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Full Length Research Paper

In vitro effects of artemisinin on inorganic phosphate-solubilizing bacteria

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In this study, four inorganic phosphate-solubilizing bacteria (PSB), that is, PSB1, PSB2, PSB14, and PSB16, were isolated from horse bean soil sampled from a farm at Southwest University, China. We evaluated the toxic effects of artemisinin (0, 24, and 48 mg L^{-1}) on PSB. To assess the antibacterial activity on PSB, the growth curves were modeled for different concentrations of artemisinin in liquid culture media. The ratio of glucose or saccharose utilization as a carbon source was measured. To further investigate the effect of artemisinin on the phosphate (P) release ability of PSB, P, organic acid, and pH in PSB culture solutions were determined. Artemisinin severely affected the growth and propagation of PSB. The amounts and carbon source utilization ratio of PSB with 48 mg L^{-1} artemisinin were significantly lower than with 0 mg L^{-1} after 6 h. The capacity of PSB for tricalcium phosphate (TCP) P solubilization was severely affected by artemisinin, the total P released by PSB1, PSB2, PSB14 and PSB16 inoculated with 48 mg L^{-1} artemisinin decreased by 31.45, 38.19, 6.73, and 12.41%, respectively, relative to 0 mg L^{-1}. The major organic acids secreted by PSB (oxalic acid and citric acid) decreased with increasing artemisinin concentrations, with consequent effects on pH. It is concluded that high concentration artemisinin inhibits the growth and organic acid secretion of PSB, with further effects on P-solubilization, which is significant when evaluating its ecotoxicity.

Key words: Artemisinin, carbon source utilization ratio, inorganic phosphate-solubilizing bacteria, growth curves, organic acids, toxic effects.

INTRODUCTION

Many plant species produce secondary metabolites that enhance their ability to defend themselves against herbivores or pathogenic microbes (Taiz and Zeiger, 2002; Hao et al., 2010). Antimicrobial compounds are mainly produced by plant roots such as terpenoids, benzoxazinone, flavonoids and isoflavonoids (Harsh et al., 2006), whereas others are produced via volatilization and eluviation of the aerial parts and through decomposition of plant body remnants in soil (Shen, 2006; Li et al., 2011). Soil may be exposed to high concentration of toxins when toxin-producing plants are continuously cultivated on a large scale.

Artemisinin is a secondary metabolite of *Artemisa annua* L., which is available commercially as an antimalarial drug in China (Klayma, 1985; Meshnick et al., 1996). However, it was recently reported that artemisinin had toxicity for plants, soil organisms and the aquatic environment. In soil, artemisinin repelled earthworms and inhibited the growth of lettuce (Jessing, 2009). The inhibitory allelopathic effects of *A. annua* on *Triticum aestivum* L., *Cucumis sativus* L., and *Raphanus sativus* L. were found to be strong (Shen, 2006). In aquatic environments, artemisinin had toxic effects on fresh water algae and duckweed (Jessing, 2009). The soil concentrations in *A. annua* field were up to 11.7 mg kg^{-1}, and it took at least 35 days before it could not be detected (< 0.36 mg kg^{-1}) at 20℃, classifying artemisinin as relatively persistent in the environment, combined with its water solubility of 49.7±3.7 mg L^{-1}, makes it potentially leachable (Jessing, 2009). However, there are no

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Table 1. The ability of the bacterial isolates to solubilize TCP.

Train	Taxonomic status	P-solubilization index
PSB1	*Aerococcus sp.*	2.86 ± 0.55[b]
PSB2	*Alteromonas sp.*	1.80 ± 0.23[c]
PSB14	*Pseudomonas mendocina*	4.58 ± 0.63[a]
PSB16	*Pseudomonas aeruginosa*	4.26 ± 0.32[a]

Colonies indicating the dissolution of tricalcium phosphate were scored after incubated seven days, and the P-solubilization index [=the ratio of the total diameter (colony +halo zone) to the colony diameter] was calculated. All the data were mean ± standard deviation of three replications. Different letter within the same column are significant at $P < 0.05$

previous reports on the ecotoxicity of artemisinin on soil microorganisms, such as phosphate-solubilizing bacteria (PSB).

Phosphorus (P) is the second most important macronutrient required for plant growth after nitrogen. Even in phosphorus rich soils, the majority of this element is present in an insoluble form and only a small proportion (0 to 0.1%) is available to plants (Stevenson and Cole, 1999). A large percentage of the phosphate fertilizers applied to soils also reprecipitates into insoluble forms thereby not being able to meet the P demand of crops (Podile and Kishore, 2006). PSB secrete organic acids and phosphatases, and organic acids convert the insoluble phosphates into soluble monobasic ($H_2PO_4^-$) and dibasic (HPO_4^{-2}) ions, a process which is referred to as mineral phosphate solubilization, phosphatases improve mineralization (hydrolysis) of organic P, which is available to plants (Richardson and Simpson, 2011). Therefore, PSB play an important role in plant nutrition by increasing phosphorus uptake (Antoun et al., 1998; Chabot et al., 1998; Rodriguez et al., 2006). Large scale cultivation of *A. annua*, incorcorpation of plant parts left over after harvest or isolation of artemisinin may transfer it to soil.

On the basis of the results of studies described earlier, it was hypothesized that high concentration of artemisinin affected soil PSB. To confirm this hypothesis, the growth, propagation, and carbon source utilization ratio of PSB were tested, as well as phosphate and organic acid concetration, and pH of culture solutions with different amounts of artemisinin.

