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Isolation, Cloning and Sequence Analysis of 1-Aminocyclopropane-1-Carboxylate Deaminase Gene from Native *Sinorhizobium meliloti*

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Background: Many plant growth-promoting bacteria including Rhizobia contain the 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme that can leave ACC, and thereby lower the level of ethylene in stressed plants. Drought and salinity are the most common environmental stress factors for plants in Iran.

Objectives: The main aim of this research was development of bio-fertilizers containing ACC deaminase enzyme which is very important in conditions of stressed drought and salinity.

Materials and Methods: In this research 168 isolates of native *Sinorhizobium meliloti* were evaluated for ACC deaminase activity. These isolates were classified in four groups based on growth rate on ACC containing medium and enzyme activity. One isolate from each group was selected for molecular characterization. The nucleotide sequence of 16S rRNA gene of the selected isolates were determined. The ACC deaminase genes (*acdS*) on total and chromosomal DNA of *S. meliloti* KYA40, and KYA71 strains were isolated and cloned in pTZ57R/T vector and the obtained recombinant plasmids were used for sequence analysis.

Results: The sequence of *acdS* genes from strains KYA71 and KYA40 and corresponding proteins were analyzed with respect to available sequences in NCBI database. The 16S rRNA gene sequences of *S. meliloti* strains submitted to the GeneBank/NCBI database. The *acdS* gene of KYA71 may be located on chromosomal DNA and in KYA40 it is located on one of the mega plasmids. These two genes have 99% similarity with three nucleotide differences which only lead to a change in one amino acid 48, threonine in KYA40 *acdS* gene and methionine in KYA71.

Conclusions: The comparison of amino acid sequences of KYA40 and KYA71 with other sequences in the database showed that the amino acids 37 to 58 in almost all strains were similar. Therefore, it was concluded that it was a conserved region in this location of *acdS* genes and any changes in this region may cause change in ACC deaminase activity.

Keywords: ACC deaminase; *acdS* gene; *Sinorhizobium meliloti*

1. Background

Ethylene is an important hormone for the normal growth and development of plants, developments such as root elongation, senescence, fruit ripening, etc. However, when this hormone is present at high concentration, for example in stressed conditions, it can be damaging for plants (1). Plant growth promoting rhizobacteria (PGPR) are a group of soil bacteria that can stimulate the growth of plants through various mechanisms such as nitrogen fixation, synthesizing phytohormones or solubilizing phosphates (2). Many PGPRs contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase. This enzyme can cleave ACC, the precursor of ethylene, to α-ketobutyrate and ammonium (3) and thereby lower the level of ethylene in stressed plants (4). This process enables microorganisms to grow on a minimal medium containing ACC as the sole nitrogen source. The low levels of ethylene by ACC deaminase is considered one of the most important mechanisms employed by plant growth-promoting bacteria to facilitate plant growth under environmental stress conditions such as salinity (5), flooding (6) and drought (7). Rhizobia such as *Sinorhizobium meliloti* are well

known as nitrogen fixing bacteria and have a symbiotic relation with root of legume plants. Some Rhizobia contain the enzyme ACC deaminase (15). Drought and salinity are the most common environmental stress factors for plants in Iran, where about 75% of the land area is semi-arid or arid and the total saline soils are approximately 55.6 Mha (about 34% of the land area) (8, 9). Therefore, development of biofertilizers containing ACC deaminase enzyme will be very important in these stressed conditions in Iran. In this research, some indigenous *Sinorhizobium meliloti* strains were investigated for ACC deaminase enzyme activity. Four strains were selected for further investigation. We sequenced 16S rRNA and ACC deaminase (*acdS*) gene of these strains. The sequence of 16S rRNA gene has been widely used as a molecular tool to estimate relationships among bacteria (phylogeny), but more recently it has also become important as a means to identify an unknown bacterium to the genus or species level. We postulated that these strains have ACC deaminase activity. Here we describe isolation, cloning and sequence of *acdS* gene of *Sinorhizobium meliloti* KYA40 and KYA71 strains.

