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# ISOLATION AND IDENTIFICATION OF *PSEUDOMONAS PUTIDA* FROM *MEDICAGO SATIVA* RHIZOSPHERE SOIL

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**Abstract**

A gas-cycle incubation system was used to isolate hydrogen-oxidizing bacteria from rhizosphere soil of alfa alfa (*Medicago sativa*). Strain WMQ-7 was able to produce ACC deaminase. The ninhydrin reaction was used to test the enzymatic activity of ACC deaminase which was 0.671 U/μg. On the basis of morphology, physiological characteristics, 16S rDNA sequence and a phylogenic tree, strain of WMQ-7 was identified as *Pseudomonas putida* (GenBank accession number EU807744). The growth and output of wheat were increased owing to the effect of strain WMQ-7. From the result it appeared that ACC deaminase was one of the plant growth promoting mechanisms.

## Introduction

Rotated crop, intercrop of legumina can enhance the fertility of soil, decrease the requirement of fertilizer, and increase the output. Recent information indicated that nitrogenous fertilizer cannot completely replace the effect of rotated crop of legumina, and it cannot be explained the 75% increased production by the already known research (Hesterman *et al*. 1986). During long-term research on the physiological and ecological characteristic of the microbe around $HUP^-$ legumina (Dong and Layzell (2001), Dong and Layzell (2002), Mclearn and Dong (2002), Dong *et al*. (2003) proposed the "theory of hydrogen fertilizer", that is, the hydrogen released from root nodule can promote the growth of the microbe around the root, as well as promote the growth of plants. Most hydrogen-oxidizing bacteria belong to the plant growth-promoting rhizobacteria (PGPR) and Hu *et al*. (2004) did research on this microbe. 1-aminocyclopropane-1-carboxylic acid (ACC) is the precursor to synthesize ethylene in plants. Many PGPR can produce ACC deaminase degrading ACC into ketobutyric acid and ammonia (Wei *et al*. 1994). The reduction of ACC cause the decrease of ethylene, therefore plants can grow rapidly (Chen *et al*. 2008). The produce of ACC deaminase is one of mechanisms that PGPR can promote the growth of plants.

This investigation deals with the isolation, identification of hydrogen-oxidizing bacteria from the rhizosphere soil of *Medicago sativa*, enzymatic activity of ACC deaminase and the plant growth promoting mechanism.

## Material and Methods

Soil samples were collected from the rhizosphere of *Medicago sativa* in Shaanxi (NW China). Mineral salt agar medium (MSA)(2.0 g $NaNO_3$, 1.2 g $K_2HPO_4$, 0.5 g $MgSO_4$, 0.5 g KCl, 0.14 g $KH_2PO_4$, 0.01 g $Fe_2(SO_4)_3 \cdot H_2O$, 0.02 g yeast extract, 15 g agar, one liter water, pH 7.2) and MSA modified medium (without nitrogen source, 0.5 g/L ACC added) was used for this experiment.

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Hydrogen-oxidizing bacteria in the soil were enriched, isolated and purified, and their ability to oxidize $H_2$ was tested. A gas-cycle incubation system was used to produce $H_2$ by brine electrolysis to enrich the hydrogen-oxidizing bacteria (Chen *et al*. 2007). The $H_2$ was mixed with clean air (280 ml/min), which contained $4.16 \times 10^{-4}$ mol/l ~ $2.42 \times 10^{-3}$mol/l $H_2$ of the mixed air. The dilute soil liquid was spread on MSA solid medium after enrichment for one month and aerated the same concentration of $H_2$ to isolate and purify hydrogen-oxidizing bacteria. A portion of $2.42 \times 10^{-3}$mol/l $H_2$ of the mixed air was aerated into the purified bacterial slope, and then gas chromatography was used to test bacterial ability to oxidize $H_2$ after 3 days (Chen *et al*. 2007).

