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ENVIRONMENTAL BIOTECHNOLOGY

Microbial diversity of mangrove sediment in Shenzhen Bay and gene cloning, characterization of an isolated phytase-producing strain of SPC09 *B. cereus*

Shengpeng Zhang · Shao-an Liao · Xiaoyuan Yu · Hongwu Lu · Jian-an Xian · Hui Guo · Anli Wang · Jian Xie

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Abstract Phytases hydrolyze phytate to release inorganic phosphate, which decreases the requirement for phosphorus in fertilizers for crops and thus reduces environmental pollutants. This study analyzed microbial communities in rhizosphere sediment, collected in September 2012 from Shenzhen Bay, Guangdong, China, using high-throughput pyrosequencing; the results showed that the dominant taxonomic phyla were *Chloroflexi*, *Firmicutes*, and *Proteobacteria*, and the proportion of the beneficial bacteria, *Bacillus*, was 4.95 %. Twenty-nine culturable, phytase-producing bacteria were isolated, their phosphorus solubilization capacity was analyzed, and they were taxonomically characterized. Their phylogenetic placement was determined using 16S ribosomal RNA (rRNA) gene sequence analysis. The result shows that most of the isolates are members of the order *Bacillales*, although seven strains of *Enterobacteriales*, two strains of *Pseudomonadales*, and one strain of *Oceanospirillales* were also identified. The phytase gene was cloned from SPC09, *Bacillus cereus*, which showed the highest phosphorus solubilizing ability among the isolated strains. The gene encoded a primary translation product of 335 amino acids. A construct including the 1005-nt ORF fragment, *Bc-phy*, was transformed into *Escherichia coli*. The recombinant phytase was produced and purified, which revealed the temperature optima at 60 °C and pH optima at 6.5. The assessment by quantitative PCR (qPCR) showed an abundance of bacteria containing the *Bc-phy* gene; the level was generally higher in the mangrove forest than in the tidal flats and in surface soil compared to bottom soil, and the highest value was obtained in June. Herein, we report on the cloning, characterization, and activity of a novel phytase isolated from a mangrove system.

Keywords Mangrove · Bacterium · Phosphorus solubilization · Phytase · Cloning

Introduction

Mangroves are widely distributed globally in tropical and subtropical regions, covering approximately 60 to 75 % of the global coastline. In 2000, the remaining mangrove forest areas of the world equaled 53,190 mi^2 (137,760 km^2), spanning 118 countries and territories. The most extensive mangrove regions are in Brazil, Indonesia, and Australia (Holguin et al. 2001). The 2010 update of the World Mangrove Atlas indicated that one fifth of the mangrove ecosystems of the world have been destroyed since 1980 (Spalding et al. 2010), predominantly because of aquaculture, timber production, and urban development. The importance of mangrove ecosystems derives from their remarkable biological productivity, which provides a habitat for rich biodiversity, ranging from bacteria, fungi and algae to invertebrates, birds, and mammals.

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S. Zhang · S.-a. Liao · X. Yu · H. Lu · J.-a. Xian · H. Guo · A. Wang (✉)
Key Laboratory of Ecology and Environmental Science of Guangdong Higher Education Institutes, Guangdong Provincial Key Laboratory for Healthy and Safe Aquaculture, College of Life Science, South China Normal University, Guangzhou 510631, People's Republic of China
e-mail: 105229213@qq.com

S. Zhang · J. Xie
South China Sea Marine Engineering and Environment Institute, State Oceanic Administration, Guangzhou 510300, People's Republic of China

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Mangroves are highly productive; however, they are frequently nutrient-poor. As in other plant communities, nutrient availability is one of the major factors influencing mangrove forest structure and productivity (Feller et al. 2003). The activity of microorganisms is the major biotic factor in the processing of essential compounds that sustain higher organisms (Holguin et al. 2001). Previous studies have shown that in nutrient-limited environments, such as mangrove forests, soil microorganisms significantly respond to phosphorus solubilization, thus playing an important role in nutrition uptake by plants (Vazquez et al. 2000). Although mangrove forests represent ecosystems of global importance, the genetic diversity and abundance of genes that are critical to their functioning have scarcely been explored (Andreote et al. 2012). Biological phosphorus solubilization is a key ecosystem process and is influenced by the resident functional microbial community as well as by environmental conditions.

