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RESEARCH ARTICLE

Zinc phosphate dissolution by bacteria isolated from an oligotrophic karst cave in central China

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Abstract Biogeochemical processes are fundamental to sustain the ecosystem in subsurface caves, but to date they are still far from well understood. To investigate microbially mediated phosphorus and zinc cycles, we isolated three bacterial strains from the dripping water in Heshang cave, central China, identified as *Exiguobacterium aurantiacum* E11, *Pseudomonas fluorescens* P35, and *Pseudomonas poae* P41, respectively. Microbial capabilities in the dissolution of phosphorus-containing minerals were tested with zinc phosphate ($Zn_3(PO_4)_2$) in batch culture at 30°C. A spectrophotometer, atomic absorption spectrum, and scanning electronic microscopy were used to measure the microbial growth, soluble Zn(II) concentration, and to observe the morphology of $Zn_3(PO_4)_2$ before and after microbial dissolution. *P. fluorescens* and *P. poae*, the well-known phosphorus solubilizing bacteria (PSB), are observed to solubilize $Zn_3(PO_4)_2$ with an efficiency of 16.7% and 17.6%, respectively. To our knowledge, *E. aurantiacum* is firstly reported in this study to dissolve phosphorous-containing minerals with a higher efficiency of 39.7%, expanding our understanding about the ubiquitous occurrence of PSB in natural environments. Aqueous Zn(II) concentration positively correlates with H^+ activity, confirming the presence of acidification mechanisms widely exploited by PSB. Few itching pits were observed on the surface of $Zn_3(PO_4)_2$ after microbial dissolution, inferring that microbial dissolution is not always associated with the direct contact with minerals. Even though the soluble Zn(II) concentration reached up to 370 mg/L in the system inoculated with *E. aurantiacum* E11, inhibition of microbial growth was not detected by spectrophotometer. Our laboratory data revealed the importance of microbially-mediated P and Zn cycles in the subsurface ecosystem.

Keywords karst cave, phosphate solubilizing bacteria (PSB), zinc toxicity, biogeochemical process, subsurface biosphere

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1 Introduction

Karst areas account for 21% terrestrial land surface area (Engel, 2010). Subsurface karst caves are usually nutrient-limited due to the lack of production from photosynthesis (Lee et al., 2012), though some nutrients might be transported into the caves by underground rivers, by dripping water from overlying soils, or by wind from the outside (Chelius et al., 2009). In particular, due to the low solubility of phosphorous (P)-containing minerals in alkaline conditions, phosphorous might be the key limiting factor for microbial growth in karst areas. Phosphorous in soils is also a limiting factor for plant growth particularly under alkaline conditions (Feng et al., 2004), which leads to the annual application of nearly 30 million tons of phosphorous fertilizer to enhance plant productivity in the world. However, as much as 80% of phosphorous fertilizer becomes immobile and unavailable due to adsorption, precipitation, or conversion to organic forms (López-Bucio et al., 2000), and phosphorus solubilizing bacteria (PSB) are proposed to play a major role in the solubilization of soil unavailable phosphorus (Cunninghum and Kuiack, 1992; Kang et al., 2002; Mikonova and Novakova, 2002; Pradhan and Sukla, 2005; Khan et al., 2007). Nevertheless, less is known whether microbes also play significant roles in phosphorus release in alkaline karst caves.

Even though the phosphorous release from P-containing minerals has been studied extensively (Peix et al., 2001; Welch et al., 2002; Pérez et al., 2007; Rosling et al., 2007; Tao et al., 2008; Singh et al., 2011; Xiang et al., 2011), the behavior of the associated cations released from P-containing minerals, which might also greatly affect the growth of plant or microbes, was not well addressed (Fasim et al., 2002; Saravanan et al., 2007). For example, Zn released from zinc phosphate in aquatic environments

and soils can affect the productivity and biodiversity of these ecosystems due to its dual characteristics; zinc is required by many enzymes in trace amounts (Hughes and Poole, 1989), but is toxic in many instances even in relatively low concentrations (McGrath et al., 1995; Atmaca et al., 1998; Bong et al., 2010).

