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Isolation and identification of new beneficial bacterial strains from rhizosphere of *Citrus sinensis* orchards

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ABSTRACT

The rhizosphere is the area around the root of a plant occupied by a unique population of useful bacteria known as plant growth promoting rhizobacteria (PGPR). In this study, the isolation and identification of rhizobacteria from orange (*Citrus sinensis*) orchards using 16S rRNA gene, as well as biological and biochemical assays is reported. Analysis of 16S rRNA gene was confirmed by biological and biochemical assays and showed that the isolated bacteria belong to the genera *Bacilli, Enterobacter* and *Pseudomonas*. Accordingly, *Enterobacterkobei* MR-R2, *Pseudomonas putida* MR-R5, *Bacillus cereus* MR-R, *Bacillus thuringiensis* MR-R1 and *Bacillus mycoides* MR-R4 were identified and registered in GenBank. The results of phosphate solubilization testing revealed that the maximum rate of phosphate solubilization was observed for *B. thuringiensis*, and *E. kobei;* however, *B. cereus*, *B. mycoides*, and *P. putida* indicated the least activity. It can be concluded that the isolated strains (especially *B. thuringiensis)* are newly identified phosphate solubilizing bacterial strains, among which *B. thuringiensis* can be used for growth promotion of orange trees.

Keywords: *Citrus sinensis*, phosphate solublization, Rhizobacter, Rhizosphere, 16S rRNA.

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Introduction

Agriculture is one of the most effective factors in the economy and human lifestyle [1]. Improvement of soil quality is considered as the most vital factor for agricultural development. Different types of soil contain some species of microbial flora, which affects the growth and development of different types of plants, microbes play a unique role in ecosystems [2]. Studying the coexistence of microorganisms with plants is very important for agricultural development and can be exploited in plant biotechnology; for example, plant growth enhancement, diseases-fighting plants, immune system resistance enhancement, useful compound extraction and siderophore production [3].

Rhizobacteria is among the known useful microbes that directly or indirectly assist plant growth [2]. Their ability to solubilize phosphate and produce phytohormones are the direct effects of these bacteria, whereas the indirect effects include inducing siderophore, antibiotic, degradation of enzymes and stimulation of plant defense systems [4, 5]. Some reported bacterial genera which are effective in the growth of different plants are as follows: *Azotobacter*, *Bacillus*, *Azospirillum*, *Serratia*, *Burkholderia*, *Enterobacter*, *Arthrobacter*, *Herbaspirillum*, *Pseudomonas* and *Rhizobium* [6].

Conversely, some genera of bacteria such as *Enterobacter*, *Bacillus* and *Pseudomonas* isolated from *Citrus sinensis* have been reported as beneficial bacteria that coexist with host plants [7]. Several studies were carried out to control pests and protect plants against diseases and insects, in order to enhance plant growth [8]. The main objectives of this study were to isolate and identify rhizobacteria from orange orchards and investigate their ability to esolubilise phosphate and hydrolyse alpha amylase.

Materials and Methods

Isolation of rhizosphere bacteria

Soil samples were collected from 8 to 10-year-old orange tree roots (of *C. sinensis*) from Dalfard –Jiroft located in Kerman Province, Iran. Surface soil (5 to 15 cm depth) was collected and added to plastic containers and transferred to the laboratory. In this research, 0.5 mg soil was dissolved in 5 ml sterile water and then the resultant suspension was centrifuged at 5000 rpm for 30 min at 4°C. To isolate bacterial strains, 100 µl of centrifuged supernatant was cultured on Pikovskaya's agar. The isolation was carried out after 9-fold serial dilutions on Pikovskaya's agar (PSB) by the Pour-plate method and subsequently incubated for 72 h at 30°C. Then, colonies which were grown on the PBS plates with clear halos were selected[9]. The colonies were identified after DNA extractions using Polymerase Chain Reaction (PCR) analysis. Also, in order to isolate *Bacillus*, *Pseudomonas* and *Enterobacter* genera from *C. sinensis* orchard, starch containing Nutrient agar (NA), *Pseudomonas* Isolation Agar (PIA) and Eosin Methylene Blue agar (EMBA) media were used, respectively.