Hydrogen-oxidizing bacteria were inoculated in MSA modified liquid medium, and incubated them for 7 days in shaking table incubator (120r/min, 30°C). After the incubation period, the bacteria were isolated for 30 seconds (10000r/min, –4°C). The supernatant was spotted on silica gel plate. Developing solvent was n-butanol/acetic acid/distilled water = 75: 15: 10 (v/v/v). Color developing reagent was 0.5% ninhydrin. Developing time was 2.5 hrs, colouration temperature was 100°C and the colouration time was 20 min. The process was repeated without ACC to serve as control.

Screening of the positive ACC deaminase was strain WMQ-7. The morphology and physiological characteristics was tested according to the method described by Dong and Cai (2001). Amplification and the sequencing of 16S rDNA about the strain WMQ-7 were performed at TaKaRa (Dalian, China).

Phylogenic tree based on 16S rDNA sequences of strain WMQ-7 was done. 16S rDNA sequences of strain WMQ-7 was submitted to NCBI and compared with other homology sequences. DNASTAR (MegAlign) and MEGA3.1 were used to analyze the base sequences, content of GC and compute the distance of heredity with parameter Kimura-2 (Xiao *et al*. 2008). The phylogenic tree was built by NJ method (Xiao *et al*. 2008).

WMQ-7 was inoculated in MSA modified liquid medium and cultured for 1 day in shaking table incubator (120r/min, 30°C). The cultured cells were centrifuged in a high speed centrifuge (10000r/min, 4°C).The supernatant and deactivated enzyme was decanted. Ninhydrin reaction was used to test the enzymatic activity of ACC deaminase (Wang *et al*. 2005). The unit enzymatic activity was defined as the ability to consume 1 μg ACC at 30°C, pH 7.2 in 1 min.

The seeds of wheat were soaked in distilled water at room temperature for 7 ~ 10 hrs and were sterilized after germination (Kang *et al*. 2008). The sterilized seeds and 15 ml hydrogen-oxidizing bacterial suspension was placed in sterile plates. The seeds were cultured at 25°C for 5 ~ 7 days and the parts above the roots were harvested, dried at 100°C for 32 hrs and weighed.

The seeds were sowed into flower pots in October 2015 and cultured at natural conditions. The bacteria determined to promote the growth of wheat in plate test were chosen and cultured for 1 day (120 r/min, 30°C). The seeds in flower pots were maintained at natural conditions. The process was repeated for one without bacteria and the other with tap water as blank control. The wheat was harvested in May 2016 and analyzed for typical characteristics such as the height of plants, number of wheat ear and wheat grains.

## Results and Discussion

The bacteria were grown in single colonies after cultured for 7 days. The colonies were examined under light microscope and 37 purified strains were obtained. The results of $H_2$ absorbing value of the bacteria are presented in Table 1. There were 8 strains that $H_2$ absorbing value in excess of $2.44 \times 10^{-4}$ mol/l and accounted for 25.93%, which was determined as the hydrogen-oxidizing bacteria (Chen *et al*. 2007). The $H_2$ absorbing value of strain WMQ-7 and FMG-5 were both in excess of $12.28 \times 10^{-4}$ mol/l, but WMQ-7 had stronger ability to oxidize.

**Table 1. $H_2$ concentration consumed by different strains.**

| Strains | Initial $H_2$ concentration | Surplus $H_2$ concentration | Net consumed $H_2$ concentration |
|---|---|---|---|
| WMQ-7 | 19.90 | 1.31 | 18.59 |
| FMG-3 | 12.64 | 3.90 | 8.74 |
| FMG-5 | 24.24 | 10.81 | 13.43 |
| WMQ-8 | 19.50 | 10.57 | 8.93 |
| LD-WMQ | 13.35 | 7.40 | 5.96 |
| WMG-8 | 18.02 | 11.27 | 6.75 |
| FMQ-3 | 16.15 | 13.48 | 2.68 |
| WMG-7 | 15.73 | 14.14 | 1.59 |
| Blank | 22.41 | 22.08 | 0.33 |

The unit indicating the hydrogen concentration is $10^{-4}$ mol/l.