As the important bioavailable phosphorous producers, PSB were hypothesized to exist in dripping water because they are required to sustain karst cave ecosystems. The associated cations released due to the activity of PSB will also affect the microbial activity when the cation concentration reaches a certain level. We thus isolated bacteria from the dripping water in Heshang cave, Hubei, China to investigate their capability to decompose P-containing minerals and to evaluate the cation effect on microbial growth. The results will provide insights into the ongoing biogeochemical process in subsurface environments and help us to understand the maintaining of the ecosystems.

2 Materials and methods

2.1 Sampling

The bacterial strains were isolated from the dripping water of Heshang cave, which is situated in central China (30°27′N, 110°25′E; 294 m asl) in the middle reaches of the Yangtze Valley, a region strongly impacted by the East Asian Monsoon (Hu et al., 2008). Dripping water was collected into a 10 L sterilized plastic cask by an aseptic funnel in March 2010 and taken back to the geomicrobiology laboratory at the China University of Geosciences (Wuhan) at 4°C in a cooler. Isolation of bacteria from the water sample was conducted as soon as possible.

2.2 Culture media

An oligotrophic medium (Liu et al., 2010) was used for the isolation of bacteria from dripping water in Heshang cave, which contains nystautin (0.006 g/L), pyruvate (0.05 g/L), and agar (15 g/L). The filtered dripping water from Heshang cave was used to make this medium.

R_2A medium (Bidle, 2007) was used for the purification of bacterial isolates from oligotrophic conditions. It contains (per liter) 0.5 g yeast extract, 0.5 g peptone, 0.5 g tyrosine, 0.5 g glucose, 0.5 g soluble starch, 0.5 g sodium pyruvate, 0.3 g K_2HPO_4, and 0.05 g $MgSO_4$. The BTG medium employed for the PSB screening contains (per liter) 10 g peptone, 10 g D-glucose, and 15 g agar. When required, a proper amount of finely ground zinc phosphate was added with thorough stirring to obtain a homogeneous suspension with a final concentration of 5 mmol/L. Experiments in liquid BTG medium amended with $Zn_3(PO_4)_2$ were also performed to screen the PSB. The dissolution of zinc phosphate was performed in a defined mineral salts medium (MSM), with glucose as the sole carbon source and 5 mmol/L suspended zinc phosphate was added as well. The MSM medium contains (per liter): glucose 10 g, $(NH_4)_2SO_4$ 5 g, KH_2PO_4 2 g, K_2HPO_4 2 g, $MgSO_4 \cdot 7H_2O$ 250 mg, $FeSO_4 \cdot 7H_2O$ 10 mg, $MnSO_4 \cdot 7H_2O$ 10 mg, $CaCl_2 \cdot 2H_2O$ 10 mg, $ZnSO_4 \cdot 7H_2O$ 30 mg, H_3BO_3 100 mg, $CoCl_2 \cdot 6H_2O$ 117 mg, $CuCl_2 \cdot 2H_2O$ 30 mg, $NiCl_2 \cdot 6H_2O$ 10 mg, and $NaMoO_4 \cdot 2H_2O$ 100 mg (Di Simine et al., 1998).

2.3 Isolation and identification of bacteria from dripping water

A 10 mL water sample was filtered through 0.22 μm cellulose acetate membrane, followed by immediate attachment to the oligotrophic medium. After three weeks' incubation at 25°C, genome DNA was extracted from visible individual colonies in different plates for 16S rDNA sequencing. The 16S rDNA gene was amplified with 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGCTACCTTGTTACGACTT-3′) primers. The reaction conditions were as follows: 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 48°C for 1 min and primer extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. The gene sequencing was performed at Nanjing Genscript Company with the ABI3730 machine. Taxonomic analysis was conducted with RDP 10[1)] and the NCBI database[2)] with 97% similarities. Phylogenetic analyses were conducted using MEGA version 5.0[3)]. The phylogenetic tree was constructed by the neighbor-joining method using the distance matrix from the alignment. Distances were calculated using the Kimura method.

2.4 Screening of phosphate-solubilizing bacterial strains

Strains from R_2A medium were streaked on the BGT plates and transferred into BGT liquid medium, both of which were amended with zinc phosphate to screen PSB. The plates were incubated at 30°C for 72 h. The liquid was incubated at 180 r/min and 30°C. Visible transparent dissolution halos on BGT plates will form, and aqueous Zn(II) concentration will increase in the liquid culture if the strains are able to solubilize zinc phosphate.