DNA extraction

To extract bacteria DNA, a colony of each strain was cultured in nutrient broth at 37°C for 24 h. Subsequently, DNA was extracted using Vivantis DNA Extraction Kit, according to the manufacturer's instructions.

16SrRNA Amplification

The 16S rRNA was amplified using forward (5´-AGTTGATCCTGGCTCAG-3´) and reverse (5´-GGCTTACCTTGTTACGAC-3´) primers. PCR conditions were as follows: Initial

denaturation temperature at 94°C for 5 min and 30 cycles including a denaturation step at 95°C for 1 min, an annealing step at 95°C for 1 min and an extension step at 72°C for 2 min. Finally, PCR product was analyzed on 1% agarose gel, stained with ethidium bromide, and visualized under UV illumination and sequencing of the16S rRNA was performed by Bioneer Company, South Korea.

Phylogenetic analysis

The obtained 16S rRNA sequence was compared with the submitted sequence in the GenBank. The phylogenetic tree and molecular analysis were carried out using the Molecular Evolutionary Genetics Analysis (MEGA5) software. To produce a tree for the 16S rRNA sequence isolated from the *Enterobacter*, *Pseudomonas* and *Bacillus* genera, the neighbor-joining method and bootstrap test were used (the bootstrap value used was 1000). All test, newly identified bacterial genera and species, were registered in the GenBank.

Morphological, Physiological, and Biochemical characterization of isolates

Morphological, biological and physiological characterization of bacterial isolates were performed using conventional and routine techniques based on Bergey's Manual of Systematic Bacteriology [10]. Shape and color of the colonies, which were grown on nutrient agar, were determined after 48h at 30°C. Biochemical characteristics were determined by Simmons Citrate agar, Egg yolk agar (*Bacillus* species), urease test, Triple Sugar Iron (TSI) agar and Catalase test (adding a drop of hydrogen peroxide to selected colonies). Oxidase disc was used to assay oxidative activity of Gram-negative isolated bacterial strains and also, Gram staining was accomplished as a standard method to study the strains. Furthermore, in order to precisely identify *Enterobacter*, the strain was cultured for 48 h in an anaerobic medium. Also, gelatin liquefaction test was used to identify certain *Pseudomonas* spp. Optimum pH for the growth of strains measured at 30°C ranged from 4.5 to 8.

Growth curve

The growth curve of the isolated bacteria at 30°C was drawn. At first, a concentration equal to 0.5 McFarland

Turbidity Standard was prepared for each strain. Then, 100 µL of the mixture was added to 50 ml nutrient broth medium and finally, bacterial density was measured by spectrophotometer at 600 nm.

Qualitative estimation of phosphate solubilization

Qualitative analysis of phosphate solubilization was carried out using Petri dishes containing Pikovskaya's agar [9]. For this purpose, isolated bacteria were cultured in the medium in spot inoculation, using a sterile needle. Petri dishes were subsequently incubated at 30°C for 72h and then, the diameter of clear halo around the bacteria was measured using a ruler.

Results

In this research, in order to isolate *Bacillus*, *Pseudomonas*, and *Enterobacter* genera from *C. sinensis* orchard, starch containing NA, PIA and EMBA were used, respectively. The 16S rRNA amplification revealed that these strains belong to three genera and five species. The 16S rRNA sequence BLAST search showed that MR-R2 is more than 97% similar to *E. kobei* and also the resultant phylogenic tree indicated that MR-R2 and *E. kobei* (NR028993) are closely related and these strains lie on the same branch (Fig. 1).