MATERIALS AND METHODS

Collection and characterization of soil samples

Soil samples were collected from horsebean soil on the farm of Southwest University in China (Beibei, Chongqing, China; 29°81′34.38″N, 106°41′44.00″E). The physicochemical properties of the samples (a purple sand soil) were as follows: pH, 6.30; organic matter, 16.72 g kg^{-1}; total N, 1.03 g kg^{-1}; $NH4^+$-N, 2.85 mg kg^{-1}; $NO3^-$-N, 128.45 mg kg^{-1}; available P (extracted by 0.5 mol L^{-1} NaOH; molybdenum blue colorimetric method), 18.71 mg kg^{-1}; total P, 0.56 g kg^{-1}; available K, 89.49 mg kg^{-1}; and total K 18.23 g kg^{-1}. Soil samples (maximum depth, 20 cm) were collected from 10 cores at the site and transferred to the laboratory in sterile plastic bags where were stored at 4℃ until use. Each sample was passed through a 2 mm sieve to remove small stones and root fragments. Samples were pooled randomly to produce one working unit. Half of the unit was air-dried to determine the physicochemical properties, whereas the other half was stored in sterile plastic bags at 4℃ and used for the isolation of bacteria.

Isolation of bacteria

All microbiological media were prepared with deionized water (model 70; corporation water purified in Shanghai, China). To enhance the activities of phosphate-solubilizing bacterial populations, 10 g soil samples (three replicates) were added to Erlenmeyer flasks containing 90 mL of Jensen's nitrogen-free glucose liquid medium (JM). The medium consisted of: glucose (20 g), $K_2HPO_4 \cdot 3H_2O$ (1 g), $CaCO_3$ (2 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), NaCl (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), and Na_2MoO_4 (0.005 g) in 1 L of deionized water (Jensen, 1951), and it was amended with 10 g L^{-1} tricalcium phosphate (TCP) (Sigma Chemical Company, St Louis, MO, USA). Samples were then incubated on a rotary shaker (THY-111B; Tintan, Jiangsu Province, China) at 121 rpm at 28℃ in the dark for one week (Gaind and Gaur, 1991; Khaled et al., 2010). The supernatant was then serially diluted with sterile water, followed by homogenization (0.2 mL). Dilutions from 10^{-4} to 10^{-6} were plated using the spread method on Pikovskaya's agar medium (Pikovskaya, 1948) which contained: glucose (10.0 g), $Ca_3(PO_4)_2$ (5 g), $[NH_4]_2SO_4$ (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.25 g), NaCl (0.2 g), KCl (0.2 g), $MnSO_4$ (0.002 g), $FeSO_4 \cdot 7H_2O$ (0.002 g), yeast extract (Difco) (1.0 g), deionized water (1000 mL), and agar (20 g). Nine plates per dilution were air-dried in a laminar flow-cabinet for 15 min and the inoculated plates were then incubated at 28℃ in the dark for seven days. Fifty (50) bacterial colonies were isolated from the soil samples.

After purity control, four bacterial colonies (Voucher Specimen Nos PSB1, PSB2, PSB14 and PSB16) were selected based on their P-solubilization index, that is, the ratio of the total halo diameter (colony + halo zone) to the colony diameter (Premono Edi et al., 1996; De Freitas et al., 1997; Seshadri et al., 2002). PSBs isolated from horsebean soils were identified to taxonomic status based on morphological and biochemical tests as specified in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) (Table 1). The bacteria were stored at 4℃ in the laboratory (College of Resources and the Environment, Southwest University of China), before inoculating them onto fresh medium every three months, for further studies. All media were adjusted to pH 7.0 to 7.2 and sterilized at 121°C for 30 min.

Antibacterial activity assays

The optical density (OD) or degree of turbidity in the broth culture is directly related to the number of microorganism present or colony-forming units (CFU) (Domíngueza et al., 2001; Brewster, 2003), antibacterial activity assays were carried out using growth curve method with Pikovskaya's liquid medium with K_2HPO_4 (0.5 g L^{-1}). Bacterial suspensions were adjusted to 10^8 CFU mL^{-1} (Winslow et al., 1931) and 2 mL of the suspension was then poured into 150 mL Erlenmeyer flasks containing 50 mL Pikovskaya's liquid medium with K_2HPO_4. Control treatments without the suspension were treated in the same way. Amounts of 480, 240 and 0 mg of artemisinin (Sichuan Youyang Company) were individually dissolved in 100 mL of ethanol and these solutions were sterilized

using 0.22 µm millipore filters. Pikovskaya's liquid medium (50 mL) was mixed with 0.5 mL of the artemisinin solutions to produce 48, 24 and 0 mg L^{-1} dilutions (Kotan et al., 2008). These samples were incubated at 28℃ on a rotary shaker at 128 rpm. Growth of the desired organism was measured by recording the OD at 600 nm at regular intervals until the 0 mg L^{-1} (untreated) culture reached its maximum OD (Brewster, 2003).

The OD of bacterial culture suspensions (3 mL) were tested using a spectrophotometer (Model 721) (Jayashree et al., 2011) at 600 nm after incubating for 0, 1.5, 3.5, 6, 8, 12, 16, 18 and 20 h. All tests were repeated in triplicate at each dose (n = 27), while triplicates were tested once. In order to eliminate the interference produced by bacterial metabolism, the amount of bacteria (10 µL bacterial culture suspension) was counted using a microscope (XSP-6C, Dinolite Corporation, Shanghai, China) after incubating the bacterial suspension for 6 h.

Assays of carbon source utilization ratio

Saccharose (10 µg L^{-1}) and glucose (10 µg L^{-1}) liquid media were prepared to determine the carbon source utilization ratio of isolates. Solutions contained the following: glucose (10 µg) or saccharose (10 µg), $[NH_4]_2SO_4$ (0.5 g), K_2HPO_4 (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.25 g), NaCl (0.2 g), KCl (0.2 g), $MnSO_4$ (0.002 g), $FeSO_4 \cdot 7H_2O$ (0.002 g), yeast extract (Difco) (1.0 g), and deionized water (1000 mL). Individual 150 mL flasks, each containing 50 mL of liquid medium, were inoculated with 2 mL of suspension of each isolate (approximately 10^8 CFU mL^{-1}). Mixtures were then treated with 0.5 mL of artemisinin solutions and incubated at 28°C on a rotary shaker at 121 rpm for 6 h in the dark. Control treatments without the suspension were treated in the same way. The bacterial suspension (15 mL) from each flask was centrifuged for 10 min at 10.08 x 10^4 g. The supernatant was then filtered through a sterile 0.22 µm millipore membrane to remove cellular debris. The filter-sterilized cell-free culture fluid was collected in sterile McCartney vials before testing using the total anthrone-sulfuric acid colorimetric method (**Doutre et al.,** 1978). The carbon source utilization ratio of isolates was calculated using the following formula: carbon source utilization ratio (µg L^{-1} h^{-1}) = [C sources (µg L^{-1}) in control - C sources (µg L^{-1}) in culture fluid] / 6 h (Ghose, 1987).

