JOURNAL OF PURE AND APPLIED MICROBIOLOGY, APRIL 2014. Vol. **8**(2), p. 1327-1338

Physiological Characteristics and Phylogeny of Hydrogen-Oxidizing Bacteria in *Medicago sativa* Rhizosphere with Plant Growth Promoting Mechanism

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(Received: 09 January 2014; accepted: 18 March 2014)

A gas-cycle incubation system (H_2 treatment system) and mineral salt agar medium was used to isolate and culture hydrogen-oxidizing bacteria in *Medicago sativa* rhizosphere, and 37 strains were finally isolated and cultured, 8 strains of which could absorb H_2 more than 2.44×10^{-4} mol/L was identified as *Pseudomonas sp.*, *Plesiomonas sp.*, *Pimelobacter sp.*, *Xanthobacter sp.*, *Leminorella sp.*, *Terrabacter sp.*, *Rarobacte sp.* according to the cell, colony form and biochemical characteristics. The heterotrophy culture condition of strain WMQ-7 was also studied. The enzyme activity of strain WMQ-7 was 0.671 U/μg and the content of siderophore produced by strain WMQ-7 was 7.1996 μg/mL. 16S rDNA sequence of strain WMQ-7, FMG-3, FMG-5 was analyzed and built the phylogenic tree. From the physiology characteristic and inherited characteristic, strain WMQ-7 was finally identified as *Pseudomonas putida* (GenBank accession number EU807744). Stain FMG-5 was 94 % similar to the *Rhizobium etli*, while the 16S rDNA sequence of strain FMG-3 could not find a similar comparison in GenBank. In the experiments of the 8 hydrogen-oxidizing bacteria effect on wheat, the results showed that strain WMQ-7, FMG-3 and FMG-5 had strong ability to promote the growth of wheat. It is possible that the plant growth promoting mechanism of strain WMQ-7 was due to its ACC deaminase activity and the siderophore.

Key words: *Medicago sativa*, Hydrogen-Oxidizing Bacteria, Siderophore.

Rotated-crop, intercrop of legumina can enhance the fertility of soil, decrease the requirement of fertilizer, and increase the output, which is a universally recognized agricultural practice (Baldock and Musgrave, 1980). Because of the nitrogen fixation of legumina, residual N_2 is usually considered to be the reason why rotated-crop and intercrop of legumina have lots of benefits. But new results indicate that nitrogenous fertilizer cannot completely replace the effect of rotated crop of legumina, and it still cannot be explained the 75% increased production effect by the already known (Baldock *et al*.,1981;Bolton *et al*.,1976; Hesterman *et al*.,1986; Zumft *et al*.,1975). H_2 is released during nitrogen fixation of legumina and rhizobium (La Favre and Focht, 1983), and then is oxidized in nodules rhizosphere. H_2 oxidizing capacity of soil diminishes in exponentially as the increasing distance from nodules, which is also related to the number of hydrogen-oxidizing

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bacteria (Häring *et al*., 1994). Researches show that the one oxidized O_2 is bacterium (Irvine *et al*., 2002). Dong hypothesized a H_2 fertilizer theory (Dong *et al*., 2003; Irvine *et al*., 2002)which indicated that H_2 released from root nodule could increase the microbe around the root and promote the growth of plants. These bacteria can utilize H_2 that released from rhizobia nitrogen fixation and then assimilate CO_2. They are autotrophic or facultative autotrophic growth so called as hydrogen-oxidizing bacteria. Because the uniqueness of hydrogen-oxidizing bacteria, they are different to be isolated from the soil. Cultured equipment, concentration of mixed gas, nutrition of medium, temperature, pH and salt concentration may affect the growth of them. *Pseudomonas facilis* (Meyer *et al*., 1978), *Alcaligenes ruhlandii* (Savel'eva and Zhilina, 1968), *Alcaligenes eutrophus* (Packer and Vishniac,1955), *Pseudomonas carboxydovorans* and *Bacillus schlegelii* (Schatz and Bovell,1952)were isolated from soil, when the culture medium is nearly neutral (pH6.2-7.8), temperature at 28-32°C, 85% H_2, 5% O_2 and 10% CO_2 was mixed. But these bacteria which grow relying on H_2 still have not been completely separated (Dean *et al*., 2006; Chen *et al*., 2007), and the involved leguminous species were very limited.

