ISSN 0026-2617, Microbiology, 2009, Vol. 78, No. 6, pp. 769–775. © Pleiades Publishing, Ltd., 2009.

EXPERIMENTAL ARTICLES

Characterization of Phosphobacteria Isolated from Eutrophic Aquatic Ecosystems[1]

Genfu Wu[a,2], Jie Liu[a], and Zhaoxia Ye[b]

[a] *College of Life Sciences, Zhejiang University, Hangzhou, 310058 China*

[b] *Environmental Monitoring Center of Jiaxing, Zhejiang Province, Jiaxing, 314001 China*

Received November 20, 2008

Abstract—Phosphobacteria are able to enhance phosphorus availability in soil and improve crop yields. To develop such biofertilizers, 14 predominant phosphobacteria were isolated from eutrophic aquatic ecosystems. Molecular identification and phylogenetic analysis revealed three groups among the nine isolates of inorganic phosphate-solubilizing bacteria (IPSB): IPSBl and IPSB2 belonged to the actinobacteria and flavobacteria, respectively, and the other seven belonged to the γ-proteobacteria. Among five isolates of organic phosphorus-mineralizing bacteria (OPMB), two groups were present: OPMB1 and OPMB3 belonged to the β-proteobacteria, while the other three belonged to the γ-proteobacteria. The IPSB isolates released 62.8–66.7 mg P l^{-1} from tricalcium phosphate under shaking conditions, and 26.8 to 43.7 mg P l^{-1} under static conditions; the OPMB strains released 23.5–30.2 mg P l^{-1} from lecithin under shaking conditions, and 16.7–27.6 mg P l^{-1} under static conditions. To the best of our knowledge, this is the first report indicating that IPSBl (designated *Aureobacterium resistents*) as a tricalcium phosphate-solubilizing bacterium and OPMB1 and OPMB3 (designated *Acidovorax temperans* and *Achromobacter xylosoxidans*, respectively) are lecithin-mineralizing bacteria. This investigation demonstrated that a eutrophic aquatic ecosystem is a selective source of phosphobacteria and the screened phosphobacteria are a potential alternative to the development of biofertilizers.

Key words: inorganic phosphate-solubilizing bacteria, organic phosphorus-mineralizing bacteria, phosphorus-releasing ability, biofertilizer, phylogenetic analysis.

DOI: 10.1134/S0026261709060149

Phosphorus is one of the macronutrients essential for plant growth. Worldwide, approximately 30 million tons of chemical phosphate fertilizers are applied annually [1]. However, only a relatively small amount of the applied phosphorus is actually consumed by plants and up to 80% is either lost through flushing into aquatic environments by heavy rains or is immobilized into insoluble phosphate by soil metal ions. The extravagant application of chemical phosphate fertilizers not only results in the accumulation of unavailable phosphorus in arable fields, but also leads to the eutrophication of aquatic ecosystems.

In fact, arable soils are not characteristically low-phosphorus environments. Total phosphorus content is approximately 400–1200 mg kg^{-1} of soil, while only 1.0–2.5% can actually be directly assimilated by plants [2]. Microorganisms are able to improve soil phosphorus availability, and therefore, increase crop yields. Chen et al. [1] reported that the phosphobacterium 9320-SD was capable of converting unavailable forms of phosphorus into accessible forms for plants and significantly increased both total phosphorus content and plant biomass of winter wheat under laboratory and field conditions. Turan et al. [3] demonstrated that application of phosphate-solubilizing bacteria significantly increased the shoot and root weight of tomato plants. To protect the environment and avoid eutrophication of aquatic ecosystems, bacterial phosphorus fertilizers should be applied to arable fields instead of chemical phosphate fertilizers. For this purpose, many phosphobacteria have been screened and several commercial biofertilizers have been developed [4, 5]. The unavailable phosphorus in soils exists either in the form of inorganic phosphate, such as ferrous phosphate, calcium phosphate, and aluminum phosphate, or in the form of organic phosphorus, such as phytate, nucleic acids, and lecithin [6, 7]; phosphorus biofertilizer should therefore contain both inorganic phosphate-solubilizing bacteria (IPSB) and organic phosphorus-mineralizing bacteria (OPMB). However, almost all of the previously reported phosphobacteria have been screened from arable soils, especially from rhizospheres, and the development of other screening resources is anticipated in the future.

Eutrophic shallow rivers (or lakes) in arable areas are rich in both insoluble inorganic phosphate and organic phospholipids [8], and thus are alternative resources for phosphobacteria screening. In this study, tricalcium phosphate-solubilizing bacteria and lecithin-mineralizing bacteria were isolated from eutrophic aquatic ecosystems, their phylogenetic analysis was performed based on 16S

[1] The article is published in the original.