2.5 Zinc phosphate dissolution by different PSB strains

Zinc phosphate tetrahydrate powder was commercially

1) http://rdp.cme.msu.edu/
2) http://www.ncbi.nlm.nih.gov/
3) http://www.megasoftware.net/

purchased (Sinopharm Chemical Reagent Co. Ltd) and subjected to the dissolution experiment conducted in a MSM medium in a batch culture. The inoculum (2.5%) was transferred from R_2A overnight culture to 400 mL of liquid MSM medium supplemented with 5 mmol/L zinc phosphate. The cultures were incubated in an orbital incubator at 150 r/min and 30°C for approximately 12 days. Samples were collected at 12 h intervals for the measurement of pH, optical density (600 nm), and Zn content. All experiments were run in duplicate, and data presented here are the average of the duplicates. OD600 of the cultures were used as parameters for microbial growth. Zn content in the solution was indicative of the dissolution of zinc phosphate.

2.6 Analytical methods

An aliquot of 10 mL culture was collected periodically for pH measurement with a pH meter (Delta 320, Mettler-Toledo, Columbus, OH). One milliliter of sample was used for OD measurement with a spectrophotometer (TU-1800, Beijing pgeneral Company Limited). About 10 mL of the cultures were centrifuged for 20 min at 5000 r/min, and the supernatant was used for aqueous Zn analysis with Atomic Absorption Spectrometry (AAS, Hitachi 180-70, Japan). Samples of zinc phosphate residue were mounted on Al-stubs with double-sided carbon tabs and coated with a thin layer of gold. The morphology images were collected by Scanning Electronic Microscope (SEM, JSM-35CF with 15–39 mm of working distance, 0–39 kV and 6 nm of resolution, Japan).

The AAS and SEM analyses were conducted at the State Key Laboratory of Geological Processes and Mineral Resources in China University of Geosciences (Wuhan). Other analyses were conducted in the geomicrobiology laboratory in the State Key Laboratory of Biogeology and Environmental Geology, China University of Geosciences (Wuhan).

3 Results

3.1 Strain screening and identification

Isolated strains of E11, P35 and P41 showed potential ability to dissolve $Zn_3(PO_4)_2$ by forming transparent halos on BGT plates and increasing the concentration of aqueous Zn in BGT liquid medium. The 16S rDNA gene sequences of the three strains showed 99%, 100%, and 98% identity with the typical strains of *Exiguobacterium aurantiacum* (M-4), *Pseudomonas fluorescens* Pf-5 and *Pseudomonas poae* NBB19, respectively (Fig. 1). The three strains were