Phosphate assays

Isolates solubilized from TCP by phosphates that were quantified using vanadate-molybdate-yellow colorimetry (Chapman and Pratt, 1979). Solubilization of insoluble inorganic phosphates was determined in Pikovskaya's liquid medium prepared according to Harrison et al. (1972). The medium was buffered using NaOH (2 mol L^{-1}) to pH 7.08. Individual 150 mL flasks, each containing 50 mL of amended Pikovskaya's liquid medium with TCP (2 g L^{-1}), were inoculated with a 2 mL suspension of each isolate (approximately 10^8 CFU mL^{-1}) and treated with 0.5 mL of artemisinin solutions. Deionized water media inoculated with 0.5 mL ethanol was treated with artemisinin (0 mg L^{-1}). Samples were then incubated at 28℃ on a rotary shaker at 121 rpm in the dark. After seven days, samples were allowed to settle for 2 h at 4℃, before the bacterial suspension (15 mL) from each flask was centrifuged for 10 min at 10.08 x 10^4 *g* to separate the supernatant from cell debris. The supernatant fluid was then filtered through sterile 0.22 µm millipore membranes. The filter-sterilized cell-free culture fluid was collected in sterile McCartney vials and allowed to settle for 15 min at room temperature. Then it was used for the determination of water-soluble P (Khaled et al., 2010). The control consisted of sterile non-inoculated liquid medium. The sedimentations were washed using sterile deionized water three times and digested with H_2SO_4 and H_2O_2, before the determination of bacterial phosphates after sterilizing at 121°C for 30 min.

Total solubilizing phosphates (mg L^{-1}) = available P in the culture solution (mg L^{-1}) + bacterial phosphates (mg L^{-1}).

Assays of organic acids and pH

To record the change in pH and quantify the amount of organic acids released into the medium, a 0.5 mL aliquot of the culture from the previous TCP solubilization experiment was mixed with an equal volume of 0.05 mol L^{-1} sulfuric acid, and centrifuged for 10 min at 10.08 x 10^4 g. It was then filtered through sterile 0.22 µm millipore membranes. Oxalic acid and citric acid in the culture filtrates were determined using a high performance liquid chromatography (HPLC) system (LC-20AT series, Shimadzu, Japan), equipped with ChemStation software (Shimadzu). The system comprised two pumps, an online vacuum degasser, a thermostatted column compartment, and a diode array detector, used for chromatographic analysis. All separations were carried out using a reverse-phase Diamonsil C_{18} column (4.6 mm × 250 mm, 5 µm). A linear gradient elution using eluent A (methanol) and eluent B (0.1% phosphoric acid) was used for the separations. The elution program was well optimized and it was conducted as follows: an isocratic elution of 95% B for the first 20 min; solvent flow rate was 1.0 mL min^{-1}; the ion detection wavelength was set at 210 nm; the injection volume was 20 µL; and the column temperature was maintained at 30℃ (Kim et al., 1997a; Yang, et al., 2009). The total organic acid in the culture filtrates was determined by titration, with phenolphthalein as indicator. The pH of the culture medium was measured with a pH meter equipped with a glass electrode.

Statistical analysis

To evaluate the effect of different artemisinin concentrations on growth, propagation, carbon source utilization ratio, soluble P concentration, organic acid release, and pH of IPSB, data were tested using a one-way analysis of variance (ANOVA) and significant differences between means were compared using Fisher's Protected LSD test (at $P = 0.05$). SuperANOVA® (Abacus Concepts Inc., Berkeley, CA, USA) was used for all analyses. Regression analyses shown in all figures were conducted using SigmaPlot 7.101 (SPSS Inc., Chicago, IL, USA).

RESULTS

Effects of artemisinin on growth and propagation of PSB

Most PSB species grow satisfactorily in Pikovskaya's medium. Yeast extract-based mediums supply a variety of sources for nutrients and growth factors (Elkan and Kwik, 1968; Graham, 1963), whereas a minimal medium made with only specific additives is more likely to highlight metabolic disorders of bacteria resulting from artemisinin, that is,induced metabolic damage. Therefore, to enhance isolates growth, K_2HPO_4 (0.5 g L^{-1}) was added to Pikovskaya's medium. Based on the OD of the isolates, growth rates were as follows: PSB2＞PSB1＞PSB14＞PSB16.