[2] Corresponding author: e-mail: wugenfu@zju.edu.cn

rRNA gene sequences, and the phosphorus-releasing abilities of these isolates were evaluated. We found that eutrophic aquatic ecosystems are selective sources for screening phosphobacteria and that the screened phosphobacteria supply potential alternatives to the development of biofertilizers.

MATERIALS AND METHODS

Isolation of tricalcium phosphate-solubilizing bacteria and lecithin-mineralizing bacteria. Bacterial strains were isolated from water or sediment samples collected in eutrophic shallow rivers of eastern China using the traditional dilution and smearing method. IPSB were first enriched in IP medium with tricalcium phosphate as the sole phosphorus source and then screened on the same medium with 20.0 g/1 agar added. The IP medium contained the following (g/1 distilled water): glucose, 10.0; NaCl, 0.3; $(NH_4)_2SO_4$, 0.5; $MgSO_4$, 0.3; KCl, 0.3; $MnSO_4$, 0.03; and $FeSO_4$, 0.03; (pH 7.2). After autoclaving, the medium was supplemented with 3.0 g/1 of sterilized $Ca_3(PO_4)_2$. The phosphate-solubilizing abilities were preliminarily evaluated based on the formation of clear zones around the colonies after five days of incubation at 30°C; those colonies with unique morphologies were picked and purified.

OPMB were first enriched in OP medium with lecithin as the sole phosphorus source (similar to IP medium, but with 0.025 g/1 lecithin and 3.0 g/1 $CaCO_3$ added instead of calcium phosphate) and then screened on yolk agar (similar to IP agar, except 40 ml/1 yolk and 3.0 g/1 $CaCO_3$ were added instead of calcium phosphate), based on the formation of a hydrolysis zone. After five days of incubation at 30°C, the OPMB strains with unique colony morphologies were isolated and purified.

Identification of predominant phosphobacteria. Cell morphology and biochemical characteristics of the isolated bacteria were determined according to the Bergey's manual of determinative bacteriology [9]. DNA extraction was performed using a freeze-thaw method as described in [10]. A loopful of bacterial cells was suspended in 0.4 ml of 0.2 M NaOH, boiled, and frozen twice for 5 min to disrupt the cells. Then, an equal volume of 0.2 M HC1 was added to neutralize the bacterial lytic solution. After centrifugation at 10000 *g* for 5 min to remove the cellular debris, 2 µl of the supernatant was directly used as a DNA template for amplification of the 16S rDNA sequence.

Polymerase chain reaction (PCR) amplification of 16S rDNA was performed in a Mastercycler using 8F and 1492R primers [7]. Each PCR mixture (50 µl) contained: 1.5 mM Mg^{2+}, 50 mM K^+, 200 µM dNTPs, 400 nM of each primer, and 1U *Taq* polymerase (Takara, Japan). Amplification was performed using the method of Wu et al. [11] as follows: an initial denaturation at 94°C for 3 min; 30 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min; and a final elongation at 72°C for 10 min. The products were analyzed by electrophoresis in 1.0% agarose gel and purified with the Biospin Gel Extraction Kit (BioFlux, China). The 16S rRNA gene sequences were detected in a MegaBACE 1000 DNA automated sequencer using 27F as the sequencing primer. The sequencing results were compared to those in the NCBI database using the BLAST software package (http://www.ncbi.nlm.nih.gov). Phylogenetic trees were constructed using the DNAMAN software package (Lynnon Biosoft, Quebec, Canada). The determined partial 16S rRNA gene sequences were deposited in the EMBL Nucleotide Sequence DataBase.

Determination of phosphorus-releasing ability. Phosphorus-releasing ability was quantitatively evaluated based on the water-soluble inorganic phosphorus (WSIP) content in culture liquids combined with the total phosphorus (TP) assimilated by bacterial cells. The isolated strains were inoculated into IP or OP medium and cultivated under static or shaking (180 rpm) conditions at 30°C for a defined time, then 5 ml of the culture liquid was centrifuged at 10000 *g* for 15 min. The pellets were washed twice with 0.1 M HCl to remove calcium phosphate, and then resuspended in 5 ml of 0.85% NaCl. The bacterial biomass was estimated by determining the optical density at 560 nm (OD_{560}), and the phosphorus that was immobilized in the cells was evaluated by analyzing the TP of the cell suspension using the persulfate oxidation method [8]. The phosphorus in the culture liquid was determined by measuring the WSIP of the supernatant using the Mo-Sb-Ascorbic acid colorimetric method [8].