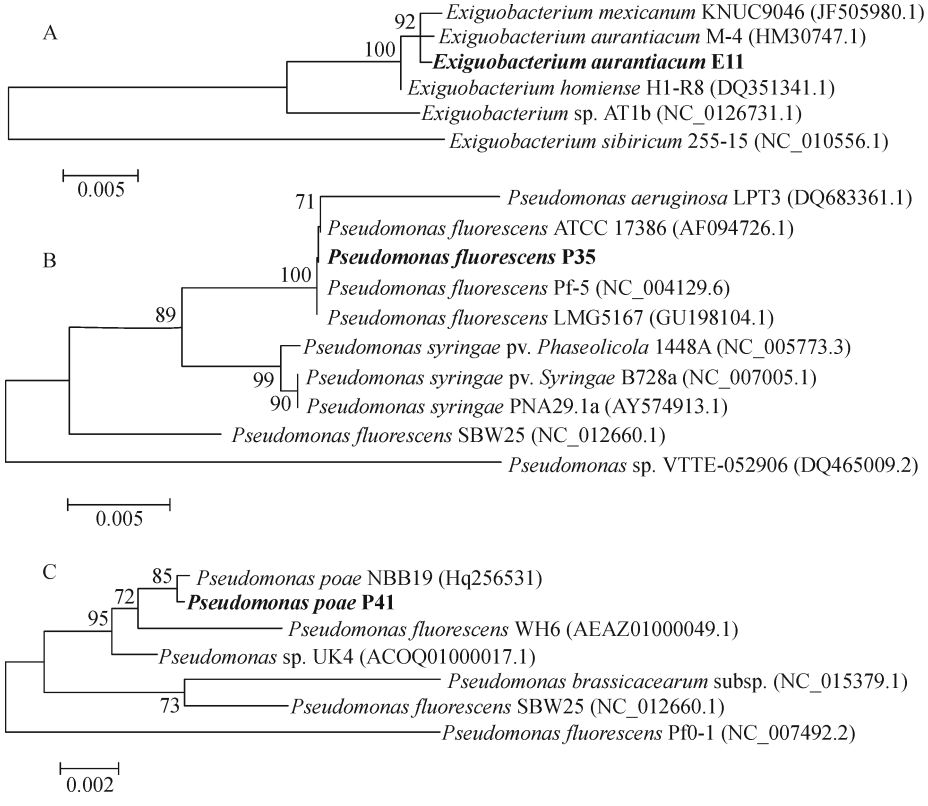

Fig. 1 Neighbor-joining phylogenetic analysis of the three bacterial isolates from the dripping water in Heshang cave, Hubei, central China. The isolates were bolded in the figure. A. *Exiguobacterium aurantiacum* E11; B. *Pseudomonas fluorescens* P35; C. *Pseudomonas poae* P41.

used for further investigation on P-containing mineral dissolution experiments.

3.2 Microbial growth and pH variation

P. poae P41 started to grow after 55 h' incubation, and then increased exponentially in MSM amended with $Zn_3(PO_4)_2$. It reached the plateau after 126 h (Fig. 2A). *E. aurantiacum* E11, *P. fluorescens* P35 grew slowly for the first 137 h and 156 h, respectively. The growth leveled off after 211 h for *E. aurantiacum* E11 (Fig. 2A). The growth of *P. fluorescens* P35 reached a plateau at 201 h and lasted 25 h. After that a re-growth was observed in *P. fluorescens* P35 and continued to the end of the experiment (Fig. 2A).

In the abiotic system, pH remained quite stable around 6.0 (Fig. 2B). The pH values in microbial inoculated systems were close to those in the abiotic control at the initial microbial growth, but decreased sharply within the exponential phase, with the final pH being 3.87, 4.01 and 4.51 in *E. aurantiacum* E11, *P. fluorescens* P35 and *P. poae* P41 inoculated systems, respectively (Fig. 2B).

3.3 Aqueous Zn(II) concentration

The initial Zn(II) content was around 1 mg/L for the medium (Fig. 2C). Variation of Zn(II) concentration in microbial inoculated systems shows good coupling with the microbial growth and pH (Fig. 2). In *P. poae* P41 inoculated system, the Zn(II) concentration was observed to keep unchanged before 42 h when pH was around 6.0, followed by a slow increase to 89.0 mg/L (90 h) and a sharp increase to 164 mg/L (126 h, Fig. 2C) which is consistent with the pH decrease in the system (Fig. 2B). The Zn(II) content leveled off after 126 h when the pH values stabilized in the system.

The aqueous Zn(II) content in the other two inoculated systems shows that the variation trends were comparable with that in *P. poae* P41 inoculated system. However, the onset time of Zn(II) increase and the final concentration of aqueous Zn(II) were different. In *E. aurantiacum* E11 inoculated systems, the Zn(II) concentration started to increase at 108 h with the value of 370 mg/L at the final stage (Fig. 2C). In contrast, the Zn(II) concentration in *P. fluorescens* P35 inoculated systems was found to increase at 157 h and maintain 163.5 mg/L at the end (Fig. 2C).

The aqueous Zn(II) concentration was found to show a positive correlation with the H^+ activity calculated from pH values measured, and the correlative coefficient R^2 are 0.848, 0.842 and 0.990 for *E. aurantiacum* E11, *P. fluorescens* P35, and *P. poae* P41, respectively (Fig. 3). In comparison, a stronger positive correlation ($R^2 = 0.994$) was observed in the abiotic control when HCl was used to acidify the solution to release Zn(II) from zinc phosphate (Fig. 3).