Most hydrogen-oxidizing bacteria belong to the plant growth-promoting rhizobacteria (PGPR), and colonize around roots of plants. They usually produce antibiotics, ACC deaminase, siderophore and HCN, which can directly promote indoles acetic acid or restrain rhizosphere pathogens and adjust rhizosphere beneficial microorganisms. Thereby, the yield of crops can be kept stable and high (Hu *et al*., 2004). 1-aminocyclopropane-1-carboxylic acid (ACC) is a precursor to ethylene synthesis in plants. Many PGPR can produce ACC deaminase, which degrade ACC into ketobutyric acid and ammonia (Häring *et al*., 1994). The reduction of ACC cause the decrease of ethylene, therefore plants can grow rapidly (Irvine *et al*., 2002). ACC deaminase is one of mechanisms that PGPR can promote the growth of plants (Ramamoorthy *et al*., 2001; Fu *et al*., 2009).

Medicago sativa rhizosphere from the Central Shaanxi Plain was designated as study material. Gas-cycle incubation system was used to isolate the hydrogen-oxidizing bacteria. Initially cleared the taxonomy position of hydrogen-oxidizing bacteria from *Medicago sativa* and constructed the phylogeny tree. This study reported the plant-growth promoting effect of these strains. Superficial characteristics of germinal wheat were tested by small scale cultivated experiments. ACC deaminase and siderophore were studied the mechanisms that could offer new approaches to increase grain production.

MATERIAL AND METHODS

Soil sampling

Medicago sativa rhizosphere soil was sampled in Central Shaanxi Plain, China.

Culture medium

Mineral salt agar medium (MSA), ACC test culture medium, MKB medium and CAS siderophore test culture medium were prepared according to literatures (Chen *et al*.,2007; Fu *et al*.,2009; Tian *et al*.,2008; Chen *et al*.,2006; Pérez-Miranda *et al*.,2007).

Isolation of strains

Gas-cycle incubation system (Chen *et al*., 2007; Fu *et al*., 2009) was used and improved. The glass needle syringe was in series instead of conical flask, and adjustable airflow control device was fixed at the entry of the mixed gases. H_2 was produced by brine electrolysis to enrich the hydrogen-oxidizing bacteria. The H_2 mixed with clean air and formed airflow of 280 mL/min, which contained H_2 of 4.16×10^{-4} mol/L-2.42×10^{-3} mol/L. Hydrogen-oxidizing bacteria from *Medicago sativa* rhizosphere were enriched in three series of glass tube, the one not treated in gas-cycle incubation system was used as control. Gas chromatography was used to test the content of H_2 in every 2d and draw corresponding curve (Chen *et al*., 2007).The mixture gas containing H_2 well-distributed in the soil because of the improved gas-cycle incubation system and cuts losses during the gas diffusion. The adjusting device could make the mixed gas keep stable. The diluents soil liquid was spread on MSA solid medium after enrichment for 1 month and aerated the same concentration of H_2 to isolate and purify hydrogen-oxidizing bacteria (Fu *et al*., 2009).

H_2 consumption ability test

A portion of 2.42×10^{-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 cultured for 3d (Chen *et al*., 2007; Fu *et al*., 2009). Screening the bacterium which had the strongest H_2 oxidizing ability, and then inoculated it in MSA liquid culture medium cultured at 30°C,120 r/min with 2.42×10^{-3} mol/L H_2 discontinuously. The one without the H_2 was served as negative control. Take samples at every 2d and test the absorbance at 600 nm, then draw the growth curve.

Morphology and physiological characteristics

The test of morphology and physiological characteristics was accruing according to literatures (Pérez-Miranda *et al.*, 2007; Shen *et al*., 2003; Dong *et al*., 2001).

Heterotrophy conditions

8 g/L glucose, maltose, soluble starch, sucrose and lactin were added respectively to MSA liquid culture medium as carbon source. Different temperature 20°C, 25°C, 30°C, 35°C, 37°C, 40°C, 45°C and different pH 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10 was set, then cultured at 120 r/min for 24h. The absorbance at 600 nm was test to check out the growth condition of the bacteria (Shen *et al*., 2003).

The growth of wheat effected by hydrogen-oxidizing bacteria

Culture plate test

Wheat seeds were soaked in distilled water for 7-10h at room temperature and made surface sterilization after germination (Kang *et al*., 2008). 15 mL suspended liquid of bacteria and the treated wheat seeds were put in aseptic plate, others put in 15 mL sterile MSA medium (including 8 g/L glucose) were used as blank control. They were cultured in thermostatic illumination incubator at 25°C for 5-7d and the part above the roots were treated at 100°C, drying for 32h then weighted.

Flowerpot experiments

Sow at October 2007 with the soil from orchard of northwest university. 10 wheat seeds were sowed in each flowerpot and cultured at natural light condition. Strains that promoted the growth of wheat obviously were selected and cultured at 30°C,120 r/min for 24h. Sterile the seeds in MSA medium (including 8 g/L glucose) and the seeds water irrigated were respectively served as blank control. Wheat was harvest in mid-May 2008 and analyzed the height, wheatear, wheat grain and so on.

