/1815, PST_1815/1816, and PST_1814/1816 when using A1501 cDNA.

orientation. The transcriptomic analysis of *P. stutzeri* A1501 on a series of growth conditions suggests that *acdS* and PST1816 are co-expressed (data not shown), and this was further confirmed by amplifying the intergenic regions between those adjacent genes. Three pairs of oligonucleotide primers were designed (Table 2). As shown in Fig. 2B, only one product with the expected size was obtained using the PF/PR primer pairs spanning the borders of *acdS*-PST1816, and no PCR product was observed when using the primer pairs spanning the borders of PST1814-*acdS* or PST1814–PST1816. These results indicated that *acdS* forms a co-expression unit with the downstream gene PST1816. Based on this result, a nonpolar insertion mutant of the *acdS* gene, named *P. stutzeri* A1815, was constructed by homologous suicide plasmid integration, as described previously [47]. We further detected the expression of PST1816 in the mutant strain A1815 by RT-PCR, and it was shown that although the *acdS* gene was disrupted, the adjacent gene, PST1816, was expressed by using the promoter from the pK18mob vector.

Properties of the *acdS* Mutant

We first tested the growth properties of the *acdS* mutant A1815 and wild-type *P. stutzeri* A1501 in different media. As shown in Fig. 2, no significant difference in the growth ability between A1501 and A1815 was observed in the liquid minimal medium DF or medium K (Figs. 2A and 2B). However, A1815 could not grow in ADF minimal medium with ACC as the sole nitrogen source, whereas the complemented strain restored the growth ability in this medium (Fig. 2C), suggesting that inactivation of the *acdS* gene resulted in loss of the ability to catalyze the cleavage of ACC into ammonia and α-ketobutyrate. Subsequently, the ACC deaminase activities of both strains were measured. When cultured in medium ADF with ACC as the sole nitrogen source, the ACC deaminase activity of *P. stutzeri* A1501 was 3.804 ± 0.096 mmol α-ketobutyrate/mg protein/h, whereas no activity was detected in the mutant strain A1815. These results are consistent with the properties of the *acdS* mutants of *P. putida* GR12-2 [19], *Burkholderia phytofirmans* PsJN [45], *B. unamae* MTl-641^T [30], and *Enterobacter cloacae* UW4 [25], suggesting that the *P. stutzeri* A1501 *acdS* gene, which encodes ACC deaminase, is essential for ACC metabolism.

