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Isolation and Characterization of A Salt-tolerant, Phosphate-solubilizing Bacterium Isolated from Yellow River Delta Saline-Alkali, China

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Phosphate-solubilizing bacteria (PSB) were isolated from the soil of Yellow River Delta saline-alkali, Shandong province (north China). 10 PSB were able to produce halo at 30° in a plate assay in the presence of solubilizing tricalcium phosphate in National Botanical Research Institute's Phosphate growth medium (NBRIP). One isolate (named as strain JP) was selected from those 10 PSB as the representative strain for the further identification. Microbiological assay showed that strain JP-1 was Gram negative, slightly curved with rods (sizes, 0.3-0.5μm wide and 1.1-1.55μm long). Biochemical tests indicated that strain JP-1 utilized glucose, glycerol, sucrose, lactose and mannose, but not urease and maltose. The optimum concentration of salts [NaCl and $CaCl_2$] for strain JP-1 to solubilize phosphate was 2.0 mg l^{-1}. Maximum concentration of soluble P released from $Ca_3(PO_4)_2$ by strain JP-1was 361 mg l^{-1} under 2.0 mg l^{-1} of salts [NaCl] in NBRIP. The solubilization of phosphate for strain JP-1was accompanied by the production of acid, pH values reducing from about 8.0 to 5.0. Strain JP-1 shared a 16S rRNA gene sequence similarity of 98% with *Bacillus thuringiensis* (DQ286305). The phylogenetic analysis proved that strain JP-1 (AB917465) was one member of the genus *Bacillus*.

Key words: Phosphate-solubilizing, Saline-alkali, Salt-tolerant, *Bacillus* sp. JP-1.

Phosphorus is second only to nitrogen as a mineral nutrient element required by both microorganisms and plants for its important role of accumulating and releasing of energy during cellular metabolism (Alexanderÿ1997). In the soil, inorganic phosphorus is present as insoluble forms and leading to very low concentration of inorganic phosphorus could be absorbed by plants since plants can only absorb the inorganic phosphorus(Abdÿ1994). PSB are considered to play an important ecophysiological role and often used as plant growth promoters in agriculture by solubilizing insoluble phosphate with the low-molecular-weight organic acids such as keto-gluconic acids and chelating oxo acids produced from sugars (Halvorson *et al*., 1990; Asea *et al*., 1998; Rodriguez *et al*., 2006), especially in P-deficient soils (Goldstein, 2007; Rodrý´guez *et al*., 2007; Jorquera *et al*., 2008; Poonguzhali *et al*., 2008). In addition, the application of PSB in the aquaculture has also been reported (Jana and De ,1990; Sahu and Jana ,2000; Chakraborty *et al*., 2004; Li and Shi,2006; Song *et al*., 2007; Wu *et al*., 2009).

Approximately 7% of the global land surface is covered with saline soil (Ruiz *et al*., 1996). Because both alkalinity and salinity in soil significantly reduce plant growth by inducing iron deficiencies and ion imbalances (Shannon,1997; López *et al*., 2006), vegetation development on alkaline-saline soil is usually poor. The Yellow River

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Delta, one of the largest deltas in China, is situated in the northeast of Shandong Province, on the southern bank of the Bohai Sea. The delta at longitude 118°05'E–119°15'E and latitude 36°55'N–38°15'N covers an area of 787,000 ha, including 670,000 ha of alkaline wasteland, with a population of 5.4 million. The very high concentration of dissolubilizing P in the soil of Yellow River Delta saline-alkali is considered to be a huge storage for plants to assimilate after being changed into soluble P. Therefore, the isolation and characterization of PSB which posses the high ability of solubilizing in Yellow River Delta saline-alkali become very urgent and necessary. The aims of this research were (1) to characterize the PSB isolated from Yellow River Delta saline-alkali and (2) to identify the phylogenetic position of PSB and (3) to test the ability of P-solubilizing under the high salt stress.

MATERIALS AND METHODS

Soil sample descriptions

Samples were collected from a depth of 15 cm at the localities of Yellow River Delta saline-alkali soil (longitude, 118°48'E–119°05'E; latitude 37°52'N–38°12'N). The soil was thoroughly mixed, placed in plastic bags and immediately transported to the research station. Total organic carbon (TOC) and nitrogen (TON) of soil were analyzed using a ligui TOC II total organic carbon (nitrogen) analyzer according to the manual instructions. The available P concentration of soil was determined using the sodium bicarbonate-extractable P colorimetric method (Olsen,1954). Soil pH value was determined with a 1:2.5 mass ratios of samples and deionized water. Soil electrical conductivity (EC) was measured with a conductivity meter (Model DDS-11A; Leizi, Shanghai, China). Water content in soil sample was estimated by drying material at 105ºC overnight.