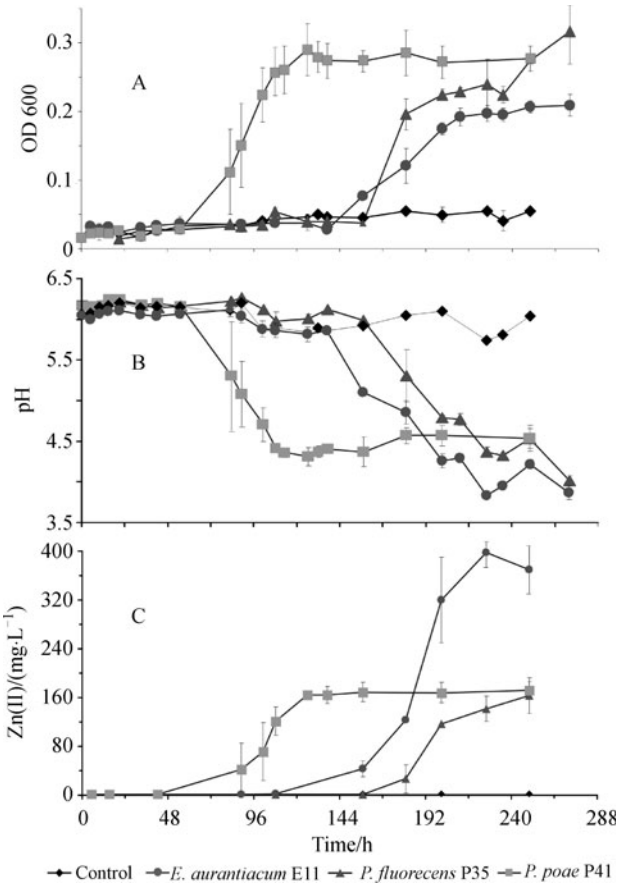

Fig. 2 Growth curves (A), variations in pH (B) and aqueous Zn(II) concentration (C) in microbial inoculated systems in MSM amended with $Zn_3(PO_4)_2$.

3.4 Morphology of zinc phosphate

Particles of $Zn_3(PO_4)_3$ collected at the end of abiotic and biotic experiments were subjected to SEM observation. Zinc phosphate particles show a large size with smooth surfaces in the abiotic system (Fig. 4A). Bacteria were found to congregate (Fig. 4B) or attach on the surface of the zinc phosphate particles (Figs. 4C and 4D). In the abiotic system, particles are characterized by relatively sharp edges, clear and neat surfaces. In the biotic systems, the particle edges became obtuse, and some etching pits with sizes close to that of bacteria were observed on the surface of some particles (Fig. 4C).

4 Discussion

4.1 Wide occurrence of PSB strains

Previously, many bacterial genera were demonstrated to be able to solubilize unavailable phosphate in soils, including *Bacillus*, *Pseudomonas*, *Erwinia*, *Agrobacterium*, *Serra-*

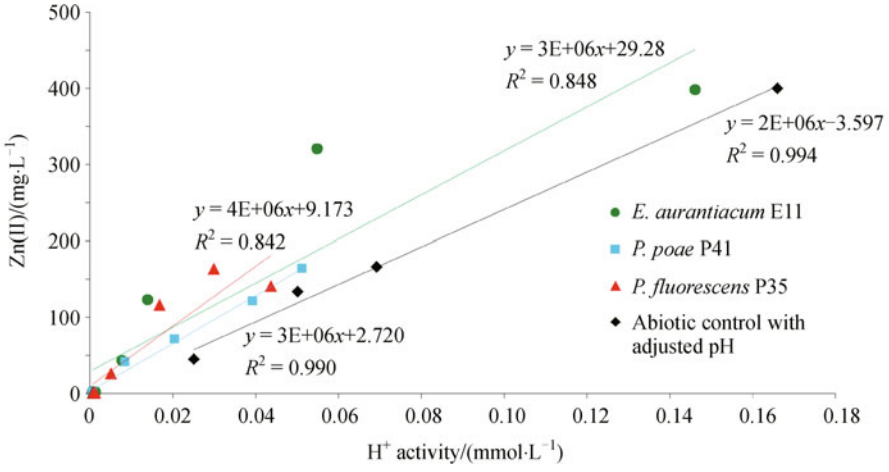

Fig. 3 The relationship between H^+ activity and soluble Zn(II) concentration in the inoculated systems with *E. aurantiacum* E11, *P. fluorescens* P35, *P. poae* P41 in MSM amended with $Zn_3(PO_4)_2$. A correlation between pH values and H^+ activity was also showed in the abiotic system in which the pH was adjusted with diluted HCl.