Phytate (myoinositol hexakisphosphate) is the major phosphorus storage compound in the seeds of higher plants, particularly cereals and legumes. This compound is the major source of organic forms of phosphorus in soil (Richardson et al. 1994). Phytases has been divided into the following four distinct classes: histidine acid phosphatases (HAPS), B-propeller phytases, purple acid phosphatases, and most recently, protein tyrosine phosphatase-like phytases (PTP-like phytases) (Mullaney and Ullah 2003). The enzymes catalyze the hydrolysis of phytate phosphomonoester bonds, creating lower forms of myoinositol phosphates and inorganic phosphate (Ullah and Phillippy 1994), which play important roles in promoting plant growth (Drakakaki et al. 2005), feeding food animals (Cao et al. 2007), and protecting plants from environmental pollution (Vats et al. 2005). The phytase gene has previously been cloned from several species of microorganisms, such as *Bacillus subtilis*, *Obesumbacterium proteus*, *Klebsiella pneumoniae*, *Aspergillus fumigatus*, *Thermomyces lanuginosus*, *Peniophora lycii*, *Agrocybe pediades*, *Trametes pubescens*, and *Ceriporia* sp.; these organisms have been isolated from forest soil, industrial crop rhizospheres, and root interiors and the gastrointestinal tracts of livestock and poultry. Few studies have reported phytase genes cloned from mangrove sediment.

Mangrove of Shenzhen Bay is located in the subtropical coast intertidal. According to our previous study, content of total phosphorus in sediment and seawater is lower than the others in China, indicating that this wetland is mainly limited by phosphorus (unpublished data). It is well worth studying the microbial diversity and the solubilizing capacity of microorganisms in these ecosystems. In this study, we screened bacterial strains belonging to several genera for extracellular phytase production, using 16S ribosomal DNA (rDNA) sequencing and phylogenetic tree analysis. A novel *Phytase* gene from the strain *B. cereus* SPC09, isolated from mangrove sediment, was cloned, sequenced, and recombinantly produced.

Materials and methods

Soil sampling

Sediment samples (22.525° N, 114.007° E) from the root zone (20–30-cm depth) of *Kandelia candel* found in the Futian Mangrove Nature Reserve in Shenzhen, China, were collected using a sterile stainless steel spatula into a sterile bottle. Three replicate samples were randomly collected from three sites (1 m apart) to assemble a composite sample, which was used for bacterial screening.

PCR amplification and pyrosequencing

Universal primers, consisting of the 27 forward primer (5′-xxxAGAGTTTGATCCTGGCTCAG-3′) and the 533 reverse primer (5′-xxxTTACCGCGGCTGCTGGCAC-3′), were selected to amplify the bacterial 16S ribosomal RNA (rRNA) genes of the appropriate size for 454 pyrosequencing. The amplification, purification, pooling, and pyrosequencing of a region of the bacterial 16S rRNA gene were performed as previously described (Fierer et al. 2008; Stackebrandt and Goodfellow 1991).

The sequence analyses were processed as previously described, using the Mothur software (Schloss et al. 2009). The sequence reads belonging to the sample were extracted from the data obtained from the 454 FLX sequencer according to the tags with no ambiguous base pairs. The sequences were trimmed to remove the primers and low-quality sequences. All of the representative sequences were aligned against a subset of the Silva database as the reference. The community level diversity of the soil sample was analyzed by comparing the number of operational taxonomic units (OTUs) with an OTU defined at the 97 % similarity level for bacteria (Kunin et al. 2010). All the OTUs clustered at the dissimilarity of 0.03 were classified. This level of the sample sequencing effort was insufficient to characterize the full extent of the bacterial variety in the collected soil. However, previous studies suggested that the overall community composition and the relative differences in diversity could be quantitatively compared with this level of sequencing (Rousk et al. 2010).