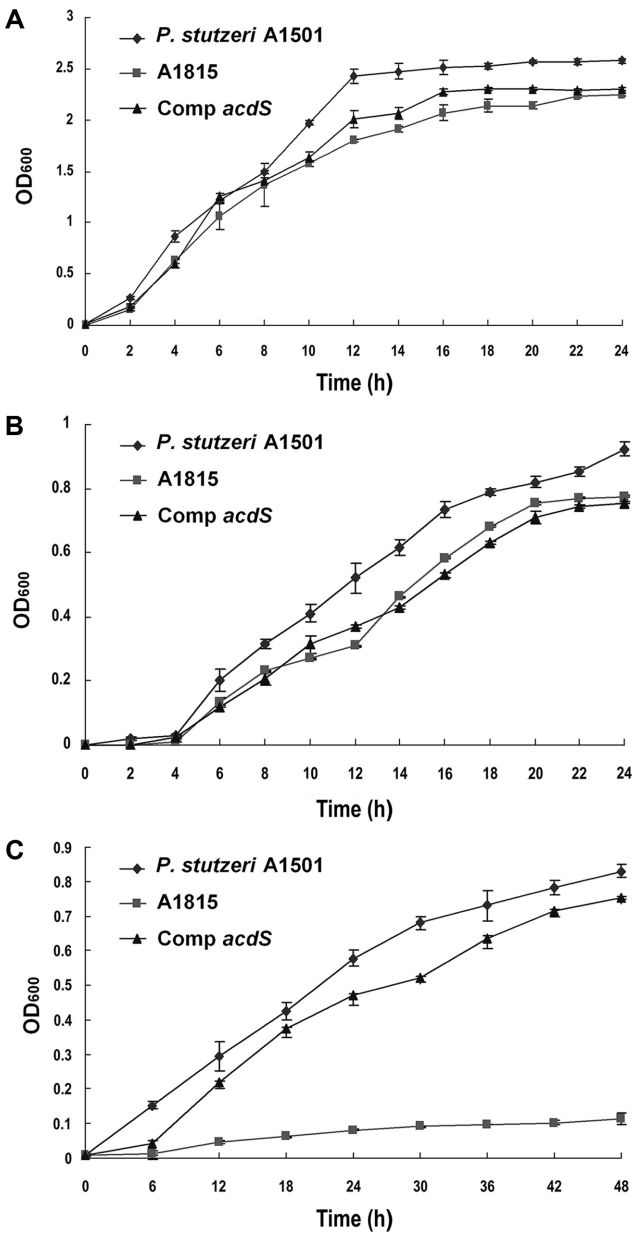

Fig. 2. Time-course growth curves of *P. stutzeri* A1501, the mutant strain A1815, and the complementary strain Comp *acdS* in the following media: (**A**) medium K, (**B**) minimal medium DF, and (**C**) minimal medium ADF (ACC as the sole source of nitrogen).
The results were obtained from three independent biological replicates. Error bars represent the SDs.

Impact of *acdS* on the Sensitivity and Nitrogenase Activity under Environmental Stresses

PGPR can directly facilitate the proliferation of their plant hosts, for example, by fixing atmospheric nitrogen. Environmental stress can also affect the strain's survival rate and nitrogenase activity, consequently affecting plant growth. To determine if strain growth and nitrogenase activity were affected by inactivation of the *acdS* gene

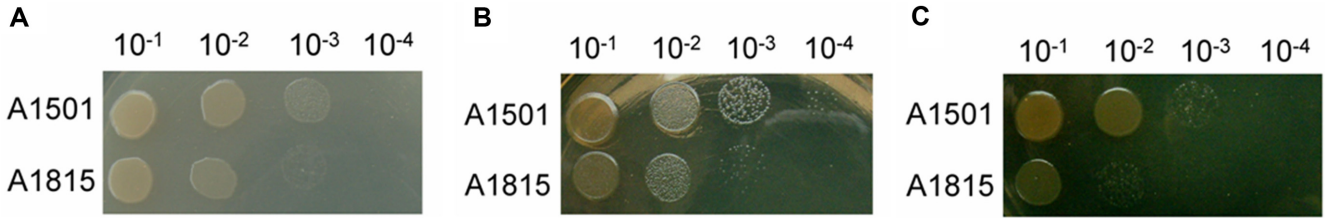

Fig. 3. Sensitivity of wild-type strain *P. stutzeri* A1501 and mutant strain A1815 to salt and heavy metal stresses.
(**A**) *P. stutzeri* A1501 and A1815 growth in LB medium for 1 h, as the negative control. (**B**) The sensitivity of *P. stutzeri* A1501 and A1815 to 1 M NaCl shock for 3 h. (**C**) The sensitivity of *P. stutzeri* A1501 and A1815 to 13.2 mM Ni shock for 3 h. The data were obtained from triplicate experiments.

under stress conditions, the sensitivity and nitrogenase activity of both strains exposed to environmental stresses were tested. As shown in Fig. 3, compared with the wild-type A1501, the *acdS* mutant A1815 became more sensitive to the environmental stresses of salt and heavy metal shock. The survival rate of A1815 was lowered by 1–2 orders of magnitude compared with the wild-type when exposed to 1.0 M NaCl treatment for 3 h and by 2–3 orders of magnitude when exposed to 13.2 mM $NiCl_2$ for 3 h.