Isolation of phosphate solubilizing bacteria

PSB were isolated from the saline soil of Yellow River Delta saline-alkali essentially according to the method (Lu *et al*.2008). In brief, PSB were enriched by inoculating 2 ml of suspensions from soil samples (5 g of fresh sample soaked with 45 ml of sterile distilled water) in NBRIP containing the following ingredients (Nautiyal, 1999) (l^{-1}): glucose 10 g, $MgCl_2 \cdot H_2O$ 5 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, KCl 0.2 g, $(NH_4)_2SO_4$ 0.1 g, $Ca_3(PO_4)_2$ 5 g. The medium was autoclaved at 121°C for 20 min for sterilization. $Ca_3(PO_4)_2$ was autoclaved first, then, the other sterile ingredients were aseptically mixed after autoclaving. The final pH was adjusted to 7.0 with sterile NaOH (1mol l^{-1}). The enriched culture was plated on NBRIP amended with 1.5% agar (w/v) and incubated at 30°C for 7 days. Colonies were selected from the plates on the basis of the appearance of a solubilizing zone. 10 isolates were purified and stored at semi-solid NBRIP agar (0.3%, w/v) medium for the future study.

Phenotypic test of strain JP-1

The cell morphology was verified by scanning electron microscope (SEM) (Hitachi, S-4800). Gram staining, colony morphology determination were essentially performed as described by Lu *et al*. (2008).

Biochemical tests of strain JP-1

Biochemical characterization for strain JP-1 consisted of catalase assay, oxidase assay, testing for growth on citrate, glucose, sucrose, soluble starch, lactose and so on. In addition, testing was done for the production of urea, indole, oxidase (catalyzed oxidation of cytochrome to H_2O_2). The biochemical tests were carried out essentially according to Bergey´s Manual of Determinative Bacteriology(Holt *et al*.,2010).

Tests on phosphate solubilizing ability

The 10 isolates from the previous purification operation, one isolate per plate, was stabbed and incubated for 24h, 48h, and 72h at 30 °C on NBRIP agar medium and their P-solubilizing zone were measured simultaneously. The experiments were repeated 3 times and the results are expressed as halo zone (Nguyen *et al*.,1992).

Quantitative test of phosphate solubilization was performed with Erlenmeyer flask (200ml) containing 100ml of NBRIP liquid medium inoculated with strain JP-1 (2ml inoculum with approximately 3 to 4×10^9 cfu ml^{-1}). Series of salt [NaCl and $CaCl_2$] concentrations (0, 2.0, 4.0 and 6.0 mg/ml) and pH (6.0, 7.0, 8.0 and 9.0) were prepared to examine the effect on P-solubilizing ability for strain JP-1 grown in NBRIP medium respectively. Autoclaved, uninoculated medium served as controls. The flasks were incubated at 30°C with shaking of 180 rpm per minute for 6 days. Soluble phosphate in culture supernatant was determined

spectrophotometrically according to the method described by Murphy and Riley at intervals of 24 hrs (Murphy and Riley,1962). Time dependent changes of pH value in NBRIP medium for strain JP-1 were measured at intervals of 12 hrs with a pH meter.

PCR amplification of 16S rRNA from the isolates

Phylogenetic assignment of strain JP-1was carried out by sequence analysis of the 16S rRNA. Genomic DNA of strain JP-1 was extracted from the cultures grown in NBRIP medium using the TIANamp Bacteria DNA DP302 Kit (Beijing Tiangen Biotech), according to its instructions. The 16S rRNA gene sequence was amplified using general primers PF5´-AGA GTT TGA TCC TGG CTC AG-3´and PR 5´-GGY TAC CTT GTT ACG ACT T-3´. PCR reactions contained 100 ng of genomic DNA, each primer at a concentration of 0.4 ìM, each dNTP at a concentration of 200ìM. PCR was performed using the following cycles: one initial denaturation at 94 °C for 3 min; 32 cycles of denaturation at 95°C for 45s; annealing at 55°C for 45s and a final extension at 72 °C for 10 min. The products were separated by running 5 ìl of the PCR reaction mixture in 1.0% (w/v) agarose gel and staining the bands with ethidium bromide.