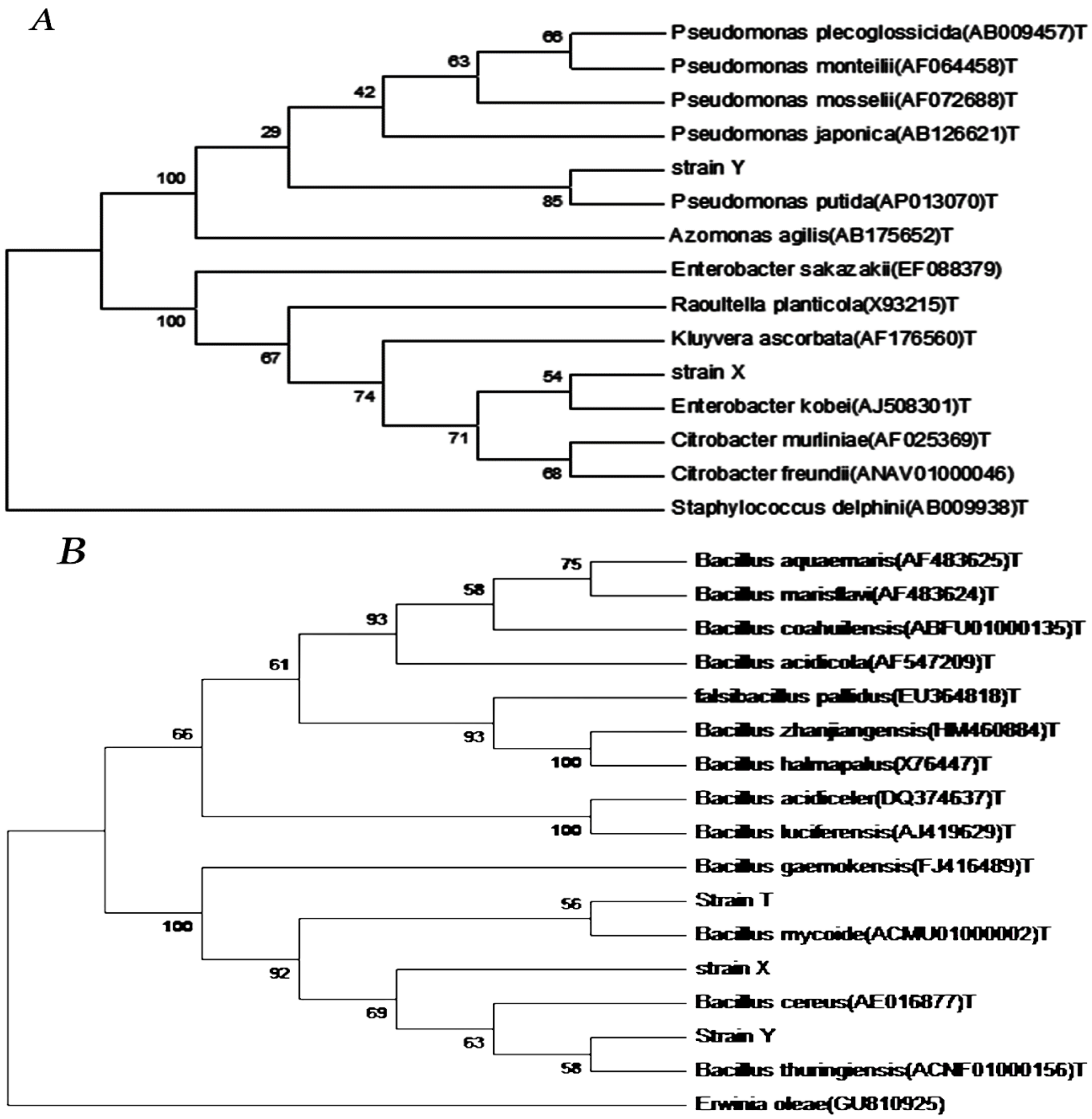

Figure 1. Phylogenetic tree based on partial sequences of the 16S rRNA gene. The tree determined by the neighbor-joining method andvalues at branching points indicate bootstrap. (a)Phylogenetic tree of bacillus cereus group and *Erwiniaoleae* as out group and *falsibacilluspallidus* as type sp of *Bacillaceae.*(b) Phylogenetic tree of strain Y and K . *Staphylococcus delphini* as out group and *Azomonasagilis* as type sp of *Pseudomonadaceae, Raoultellaplanticola* as type spof *Entrobacteriaceae.*