Reduced growth curves of PSB in broth medium were

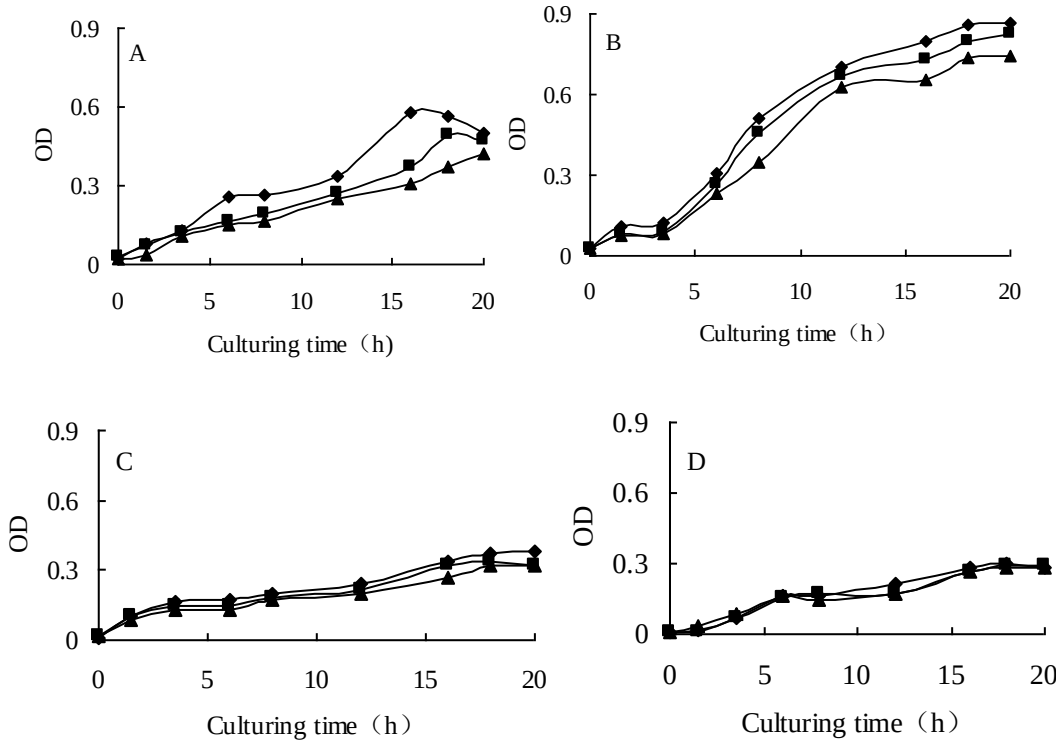

Figure 1. The influence of artemisinin on the growth of inorganic phosphate-solublizing bacterial in liquid culture mediums. (A) PSB1; (B) PSB2; (C) PSB14; (D) PSB16, artemisinin application rates are denoted as ◆, 0 mg L^{-1}; ■, 24 mg L^{-1}; ▲, 48 mg L^{-1}. Results are means of three independent trials and each carried out in triplicate.

observed with different concentrations of artemisinin (24 and 48 mg L^{-1}). This showed that increasing amount of artemisinin in liquid culture media (0, 24 and 48 mg L^{-1}) led to a reduction in the OD of IPSB (PSB1, PSB2, and PSB14) (Figure 1). The ODs of the culture solution (0, 24, and 48 mg L^{-1} artemisinin) when incubated with PSB1 were 0.259, 0.167, and 0.148, respectively, after 6 h (Figure 1A). The ODs of PSB2 were 0.309, 0.267 and 0.231, respectively (Figure 1B). The ODs of PSB14 were 0.175, 0.151, and 0.131, respectively (Figure 1C). The results were significantly different ($P < 0.05$) when comparing 0 with 24 or 48 mg L^{-1}. However, artemisinin had no significant effects on the OD of PSB16, where the ODs of isolates were 0.160, 0.157 and 0.16, respectively (Figure 1D).

Statistical analysis of the bacterial number after incubation for 6 h indicated that artemisinin affected the growth and propagation of strains PSB1, PSB2, and PSB14, while it also affected PSB 16. Table 2 shows that treatments with artemisinin significantly decreased the bacterial count of the isolates.

Effects of artemisinin on the carbon source utilization ratio by PSB

Other than vitamins, nitrogen, and amino acids, yeast extract provides carbon. To investigate the possible inhibitory effects of artemisinin on the carbon source utilization ratio of PSB, a liquid medium containing saccharose (10 μg L^{-1}) and glucose (10 μg L^{-1}) was pretreated with increasing concentrations of artemisinin ranging from 0 to 48 mg L^{-1} for 6 h. Figure 2 shows that artemisinin exhibited significant inhibitory effects on the carbon source utilization ratio of PSB in a dose-dependent manner. The carbon source utilization ratio of the isolates deceased with the increasing concentration of artemisinin, and a significant difference was found when comparing between 0 with 24 and 48 mg L^{-1}, except utilization ratio of glucose by PSB1.

Effects of artemisinin on the P-solubilizing activity of PSB

The P-solubilizing activity measures the microbial biochemical ability (Sagoe et al., 1998). However, the P-solubilizing activity of PSB was inhibited in this study. Figure 3 shows that by increasing the concentration of artemisinin from 0 to 48 mg mL^{-1}, the P-solubilizing activity of isolates was significantly decreased. Compared with 0 mg L^{-1}, the TP solubilized by PSB1 treated with 24 and 48 mg L^{-1} artemisinin (decreased by 10.96 and

Table 2. Effects of different amount (0, 24, 48 mg L^{-1}) of artemisinin on numbers(×10^6 bacteria mL^{-1}) of PSB in liquid culture media after incubation for 6 h.

Strains	Artemisinin concentration (mg L^{-1})		
	0	24	48
PSB1	237.5 ± 4.97^{bA}	204 ± 7.23^{bB}	29.5 ± 4.96^{bC}
PSB2	487.5 ± 17.82^{aA}	307.5 ± 30.21^{aB}	61.5 ± 3.76^{aC}
PSB14	97 ± 10.97^{cA}	46.5 ± 8.35^{cB}	11 ± 1.70^{cC}
PSB16	29 ± 2.81^{dA}	15 ± 2.24^{dB}	4.5 ± 0.94^{dC}

The average ± standard deviation (n = 3) following different capital on the same row indicates the values are significantly different at $P < 0.05$, different lower case letter on the same column indicates the values are significantly different at $P < 0.05$.