2. Objectives

The main objectives of this study was th evaluation of 1-aminocyclopropane-1-carboxylate (ACC) deaminase genes (acds) for further studying of environmental stresses such as dry and saline soils in future investigations.

3. Materials and Methods

3.1. Isolation of ACC-utilizing strains

One hundred and sixty eight strains of *Sinorhizobium meliloti* were obtained from the collection of Soil and Water Research Institute of Iran. Before assaying the ACC deaminase activity of strains they were screened on ACC containing Rhizobium minimal medium (RMM) and classified based on the growth rate by measuring the difference between colony diameter on ACC and control plates. Three series of plates were prepared; 1. RMM (as a negative control). 2. RMM supplemented with 150 µL of 0.3 M ACC, 3. RMM supplemented with 150 µL of 0.3 M NH_4Cl (as a positive control). The colony diameter was measured after 4 days incubation at 28ºC. One strain of each group was selected for further investigation.

Solution 1 and 2 autoclaved separately. Thesolution 3 filter-sterilized through a 0.2 ìmmembrane andadded to mixed medium at about 50ºC (Table 1).

3.2. Genomic and total DNA preparation

Pure cultures of *Sinorhizobium meliloti* isolates were grown in 50 ml conical flasks containing 25 ml of Yeast Extract Mannitol Broth medium (12). The cells from 5 ml were pelleted in a bench centrifuge (7500 g in 10 min in 4ºC) and the resuspended pellets (50 µL) were transferred into 1.5 ml centrifuge tubes, followed by washing the cells three times with 500 µL TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0). The cells were re-suspended in 100 µL of a solution containing 25% sucrose, 1 mg/ml lysosyme, 10 mM Tris HCl, pH 8.0 at 37ºC for 15 min. The lysate was mixed gently with 150 µL of 7.5 M ammonium acetate and mixture was extracted with 500 µL of chloroform/isoamyl alcohol (24:1, v/v) by mixing and centrifuging in 9500 g for 10 min. The aqueous layer was transferred to a clean 1.5 mL centrifuge tube and the DNA was precipitated by propanol. DNA was washed twice in 100 µL 70% ethanol and then resolved in 20 µL of TE buffer (13). For preparation of total DNA from *Sinorhizobium meliloti* about 100 µL of culture was centrifuged at 9500 g for 3 minute. The cells were re-suspend-

Table 1. Rhizobium minimal medium (RMM)

Solution	Compound	Amount
1)	K_2HPO_4	2.05 gr
	KH_2PO_4	1.45 gr
	Distilled water	200 ml
2)	NaCl	0.15 gr
	$CaCl_2$	0.01 gr
	$MgSO_4.7H_2O$	0.5 gr
	Mannitol	10.0 gr
	Microelements solution	0.5 ml
	Distilled water	800 ml
	Agar	15 gr
3)	Pantathonic acid	1mg/ml
	Biotin	1mg/ml
	Thiamine	1mg/ml

ed in 50 µL distilled water and boiled for 5 minutes and centrifuged at 9500 rpm for 5 minute. 2 µL of the supernatant was used as template in 25 µL PCR reaction.

3.3. Amplification and sequencing of 16S rRNA

The universal Primers, fD1 5' AGAGTTTGATCCTGGCTCAG 3' and rD1 5'AAGGAGGTGATCCAGCC 3' (14) were used for PCR amplification of 16S rRNA gene. PCR products were cleaned by High Pure PCR Product Purification Kit (Roche Molecular Biochemicals, Mannheim, Germany). The partial nucleotide sequences of the 16S rRNA gene were determined by direct sequencing of appropriate PCR products using fD1 and rD1 primers. The sequences of 16S rRNA were compared with sequences from NCBI Gene Bank database.