The 16S rRNA sequence BLAST search characteristic of the MR-R5 strain has 99% similarity to *Pseudomonas* and *Pseudomonas putida* (KX56789.1). Moreover, the 16S rRNA sequence analysis revealed that MR-R is 98% similar to *Bacillus cereus*. The 16SrRNA sequence comparison showed that MR-R1 has high similarity to *B. thuringiensis*. The 16S rRNA sequence analysis indicated that MR-R4 in the phylogenetic tree lies on the same branch close to *Bacillus mycoides* (MY.1236.1). Therefore, the five isolated strains were named and recorded in the GenBank as follows: *B. cereus* MR-R, *B. thuringiensis* MR-R1, *B. mycoide*s MR-R4, *E. kobei* MR-R2 and *P. putida* MR-R5 under accession numbers KC413033.1, KC461226.1, JX941572.1, KC413032.1 and JX843766.1, respectively.

Additionally, morphological and biological results of the isolated strains are listed in Table 1. The MR-R2 strain is a Gram-negative, anaerobic, rod shaped and

mobile bacterium with yellow colony. Based on this study's results, this bacterium is lactose- and catalase-positive, Simmons Citrate, Urease-, and Oxidase-negative. Furthermore, the results of the present study show that the strain can grow at pH=5 to 8, but the optimum pH for growth is 6. Moreover, the biological and morphological characteristics of MR-R5 revealed that it is a Gram-negative, non-mobile strain. This bacterium is an oxidase-, catalase- and a Simmons citrate-positive strain as well, but cannot consume urea and lactose. The maximum growth rate was observed at pH=6.3. The morphological and physiological results show that the three strains have similar properties (Egg Yolk agar, Gram test, and Simmons citrate agar). It is worthy of note that MR-R4 colonies are root-like and non-mobile, whereas the MR-R1 and MR-R strains are mobile. The maximum growth rate of MR-R4 and MR-R were observed at pH=6, whereas the maximum growth of MR-R1 was recorded at pH=7 (Table 1).

Table 1. Morphological and biological characteristics of the isolated strains

strains reaction	***B.cereus*** **(MR-R)**	***B.thuringiensis*** **(MR-R1)**	***B. mycoides*** **(MR-R4)**	***E. kobei*** **(MR-R2)**	***P. putida*** **(MR-R5)**
Gram test	+	+	+	-	-
Mobility	+	+	-	+	+
Oxidase	*	*	*	+	+
Citrate	-	-	-	*	+
Urease	*	*	*	-	-
Catalase	+	+	+	+	-
TSI	*	*	*	Acid/Acid	Alk/Alk
Egg yolk agar	+	+	+	*	*
Gelatinize	*	*	*	*	+
Hemolysis blood agar	+	+	+	+	*
Grow in anaerobic condition	*	*	*	+	*

Positive and negative response is shown by + and – respectively. The sign "*" indicated that the test was not performed.

Growth curve

The growth curve of the isolated strains revealed that all the strains grew to a logarithmic phase after four hours and were in a stationary phase after 48 h (Fig. 2).