tia, Flavobacterium, *Enterobacter*, *Micrococcus*, *Azotobacter*, *Bradyrhizobium*, *Salmonella*, *Alcaligenes*, *Chromobacterium*, *Arthrobacter*, *Streptomyces*, *Thiobacillus*, *Escherichia*, and *Kushneria* (Ouahmane et al., 2007; Jha et al., 2009; Selvakumar et al., 2011; Zhu et al., 2011). Here we demonstrate that the bacterial strains isolated from an alkaline, dark, and oligotrophic karst cave can also dissolve zinc phosphate. This expands our knowledge about the habitats of PSB in natural environments. It is not surprising that *P. fluorescens* P35 and *P. poae* P41 can solubilize zinc phosphate with an efficiency of 16.7% and 17.6%, respectively, based on the calculation of soluble Zn concentration. Furthermore, we also isolated a strain identified as *E. aurantiacum* E11 which is capable of zinc phosphate dissolution with a higher efficiency of 39.7%. To our knowledge, it is reported nowhere else about the capability of *E. aurantiacum* E11 to solubilize phosphorus minerals. This indicates that solubilizing P-minerals might be a universal activity of heterotrophs.

Among the reported PSB genera, *Pseudomonas* is the most studied one. The reported dissolution efficiency varied from 0.95%–26.2% for $Ca_3(PO_4)_2$ (Illmer and Schinner,1992; Hu et al., 2010; Li et al., 2010; Govindan et al., 2011). The dissolution efficiency of $Zn_3(PO_4)_2$ was reported to be 47% by *P. fluorescens* 3a (Di Simine et al.,1998), which is higher than that by *E. aurantiacum* E11 isolated in our work. However, the dissolution efficiencies of $Zn_3(PO_4)_2$ by *P. fluorescens* P35 and *P. poae* P41 are lower than that by *E. aurantiacum* E11(39.7%) in our own experiments. The efficiency might be affected by carbon source, pH, temperature, the concentrations of sodium chloride and glucose. It was suggested that optimal dissolution condition for $Zn_3(PO_4)_2$ by *P. fluorescens* 3a is at 20°C, and pH 7, with glucose as the carbon source (Di Simine et al.,1998). Our experiments were conducted at 30°C and at an initial pH of 6.2 with glucose as the sole carbon source. It is likely that *E. aurantiacum* E11 isolated from Heshang cave might dissolve $Zn_3(PO_4)_2$ with higher efficiency under the optimal conditions. Consequently, *E. aurantiacum* E11 may play a significant role on the phosphorus release in some extreme environments as observed in our work. Given that the released Zn(II) might occur as secondary precipitate or adsorb on the cell surface, the actual dissolution efficiency of zinc phosphate should be higher than the value we reported.

4.2 Microbial dissolution mechanism

Different mechanisms have been proposed to explain the microbial dissolution of P-minerals; they include the releasing of organic acids, phosphatases, chelating compounds, mineral acids and siderophores (Gaskins et al., 1985; Schippers and Chanway, 1987; Podile and Dube, 1988; Singh et al., 1989; Kim et al., 1998; Landeweert et al., 2001; Vassilev et al., 2001; Pradhan and Sukla, 2005). All of these mechanisms proposed are related to the production of PSB metabolite which can result in the decrease of environmental pH. For example, bacteria can produce various organic acids via different metabolic pathways (Lin et al., 2006; Mardad et al., 2013) to decrease the environmental pH. As a result, the level of soluble phosphorus released was positively correlated with the concentration of viable bacteria (Chen et al., 2008).