Isolation of culturable phytase-producing bacteria

The phytase-producing bacteria were isolated from the soil samples. One gram of each sample was suspended in 100 mL of a sterile saline solution (8.5 g/L of NaCl) and vigorously shaken for 10 min. The samples were then serially diluted, and 10^{-4} and 10^{-5} dilutions of each sample were spread onto phytase screening medium (PSM) (1.5 % glucose, 0.5 % NH_4NO_3, 0.05 % KCl, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.001 % $FeSO_4 \cdot 7H_2O$, 0.001 % $MnSO_4 \cdot 7H_2O$, 0.5 % Na-phytate, and 2 % agar, with pH adjusted to 5.5). The media and phytate

(Sigma) were autoclaved separately at 121 °C for 30 min (Kerovuo et al. 1998) and incubated at 28 °C for 24 h.

The bacterial isolates were cultivated individually in a PSM again and were incubated 28 °C for 72 h. To visualize the clear zone, equal volumes of 6.25 % ammonium molybdate and 0.42 % ammonium vanadate solutions were flooded onto the plates and incubated. Clear zones around individual colonies are indicative of phytase activity; for this assay, the colony diameter (CD) and the halo diameter (HD) were measured. The strains with clear zones were selected for further studies of the sequence, phylogenetic analysis, and phosphorus solubilization capacity.

Screening for phosphorus solubilization capacity

The bacterial isolates to be screened for phosphorus solubilization capacity were inoculated individually into nutrient broth to revive the culture and incubated at 28 °C for 24 h. The broth cultures were centrifuged and suspended in a sterile saline solution (8.5 g/L of NaCl) until the optical density (OD) reached 2.0 at 600 nm, using a spectrophotometer. To estimate the amount of phosphorus release, 1 mL of the cell suspension was inoculated into 100 mL of sterilized phytase screening broth (1.5 % glucose, 0.3 % $(NH_4)_2SO_4$, 0.01 % $CaCl_2$, 0.05 % $MgSO_4$, 0.01 % $FeSO_4$, 0.001 % $MnSO_4$, and 0.5 % Ca-phytate and pH adjusted to 5.5). Media and phytate were autoclaved separately at 121 °C for 30 min (Yoon et al. 1996) and dispensed into a 250-mL Erlenmeyer flask for each strain in triplicate. The flasks were incubated at 28 °C for 5 days, and the amount of phosphorus released into the broth was estimated.

To estimate the amount of phosphorus released from Ca phytate by the phytase-producing bacteria, a 1-mL sample of the culture was taken and centrifuged at 12,000×*g* for 10 min, and the supernatant was used to estimate the P release by the molybdate blue method (Murphy and Riley 1962). The strain with a higher phosphorus solubilization capacity, which belonged to *Bacillus*, was selected for the gene cloning.

Cloning of the phytase gene

The genomic DNA was purified from SPC09 (deposited in GDMCC, strain number GIM1.778), *B. cereus*, which performed well in a phosphate-dissolving assay. The forward primer, 5′-GCWGATGAYCCKGCGATTTGG-3′, and the reverse primer, 5′-TCKGTATCGCTTGTSCCGTC-3′, were designed based on the conserved sequences of the bacterial phytase proteins, including *Bacillus amyloliquefaciens* strain DSM 1061 phytase (AEC13691), *Bacillus licheniformis* strain PB-13 phytase (AFQ59979), *Bacillus sp.* DS11 phytase (AAC38573), *B. subtilis* strain US417 phytase (CAM58513), and *B. subtilis* strain ATCC 12711 phytase (AFE19189), to obtain the core region. The amplified fragment with the appropriate size was ligated into the pMD18-T vector for sequencing.

The 5′ and 3′ flanking regions of phytase were obtained using a TAIL-PCR. The nested gene-specific primers (Table 1) for the TAIL-PCR were designed according to the core region. The TAIL-PCR was performed as described by Huang (Huang et al. 2011). The amplified PCR products were subjected to sequencing. An assembled contig was generated, and the deduced amino acid sequences were compared to the NCBI protein database using a Blast (Basic Local Alignment Search Tool) search.

Expression of the recombinant phytase

The clones containing the entire phytase gene, which was designated as *Bc-phy*, were subjected to sequence analysis. The recombinant BC-PHY protein was expressed using an *E. coli* expression system. The open reading frame of *Bc-phy* was amplified using PCR and was sub-cloned into a pPROEX-HTb expression vector (Life Technologies, Burlington, Canada) between the BamHI and HindIII restriction sites, with a His-tag at the N-terminal end of the target gene. The *E. coli* strain DH5α cells were transformed with the recombinant plasmid DNA, pPRO-*Bcphy*, and the wild-type vector.