RESULTS AND DISCUSSION

Isolation and identification of phosphobacteria. Chen et al. [4] found that among 36 phosphate-solubilizing isolates, 53% were the strains *Rhodococcus erythropolis* and *Bacillus megaterium*. For full screening of phosphobacteria, more efficient strain identification, and determination of the phosphorus-releasing ability, bacterial isolates were screened based on their colony and cell morphology, halo zone diameter, and biochemical properties, such as oxidase and catalase activities. A total of 14 phosphobacteria, including 9 IPSB and 5 OPMB strains, were selected for phylogenetic analysis of the partial 16S rRNA gene sequences. The closest matches to these isolates were determined according to the BLAST results (table). IPSB1 was nearly identical to *Aureobacterium resistents*, a species that was first reported as a phosphobacterium. However, *Microbacterium* sp., which is a close relative, has already been described as a tricalcium phosphate-solubilizing bacterium [12]. IPSB2 was preliminarily classified as *Chryseobacterium* sp.; the phosphorus-solubilizing activity of this genus was previously reported [4]. The isolates IPSB3 to IPSB9 shared similar characteristics with *Pantoea* sp., *Acinetobacter* sp., *Proteus* sp., *Pseudomonas* sp., *Klebsiella* sp., *Citrobacter* sp., and *Aeromonas* sp., respectively. The strains in these genera were frequently isolated in soils as phosphate-solubilizing bacteria [2, 13–16].

Identification of IPSB and OPMB isolates by alignment with 16S rRNA gene sequences in the nucleotide database

Strain	Nucleotides*	Closest match and its EMBL accession code	Identity (%)	Gap (%)	EMBL code
IPSB1	802	*Aureobacterium resistens* Y14699	97.01	0.37	AM232724
		Microbacterium testaceum AF474325	96.37	0.48	
IPSB2	869	*Chryseobacterium scophthalmum* AJ271009	94.59	0.69	AM232725
IPSB3	532	*Pantoea agglomerans* AY691545	97.17	2.07	AM232726
IPSB4	644	*Acinetobacter seohaensis* AY633608	96.89	0.31	AM232727
IPSB5	862	*Proteus mirabilis* AF008582	99.07	0.69	AM232728
IPSB6	606	*Pseudomonas jessenii* AF501361	98.51	0.83	AM232729
IPSB7	594	*Klebsiella ornithinolytica* Y17662	97.64	1.85	AM232730
IPSB8	609	*Citrobacter freundi* DQ133536	99.18	0.82	AM232731
		Enterobacter kobei AJ508301	98.85	0.82	
IPSB9	481	*Aeromonas caviae* X74674	96.67	2.91	AM232732
OPMB1	554	*Acidovorax temperans* AF078766	99.64	0.36	AM232719
OPMB2	1010	*Klebsiella pneumoniae* AF228920	98.32	1.09	AM232720
OPMB3	793	*Achromobacter xylosoxidans* AY631060	99.62	0.38	AM232721
OPMB4	760	*Escherichia coli* AB210981	98.81	0.92	AM232722
OPMB5	732	*Pantoea agglomerans* AY315453	98.82	0.86	AM232723

* Nucleotide number corresponds to the closest sequence match.

Phylogenetic analysis demonstrated that the nine IPSB isolates were distributed in three sub-classes (Fig. 1a): IPSB1 and IPSB2 belonged to the actinobacteria and flavobacteria, respectively, and the other seven strains belonged to well-known phosphate-solubilizing genera of the γ-proteobacteria. The γ-proteobacteria are widely distributed in diverse environments; many of them are capable of producing organic acids during metabolism [9]. Acid production seems to be a common characteristic of phosphate-solubilizing bacteria.

Unlike inorganic phosphate-solubilizing bacteria, which have been studied in various arable areas, organic phosphorus-mineralizing bacteria are poorly investigated in the environment, despite the fact that approximately 30 to 70% of total phosphorus in agricultural soils is present in organic forms [6]. Five lecithin-mineralizing bacteria were screened in this study. Strains OPMB1 and OPMB3 were found to be closely related to *Acidovorax temperans* and *Achromobacter xylosoxidans*, respectively. To our knowledge, this is the first time that these two species have been reported for lecithin-mineralizing abilities; phylogenetic analysis showed that both of them belonged to the β-proteobacteria (Fig. 1b). According to their morphological and biochemical characteristics, OPMB2, OPMB4, and OPMB5 should be classified as the *Enterobacteriaceae*. Analysis of 16S rRNA genes confirmed that the three strains were highly identical to *Klebsiella* sp., *Escherichia* sp., and *Pantoea* sp., respectively. These results correspond with previous reports suggesting that different patterns of phosphatase activity are widespread in bacteria belonging to the family *Enterobacteriaceae* [2]. Although the lecithin–mineralizing activities of these three genera have been poorly reported, their phytate-mineralizing abilities are widely acknowledged [6, 17]. Lecithin is a phospholipid component of cell membranes, and these bacteria are therefore believed to act as decomposers of membrane organic debris.