The pH values measured in our experiments decreased from 6.2 to 4.51 or even lower in the inoculated systems. The aqueous Zn(II) concentration was positively correlated with H^+ activity, suggesting that pH played a key role in solubilization of $Zn_3(PO_4)_2$. Even though we did not determine the specific organic acids produced throughout the whole experimental course, the chemical control experiments with the addition of inorganic acid (HCl) further confirmed the importance of acidification in $Zn_3(PO_4)_2$ dissolution.

Bacterial dissolution can occur via indirect and direct

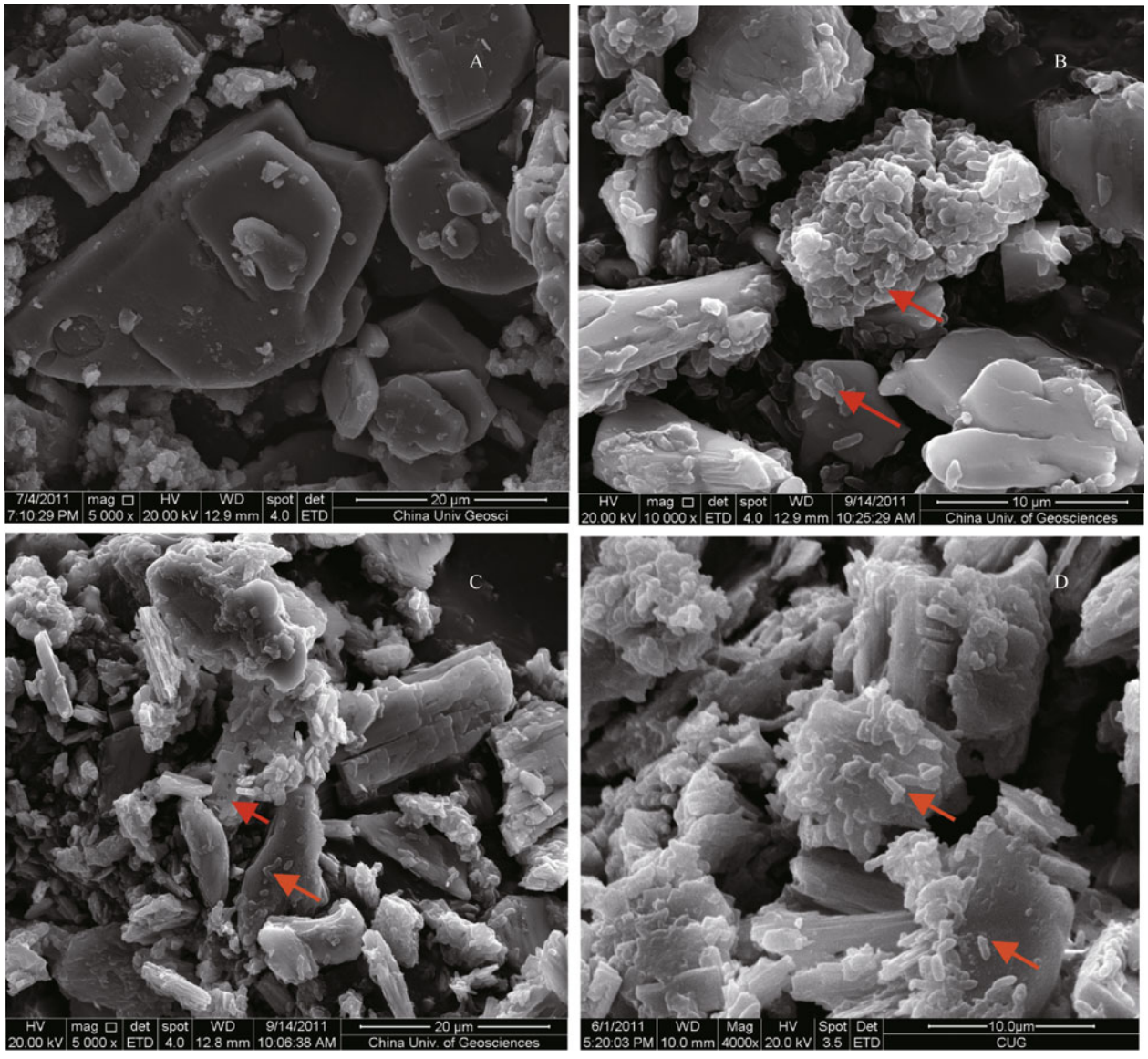

Fig. 4 SEM images of zinc phosphate in the abiotic and biotic systems collected at the end of experiments. A. Zinc phosphate particle in abiotic system; B. Bacteria assemblage and cells attached to the surface of zinc phosphate particles (indicated by the red arrow); C. The attached cells and etching pits on the surface of zinc phosphate particles (indicated by the red arrow); D. Cells on the surface of zinc phosphate particles (indicated by the red arrows).