The nitrogenase activity of wild-type *P. stutzeri* A1501 and that of the A1815 mutant were also examined. The results showed that inactivation of the *acdS* gene did not affect the nitrogenase activity of the A1815 mutant under nitrogen fixation conditions. However, when 200 mM NaCl was added to the medium, the nitrogenase activity was decreased approximately 20% in A1815 compared with that of wild-type (Fig. 4). To explore the relationship between the nitrogen fixation (*nif*) genes and the *acdS* gene, the expression profiles of the *nif* genes in *P. stutzeri* A1501 and A1815 under different conditions were determined by quantitative RT-PCR. Under nitrogen fixation conditions with 200 mmol NaCl, the expression of the *nifH* (coding for the nitrogenase Fe protein), *nifA* (coding for the nitrogen fixation positive regulatory protein), *glnA* (coding for glutamine synthetase), and *rpoN* (coding for the RNA polymerase sigma-54 factor) genes was over 3-fold lower in A1815 compared with *P. stutzeri* A1501. At present, it is difficult to determine how *acdS* affects *nif* gene expression, and the mechanism needs to be investigated. It has been predicted that the ACC deaminase gene is under the control of NifA and RpoN in *Mesorhizobium loti* ICMP3153 [44] and *M. loti* MAFF303099 [26], respectively. However, in our case, the expression of *acdS* was not increased under nitrogen fixation conditions, even though *rpoN* and *nifA* were highly induced [48]. In the RpoN mutant, *acdS* was slightly decreased, with a 64% level of that in the wild-type A1501, as shown by transcriptomic analysis (data not shown). Taken together, these data suggest that the *acdS* gene may be involved in the regulation of the *nif* gene of *P. stutzeri* A1501, in an undescribed manner, under salt stress conditions.

RpoS is a sigma factor that was reported in many bacteria to associate with the core RNA polymerase and to modify

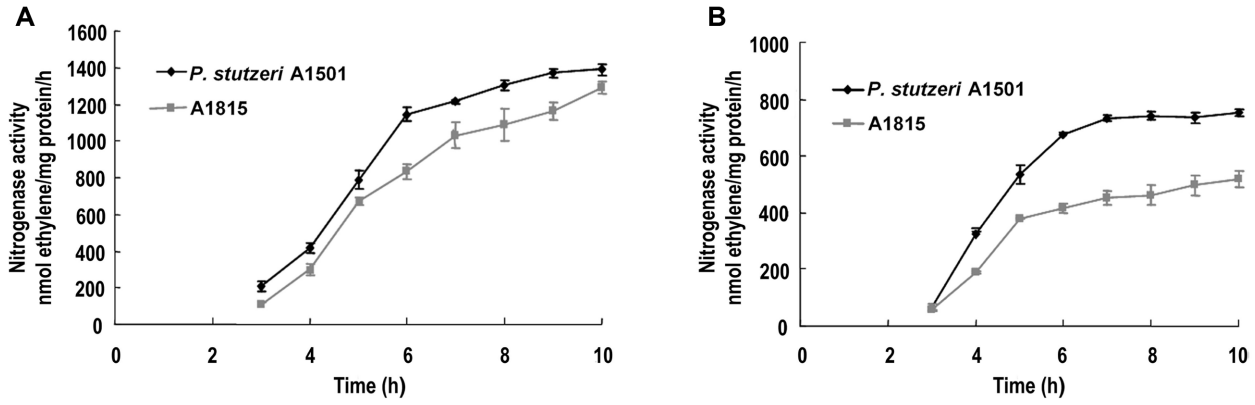

Fig. 4. Time-course experiment of nitrogenase activity.
(**A**) Nitrogenase activity of the wild-type A1501 and *acdS* mutant. (**B**) The effect of 0.2 M NaCl on the nitrogenase activity of wild-type A1501 and the *acdS* mutant. The results were obtained from three independent biological replicates. The error bars represent the SDs.

global gene transcription under stress conditions or as cells enter the stationary phase [5]. In this study, the expression of the *rpoS* gene in both strains was further detected using real-time PCR. In the *acdS* mutant, a 4-fold down-regulation of *rpoS* gene expression was observed compared with the wild-type A1501 during the stationary phase, indicating that *acdS* could affect the expression of the *rpoS* gene either directly or indirectly. Therefore, there exists one possibility that the *acdS* gene impacts the growth and nitrogenase activity of *P. stutzeri* A1501 through affecting the global regulator *rpoS*, although further investigations need to be performed.