3.4. Amplification, cloning and expression of ACC deaminase (acdS) gene

Two specific primers ACCDF: 5′-ATGTCACTGTTGGAAAAGTTCGA-3′ and ACCDR: 5′-TCAGCCGTCCCTGTAATAGC-3′ were designed for amplification of the ACC deaminase gene (Analysed by OligoAnalyzer 3.1). The genomic and total DNAs of four strains were used as a template in the PCR reaction. The following temperature profiles were used for PCR amplification: 95ºC for 5 min for an initial denaturation; 30 cycles of denaturation; at 94ºC for 1 min, annealing at 53ºC for 1.5 min, extension at 72ºC for 1.5 min, and a final extension at 72ºC for 10 min. The pfu DNA polymerase was used for DNA amplification in PCR. PCR products were purified by High Pure PCR Product Purification Kit, (Roche, Cat. No. 11732668001). The pure PCR products were evaluated by electrophoresis on 1% agarose gel. The PCR products of ACC deaminase (*acdS*) gene were extended at 72ºC for 30 min by taq DNA polymerase and dATP for A-end tailing. The high purified PCR products were ligated to pTZ57R/T cloning plasmid (Fermentas, Germany) and transformed into *E. coli* DH5α. The transformants were spreaded on LB agar medium containing IPTG, Xgal and Ampicilin and incubated at 37ºC for 16 h. Four white colonies were selected and cultured in LB medium and incubated at 37ºC for 24 h. The transformants were evaluated for inserted DNA by PCR using universal M13-pUC primers: Forward (17mer): 5′-d (GTTTTCCCAGTCACGAC)-3′, Reverse (17mer): 5′-d(CAGGAAACAGCTATGAC)-3′

One of the white colonies that received inserted fragment of the *acdS* gene was confirmed by PCR using ACCDF and ACCDR primers. The recombinant plasmids containing *acdS* were sequenced for ultimate confirmation.

3.5. ACC deaminase activity

The strains were grown in Yeast Extract Manitol Broth medium to late log phase before the cells were harvested by centrifugation (8000 rpm in 10 min), washed with 0.1 *M* Tris-HCl (pH=8.5) and incubated in RMM containing ACC as the sole source of nitrogen for 24 h. The bacterial cells were collected, re-suspended in 0.1 *M* Tris-HCl, 30 µL toluene were added to the cell suspension. The toluenized cells were divided in two aliquots. In one aliquot, ACC deaminase activity was assayed immediately by adding 0.5 *M* ACC and α-ketobutyrate is derivative as a phenyhydrazone and then the absorbance is measured at 540 nm (3, 10). In another aliquot of toluenized cells, the total protein was assayed by by Bradford method (11) using bovine serum albumin as standard. One unit of ACC deaminase activity identified as formation of 1 nmol of α-ketobutyrate per mg protein per hour under these conditions.

4. Results

4.1. ACC deaminase activity of strains

From 168 isolates, 77 isolates were able to grow on ACC containing medium. Based on the colony diameter of isolates on RMM medium, strains were categorized in four groups, zero, <2, 2-6 and >6 mm. (Table 2).

Twenty strains of 168 strains (five strains fromeach group) including high, medium, weak and nogrowth were selected for future investigation. These strains were selected based on other similar morphological and physiological characteristics (data not shown).

Different strains obtained on ACC containingmedium showed differences in their deaminase activities, as shown in Table 3. The results showed that, ACC deaminase activity of strains was 0-326 nmol of α-ketobutyrate mg^{-1} protein h^{-1} (Table 3).

Four strains of *Sinorhizobium meliloti* KYA40

(with maximum growth on ACC agar and 136 nmol α-ketobutyrate $mg^{-1}protein.h^{-1}$), KYA71 (with medium growth on ACC agar and 326 nmol α-ketobutyrate $mg^{-1}protein.h^{-1}$), KYA27 (with weak growth on ACC agar and no ACC deaminase activity), and KYA95 (with no growth on ACC agar and no ACC deaminase activity) were selected for molecular investigations.