Enzymatic activity of ACC deaminase produced by hydrogen-oxidizing bacteria

Acid thin-layer chromatography method was used to screening the strains produed ACC deaminase (Chen *et al*., 2007) and ninhydrin reaction (Fu *et al*., 2009; Shen *et al*., 2003) was used to test the enzymatic activity of ACC deaminase. The unit enzymatic activity was defined as the ability to consume 1 µg ACC at 30 °C, pH 7.2 in 1 min.

Test of siderophore produced by hydrogen-oxidizing bacteria

Screening of siderophore positive strains

The bacteria solution was diluted for coating at CAS and MKB test culture medium. The one without bacteria was served as the blank control. They were cultured at 30°C and the bacteria that could change the color of culture medium were picked.

Siderophore qualitative tests

Inoculate the above picked strains into 50 mL MSA culture medium and reference MSA culture medium with 20 µmol/L Fe_2SO_4. They were cultured at 200 r/min and 30°C for 12h, and centrifuged at 4°C 12000 r/min for 10min. Siderophore was concentrated by the extraction method. To test the absorption peaks, the siderophore scanned at 220-600 nm wavelength (Xie *et al*., 2006).

Siderophore type tests (Xie *et al*., 2006; Ratledge and Dover, 2000) The 2, 3-DHBA (0.1 g/L) was served as the standard. The wavelength of 200 nm-500 nm was chosen for scanning the siderophore type.

Siderophore quantitative test

Inoculated siderophore positive strains into 50 mL MSA culture medium consisted with 20 µmol/L $Fe_2(SO_4)_3$. The medium was cultured at 30°C,200 r/min for 12h, and then centrifuged at 4°C,12000 r/min for 10min. The supernatant was quantified and O-phenanthroline concentration spectrophotometrically was used to determine siderophore quantity (Yu, 1993).

Analyses of 16S rDNA sequence

Amplification and the sequencing of 16S rDNA (Sambrook *et al*., 1992) WMQ-7 were performed by TaKaRa (Dalian, China).

16S rDNA sequences of strain WMQ-7 was submitted to NCBI and compared with other homology sequences. DNASTAR (MegAlign) and

MEGA3.1 was used to analyze the base sequences, content of GC and compute the distance of heredity with parameter Kimura-2 (Fu *et al*.,2009). The phylogenic tree was built by Nj method (Fu *et al*., 2009).

RESULTS AND DISSCUSSION

Enrichment, isolation and purification of hydrogen-oxidizing bacteria and their ability to oxidize H_2

Medicago sativa rhizosphere soil sample was enriched under 2.42×10^{-3} mol/L H_2 continuously, and the residual H_2 content in the outlet of the setup did not change within 7 days (Fig. 1). On the 9-19th day for enrichment, the H_2 content decreased by 52.31%,which showed that hydrogen-oxidizing bacteria grew rapidly. On 21-29th day, H_2 content stayed at 0.887 mmol/L. It showed that the bacteria had reached the maximum and the enrichment was finished.

The control sample was treated under continuous air for 29 days, and concentration of H_2 was very low and couldn't be detected by gas chromatography. Thus, 2.42×10^{-3} mol/L H_2 was gassed into control sample. On the 15-23th day the content of H_2 decreased by 24.68% (Fig. 1), it showed that the hydrogen-oxidizing bacteria had been enriched. But the consumption of H_2 was 27.63% less than that of the one enriched directly. So H_2 was necessary for the autotrophic growth of hydrogen-oxidizing bacteria and directly enriched soil samples would promote their multiplication quickly.

37 strains were purified after enriched for 1 month. Test the H_2 absorbing value of the bacteria after enclosing cultured for 3d (Table 1). There were 8 strains' excess 2.44×10^{-4} mol/L and accounted for 25.93 % of all, so we decided them as the hydrogen-oxidizing bacteria. The H_2 absorbing

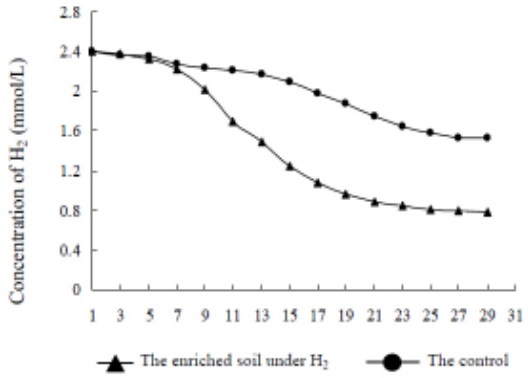


Fig. 1. Changes of the concentration of H_2 left in soil and sand mixture

Table 1. Results of oxidizing H_2

Strains	Initial H_2 concentration (10^{-4} mol/L)	Surplus H_2 concentration (10^{-4} mol/L)	Net consumed H_2 concentration (10^{-4} mol/L)	Value level
WMQ-7	19.90	1.31	18.59	IV
FMG-3	12.64	3.90	8.74	II
FMG-5	24.24	10.81	13.43	III
WMQ-8	19.50	10.57	8.93	II
LD-WMQ	13.35	7.40	5.96	I
WMG-8	18.02	11.27	6.75	II
FMQ-3	16.15	13.48	2.68	I
WMG-7	15.73	14.14	1.59	0
Blank	22.41	22.08	0.33	0