Plant Growth-Promoting Effects of *P. stutzeri* A1501 or A1815

Salt and metals are essential to plants; however, when present in excess, they can act as toxicants to plants [10, 27, 28, 41]. Salt or heavy metal stress has been shown to cause increased ethylene production and inhibit root and shoot development [8, 22, 36]. This is readily remedied in the laboratory by adding ACC deaminase-producing plant growth-promoting bacteria, which can help plants overcome many of the effects of metal or salt stress [3, 7–10, 24, 27, 35]. To test the growth-promotion ability of *P. stutzeri* A1501 and A1815 in the presence of stresses, rice inoculated or not inoculated with bacteria was subjected to salt (120 mM or 2 M NaCl) or heavy metal (0.3 mM Co^{2+}, Cu^{2+}, Zn^{2+}, or Ni^{2+}) stress for 7 days, and its growth behavior was monitored (Table 3). Inoculation with the *P. stutzeri* A1501 strain significantly promoted plant growth by increasing plant biomass compared with the control plants in all treatments, as determined by the plant height, root length, root fresh weight, and root dry weight. On the contrary, the mutant strain lost this ability. Significant differences ($p < 0.05$) were not found among control plants and plants inoculated with A1815 either in the presence of NaCl or heavy metals. These results emphasize the importance of the *acdS* gene in the plant growth-promoting effect of *P. stutzeri* A1501.

Specifically, after 7 days of salt stress driven by 120 mM NaCl, the A1815-inoculated and A1815-non-inoculated rice exhibited a 20% decrease in plant height compared with rice plants inoculated with *P. stutzeri* A1501. Other differences were observed in the biomass accumulation of

Table 3. Effect of *P. stutzeri* A1501 and the *acdS* mutant strain A1815 on rice plant growth under different conditions[a].

Treatment	Inoculated strain	Plant height (cm)	Root length (cm)	Root fresh wt (mg)	Root dry wt (mg)
0.12 M NaCl	*P. stutzeri* A1501	25.10 ± 0.57[a]	8.20 ± 0.34[a]	17.97 ± 0.48[a]	4.79 ± 0.22[a]
	A1815	20.19 ± 0.27[b]	6.24 ± 0.38[b]	14.82 ± 0.53[b]	3.77 ± 0.10[b]
	Not inoculated	19.92 ± 0.31[b]	5.91 ± 0.28[b]	14.07 ± 0.42[b]	3.43 ± 0.36[b]
2 M NaCl	*P. stutzeri* A1501	13.06 ± 0.21[a]	4.76 ± 0.31[a]	9.77 ± 0.13[a]	2.69 ± 0.19[a]
	A1815	7.62 ± 0.36[b]	2.93 ± 0.41[b]	3.31 ± 0.23[b]	1.63 ± 0.33[b]
	Not inoculated	7.46 ± 0.53[b]	2.72 ± 0.38[b]	3.07 ± 0.34[b]	1.52 ± 0.10[b]
0.3 mM Co^{2+}	*P. stutzeri* A1501	12.35 ± 0.18[a]	4.21 ± 0.16[a]	6.52 ± 0.24[a]	1.57 ± 0.14[a]
	A1815	8.97 ± 0.58[b]	2.28 ± 0.11[b]	2.68 ± 0.36[b]	1.09 ± 0.13[b]
	Not inoculated	8.64 ± 0.49[b]	2.25 ± 0.17[b]	2.51 ± 0.44[b]	1.04 ± 0.28[b]
0.3 mM Cu^{2+}	*P. stutzeri* A1501	15.25 ± 0.25[a]	5.48 ± 0.30[a]	7.44 ± 0.19[a]	1.91 ± 0.10[a]
	A1815	12.40 ± 0.22[b]	3.09 ± 0.23[b]	3.25 ± 0.10[b]	1.25 ± 0.21[b]
	Not inoculated	12.67 ± 0.19[b]	3.79 ± 0.27[b]	3.57 ± 0.16[b]	1.35 ± 0.27[b]
0.3 mM Zn^{2+}	*P. stutzeri* A1501	17.13 ± 0.67[a]	6.54 ± 0.24[a]	8.34 ± 0.58[a]	2.25 ± 0.12[a]
	A1815	12.94 ± 0.25[b]	3.80 ± 0.28[b]	3.78 ± 0.63[b]	1.37 ± 0.10[b]
	Not inoculated	12.87 ± 0.32[b]	4.03 ± 0.22[b]	3.79 ± 0.48[b]	1.39 ± 0.27[b]
0.3 mM Ni^{2+}	*P. stutzeri* A1501	14.29 ± 0.28[a]	4.60 ± 0.41[a]	7.14 ± 0.16[a]	1.77 ± 0.18[a]
	A1815	10.40 ± 0.36[b]	2.40 ± 0.11[b]	2.81 ± 0.13[b]	1.17 ± 0.23[b]
	Not inoculated	11.17 ± 0.17[b]	2.73 ± 0.25[b]	2.91 ± 0.19[b]	1.28 ± 0.31[b]