Sequence alignment and phylogenetic analysis

The 16S rRNA sequences were determined by the automatic DNA sequencer (ABI Prism Model 3700, CA, USA). The primer used for sequencing was PF as mentioned above. The sequences determined from strain JP-1 were compared with the similar sequences retrieved from the National Center for Biotechnology Information GenBank (http://www.ncbi.nlm.nih.gov/) using BLAST program (Chen *et al*.,2006). All the obtained sequences were aligned using the CLUSTAL X program (Thompson *et al*.,1997) and the phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987; Sharma *et al*.,2002; Tamura *et al*.,2011). Bootstrap analysis was performed 1000 times using the same program as above (Saitou and Nei, 1987; Sharma *et al*.,2002).

RESULTS

Identification of strain JP-1

Soil characteristics were: TOC 53.2 mg kg^{-1}, TON 250mg kg^{-1}, available P 10.4 mg kg^{-1}, pH 8.0, electrical conductivity (ECe) 40.2 dS m^{-1}. The bacteria was isolated using dilution and plating methods on NBRIP medium. 10 isolates of P-solubilizing bacteria were obtained. It could be seen from Fig. 1 that all of the tested 10 isolates possessed P-solubilizing activity. Continuous observation indicated that diameter of zone were in increasing subsequently at every day incubation and the isolate number 7 showed the most efficiency of phosphate solubilizing among the 10 isolates (Fig. 1). Therefore isolate 7 was chosen as the representative bacteria and was named as strain JP-1 for the further study. The physichemical tests proved that strain JP-1 was Gram-negative. Colonies were milk white and smooth edge in NBRIP agar medium. Electron scanning micrograph indicated that strain JP-1 was slightly curved with rods (sizes, 0.3-0.5µm wide and 1.1-1.55µm long). The other morphological and chemical properties of strain JP-1 were listed in Table 1. Based on selected characteristics, strain JP-1 was seemed to be one member of genus *Bacillus*.

Data presented in Fig.2 indicated that the alkaline environment was more suitable for strain JP-1 to solubilize phosphate. Strain JP-1 showed the nearly identical P-solubilization activities when it was cultured in NBRIP medium with pH values of 7.0, 8.0 and 9.0 (Fig.2). The clearly lower P-

Table 1. Morphological and physiological traits of strain JP-1

Characteristics	Strain JP-1
Catalase activity	+
Oxidase	+
Urea	-
H_2S production	-
Indole	+
Urease	+
Maltose	-
Citrate	-
Glucose	+
Glycerol	+
Sucrose	+
Lactose	+
Mannose	+
Voges proskauer	+
Starch hydrolyzation	-
Gelatin liquefaction test	-

+: Positive response; -: Negative response

solubilization abilities for strain JP-1 growing in NBRIP medium with pH 6.0 was also proved in Fig. 2.

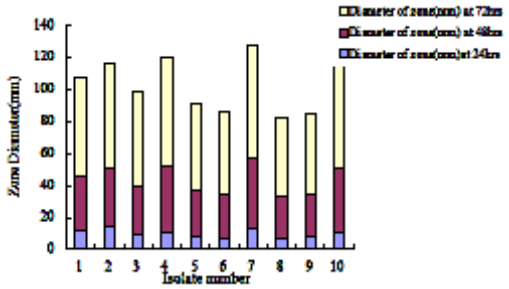

Fig. 1. Efficiency of phosphate solubilization by 10 isolates grown in NBRIP medium

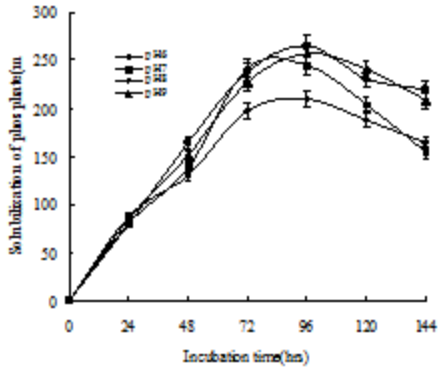

Fig. 2. Effect of pH on phosphate solubilizing ability for strain JP-1 grown in NBRIP medium. Values are means of three independent readings. *Error bars* (mean±standard deviation) are shown when larger than the symbol

The pH values were measured at 12 h intervals to find out whether solubilization of phosphate was accompanied by the production of acid or not. The clear decrease of pH values from about 8.0 to 5.0 in NBRIP medium inoculated with strain JP-1 during the incubation period (144 hrs) was proved (Fig.3).