Note: 0: $<2.44\times10^{-4}$ mol/L; I: 2.44×10^{-4} mol/L~5.96×10^{-4} mol/L; II: 6.06×10^{-4} mol/L~8.93×10^{-4} mol/L; III : 12.28×10^{-4} mol/L~13.83×10^{-4} mol/L; IV: $\geq18.59\times10^{-4}$ mol/L.

value of strain WMQ-7 and FMG-5 were both excess 12.28×10^{-4} mol/L, but WMQ-7 had stronger ability to oxidize H_2 which was 19.90×10^{-4} mol/L.

WMQ-7 grew quickly in MSA liquid culture medium, when intermittently injected H_2. The liquid culture medium changed turbid after cultured for 7 days. The logarithmic phase was on the 18th day, stationary phase was during the 22-26th day and the bacteria declined after the 26th day (Fig. 2). WMQ-7 wasn't treated under H_2 as control did not grow. It also illustrated that H_2 was necessary for the autotrophic growth of hydrogen-oxidizing bacteria.

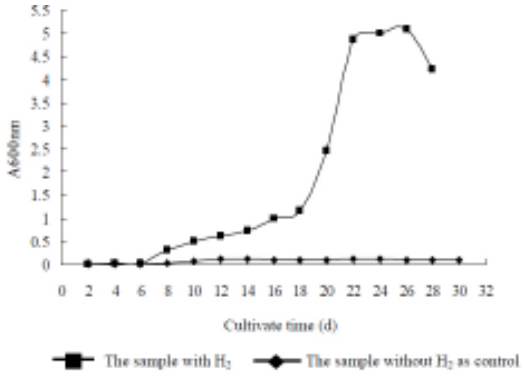


Fig. 2. Growth curve of the strain WMQ-7 in liquid MSA

Physiological and biochemical characteristics of hydrogen-oxidizing bacteria

Colonial morphology

8 strains had stronger ability to oxidize H_2 after cultured in MSA for 7 days and grew single colony significantly. Colony was round and the diameter was 0.4 mm-2 mm. The colonies were white except for the yellow colonies of FMG-5 and WMG-8. The edge of the colonies were regular except FMG-30FMG-5 and WMG-8 (Table 2). WMG-8, FMQ-3 and WMG-7 were gram-positive bacterium and the others were negative.

Table 2. Gram staining and strains morphological character

Strains	Gram staining	Cell morphology	Colonial morphology
WMQ-7	-	Short rod	White, round, small
FMG-3	-	Short rod	White, irregular colonies edge
FMG-5	-	Short rod	Yellow, irregular colonies edge
WMQ-8	-	Short rod	White, round, small
LD-WMQ	-	Short rod	Light white, round, small
WMG-8	+	Short rod	Light yellow, irregular colonies edge
FMQ-3	+	Short rod	White, round, small
WMG-7	+	Short rod	Oyster white, round, small

In scanning electron microscopy (Fig. 3) the cells can be clearly seen. 8 strains were all short rods and the size were between (0.525-0.96) μm×(0.92-2.80) μm and the cell form had no obvious difference. In transmission electron microscopy, WMQ-7 had single cluster flagellum (Fig. 4) and the flagellum length is about 2.5 times than the length of the bacterium.