Thin-layer chromatography of the 8 strains showed that strain WMQ-7 produced ACC deaminase. Other strains could not produce ACC deaminase.

The standard curve was conducted according to the method described by Wang (Wang *et al.* 2005). The light absorption value of the strain WMQ-7 was 0.4 at 570 nm. The regression equation of standard curve was that $y = 0.0011x + 0.027$, $R^2 = 0.9958$. The concentration of ACC consumed by WMQ-7 was 161μg/ml. The calculated enzymatic activity of ACC deaminase was 0.671 U/μg.

Strain WMQ-7 was short-clubbed (0.525 ~ 0.76 × 0.84 μm ~ 1.80 μm). It had flagellum and was Gram-negative$^-$. The colony on MSA solid culture medium after 7 days at 30°C was 1.5 mm. The bacterium colony was round, jagged, white and smooth. Physiological characteristics of WMQ-7 are presented in Table 2.

**Table 2. Physiological characteristics of strain WMQ-7.**

| Characteristics | Result | Characteristics | Result |
|---|---|---|---|
| Oxidase | + | Gelatin liquefaction | – |
| Catalase | + | Nitrate reduction | + |
| Oxidative fermentationofglucose | – | Tryptophan deaminase test | + |
| Voges-Proskauer test | – | Indole production | – |
| Methyl red test | – | Urease test | – |
| Hydrolysis of starch | + | $H_2S$ generation testing | – |
| Cellulose decomposing | – | L-Phenylalaninase | – |
| Use of citrate | + | Arginine dihydrolase | + |

The 16S rDNA sequence of strain WMQ-7 was 1451 bp, the content of GC was 53.8% and the GenBank accession number was EU807744. Phylogenic tree based on 16S rDNA sequences of strain WMQ-7 showed that there were three phylogenetic developing branches (Fig. 1). Strain WMQ-7 was clustered together with *Pseudomonas putida* in phylogenetic tree, with the sequence

identity of 99%. Together with the results of morphology and physiological characteristics, strain WMQ-7 was identified as *Pseudomonas putida*.

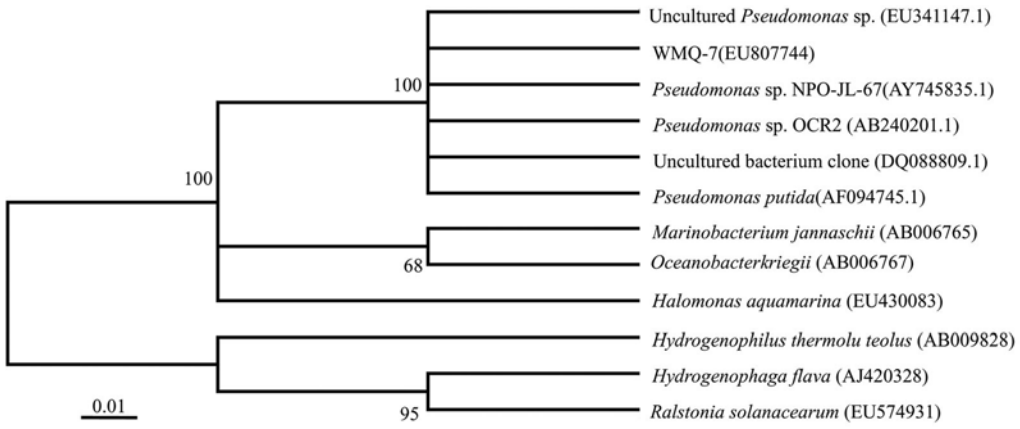


Fig. 1. Phylogenic tree based on 1451-bp fragment of 16S rDNA sequences of strain WMQ-7. Number sat each branch points indicate the percentage supported by bootstrap based on 1000 resampled data sets. After each bacterial name, the GenBank accession numbers are shown in parentheses. Bar, 0.01 substitutions per nucleotide.