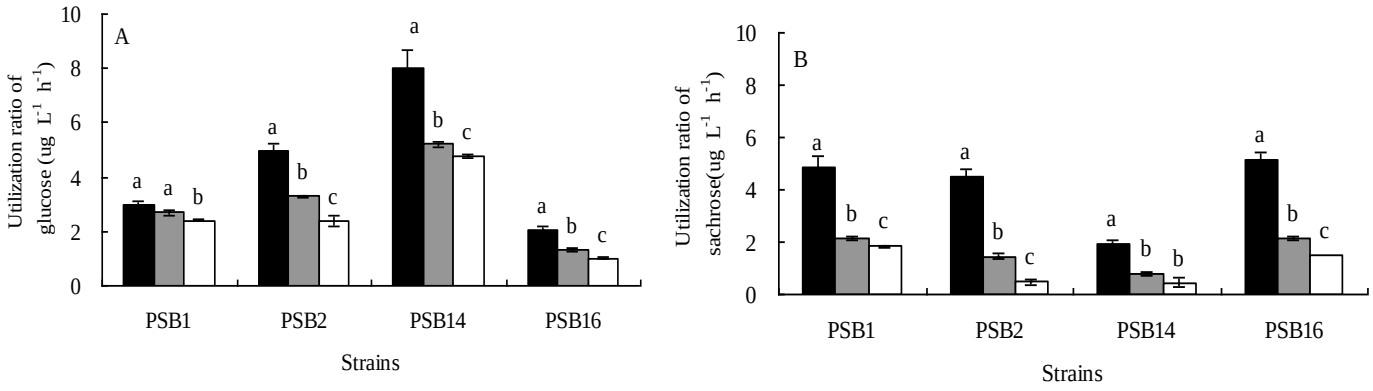

Figure 2. After treatment with artemisinin for 6 hours, carbon utilization ratio of PSB in culture solutions were measured by anthrone-sulfuric acid colorimetric method. Artemisinin application rates are denoted as 0 mg L-1 (■), 24 mg L-1 (■) and 48 mg L-1 (□). (A) Utilization ratio of glucose, (B) Utilization ratio of saccharose, Data are representative of three independent experiments (average ± Standard deviation). Different letter within the column are significant at $P<0.05$.

31.45%, respectively) (Figure 3A); with PSB2 it decreased by 16.93 and 38.19%, respectively (Figure 3B); with PSB14 it decreased by 5.22 and 6.73%, respectively (Figure 3C); and with PSB16 it decreased by 6.35 and 12.41%, respectively (Figure 3D). Phosphate of the culture solutions and PSB incubated with artemisinin (48 mg L^{-1}) also decreased significantly.

Effects of artemisinin on the organic acid secretory activity of PSB

Secretion of oxalic acid by isolates was severely affected by artemisinin (Figure 4A), as the concentration of artemisinin increased from 0 to 48 mg mL^{-1}. The amounts of oxalic acid in the treated culture solution decreased significantly with increasing artemisinin. Oxalic acid levels in PSB1, PSB2, PSB14, and PSB16 treated with 0 mg mL^{-1} artemisinin were 4.21, 2.97, 8.47 and 8.40 g L^{-1}, respectively, whereas with 24 mg mL^{-1} artemisinin the levels were 3.35, 1.07, 7.92, and 7.99 g L^{-1}, respectively. With 48 mg mL^{-1} artemisinin, the levels were 2.39, 0.63, 7.27 and 7.42 g L^{-1}, respectively. Compared with 0 mg L^{-1} artemisinin, the concentration of oxalic acid in culture solutions treated with 48 mg L^{-1} artemisinin decreased by 43.16, 78.76, 14.15, and 11.73%, respectively.

Citric acid secretion was also severely affected when the isolates were treated with different concentrations of artemisinin (Figure 4B). The results show that strains PSB1 and PSB2 secreted large amounts of citric acid, whereas PSB14 and PSB16 secreted little or no citric acid.

When strain PSB1 was grown with different concentrations (0 to 48 mg L^{-1}) of artemisinin, citric acid secretion decreased from 3.01 to 2.50 g L^{-1} and the same trend was found with PSB2, the secretion of which dropped from 3.02 to 1.77 g L^{-1}.

Total organic acid secretion (Figure 4C) by the four isolates decreased with increasing artemisinin. Compared with 0 mg L^{-1} artemisinin, the total organic acid concentration in culture solutions treated with 48 mg L^{-1} artemisinin decreased by 2.62, 3.56, 1.20 and 1.06 g L^{-1} for BSP1, PSB2, PSB14 and PSB16, respectively.