Identification of phosphobacteria demonstrated that most strains isolated from aquatic ecosystems were also reported in arable fields. Due to the differences in ecological niche specialization, *Enterobacteriaceae*, which are considered the dominant bacterial group in sewage, were frequently found in aquatic ecosystems, while the stress-resistant *Bacillus* spp. were usually isolated from arable fields [2]. Although in laboratory experiments *Bacillus* spp. were found to be excellent biofertilizers with strong phosphorus-releasing abilities [3], these bacteria survive unsuitable environmental conditions in spore forms with

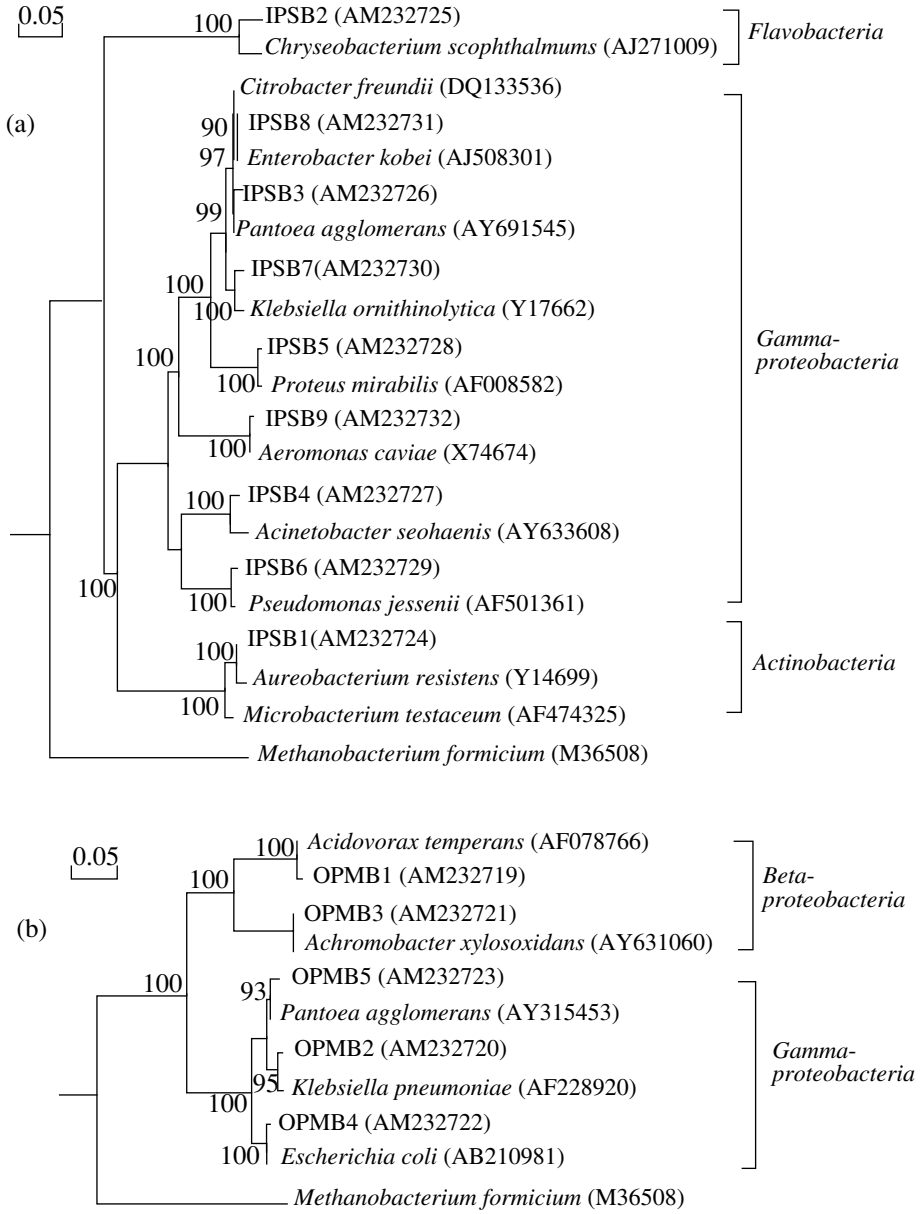

Fig. 1. Phylogenetic affiliations of predominant tricalcium phosphate-solubuilizing bacteria (IPSB, a) and lecithin-mineralizing bacteria (OPMB, b), as revealed by comparative analysis of partial 16S rRNA gene sequences and those in the NCBI nucleotide databases. The sequences were aligned and the phylogenetic trees were constructed with the DNAMAN software package (distance method, 1000 bootstrap trails). Division level groupings are bracketed at the right of the figure. The scale bar represents 5% estimated sequence divergence. Numbers indicate bootstrap confidence values as the percentage of 100 bootstrap replications. *Methanobacterium formicium* was used as an outgroup.

no metabolic function, which is not in line with sustainable growth in extreme environments, such as barren fields or arid lands.