4.2. Analysis of 16 S rRNA genes

PCR with universal Primers, fD1 and rD1 resulted in 1500 bp fragment of 16S rRNA gene sequence (Figure 1.). The sequences were subjected to BLAST (Basic Local Alignment Search Tool) in NCBI data base to compare a DNA sequence with DNA sequences in the database which showed that these strains are *Sinorhizobium meliloti*.

The 16S rRNA gene sequences of *Sinorhizobium meliloti* strains were submitted to the GeneBank/NCBI database under the accession numbers of *EU625296* (*S. meliloti* KYA27), EU603723 (*S. meliloti* KYA40), EU603721 (*S.* meliloti KYA71) and EU625297 (*S. meliloti* KYA95).

A 1.5 kb fragment of 16S rRNA for each strain was detected. Ethidiombromid stained agarose gel of the PCR products of 16S rRNA showed in Figure 1.

4.3. ACC deaminase genes

PCR with the primers ACCDF and ACCDR resulted in 1020 bp fragment for strains KYA40 and KYA71. The Ethidium bromide stained Agarose gel of the PCR products of ACC deaminase gene in strain KYA40 and KYA71 are shown in Figure 2 for colony PCR and in Figure 3 for genomic DNA.

The sequence analysis and BLAST search in NCBI revealed that these fragments are ACC deaminase gene. The sequence of *acdS* genes from strains KYA71 and KYA40 submitted to the GeneBank/NCBI database under the accession numbers of KYA71 (EU003994) and KYA40 (EU603722).

5. Discussion

The 16S rRNA evaluation of the strains revealed that the bacteria used in this study are *sinorhizobium melioloti* and this means that the previous biochemical tests for identification of these bacteria were accurate. The results of the present study clearly demonstrated that some of *Sinorhizobium meliloti* strains native to soils of Iran contain ACC deaminase enzyme. The first presence of ACC deaminase in Rhizobia was reported by Ma and colleague (15). Peters and Crist-Estes reportedthat ethylene inhibits nodule development in alfalfa (*Medicago sativa*) (16). It was reported that strains of rhizobia that have ACC deaminase enzyme may have the ability to lower ethylene levels in their host specific legumes and overcome some of the negative effects of ethylene on nodulation. Ma *et al*., (17) reported that *Sinorhizobium meliloti* containing ACC deaminase-producing activity showed 35-40% greater efficiency in modulating of alfalfa than strain without ACC deaminase activity. Hence the ACC deaminase containing *Sinorhizobium meliloti* strains obtained from this research can be used for future investigation and inoculation of alfalfa in salinity and drought stress conditions in Iran. Therefore these genes may be

Table 2. Classification of isolates based on growth on ACC containing medium

Bacterial group	Colony diameter	Growth rate on ACC agar	No of Isolates	Selected strains				
1	>6	High	5	KYA166	KYA130	KYA44	KYA40	KYA5
2	2-6	Medium	23	KYA49	KYA62	KYA97	KYA71	KYA 60
3	<2	Weak	49	KYA74	KYA45	KYA15	KYA64	KYA27
4	0	No growth	91	KYA72	KYA43	KYA37	KYA58	KYA95

Table 3. ACC deaminase activity

Bacterial strain	ACC deaminase activity*	Bacterial strain	ACC deaminase activity*
KYA5	125	KYA60	200
KYA15	0	KYA62	192
KYA27	0	KYA64	0
KYA37	0	KYA71	326
KYA40	136	KYA72	0
KYA43	0	KYA74	0
KYA44	201	KYA95	0
KYA45	0	KYA97	135
KYA49	127	KYA130	135
KYA58	0	KYA166	135

used for transferring to other bacteria or producing transgenic plants. Based on the PCR amplification of *acdS* gene by two methods colony PCR and genomic DNA, it is concluded that the *acdS* gene of KYA71 may be located on chromosomal DNA and in KYA40 is on one of mega plasmids. (18) reported that *acdS* gene of *R. leguminisarum* bv *viciae* 128C53K is located on one of the endogenous large plasmids.