To determine the effect of various salts [NaCl and $CaCl_2$] on P-solubilizing ability for strain JP-1, strain JP-1 was cultured in NBRIP medium containing salts [NaCl and $CaCl_2$] 0, 2.0, 4.0 and 6.0 mg ml^{-1} respectively. Data was presented in Fig. 4 (for NaCl) and Fig. 5 (for $CaCl_2$) respectively. Fig. 4 and Fig. 5 indicated that 2.0 mg l^{-1} of NaCl or $CaCl_2$ seemed to be the optimum concentration for strain JP-1 to solubilize phosphate respectively. Maximum concentration of soluble P released from $Ca_3(PO_4)_2$

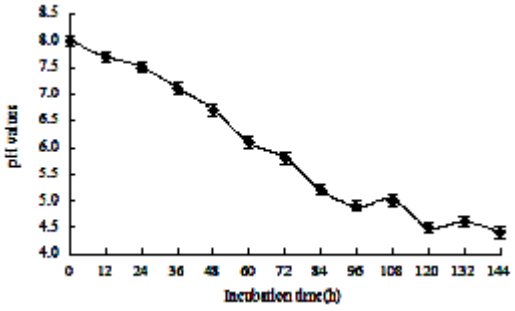

Fig. 3. Time dependent changes of pH value in NBRIP medium for strain JP-1. Values are means of three independent readings. *Error bars* (mean±standard deviation) are shown when larger than the symbol

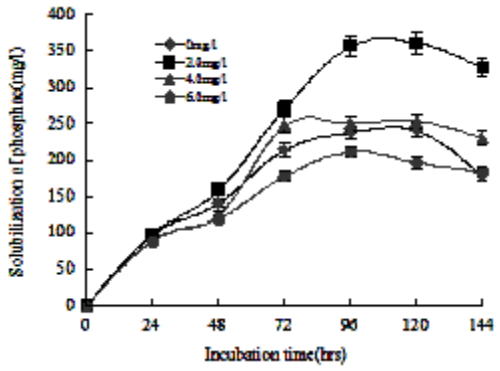

Fig. 4. Effect of NaCl supplements at 30°C on phosphate solubilization ability for strain JP-1 grown in NBRIP medium. Values are means of three independent readings. *Error bars* (mean±standard deviation) are shown when larger than the symbol

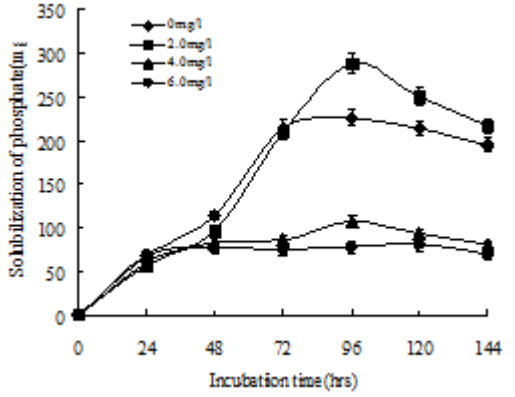

Fig. 5. Effect of $CaCl_2$ supplements at 30°C on phosphate solubilization ability for strain JP-1 grown in NBRIP medium. Values are means of three independent readings. *Error bars* (mean±standard deviation) are shown when larger than the symbol

by strain JP-1was 361 mg l^{-1} under 2.0 mg l^{-1} of salts [NaCl] in NBRIP (Fig. 4). The P-solubilizing ability was clearly inhibited when NaCl concentration was more than 2.0 mg l^{-1}(Fig. 4 and Fig. 5). The inhibition of $CaCl_2$ on P-solubilizing was more obvious than NaCl under the same concentrations(4.0 and 6.0 mg ml^{-1}) (Fig. 4 and Fig. 5).

Phylogenetic analysis

The 16S rRNA sequences of strain JP-1 has been submitted to DNA data bank of Japan (DDBJ) and assigned accession number AB917465. The DDBJ database was used to search for 16S rRNA sequences of all the sequences used in constructing neighbor-joining phylogenetic tree (Fig. 6). Phylogenetic tree showed that strain JP-1was allocated to cluster of *Bacillus*, most closed to *Bacillus thuringiensis* (DQ286305) with a highest similarity (98%). The result (Fig. 6) indicated that strain JP-1 was seemed to be one member of genus *Bacillus.*