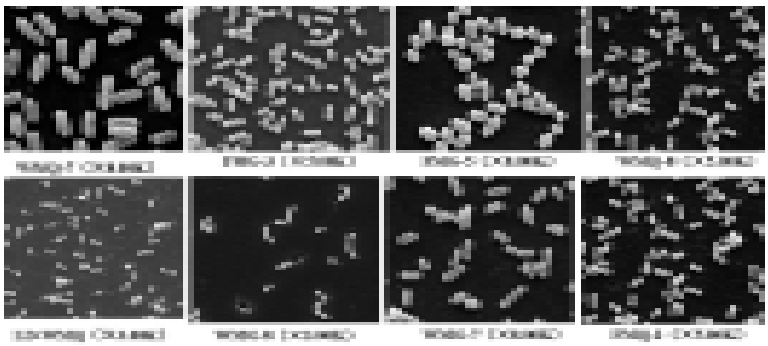


Fig. 3. Stereoscan photographs of 8 strains

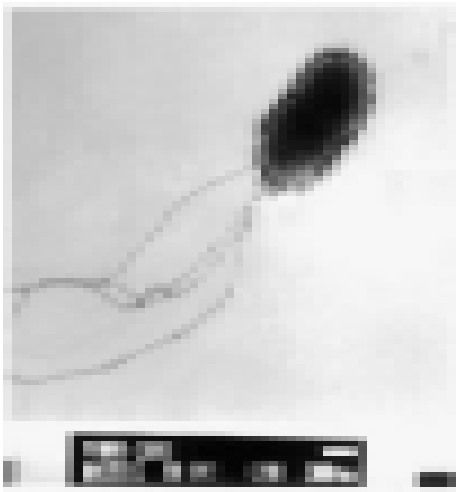


Fig. 4. Morphological character of strain WMQ-7 under transmission electron microscope (20000x)

Physiological characteristics

Physiological characteristics results showed (Table 3) the 8 strains were quite different and they were assigned to 7 different genera (Table 4) according to the common bacteria system identification handbook (Dong and Cai, 2001). Heterotrophic experiments of different carbon sources added in liquid medium showed that WMQ-7 grew well and could maintain a longer exponential phase with glucose and maltose. So the glucose and maltose was the better carbon source for WMQ-7. The best culture temperature was 30°C and the best pH was 7.0.

Growth of wheat promoted by hydrogen-oxidizing bacteria

Wheat seeds treated under light for 7 days and by 8 strains of hydrogen-oxidizing bacteria were observed and recorded for the wheatgrass, roots and dry weigh. SPSS was used for the single factor analysis of variance. The results showed (Table 5) the average length of wheat roots treated by strains FMG-5, WMQ-7 and FMG-3 were increased by 58.26%, 73.92% and 74.78% respectively than blank control. The average length of wheatgrass treated by strains WMQ-8, WMG-8, FMQ-3, LD-WMQ, WMQ-7, FMG-5 and FMG-3 were all increased by above 34.64% than blank control. The length of wheatgrass treated by strain

Table 3. Physiological and biochemical characteristics of 8 hydrogen-oxidizing bacteria

Characteristics	WMQ-7	FMG-3	FMG-5	WMQ-8	LD-WMQ	WMG-8	FMQ-3	WMG-7
1	+	+	-	-	+	-	+	-
2	+	+	+	+	+	+	+	+
3	-	+	+	-	-	+	-	+
4	-	-	-	-	-	-	-	-
5	-	-	-	-	-	+	-	-
6	+	+	+	+	-	+	+	+
7	-	-	-	-	-	-	-	-
8	+	+	+	+	-	+	+	+
9	+	-	-	-	+	+	-	+
10	+	-	-	+	-	+	+	+
11	-	-	-	-	-	-	-	-
12	-	-	-	-	+	-	-	-
13	+	-	+	+	+	-	+	-
14	-	-	-	-	-	-	-	-
15	+	-	+	-	+	+	-	+

Note: 1. Oxidase, 2. Catalase, 3. Acid and gas production from glucose, 4. V.P test, 5. M.R test, 6. Hydrolysis of starch, 7. Cellulose decomposing, 8. Gelation liquefaction, 9. Nitrate reduction, 10. Tryptophan deaminase, 11. Indole production, 12. Urease test, 13. H_2S production, 14. L-Phenylalaninase, 15. Use of citrate

Table 4. Strains' genus position

Strains	Species
WMQ-7	*Pseudomonas*.sp
FMG-3	*Pseudomonas*.sp
FMG-5	*Xanthobacter*.sp
LD-WMQ	*Plesiomonas*.sp
WMQ-8	*Leminorella*.sp
WMG-7	*Pimelobacter*.sp
WMG-8	*Terrabacter*.sp
FMQ-3	*Rarobacter*.sp

Table 5. 8 strains of hydrogen-oxidizing bacteria had different effect on wheat roots, wheatgrass and dry weight

Strains	Length of wheat roots (cm)	Length of wheatgrass (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