These two genes had 99% similarity with three

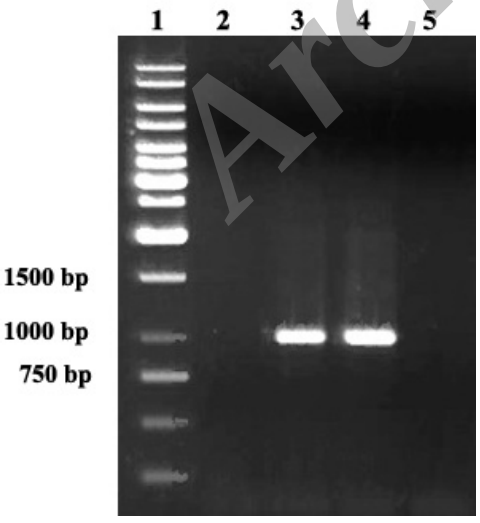

Figure 2. Ethidium bromide stained agarose gel of the PCR products of ACC deaminase gene of colony PCR, Lane 1:1 kb DNA ladder, lane 2: KYA27, lane 3: KYA40, lane 4: KYA71, lane 5: KYA95

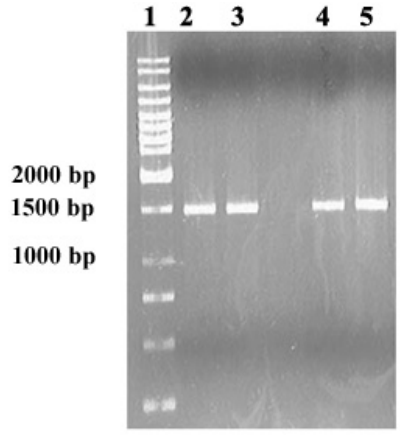

Figure 1. Ethidium bromide stained agarose gel of the PCR products of 16S rRNA (Lane 1:1 kb DNA ladder, Lane 2: YYA27, Lane 3: KYA40, Lane 4: KYA71, Lane 5: KYA95)

nucleotide differences (Table 4), which only lead to change in one amino acid 48, threonine in KYA40 *acdS* gene and methionine in KYA71.

Two genes has 86% identity with *Sinorhizobium meliloti* plasmid pSmeSM11a and 85% with *Rhizobium leguminosarum* bv. *viciae* plasmid pRL10. Hontzeas *et al*., (19) reported that substitution of Asp44 with Gly44 by point mutation causes deactivation ACC deaminase activity. They concluded that the highly conserve Gly44 is important in gating ACC (substrate) entry enzyme's active site.

The comparison of amino acid sequences of *acdS* gene of KYA40 and KYA71 with other

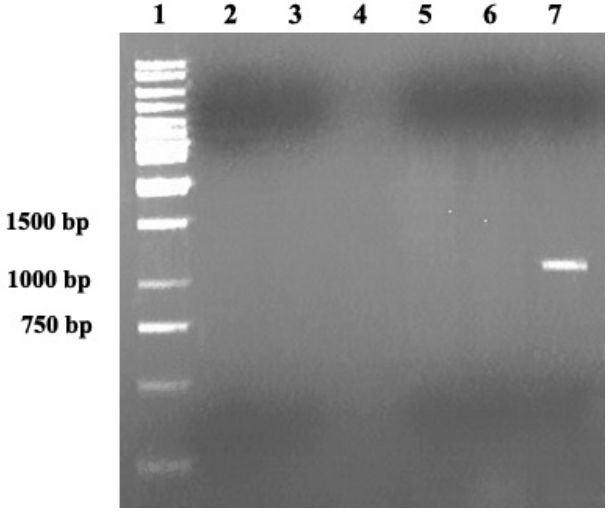

Figure 3. Ethidium bromide stained agarose gel of the PCR products of ACC deaminase gene of Genomic DNA, Lane 1:1 kb DNA ladder, lane 2 and 3: KYA95, lane 4 and 5: KYA27, lane 6: KYA40, lane 7: KYA71

Table 4. Nucleotide differences between acdS genes of KYA40 and KYA71

Nucleotide differences		Nucleotide number
acdS40	acdS71	
T	C	143
C	T	195
T	C	879

sequences in database showed that the amino acids 37 to 58 in almost all strains are similar (Figure 4).