The data are the averages ± standard deviations of three pot replicates, with five seedlings per pot for each treatment. Plant data followed by the same letter in a column for each treatment do not differ significantly at $p \leq 0.05$, as determined using Student's t test. Significant differences ($p \leq 0.05$) among control plants without stress and plants grown in the presence of NaCl or heavy metal treatments were not found using one-way analysis of variance, followed by Tukey's analysis.

root. Rice inoculated with A1815 and the control showed lower root length (30%), root fresh weight (22%), and root dry weight (28%) compared with those inoculated with *P. stutzeri* A1501. More significant differences were observed under salt stress conditions driven by 2.0 M NaCl, and more than a 40% decrease in the promoting effects on plant height, root length, root fresh weight, and root dry weight was caused by mutation of the *acdS* gene.

The effect of ACC deaminase under heavy metal conditions was also tested. In the presence of 0.3 mM heavy metal (Co^{2+}, Cu^{2+}, Zn^{2+}, or Ni^{2+}), rice plants inoculated with *P. stutzeri* A1501 showed increased plant height (19%–30%), root length (39%–47%), root fresh weight (54%–61%), and root dry weight (32%–39%) than the non-inoculated rice, whereas inoculation with the mutant A1815 strain did not cause observable changes.

These results indicated that ACC deaminase plays an important role in the growth promotion of rice plants under salt or heavy metal stress conditions. As described in *P. putida* GR12-2 [19], *B. phytofirmans* PsJN [45], *B. unamae* MTl-641^T [30], and *E. cloacae* UW4 [25], the impaired activity of A1815 was attributable to the loss of ACC deaminase activity. In summary, the combined data show that the addition of *P. stutzeri* A1501 significantly improves rice growth under stresses (salt and heavy metals), in which the presence of the *acdS* gene is necessary, and these results were highly reproducible. Moreover, significant differences ($p < 0.05$) were not found among control plants and plants inoculated with the A1815 strain, either in the presence of NaCl or heavy metals.

A model describing the role of ACC deaminase in plant growth-promoting bacteria suggests that rhizobacteria, when attached to the surface of plant seeds or roots, can take up some of the ACC exuded from the plants and cleave it, thereby decreasing the level of ethylene in the plant and, as a consequence, reducing its inhibitory effect on root elongation in particular and on plant growth in general [15, 20]. Our study supports this model by showing that the increased root fresh weight and dry weight of rice plants that resulted from inoculation with *P. stutzeri* A1501 under salt and heavy metal stress conditions require the presence of an ACC deaminase gene, as demonstrated by the profound difference in plant growth caused by the mutation of this gene in the bacterium. A beneficial effect on rice root elongation was consistently observed in plants inoculated with *P. stutzeri* A1501 [15, 18, 21]. Thus, the essential role of ACC deaminase in the growth-promoting effect exerted by *P. stutzeri* A1501 can inspire potential applications in a wide range of crops.

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