Treatment of the four isolates with different concentra-

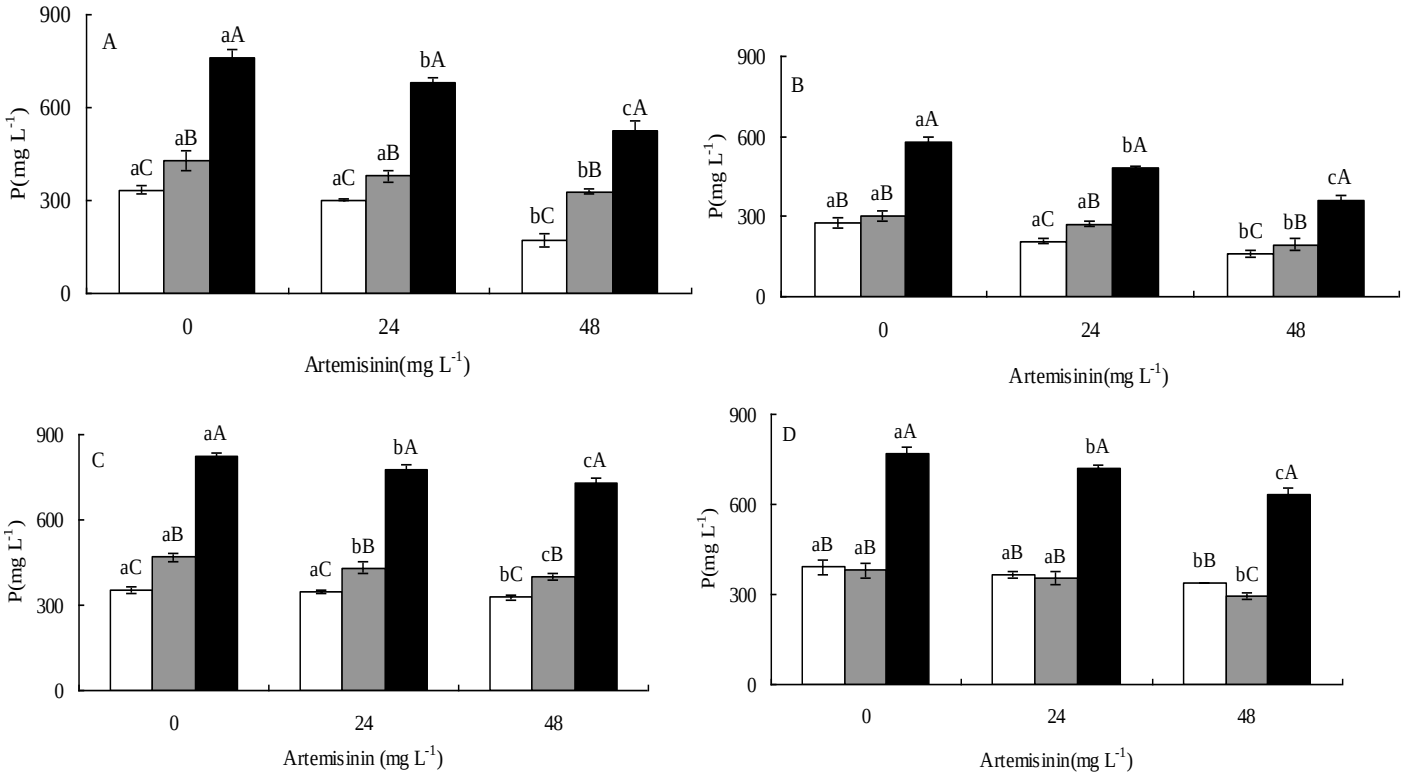

Figure 3. Concentration of different phosphorus. (□)Available P in the culture solutions, phosphorus of inorganic phosphorus-solubilized bacteria (■P of IPSB), and total phosphorus (■TP= P of PSB + available P) after inoculated seven days in liquid culture mediums with the different amounts (0, 24, 48 mg L^{-1}) of artemisinin. (A) PSB1; (B) PSB 2; (C) PSB14; (D)PSB 16. The average ± standard deviation(n=4) followed different capital letter of the same artemisinin concentration indicating the values of different phosphorus are significantly different at $P < 0.05$, different lowercase letter of the different artemisinin concentration indicates the values of same phosphorus are significantly different at $P < 0.05$.

tions of artemisinin significantly reduced pH in culture solutions amended with TCP compared with the control (pH 7.08). A significant reduction in pH (Figure 4D) and a significant increase in the concentration of released P were detected in the culture solution of all isolates treated with different concentrations of artemisinin (Figure 3). In contrast, pH of culture solutions increased with increasing concentrations of artemisinin. The pH ranges for culture solutions of PSB1, PSB2, PSB14, and PSB16 incubated with artemisinin (from 0 to 48 mg L^{-1}) were 4.7 to 5.6, 5.5 to 5.8, 3.2 to 3.6, and 3.3 to 3.6, respectively.

DISCUSSION

OD of PSB16 was not affected by artemisinin after treated 6 h (Figure 1), but the numbers were severely affected by it (Table 2). This suggests that artemisinin may have disrupted the cell envelope of PSB16, OD was interfered by the contents released out from cells, which is an effect also found with synthetic pesticides, such as diquat, paraquat, glyphosate, and chlorsulfuron (Eberbach and Douglas, 1989).

The carbon source utilization ratio of the isolates deceased significantly when incubated with artemisinin. This suggests that artemisinin may affect the carbon source metabolism of PSB, which further affects the growth and propagation. It was similar to herbicides, such as Herbogil (dinoterb) and Goltix (metamitron), which effected respiration and dehydrogenase activity, as well as carbon and nitrogen mineralization (Engelen et al., 1998).

Results of Figure 3 show that artemisinin affected the P-solubilizing activity of PSB, which may indirectly affect phosphate absorption by plants or other organisms. The synthesis inhibitory effect of *A. annua* on *Triticum aestivum* L., *Cucumis sativus* L., and *Raphanus sativus* L. was previously demonstrated (Shen, 2006). As found with herbicides, soil may reduce the activity of artemisinin, whereas these results obtained in this study where based on PSB grown in culture solution. The results indicate the potential of artemisinin to interfere with the P-solubilizing activity of PSB (Fletcher et al., 1956; 1957).