Phosphorus-releasing abilities of representative isolates. Phosphorus-releasing ability is an important parameter of phosphobacteria in biofertilizer development. Fig. 2 shows the calcium phosphate-solubilizing activities of three representative IPSB isolates under shaking or static conditions. When IPSB isolates were cultivated under shaking conditions, they grew steadily for the first 36 hours and used released phosphorus, until reaching the stationary phase (Fig. 2a), with an accumulation of 36.0–46.1 mg P l^{-1} in culture liquids (Fig. 2b). Phosphorus determination demonstrated that the intracellular phosphorus of IPSB2, IPSB4, and IPSB6 were approximately 16.7, 30.2 and 21.3 μg per ml of the culture liquid, respectively. Therefore, the total released phosphorus from tricalcium phosphate should be 62.8–66.7 mg P l^{-1}. Under static conditions, the bacteria grew slowly, particularly strain IPSB4 (Fig. 2c). The amounts of intracellular phos-

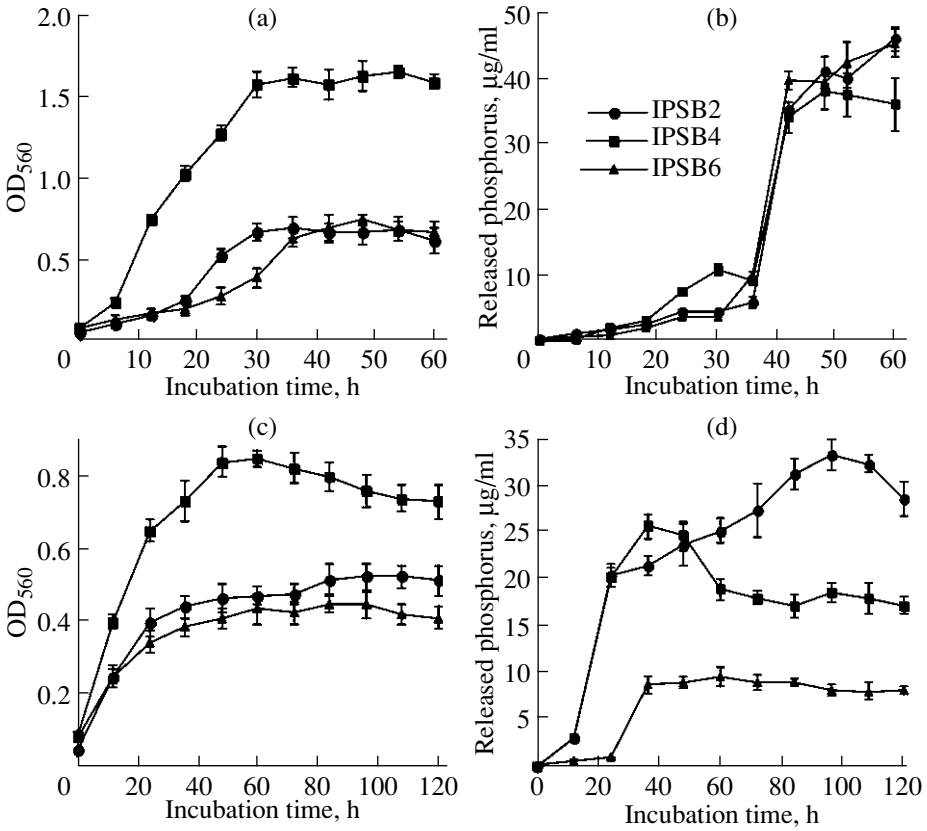

Fig. 2. The calcium phosphate-solubilizing abilities of IPSB isolates under shaking or static conditions: (a) growth curves of shaking cultures; (b) phosphorus release curves of shaking cultures; (c) growth curves of static cultures; and (d) phosphorus release curves of static cultures.

phorus in the strains of IPSB2, IPSB4, and IPSB6 were approximately 15.2, 16.5 and 18.7 μg per ml of the culture liquid, respectively, and the accumulated WSIP contents in the culture liquids were also relatively low (Fig. 2d). Taking both phosphorus types into consideration, the total released phosphorus of the three isolates ranged from 26.8 to 43.7 mg P l^{-1}, which was much lower than the total amount released under shaking conditions. Our results also verified that phosphate-solubilizing activities of the IPSB strains highly correlated with culture pH (the correlation coefficient was 0.876, $n = 180$), in agreement with the potential for acid metabolites produced by IPSB to play a major role in tricalcium phosphate solubilization [6, 15, 16].