Therefore, it is concluded that it may be a conserve region in *acdS* genes and any changes in this region may cause change in ACC deaminase activity. Shah *et al*., (20) showed that the codons used by ACC deaminase genes of *Pseudomonas putida*, *P. fluorecens* and *Enterobacter cloacae* are highly conserved. Duan *et al*., (22) reported relatively little diversity in ACC deaminase genes of some Rhizobium strains isolated from a wide geographic area.

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Authors Contribution

The study concept, design, technical material and analysis of data within this study was conducted by Houshang Khosravi, Hossein Ali Alikhani, Bagher Yakhchali and some technical and material assistance was provided by Ali Asghar Kharkhane

Financial Disclosure

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References

Sinorhizobium meliloti KYA40	AKRDDCNSGLATGGNKLRKLEY 58
Sinorhizobium meliloti KYA71	AKRDDCNSGLAMGGNKLRKLEY 58
Sinorhizobium meliloti nodulating alfalfa	AKRDDCNSGLAMGGNKLRKLEY 57
Sinorhizobium medicae WSM419	AKRDDCNSGLAMGGNKLRKLEY 57
Sinorhizobium sp.BL3	AKREDCNSGLAMGGNKLKKLEY 58
Rhizobium leguminosarum PB62	AKRDDCNSGLAMGGNKLRKLEY 57
Rhizobium leguminosarum PB45	AKRDDCNSGLAMGGNKLRKLEY 57
Rhizobium leguminosarum. bv. *viciae* 3841	AKRDDCNSGLAMGGNKLRKLEY 57
Rhizobium gallicum PB2	AKRDDCNSGLAMGGNKLRKLEY 57
Bradyrhizobium sp. BTAi1	AKRDDCNSGLAMGGNKLRKLEY 57
Bradyrhizobium sp. ORS278	AKRDDCNSGLAMGGNKLRKLEY 57
Bradyrhizobium japonicum USDA110	AKREDCNSGLA YGGNKLRKLEY 55
Azorhizobium caulinodans ORS 571	AKREDCNSGLA YGGNKLRKLEY 55
Mesorhizobium loti R7A	AKREDCNSGLA FGGNKLRKLEY 55
Mesorhizobium loti MAFF303099	AKREDCNSGLA FGGNKLRKLEY 55
Phyllobacterium brassicacearum STM19	AKREDCNSGLA FGGNKLRKLEY 55
Burkholderia cenocepacia PC184	AKREDCNSGLA FGGNKTRKLEY 36
Pseudomonas sp. strain 6G5	AKREDCNSGLA FGGNKTRKLEY 56
Pseudomonas fluorescens 2P24	AKREDCNSGLA FGGNKTRKLEY 56
Pseudomonas putida UW4	AKREDCNSGLA FGGNKTRKLEY 56
Methylobacterium sp. 4-46	AKREDCNSGLA FGGNKLRKLEY 59
Methylobacterium nodulans ORS 2060	AKREDCNSGLA FGGNKLRKLEY 55
Ralstonia pickettii 12J	AKREDCNSGLA FGGNKTRKLEY 56
Ralstonia eutropha H16	AKRDDCNSGLA FGGNKTRKLEY 56
Acidovorax avenae subsp. *citrulli* AAC00-1	AKREDCNSGLA FGGNKTRKLEY 56
Pseudomonas syringae pv. *syringae* B728a	AKREDCNSGLA FGGNKTRKLEY 56
Enterobacter cloacae UW4	AKREDCNSGLA FGGNKTRKLEY 56
Polaromonas sp. JS666	AKREDCNSGLA FGGNKTRKLEY 59
Mycobacterium abscessus	AKREDCNSGLA FGGNKVRKLEY 57
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Figure 4. The comparison of amino acid sequences of KYA40 and KYA71 with other sequences in database