After being cultured under light for 7 days in the plate test, wheat seeds were analyzed for the effect of the 8 strains. The length of sprouts, roots and dried weight were recorded. SPSS software was used for information analysis and the results showed the effect of stain FMG-5, WMQ-7, FMG-3 to the length of wheat roots which was 58.26, 73.92 and 74.78% longer than the blank control without bacteria. While under the effect of stain WMQ-8, WMG-8, FMQ-3, LD-WMQ, WMQ-7, FMG-5, FMG-3 the length of wheat sprouts were 34.64% longer than the blank control. Strain FMG-3 had the greatest effect, which was 67.64% longer than the blank control. Under the effect of stain WMQ-8, FMG-5, FMG-3, WMQ-7 the dried weight was 38.1, 47.62, 57.14 and 95.24% more than the blank control.

**Table 3. Effect of the hydrogen-oxidizing bacteria on wheat.**

| Strain | Length of roots (cm) | Height of seedling (cm) | Dry weight (g) |
|---|---|---|---|
| WMQ-7 | 6.6667** | 12.0333** | 0.2050* |
| FMG-3 | 6.7000** | 13.4667** | 0.1650* |
| FMG-5 | 6.0667** | 12.8000** | 0.1550* |
| LD-WMQ | 3.5167 | 11.8833 | 0.1100 |
| WMQ-8 | 4.2167 | 10.8167 | 0.1450* |
| WMG-7 | 4.1833 | 7.3667 | 0.1050 |
| WMG-8 | 3.3000 | 11.1333 | 0.0950 |
| FMQ-3 | 3.6667 | 11.7000 | 0.1000 |
| Blank control | 3.8333 | 8.0333 | 0.1050 |

*p = 0.004;**p< 0.001.

The flower pot test showed the effect of strain FMG-5, WMQ-7 and FMG-3 on number of wheat grain which was 88, 138.4, 112% more than the blank control without bacteria and 106.1, 161.4, 132.5% more than the blank control with tap water. This illustrates that hydrogen-oxidizing bacteria may increase the number of germinated wheat grain.

Research on plant growth promoting mechanisms of hydrogen-oxidizing bacteria, which is a special physiological group of PGPR, is beginning. It was found that soils treated with $H_2$ could promote the growth of wheat, rape, barley and soybean (Dean *et al*. 2006). The dry weight and tiller number could increase 15 to 48% of wheat and barley at 4~7 weeks (Dean *et al*. 2006). Chen *et al*. (2007) found that the length of wheat roots could increase 111 to 397% when the wheat seeds were treated with hydrogen-oxidizing bacteria.

In the present investigation hydrogen-oxidizing bacteria WMQ-7 isolated from *Medicago sativa* rhizosphere were identified as *Pseudomonas putida*. WMQ-7 promoted the weight of wheat seeds. Jacobson *et al*. (1994), Hontzeas *et al*. (2004) verified that *Pseudomonas putida* contained ACC deaminase and could promote the growth of plants, especially after sowed for several days, and the survival rates of seedlings increased. Shen *et al.* (2008) studied the enzyme production conditions and factors affecting the enzyme of strain XG32, whose enzymatic activity of ACC deaminase was 0.442 U/μg. Chen *et al*. (2007) isolated an ACC deaminase positive strain from soybean rhizosphere, but did not report the enzymatic activity. This study reported that the enzymatic activity of ACC deaminase produced by strain WMQ-7 was 0.671 U/μg. The plant growth promoting mechanism of WMQ-7 was due to the ACC deaminase which could adjust the synthesis of ethylene and promote the growth of plants (Chen *et al*. 2008).

Isolation and identification of hydrogen-oxidizing bacteria from *Medicago sativa* rhizosphere merits further study to build perfect physiological group and analysis of the species structure. This new information may provide experimental basis for the study on plants promote mechanism of hydrogen-oxidizing bacteria belonging to PGPR.

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