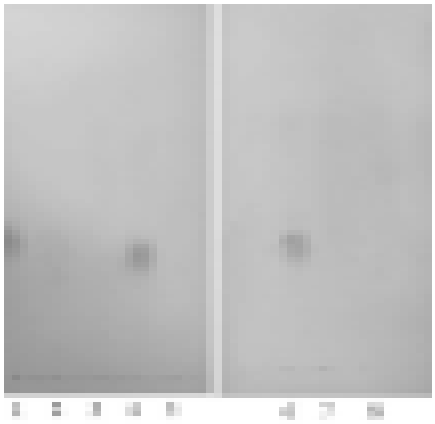
*p=0.004;**p<0.001

Table 6. Contrast of (G+C)% according to different but proximal species

Strains	(G+C)%	Strains	(G+C)%
WMQ-7	53.8	*Hydrogenophaga flava*	55.6
FMG-3	51.7	*Hydrogenophilus thermoluteolus*	58.7
FMG-5	54.45	*Rhizobium etli* CIAT 652	54.5
Pseudomonas putida	53.6	*Sinorhizobium meliloti* 1021	55.4
Pseudomonas putida strain S1	53.6	*Sinorhizobium medicae* WSM419	55.3
Pseudomonas sp. NPO-JL-67	53.5	*Brucella suis* 1330 chromosome I	55.6
Pseudomonas putida strain ATCC 11172	53.5	*Oceanobacter kriegii*	53.9
Uncultured bacterium clone MP104-0916...	53.4	*Ralstonia solanacearum* strain CPBF 1192	53.5
Uncultured *Pseudomonas sp*. clone AV 2...	53.6		

FMG-3 was the longest among them and increased by 67.64% than blank control. Dry weight of wheat treated by strains WMQ-8, FMG-5, FMG-3 and WMQ-7 were increased by 38.1%, 47.62%, 57.14% and 95.24% respectively than blank control.

Flowerpot experiments showed the number of wheat grains treated by strains FMG-5, WMQ-7, FMG-3 were increased by 88%, 138.4%,



1. Standard substance; 2. WMQ-7; 3. WMQ-7 without ACC; 4. FMG-3; 5. FMG-3 without ACC; 6. WMQ-8; 7. WMQ-8 without ACC; 8. Blank control

Fig. 5. The thin-layer chromatography results of 3 strains

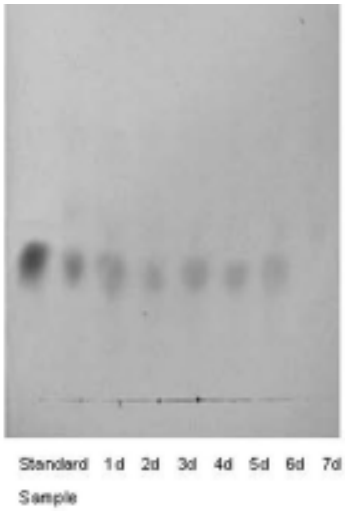


Fig. 6. Ability of WMQ-7 to consume ACC

12% respectively than culture medium blank control and 106.1%, 161.4%, 132.5% than water blank control. So the wheat treated by hydrogen-oxidizing bacteria could increase the yield of wheat but had little effect on plant height, spike length or spike number.

Enzymatic activity of ACC deaminase produced by strain WMQ-7

ACC deaminase stains

Thin-layer chromatography of srain WMQ-7, FMG-5, FMG-3 showed (Fig. 5) only

Fig. 7. Results of culture plate test on sierophore of strain WMQ-7

WMQ-7 strain could produce ACC deaminase and utilize it as the only nitrogen source. ACC was exhausted after 7 days.

Supernatants of strain WMQ-7 cultured at different time were tested by thin-layer chromatography. The results (Fig. 6) showed the longer cultured, the lighter of the color spots. There was no spot on the seventh day, which showed the content of ACC in the supernatant was little or had been exhausted.

Enzymatic activity of ACC deaminase

The regression equation of standard curve was y=0.0011x+0.0271, r=0.9958. After strain WMQ-7 was cultured in MSA modified liquid medium for 24 h, the light absorption value was 0.4 at 570 nm. The concentration of ACC in the strain

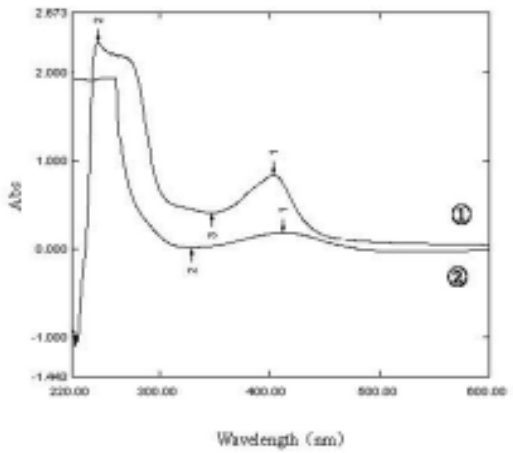


Fig. 8. UV wavelength scanning curve of siderophore produced by strain WMQ-7

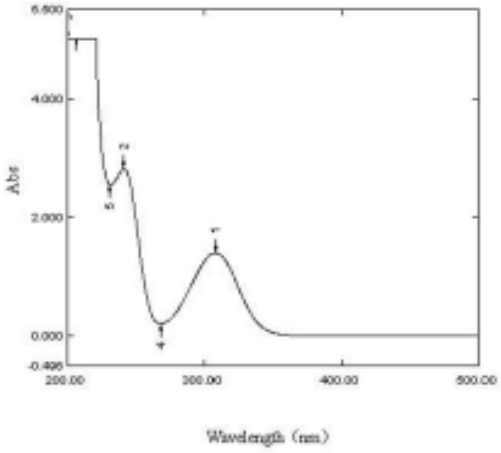


Fig. 9. UV wavelength scanning

WMQ-7 consumed was 161 µg/mL, and calculated its enzymatic activity of ACC deaminase was 0.671 U/µg.