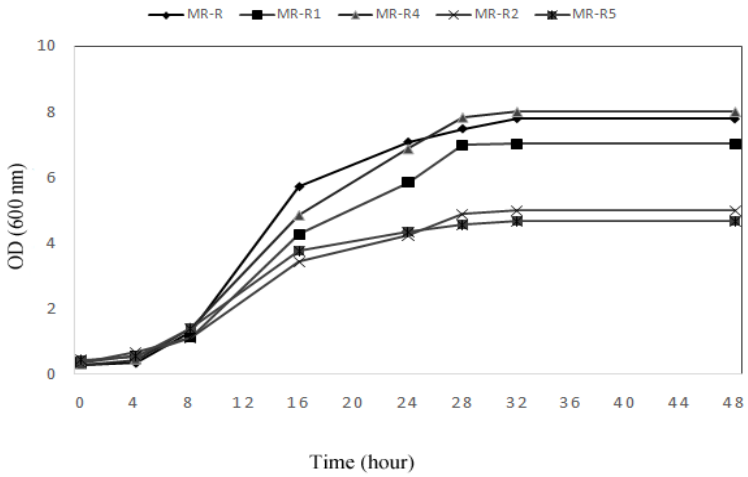

Figure 2. Growth curve for *B. cereus* MR-R, *B. thuringiensis* MR-R1, *E. kobei* MR-R2, *B. mycoides* MR-R4, *P. putida* MR-R5 at 30°C for 48h

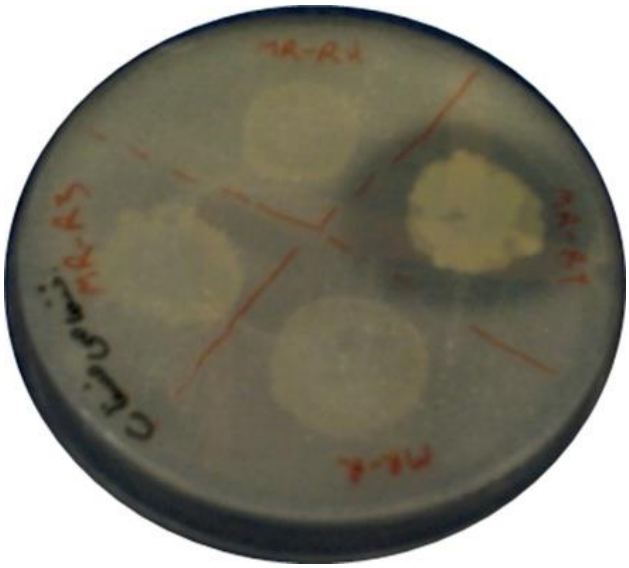

Figure 3. Phosphate solubilization of *Bacillus* group

Phosphate solubilization analysis

As shown in Figure 3, a clear 7 mm halo around the bacteria implies that the highest phosphate solubilization was by *B. thuringiensis* MR-R1. Other *Bacillus* solubilizing bacteria include *B. cereus* MR-R and *B. mycodes* MR-R4 which produced a halo of 1 mm in diameter. While *E. kobei* MR-R2 showed a halo with 4 mm diameter around its colonies, *P. putida* MR-R5 could not produce a halo around its colonies (data not shown) (Fig. 3).

Discussion

The rhizosphere is an area around the plant root that is occupied by a unique population of useful bacteria known as the PGPR [11, 12]. Due to the production of growth hormones, siderophores, antibiotics, and phosphate solubilization capability, these bacteria play an important role in promoting plant growth [4, 5]. This study reports the isolation and identification of rhizosphere bacteria population from *C. sinensis* located at Dalfored-Jiroft (Iran).

Biochemical and morphological experiments indicate (Table 1) that the MR-R2 strain isolated from the surface of the larvae belongs to *Enterobacter*. The tests show that the classification of MR-R2 at the genus level is consistent with lactose consumption and anaerobic growth condition of this bacterium. Furthermore, MR-R2 16SrRNA analysis showed that the strain is more than 97% similar to *E. kobei*. To the best of our knowledge, it is the first time that MR-R2 is reported from Iranian orange tree orchards. Furthermore, based on the investigation of morphological characteristics, which includes the MR-R5 colony shape and biochemical test, such as the Simmons citrate and oxidase test, it can be concluded that the strain belongs to the genus *Pseudomonas*. Based on the phylogenetic tree and biochemical tests, MR-R5 is classified as a gram negative bacterium and is a member of the genus *Pseudomonas*, thus the strain was named as *P. putida* (strain MR-R5). Due to mobility, lactose consumption, gram staining tests, and colony morphology of MR-R1, MR-R and MR-R4, these bacterial strains are members of the