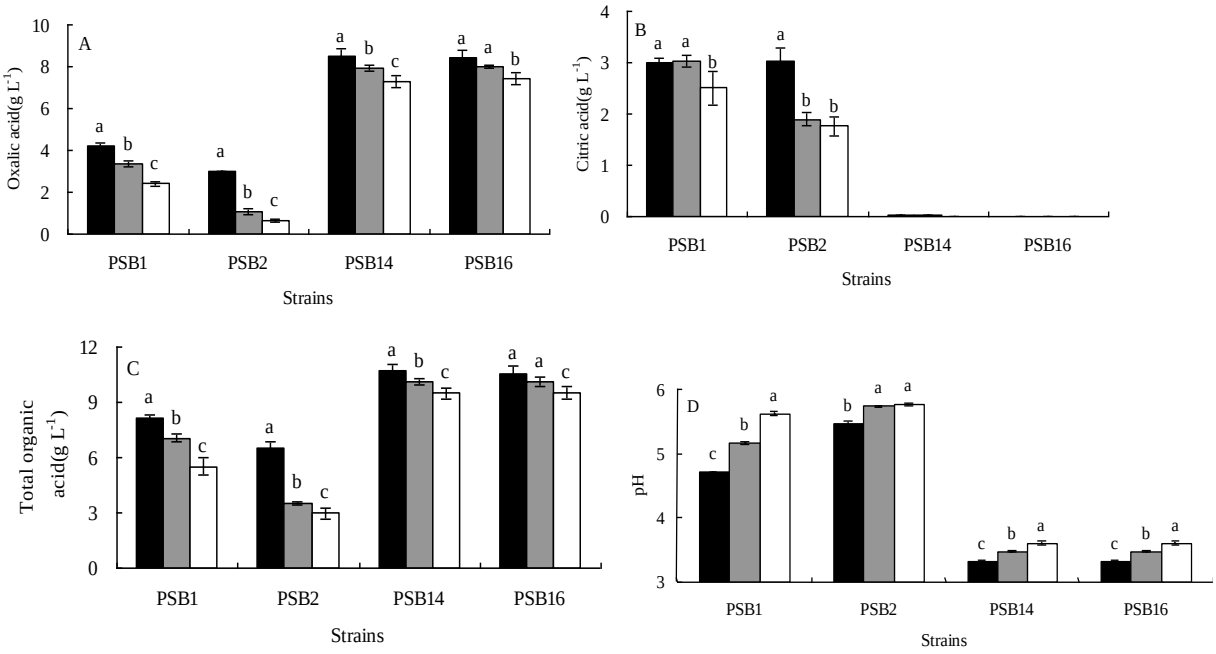

Figure 4. Concentration of organic acid and pH in liquid culture mediums with the different amounts of artemisinin, 0 mg L^{-1} (■), 24 mg L^{-1} (■) and 48 mg L^{-1} (□). (A) oxalic acid, (B) citric acid, (C) total organic acid, (D) pH. Data are representative of four independent experiments, average ± standard deviation followed by different letter(s) of the different artemisinin concentration indicates that the values are significantly different $P < 0.05$.

It has been reported that the main mechanism of mineral phosphate solubilization by PSB strains is associated with the release of low molecular weight organic acids (Goldstein, 1995; Kim et al., 1997a; Kim et al., 1997b). The hydroxyl and carboxyl groups of these organic acids chelate the cations bound to phosphate, thereby converting them into soluble forms (Kpomblekou and Tabatabai, 1994). However, P-solubilization is a complex phenomenon that depends on many factors such as the nutritional, physiological, and growth conditions of the culture (Reyes et al., 1999). There is experimental evidence to support the role of organic acids in mineral phosphate solubilization (Halder et al., 1990). While, secretion of oxalic acid by isolates was severely affected when incubated with high concentration artemisinin.

Farhat et al. (2009) showed that phosphate solubilization matched the drop in pH. Khaled et al. (2010) also reported that *Oceanobacillus picturae* grown in Pikovskaya's liquid medium amended with rock phosphate (RP) led to the highest percentage of RP solubilization (97%), among 129 RP solubilizing bacteria. The most pronounced drop *in vitro* was with a medium pH among isolates, which was the reason that PSB produced a variety of organic acids including citric, oxalic, gluconic, succinic, acetic, and lactic acids. However, the secretion of organic acids was connected with a pronounced drop in the medium pH that was inhibited by artemisinin. Thus, artemisinin may affect the expression and activity of organic acid biosynthetic enzymes.

In a word, high concentration artemisinin badly affected PSB, thus, it was suggested that waste products of *A. annua*,such as the stalks left over after harvest and the residue after artemisinin extracted, should not be stacked in large scale.

Conclusion

This study strongly suggested that high concentration artemisinin had effects on inorganic phosphate-solubilizing bacteria. Therefore, further investigations are required to determine whether artemisinin affects other microorganisms in soil. Additional studies should evaluate its safety in soil environment.

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REFERENCES

Antoun H, Beuchamp CJ, Goussard N, Chabot R, Lalande R (1998). Potential of *Rhizobium* and *Bradyrhizobium* species as growth promoting bacteria on non-legumes: effect on radishes (*Raphanus*