Siderophore and its ability to transport Fe^{3+}

The color of medium near WMQ-7 colony was changed obviously after cultured for 4 days in CAS medium and 7 days in MKB medium (Fig. 7). Blue changed into pink in CAS medium, while the blue was changed into yellow in MKB medium. These were showed strain WMQ-7 could produce

siderophore and had ability to chelate Fe^{3+}.

Scanning between 220 nm-600 nm wavelengths the chromatography (Fig. 8) showed that there was an absorption peak at 404 nm and the absorbance was 0.834 when strain WMQ-7 cultured without Fe^{3+}. As Fe^{3+} was added in the contrast culture, the absorption peak was declined and the absorbance was only 0.209. This showed siderophore produced by strain WMQ-7 having the specific absorption peak at 404 nm (Ratledge and Dover, 2000). Absorption peak of standard substance 2, 3-DHBA was at 307.60 nm and 240.60 nm (Fig. 9), which showed the siderophore produced by strain WMQ-7 was not catecholamine type. It probably could be hydroxamic acid type or citric acid type.

The O-phenanthroline spectrophotometry was used to test the siderophore, indirect calculated the siderophore concentration was 7.1996 μg/mL and the regression equation was y=0.0638x+0.0227, r=0.9977.

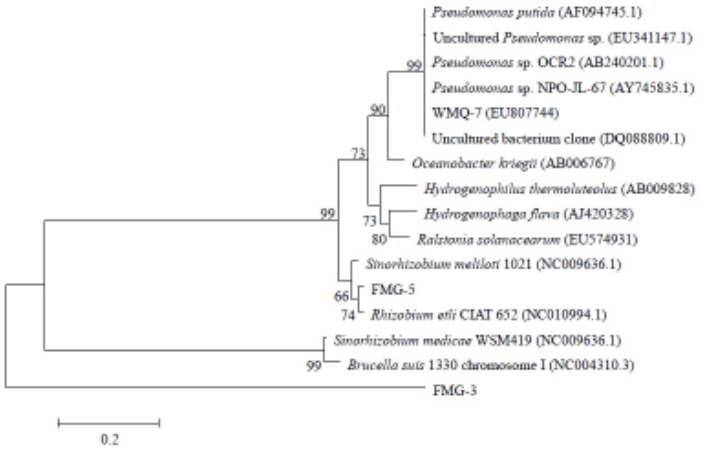


Fig. 10. Phylogenic tree based on 16S rDNA sequences of strain WMQ-7, FMG-3, FMG-5 and some relative bacteria

Analysis of 16S rDNA sequence

The 16S rDNA sequence of WMQ-7, FMG-3 and FMG-5 was separately 1451 bp, 1034 bp and 1394 bp. The content of GC was separately 53.8%, 51.7% and 54.45%. Contrast the content of GC of WMQ-7 to proximal species' (Table 6), and found that it's close to the *Pseudomonas putida*'s. While FMG-5 was close to *Rhizobium*, but the GC content of FMG-3 was very small and couldn't find a proximal species.

Phylogenic tree based on 16S rDNA sequences of WMQ-7, FMG-3 and FMG-5 (Fig. 10) showed that 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*.

Between FMG-5 and *Rhizobium* the sequence identity was 94% and they were clustered at the same branches in phylogenetic tree. But it couldn't be identified as *Rhizobium* because of the different colonial morphology and physiological characteristics. 16S rDNA of FMG-3 couldn't be found in GenBank and was a single branch in phylogenetic tree, which maybe a novel species.

CONLUSION

The capable of H_2 consumption was the basis of screening hydrogen-oxidizing bacteria. Chen (2007) isolated 22 strains of hydrogen-oxidizing bacteria from soybean rhizosphere. 55% of the strains absorbed H_2 over 1.25×10^{-4} mol/L and 40.9% of the strains had exhausted the initial concentration H_2 of 1.66×10^{-4} mol/L completely. This research in screening *Medicago sativa* rhizosphere of hydrogen-oxidizing bacteria with the initial concentration of H_2 of 2.42×10^{-3} mol/L and 8 strains absorbed H_2 over 2.44×10^{-4} mol/L, it's 25.93% of the total strains. Increasing the

concentration of H_2 would be conducive to isolate the hydrogen-oxidizing bacteria.