OPMB strains typically grew slower than the IPSB strains, and liberated less phosphorus into the culture liquid. Regarding intracellular phosphorus, OPMB1, OPMB3, and OPMB5 released 30.2, 23.5, and 26.4 mg P l^{-1} after cultivation for 60 hours under shaking conditions (Fig. 3a and 3b), and 27.1, 16.7, and 27.6 mg P l^{-1} after cultivation for 120 hours under static incubations (Fig. 3c and 3d).

It has been reported that the lecithin-mineralizing abilities of the OPMB strains were modulated by OPMB phosphatase activities [18]. Fig. 3 shows that strains OPMB3 and OPMB5 accumulated less than 10 mg P l^{-1}, and strain OPMB1 accumulated approximately 18 mg P l^{-1} under both static and shaking conditions, while WSIP did not further increase in the culture solution after the stationary phase of growth. These data indicate that the phosphatases of the OPMB isolates can only catalyze the dephosphorylation of small amounts of lecithin to maintain growth, and will quickly be repressed or inhibited by the accumulation of WSIP in the solutions.

Fig. 2 and Fig. 3 show that the phosphobacterial isolates could release 16.7–66.7 mg P l^{-1} under the experimental conditions. The results corroborate the report of Tao el al. [5], who demonstrated that five IPSB strains isolated from subtropical soils exhibited inorganic phosphorus-solubilizing abilities ranging between 25.4 to 41.7 mg P l^{-1}, and 10 OPMB strains exhibited lecithin-mineralizing abilities ranging from 13.8 to 62.8 mg P l^{-1}. Thus, the phosphobacteria isolated from aquatic ecosystems have similar phosphorus-releasing abilities as the bacteria isolated from rhizospheres. However, a wider scope of phosphorus-releasing abilities was reported in other publications. Oliveira et al. [18] isolated 13 IPSB strains and seven OPMB strains from rhizospheres and demonstrated that the IPSB strains released 8.9 to 211.1 mg P l^{-1} from calcium phosphate medium, while the OPMB strains liberated 10.8 to 38.4 mg P l^{-1} from lecithin medium. Chen et al. [4] screened 36 IPSB isolates from undisturbed soils;