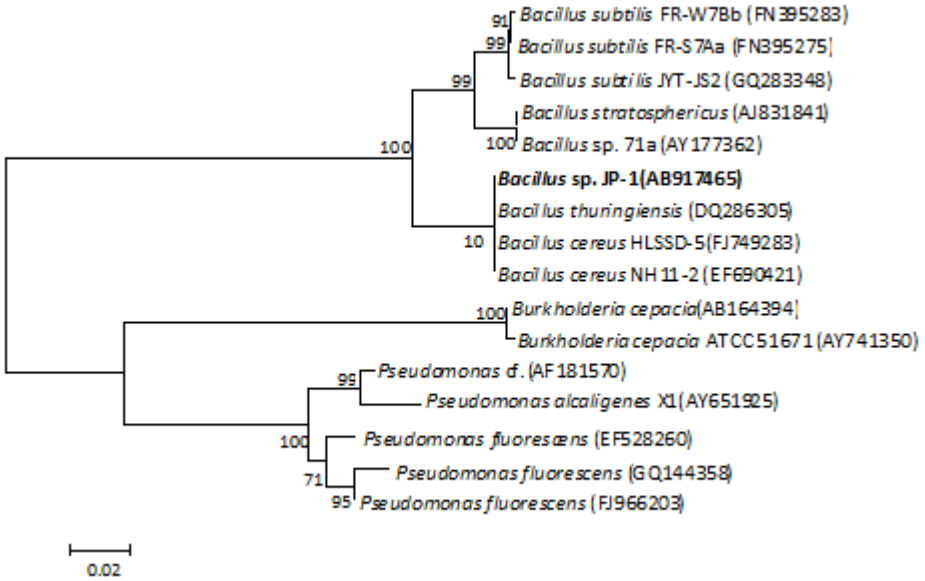

Fig. 6. Phylogenetic tree of strain JP-1 (AB917465) and its closest bacterium based on the 16S rDNA sequences constructed by the neighbor-joining method, MEGA version 5.1. The GenBank accession numbers of 16S rDNA are shown in parentheses. The scale bar indicates 0.1 substitutions per nucleotide position

DISCUSSION

Among the factors of influencing bacteria to solubilize phosphate compounds in saline-alkali soil, pH and salinity are the key ones which affect the growth of bacteria. As a result, we tested the P-solubilizing efficiency under different culture conditions including pH, $CaCl_2$ and NaCl for strain JP-1. The results in this study suggested that strain JP-1 isolated from alkaline soils have been able to solubilize phosphate in high pH and salt conditions. The suitable pH and salts [$CaCl_2$ and NaCl] concentration were 8.0-9.0 and 2.0 ml l^{-1} respectively. This result in our present study was consistent with the study of Johri *et al*. (1999), in which the improvement of low salt on P-solubilizing was also reported. What's more, the tolerance on too high concentration of salts was higher in our study for strain JP-1 than that in the study of Johri *et al.* (1999).

Isolation and characterization of microorganisms is one of prerequisites for the study of microbial systems. 16S rRNA sequence analysis as an important research method is widely used to identify the phylogenetic positions of bacteria (Weisburg *et al*., 2001; Bertrand *et al*., 2001; Ayyadurai *et al*., 2006). In present study, strain JP-1 (AB917465) was identified as *Bacillus* sp. using 16S rRNA sequences analysis. From the phylogenetic tree (Fig. 6), strain JP-1 could be regarded as one member of genus *Bacillus* for being grouped with other closet *Bacillus* strains. Further identification including DNA-DNA homology assay, G+C contents determination and

fat acids composition tests would be needed to find out the more exact phylogenetic positions for strain JP-1 (AB917465).

Microorganisms capable of producing a halo due to the solubilization of organic acids in the surrounding medium were selected as potential phosphate solubilizers. In our study, 10 isolates were found to produce the clear haloes and strain JP-1 (AB917465) (as the representative bacteria) has been proved to utilize glucose (Table 1). It could be interpreted by the report of Rodrý´guez *et al*. (2007) that gluconic acid was needed in solubilizing phosphate produced by the way of synthesizing from glucose in oxidation reactions. The clear decrease of pH (Fig. 3) also could provide the consistent conclusion with what has been described previously.

The stress tolerance towards high salt and pH for strain JP-1 (AB917465) could make it as an excellent model to study the physiological, biochemical, and molecular mechanism(s) of P-solubilizing under stress environments. Since the conditions in soil are much more complex than those in vitro, further study of affecting phosphate solubilization abilities by many more environments factors in alkaline soils seemed to be very necessary for us to make the more clear pictures of phosphate solubilization by bacteria.

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