contact with minerals. By direct contact, bacteria can attach to the surface of minerals, leaving abundant etching pits when mineral dissolution occurs (Liu et al., 2011; Lu and Wang, 2012). Few etching pits were observed by SEM in the residues collected in our experiments, inferring that microbes are not necessary to directly attach to the surface of $Zn_3(PO_4)_2$ during the dissolution.

4.3 Biogeochemical and environmental implication

Due to the thick overlying rocks (500–600 m) and the potential microbial activities in the long transportation of groundwater, most photosynthetic organic matter derived from the overlaid soil might be consumed before reaching Heshang cave. The organic matter transported into the cave is predominantly refractory, which constitutes the dominant organic matter to sustain the subsurface heterotrophic biota. In addition to the organic matter, phosphorus might be one of the key limiting factors for both heterotrophic and autotrophic microbial growth under the alkaline condition due to the low solubility of P-containing minerals. The isolation of the three PSB strains from the alkaline, dark, and oligotrophic Heshang cave strongly suggests that some bacteria in dripping water may play an important role in sustaining the dark ecosystem by offering soluble phosphorus. Our results greatly enhance our understanding of the ecosystem function in dark caves.

The increase of Zn concentration in the solution appears

to cause no inhibition to microbial growth as indicated by the spectrophotometer measurements in our work. Previously, it was demonstrated that Zn(II) was toxic to various microbes in overdoses (McGrath et al., 1995; Di Simine et al., 1998; Nweke et al., 2007). For *Bacillus* sp. SED1 and *Arthrobacter* sp. SED4 isolated from the fluvial sediment at Choba, Zn(II) would cause an inhibition of 50% activity of dehydrogenase with a concentration ranging from 13.47±1.96 to 52.79±4.32 mg/L, and a complete inhibition of dehydrogenase activity with the concentration ranging from 78.43±2.73 to 94.32±4.06 mg/L (Nweke et al., 2007). Strains isolated from a long-term Zn contaminated area in a Hungarian (Nagyhörcsök) experimental field can tolerant 75 and 100 mg/L Zn(II) (Vivas et al., 2006). The viable counts of *P. fluorescens* 3a started to decrease at the Zn concentration around 98.12 mg/L, followed by a sharp decrease at 228.94 mg/L indicating the Zn toxicity (Di Simine et al., 1998). However, the protein content kept increasing throughout the experimental course, contradictory to the viable counts (Di Simine et al., 1998). It is difficult to make an assessment about the Zn toxicity to our isolates due to unavailable viable counts in our experiment, which awaits further investigation. Nevertheless, it is well accepted that an over exposure to Zn(II) will have negative effects on microbes as well as on humans.

5 Conclusions

As an important nutrient carrier in oligotrophic karst caves, dripping water is found to harbor some phosphorus solubilizing bacteria identified to be *E. aurantiacum* E11, *P. fluorescens* P35 and *P. poae* P41 in Heshang cave in central China. Here we reported *E. aurantiacum* E11 is able to solubilize P-minerals with much higher dissolution efficiency than those of *P. fluorescens* P35 and *P. poae* P41, two species of the most studied genus, *Pseudomonas*. The correlation between H^+ activity and released aqueous Zn(II) concentration demonstrated the presence of PSB acidification during the zinc phosphate dissolution. This is further supported by the few etching pits on zinc phosphate particles shown by SEM images. No inhibition was observed on the microbial growth even when the soluble Zn(II) reached to 370 mg/L in *E. aurantiacum* E11 inoculated system. Our work on the bacterial isolates from extreme environments expands our knowledge about the natural habitats of PSB in addition to soils and sediments reported before, and sheds light on the understanding of the microbially-mediated elemental geochemical cycles in karst caves.

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