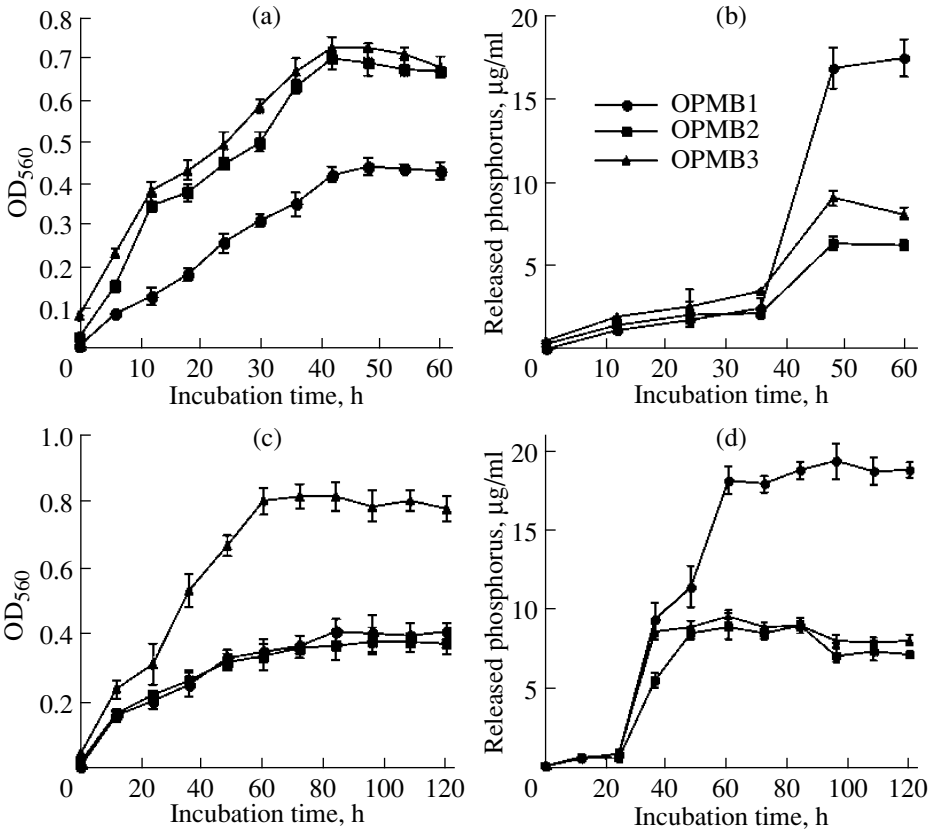

Fig. 3. The lecithin-mineralizing abilities of OPMB isolates under shaking or static conditions. (a) growth curves of shaking cultures; (b) phosphorus release curves of shaking cultures; (c) growth curves of static cultures; and (d) phosphorus release curves of static cultures.

their phosphorus-solubilizing ability varied from 31.5 to 517.9 mg P 1^{-1}. The different phosphorus-releasing abilities resulted not only from diverse genetic properties of the isolated bacteria, but were also due to the use of different methods to evaluate phosphorus release. Chen et al. [4] cultivated the isolates in liquid medium under static conditions, while Tao et al. [5], Sharan et al. [19] and Oliveira et al. [18] cultivated isolates under various shaking conditions (from gentle shaking to 200 rpm). In regards to the released phosphorus, Sharan et al. [19] only analyzed the released WSIP in culture solutions, while Tao et al. [5] also evaluated the phosphorus immobilized by bacterial cells, and Oliveira et al. [18] even enumerated the amount of phosphorus incorporated into the organic matrix of polysaccharides. Cultivation time also influenced the evaluations of phosphorus release. Chen et al. [1] determined phosphorus concentration after three days of incubation, while Sharan et al. [19] harvested the culture after five days, and Trivedi and Sa [20] harvested the cultures after 30 days.

Although phosphobacteria are widespread in nature, the phosphorus-releasing activities of these organisms are dependent on the surrounding environments. Available carbon and nitrogen sources significantly alter the metabolic pathways of phosphobacteria. Glucose is often used as a carbon source in laboratory studies, although xylose and cellobiose are usually the dominant carbon sources in many arable fields. The release of phosphorus by *Serratia marcescens* and *Pseudomonas* sp. CDB35 were both found to be the highest with glucose and the lowest with cellobiose as the carbon source [15]. Therefore, phosphorus-releasing activities of phosphobacteria in natural environments should be lower than those determined under laboratory conditions. Furthermore, the buffering capacity of soils would balance environmental pH and inhibit phosphate solubilization. These collective results indicate that creating a suitable microenvironment for bacterial metabolism is of equal importance with inoculation of large numbers of functional microorganisms, and that the future development of proficient biofertilizers must be comprised of developing both bacterial inoculates and the required auxiliary materials for optimal phosphorus degradation.

ACKNOWLEDGMENTS

We thank Jie Wu from the Hangzhou Environmental Monitoring Center for collecting water and sediment samples.

This report is based upon work funded by the Zhejiang Provincial Natural Science Foundation of China under Grant no. M303106.