sativus L.). Plant Soil 204:57–67
Brewster, JD (2003). A simple micro-growth assay for enumerating bacteria. J. Microbiol. Meth. 53:77–86
Chabot R, Beuchamp CJ, Kloepper JW, Antoun H (1998). Effect of phosphorus on root colonization and growth promotion of maize by bioluminiscent mutants of phosphate-solubilizing *Rhizobium leguminosarum* biovar phaseoli. Soil Biol. Biochem. 30:1615–1618
Chapman HD, Pratt PF (1961). Methods of Analysis for Soil, plants, and Water: University of California, Division of Agriculture, Berkeley, pp 169-170.
De Freitas JR, Banerjee MR, Germida JJ (1997). Phosphate solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.). Biol. Fertil. Soils 24:358–364.
Domíngueza MC, Rosaa M, Borobiob MV(2001). Application of a spectrophotometric method for the determination of post-antibiotic effect and comparison with viable counts in agar. J. Antimicrob. Chemother. 47:391-398.
Doutre DA, Hay GW, Hood A, Vanloon GW (1978). Spectrophotometric methods to determine carbohydrates in soil. Soil Biol. Biochem. 10(6):457-462.
Eberbach PL, Douglas LA (1989). Herbicide effects on the growth and nodulation potential of *Rhizobium trifolii* with *Trifolium subterraneum L.* Plant Soil 119:15–23.
Elkan GH, Kwik I (1968). Nitrogen, energy and vitamin nutrition of *Rhizobium japonicum.* J. Appl. Bact. 31:399-404.
Engelen B, Meinken K, Wintzingerode FV, Heuer H, Malkomes HP, Backhaus H (1998). Monitoring Impact of a Pesticide Treatment on Bacterial Soil Communities by Metabolic and Genetic Fingerprinting in Addition to Conventional Testing Procedures. Appl. Environ. Microbiol. 64:2814-2821
Farhat MB, Farhat A, Bejar W, Kammoun R, Bouchaala K, Fourati A, Antoun H, Bejar S, Chouayekh H (2009). Characterization of the mineral phosphate solubilizing activity of *Serratia marcescens* CTM 50650 isolated from the phosphate mine of Gafsa. Arch. Microbiol. 191:815–824
Fletcher WW, Dickenson PB, Forrest JD, Raymond JC (1957). The effect of soil applications of certain substituted phenoxyacetic and phenoxybutyric acids on the growth and nodulation of *Trifolium repens* Sylvester. Phyton 9:41–46.
Fletcher WW, Dickenson PB, Raymond JC (1956). The effect of certain hormone herbicides on the growth and nodulation of *Trifolium repens* Sylvester in aseptic culture. Phyton 7:121–130.
Gaind S, Gaur AC (1991). Thermotolerant phosphate solubilizing microorganisms and their interaction with mung bean. Plant Soil 133:141–149
Ghose TK (1987). Measurement of cellulose activities. Pure Appl. Chem. 59:257–268.
Goldstein AH (1995). Recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilization by Gram negative bacteria. Biol. Agric. Hortic. 12:185–193.
Graham PH (1963) .Vitamin requirements of root nodule bacteria. J. Gen. Microb. 30:245–248.
Graham PH, Ocampo G, Ruiz LD, Duque A (1980). Survival of Rhizobium phaseoli in contact with chemical seed protectants. Agron. J. 12:625–627.
Halder AK, Mishra AK, Bhattacharya P, Chakrabarthy PK (1990). Solubilization of rock phosphate by Rhizobium and Bradyrhizobium. J. Gen. Appl. Microbiol. 36:81–92.
Hao W, Ren L, Ran W (2010). Allelopathic effects of root exudates from watermelon and rice plants on *Fusarium oxysporum* f.sp. *niveum*. Plant Soil 336:485-497
Harrison MJ, Pacha RE, Morita RY, (1972). Solubilization of inorganic phosphates by bacteria isolated from upper Klamath Lake sediment. Limnol. Oceanogr. 17:50–57
Harsh PB, Tiffany LW, Laura GP, Simon G, Jorge MV (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. Annu. Rev. Plant Biol. 57:233–266.
Holt JG, Kreig WR, Sneath PHA, Staley JT, William ST (1994). Bergey's manual of determinative bacteriology, 9th edn. Williams and Wilkin Copany, Baltimore, pp. 154-157
Jayashree S, Vadivukkarasi P, Anand K, Kato Y, Seshadri S (2011). Evaluation of pink-pigmented facultative methylotrophic bacteria for phosphate solubilization. Arch. Microbiol. 193(8):543-552.
Jensen V (1951). Notes on the biology of Azotobacter. Appl. Bacteriol. 14:89–93
Jessing KK, Cedergreen N, Jensen J, Hansen HCB (2009). Degradation and ecotoxicity of the biomedical drug artemisinin in soil. Environ. Toxicol. Chem. 28:701–710.
Khaled A, El-Tarabily, Tarek Y (2010). Enhancement of morphological, anatomical and physiological characteristics of seedlings of the mangrove *Avicennia marina* inoculated with a native phosphate-solubilizing isolate of *Oceanobacillus picturae* under greenhouse conditions. Plant Soil 332:147–162.
Kim KY, Jordan D, Krishnan HB (1997a). *Rahnella aqualitis*, a bacterium isolated from soybean rhizosphere, can solubilize hydroxyapatite. FEMS Microbiol. Lett. 153:273–277.
Kim KY, McDonald GA, Jordan D (1997b). Solubilization of hydroxypatite by *Enterobacter agglomerans* and cloned *Escherichia coli* in culture medium. Biol. Fertil. Soils 24:347–352.
Klayma DL (1985). Qinghaosu (Arteminin) - an antimalarial drug from China. Science 228:1049-1055.
Kotan R, Kordali S, Cakir A (2008). Antimicrobial and insecticidal activities of essential oil isolated from Turkish *Salvia hydrangea* DC. ex Benth. Biochem. Syst. Ecol. 36:360–368.
Kpomblekou K, Tabatabai MA (1994). Effect of organic acids on release of phosphorus from phosphate rocks. Soil Sci. 158:442–453.
Li J, Liu X, Dong F, Xu J, Li Y, Shan W, Zheng Y (2011). Potential allelopathic effects of volatile oils from *Descurainia sophia* (L.) Webb ex Prantl on wheat. Biochem. Syst. Ecol. 39:56–63.
Meshnick SR, Taylor TE, Kamchongpaisa S (1996). Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. Microbiol. Rev. 60:301-315.
Pikovskaya RI （1948）. Mobilization of phosphates in soil in connection with the vital activities of some microbial species. Mikrobiologiya 17:362–370
Podile AR, Kishore GK (2006). Plant growth-promoting rhizobacteria. In Plant-Associated Bacteria , Part 2, Gnanamanickam, *S.S* Netherlands Springer, pp. 195–230.
Premono Edi M, Moawad MA, Vlek PLG(1996). Effect of phosphate solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. Indones. J. Crop Sci. 11:13–23.
Reyes I, Bernier L, Simard R, Antoun H (1999). Effect of nitrogen source on solubilization of different inorganic phosphates by an isolate of *Pencillium rugulosum* and two UV-induced mutants. FEMS Microbiol. Ecol. 28:281–290.
Rodriguez H, Fraga R, Gonzalez T, Bashan Y (2006). Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. Plant Soil 287:15–21.
Richardson AE, Simpson RJ (2011). Soil Microorganisms Mediating Phosphorus Availability Update on Microbial Phosphorus. Plant Physiol. 156:989–996.
Sagoe CI, Ando T, Kouno K, Nagaoka T (1998). Relative importance of protons and solution calcium concentration in phosphate rock dissolution by organic acids. Soil Sci. Plant Nutr. 44:617–625.
Seshadri S, Ignacimuthu S, Lakshminarsimhan C (2002). Variation in heterotrophic and phosphate solubilizing bacteria from Chennai, southeast coast of India. Indian J. Mar. Sci. 31:69-72.
Shen H (2006). Release route and allelopathic mechanism for allelochemicals of *Artemisia arlnud* L. PhD thesis. Gansu Agricultural University of China.
Stevenson FJ, Cole MA (1999). Cycles of soil: carbon, nitrogen, phosphorus, sulfur, micronutrients. 2 edn, Wiley Publisher, New York, pp. 219-318.
Taiz L, Zeiger E (2002). Plant Physiology. 3edn, Sinauer Associates Inc. Publishers, pp. 15–19.
Yang ZN, Peng QC, Luo SQ, Yu ZW, Zhao C, Zhu GS, Gui Y, Xia PH (2010). Central properties of the metabolites of *Houttuynia cordata* thunb.populations from different altitudes in Guizhou. Chinese Food Sci. 31(16):261–269
Winslow CEA, Walker HH, Sutermeister M (1932). The influence of aeration and of sodium chloride upon the growth curve of bacteria in various media. Bacteriology 10:185-208.