The researches on physiological and biochemical characteristics of hydrogen-oxidizing bacteria would help people deeply understand about their community structure and ecological functions, but the materials were scarce. Maimaiti et al. (2007) isolated hydrogen-oxidizing bacteria and identified them as *Variovorax paradoxus*, *Flavobacterium johnsoniae* and *Burkholderia sp.* from HUP^- (lack the hydrogenase enzyme) root nodule rhizosphere, and 16S rDNA sequences were analyzed. Chen (2007) isolated 20 strains of hydrogen-oxidizing bacteria from soybean rhizosphere and tested their physiological and biochemical characteristics, only one strain had ACC deaminase and identified as *Variovorax paradoxus*. 8 strains of hydrogen-oxidizing bacteria were isolated from *Medicago sativa* rhizosphere and were identified to 7 different species (Table 4). *Plesiomonas*, *Pimelobacte*, *Leminorella*, *Terrabacter* and *Rarobacter* were never reported before, compared with the reported species (Madigan *et al*., 2001). Hydrogen-oxidizing bacteria were such physiological large groups in soils.

Hydrogen-oxidizing bacteria are special physiological groups that gradually become the research focus because some of them can promote the growth of wheat and increase the yield when utilizes the H_2 released during nitrogen-fixing by legumes (Maimaiti et al., 2007). Dong (2003) found the soil treated by H_2 could obviously promote the growth of wheat, barley, rape and soybean. In the H_2 treated soil, dried weight of 4-7 weeks wheat and barley can increase by 15% to 48% (Fyson and Oaks, 1990). In the plant growth promoting experiments of hydrogen -oxidizing bacteria in soybean rhizosphere, Chen (2008) found the treated wheat roots were all longer than blank control and the growth rate was 111%-397%. Culture plate test in our research showed that the root length, wheatgrass and dry weight were obviously different as the different treated by strains of hydrogen-oxidizing bacteria on wheat seeds. Strains WMQ-7, FMG-3 and FMG-5 were the most obvious to promote the growth of wheat. The results of plant growth promoting experiments were consistent with previous reports (Chen *et al*., 2008).

Jacobson (1994) and Hontzeas (2004), respectively, 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 were raised too. Shen etc (2008) studied on factors affecting the enzymatic activity of ACC deaminase of strain XG32 was 0.442 U/μg. Chen etc (2008) isolated ACC deaminase positive strain from soybean rhizosphere, but didn't report the enzymatic activity. This study reported the enzymatic activity of ACC deaminase produced by strain WMQ-7 was 0.671U/μg. It is 1000 times more than the strain XG32's that reported before.

Siderophores are small molecular organic compounds (500-1500 D) and have high affinity to Fe^{3+}. It produced by nearly all the aerobic and facultative anaerobic bacteria when there are experiencing with iron stress. Siderophores can help the bacteria to obtain iron elements in the environment. Siderophores are not only the carrier of iron to bacteria but also play a very important role at certain plant pathogen pathogenicity aspects, and it's the mechanism of PGPR to inhibit soil-borne (Xie *et al*., 2006). Chen etc designed MSA-CAS detection culture plate and used aspartic acid instead of asparagine to test the siderophores produced by *Pseudomonas* (Chen *et al*., 2006). Pérez-Miranda etc used CAS dye liquor to test the siderophores (Pérez-Miranda *et al*., 2007). We designed the MSA-CAS and MKB-CAS test plates and found the strain WMQ-7 could produce siderophore with the absorption peak at 404 nm. When Fe^{3+} was added in the contrast culture condition, the absorption peak was declined obviously; this showed siderophore produced by strain WMQ-7 had the ability to transport Fe^{3+}. The O-phenanthroline spectrophotometry was used to test the siderophore and indirect calculate the siderophore concentration was 7.1996 μg/mL. This method was not reported yet and it could be a feasible way to make a quantitative detection of siderophore.

In this research we analyzed on molecular level and built phylogenic tree based on 16S rDNA sequences of WMQ-7, FMG-3 and FMG-5. The results showed hydrogen-oxidizing bacteria were quite different from each other, they clustered in different branches and the genetic distance was very far. Hydrogen-oxidizing bacteria were

physiological groups of different species and different taxonomic positions. Because of the close relationships between hydrogen-oxidizing bacteria and the growth of plants, plants growth promoting mechanisms would be a very important subject in the future.

ACKNOWLEDGEMENTS

This research was supported by West Light Talent Cultivation Plan of China (2013DF05) and the Natural Science Foundation of Shaanxi Province (SJ08-ZT03).

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