genus *Bacillus*. The results obtained from 16S rRNA sequencing show that the isolated *Bacillus* strains belong to the *B. cereus* group. This group includes *Bacillus* sp. that are very close to *B. cereus*, precise identification is feasible using 16S rRNA sequencing. Phylogenic analysis showed (Figure 1) that *B. thuringiensis* MR-R1, *B. cereus* MR-R, and *B. mycoides*MR-R4 lie on branches close to *B. cereus* (KF31795), *B. mycoides* (AM747228), and *B. thuringiensis* (JX941572). Thus, these strains were named as MR-R1 (*B. thuringiensis*), MR-R (*B. cereus*), and MR-R4 (*B. mycoides*) and recorded in the GenBank.

Gram negative bacteria such as *Pseudomonas* and *Enterobacter*, and Gram positive bacteria such as *Bacillus* can promote plant growth due to production of siderophore, having phosphate solubilization capacity, and production of different antibiotics [13]. Plant growth-promoting bacteria present in the rhizosphere, are able to solubilize phosphate [14]. Phosphorus in soil is an essential element for plant growth and development, and can be found in both organic and inorganic forms. Although, plant roots can only take up water-soluble phosphorus [15], PGPR can solubilize phosphate in the soil because most bacteria are able to solubilize phosphate. Basically, some bacterial genera such as *Bacillus, Pseudomonas* and *Aspergillus* are powerful phosphate solubilizing strains that have been isolated from natural sources and their useful aspects have been investigated [16].

Moreover, in the present study, the ability of the isolated bacteria to solubilize phosphate was investigated. According to previous studies, any strain that can produce a halo around its colonies may serve as a good phosphate solubilizing strain [17]. The results of this study show that MR-R1 had the highest level of phosphate solubilization when compared with other strains. This finding is consistent with the results reported by De freitas [18]. Two other species of this genus include *B. mycoide*s and *B, cereus*, which show weak phosphate solubilization activity, respectively followed by *B. thuringiensis*. These results are also consistent with previous studies by Seshadri et al. [19]. It was shown that this strain is capable of solubilizing large amounts of phosphate in the Pikovskaya's liquid medium (Fig. 3). It has been reported that *B. cereus* are human pathogens, as they can cause food poisoning. *B. mycoide*s and *B. thuringiensis* can cause diseases due to the production of enterotoxin [20]. In contrast to the investigation by PANDEY (2005), during which it was shown that the *Pseudomonas* genus can properly solubilize phosphate in the Pikovskaya's agar culture medium, the MR-R5 strain could not properly solubilize phosphate in a solid culture medium.

Based on previous studies, *Enterobacter* are among the phosphate solubilizing bacteria [21]. It has been shown that *Enterobacter* sp. such as *E. intermedium*and and *E. agglomerans* can solubilize phosphate [22], although there is no detailed reported regarding the ability of *E. kobei* to solubilize phosphate. In this research, the ability of *E. kobei* to solubilize phosphate has been reported, as results show that MR-R1 can solubilize phosphate more than other understudy strains.

In general, *Enterobacter* members are pathogenic strains [23]. Recently, *Enterobacter* has been isolated from environmental sources and some of its useful activities such as promoting plant growth and biological control of plant diseases have been reported [24, 25]. This study emphasizes *E.*

kobei as a useful bacterium to improve agricultural production.

It can be concluded that the isolated strains (especially *B. thuringiensis*) are newly identified phosphate solubilizing bacterial strains, among which *B. thuringiensis* has been suggested to be used for the growth promotion of orange trees. Previous studies have shown that *B. thuringiensis* is an insecticide strain that can be employed in forestry and control of beetle pest [26]. Furthermore, due to isolation and identification of orange orchards rhizosphere-derived *Bacillus* and *Entrobacter* sp. (with phosphate-solublizing ability from orange trees), it can be concluded that the presence of these species in the plant root region promotes plant growth.

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