REFERENCES

1. Chen, Z., Ma, S. and Liu, L., Studies on Phosphorus Solubilizing Activity of a Strain of Phosphobacteria Isolated from Chestnut Type Soil in China, *Biores. Technol.*, 2008, vol. 99, pp. 6702–6707.
2. Rodriguez, H. and Fraga, R., Phosphate Solubilizing Bacteria and Their Role in Plant Growth Promotion, *Biotechnol. Advances*, 1999, vol. 17, pp. 319–339.
3. Turan, M., Ataoglu, N. and Sahin, E, Effects of *Bacillus* FS-3 on Growth of Tomato Plants and Availability of Phosphorus in Soil, *Plant Soil Environ.*, 2007, vol. 53, no. 1, pp. 58–64.
4. Chen, Y.P., Rekha, P.D., Arun, A.B , Shen, F.T., Lai, W.A. and Young, C.C., Phosphate Solubilizing Bacteria from Subtropical Soil and Their Tricalcium Phosphate Solubilizing Abilities, *Appl. Soil Ecol.*, 2006, vol. 34, no. 1, pp. 33–41.
5. Tao, G.C., Tian, S.J., Cai, M.Y. and Xie, G.H., Phosphate-Solubilizing and Mineralizing Abilities of Bacteria Isolated from Soils, *Pedosphere*, 2008, vol. 18, no. 4, pp. 515–523.
6. Jorquera, M.A., Hernandez, M.T., Rengel, Z., Marschner, P. and Mora, M.L., Isolation of Culturable Phosphobacteria with Both Phytate-Mineralization and Phosphate-Solubilization Activity from the Rhizosphere of Plants Grown in a Volcanic Soil, *Biol. Fertil. Soil*. 2008, vol. 44, pp. 1025–1034.
7. Ermakova, I.T., Shushkova, T.V. and Leontevskii, A.A., Microbial Degradation of Organophosphonates by Soil Bacteria, *Microbiology*, 2008, vol. 77, no. 5, pp. 615–620.
8. Wu, G.F. and Zhou, X.P., Characterization of Phosphorus-releasing Bacteria in a Small Eutrophic Shallow Lake, Eastern China, *Wat. Res.*, 2005, vol. 39, pp. 4623–4632.
9. John, G.H., Noel, R.K., Peter, H.A.S. and Bergy, D., *Bergey's Manual of Determinative Bacteriology*, *9th ed*, Lippincott: Williams & Wilkins, 1994.
10. Wu, G.F. and Zhou, X.P., Distribution and Characterization of Nitrogen-Utilising Bacteria in a Eutrophic Shallow Lake in Eastern China, *Int. J. Environment and Pollution*, 2008, vol. 32, no. 1, pp. 90–103.
11. Wu, G., Hu, J. and Wu, J., Distribution of Cultivable Bacteria Communities in Two Eutrophic Ecosystems, Eastern China, *Hydrobiologia*, 2009, vol. 618, pp. 65–76.
12. Rivas, R., Trujillo, M.E., Sanchez, M.S., Mateos, P.F., Martinez-Molina, E. and Velazquez, E., *Microbacterium ulmi* sp. nov., a Xylanolytic, Phosphate Solubilizing Bacterium Isolated from Sawdust of *Ulmus nigra*, *Int. J. Sys. Evol. Microbiol.*, 2004, vol. 54, pp. 513–517.
13. Chung, H., Park, M.; Madhaiyan, M., Seshadri, S., Song, J., Cho, H. and Sa, T., Isolation and Characterization of Phosphate Solubilizing Bacteria from the Rhizosphere of Crop Plants of Korea, *Soil Biol. Biochem.*, 2005, vol. 37, pp. 1970–1974.
14. Kuklinsky-Sobral, J., Araujo, W.L., Mendes, R., Geraldi, I.O., Pizz-irani-Kleiner, A.A. and Azevedo, J.L., Isolation and Characterization of Soybean-Associated Bacteria and Their Potential for Plant Growth Promotion, *Environ. Microbiol.*, 2004, vol. 6, pp. 1244–1251.
15. Hameeda, B., Reddy, Y.H.K., Rupela, O.P., Kumar, G.N. and Reddt, G., Effect of Carbon Sustrates on Rock Phosphate Solubilization by Bacteria from Composts and Macrofauna, *Curr. Microbiol.*, 2006, vol. 53, pp. 298–302.
16. Patel, D.K., Archana, G. and Kumar, G.N., Variation in the Nature of Organic Acid Secretion and Mineral Phosphate Solubilization by *Citrobacter* sp. DHRSS in the Presence of Different Sugars, *Curr. Microbiol.*, 2008, vol. 56, pp. 168–174.
17. Golovan, S., Wang, G., Zhang, J. and Forsberg, C.W., Characterization and Overproduction of the *Escherichia coli* appA Encoded Bifunctional Enzyme that Exhibits Both Phytase and Acid Phophatase Activities, *Can. J. Microbiol.,* 2000, vol. 46, pp. 59–71.
18. Oliveira, C.A., Alves, V.M.C, Marriel, I.E., Gomes, E.A., Scotti, M.R., Carneiro, N.P., Guimaraes, CT., Schaffert, R.E. and Sa, N.M.H., Phosphate Solubilizing Microorganisms Isolated from Rhizosphere of Maize Cultivated in an Oxisol of the Brazilian Cerrado Biome, *Soil Biol. Biochem.*, 2008, Doi: 10.1016/j.soilbio. 2008.01.012.
19. Sharan, A., Darmwal, S.N.S. and Gaur, R., *Xanthomonas campestris*, a Novel Stress Tolerant, Phosphate-Solubilizing Bacterial Strain from Saline-Alkali Soils, *World J. Microbiol. Biotechnol.*, 2008, vol. 24, no. 753–759.
20. Trivedi, P. and Sa, T., *Pseudomonas corrugate* Mutants Increased Phosphate Solubilization, Organic Acid Production, and Plant Growth at Low Temperature, *Curr. Microbiol.*, 2008, vol. 56, pp. 140–144.