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جداسازی، کلونینگ و تجزیه و تحلیل توالی ۱-آمینو سیکلوپروپان-۱-کربوکسیلات دآمیناز از *Sinorhizobium meliloti* بومی

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اطلاعات مقاله

نوع مقاله
مقاله پژوهشی

کلمات کلیدی:
ژن *acdS*
Sinorhizobium meliloti
ACC دآمیناز

خلاصه مقاله

مقدمه: بسیاری از باکتری‌های محرک رشد گیاه از جمله ریزوبیوم ها حاوی آنزیم ۱-آمینوسیکلوپروپان-۱-کربوکسیلات (ACC)دآمیناز می‌باشند که به این وسیله قادرند ACC را تجزیه و از طریق آن سطح اتیلن در گیاهان تحت تنش را کاهش دهند. خشکی و شوری از معمول‌ترین عوامل ایجاد کننده تنش‌های محیطی در ایران محسوب می‌شوند.
اهداف: هدف اصلی از این پژوهش، توسعه کودهای زیستی حاوی آنزیم ACCدآمیناز بود که در شرایط تنش شوری و خشکی دارای اهمیت فراوان هستند.
مواد و روش‌ها: در این پژوهش ۱۱۸ جدایه باکتری *Sinorhizobium meliloti* بومی از نظر میزان رشد بر روی محیط کشت حاوی ACC و فعالیت آنزیمی در چهار گروه طبقه بندی و از هر گروه یک جدایه برای بررسی مشخصات مولکولی انتخاب شدند. توالی نوکلئوتیدی ژن ۱۶S rRNA از جدایه های انتخابی تعیین شدند. ژن های ACCدآمیناز (acdS) بر روی DNA کل و کروموزومی *S. meliloti* KYA۴۰ و KYA۷۱ جداسازی شدند. ژن ACCدآمیناز در پلاسمید pTZ۵۷R/T کلون شد. پس از استخراج از اشریشیا کلی سویه DH۵a، پلاسمید های نوترکیب برای تعیین توالی مورد استفاده قرار گرفتند.
یافته ها: ژن هایacdS از سویه‌های KYA۴۰ و KYA۷۱ و همچنین پروتئین‌های مربوطه بر اساس بانک توالی‌های موجود در NC I تجزیه توالی شدند. توالی ژن‌های ۱۶S rRNA و acdS جدایه های *S. meliloti* در بانک ژن NC I ثبت شدند. ژن acdS مربوط به KYA۷۱ ممکن است بر روی DNA کروموزومی و در KYA۴۰ بر روی یکی از مگاپلاسمیدها باشد. دو ژن دارای ۹۹ درصد مشابهت با اختلاف سه نوکلئوتید که فقط منجر به تغییر در فقط یک اسید آمینه ،۴۸ ترئونین در KYA۴۰ و متیونین در KYA۷۱ شد.
نتیجه گیری: مقایسه توالی‌های اسید آمینه KYA۴۰ و KYA۷۱ با دیگر توالی های موجود در بانک ژن نشان داد که اسید آمینه‌های ۳۷ تا ۵۸ تقریبا در همه سویه‌های مورد آزمایش مشابهت دارند. بنابراین نتیجه‌گیری شد که ممکن است این قسمت یک ناحیه حفاظت شده باشد و هرگونه تغییری در این ناحیه ممکن است موجب تغییر در فعالیت آنزیم ACCدآمیناز شود.

* نویسنده مسئول