Luria-Bertani (LB) medium containing 100 mg/L of ampicillin was inoculated with 1 mL of each of the overnight cultures of the different clones and agitated at 220 rpm at 37 °C. Protein expression was induced using 1 mM of isopropyl *b*-D-thiogalactopyranoside (IPTG) when the OD600 of the cultures reached 0.8 (log phase), and they were allowed to grow under identical conditions. The uninduced and 4 h post-induction samples were collected. The total protein content of the cell lysates was determined by the Coomassie blue G-250 dye-binding method using bovine serum albumin as a standard (Bradford 1976) and then electrophoresed on a 12 %

Table 1 The primers used in phytase cloning

Primer ID	Primer sequence
BAC-PHY-52	GCWGATGAYCCKGCGATTTGG
BAC-PHY-941	TCKGTATCGCTTGTSCCGTC
Phy-tail-3′-1	GGGTACTGGGCACCGACAAACG
Phy-tail-3′-2	AAGGGCAGATACAGGTGTTTGTGAACGA
Phy-tail-3′-3	GGAGAAGAAGACGTGGGCATCTGG
Phy-tail-5′-1	ACGCCACGTAGCTGTCGTTACCCT
Phy-tail-5′-2	CAGATGCCCACGTCTTCTTCTCCC
Phy-tail-5′-3	CGGTGCGATGGCAAAGACGCTAAT
Phy-ORF-5-bamh1	ggatccATGCAGGTGCTGCCCAAACT
Phy-ORF-3-hind3	aagcttTAAGACGATGCTTATTGTTGG
Bcphy-q-F	CATCTACGGCCTTTGCATGT
Bcphy-q-R	CGTACCAGGGTGCCTTTAAT

SDS-PAGE gel with a standard protein marker to determine the apparent protein molecular mass. The His-tagged recombinant BC-PHY protein was purified using His-tag resin according to the manufacturer's instructions (Novagen, Darmstadt, Germany). The crude protein was loaded onto a 1 mL Ni^{2+}-NTA Agarose column equilibrated with buffer A (20 mM of Tris–HCl, 500 mM NaCl, pH of 7.9). The protein was eluted with a linear gradient of 20 to 300 mM imidazole in buffer A. The fraction containing the purified BC-PHY was collected and refolded by dialysis for the following enzymatic assay.

Enzyme activity and properties

Enzyme assays were performed to determine the pH and temperature influencing the enzyme activity, and all the assays were run in triplicate with an average of three independent experiments. The phytase activity was measured by incubating 1 μg of the refolded enzyme with 600 μL of 2.0 mM sodium phytate (Sigma P3168, USA) in 100 mM Tris-HCl buffer (pH 7.0), supplemented with 2.0 mM $CaCl_2 \cdot 2H_2O$. The enzyme reaction was carried out at 37 °C for 30 min, and the reaction was stopped by adding an equal volume of 5 % trichloroacetic acid. The release of phosphorus from the sodium phytate was estimated using the modified ammonium molybdate method (Heinonen and Lahti 1981). One phytase unit is defined as the amount of enzyme that can liberate 1 μmol P/min. The specific phytase activity is defined as milliunits per milligram protein.

The phytase pH optima were determined using the following buffers at a concentration of 0.1 M: glycine-HCl at pH 3.0 to 3.5, sodium acetate at pH 4.0 to 5.5, morpholino ethane sulfonic acid (MES) at pH 6.0 to 6.5, and Tris-HCl at pH 7.0 to 9.0 at 37 °C (Lassen et al. 2001). The temperature optima were determined at pH 6.0 to 7.5, with temperature values ranging from 30 to 80 °C.

16S rDNA amplification and sequencing

DNA was obtained from 29 strains of phytase-producing bacteria; extraction was performed using the Bacteria DNA Extraction Kit (Tiangen, China). The extracted genomic DNA was used as a template for the PCR amplification of the 16S rRNA genes using the bacterial universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTT ACCTTGTT ACGACTT-3′). The bacterial 16S rRNA gene sequences were amplified with ExTaq DNA polymerase (TaKaRa Bio, Inc., Otsu, Japan) as follows: 1 cycle of 96 °C for 3 min, then 27 cycles of 95 °C for 1 min, 61 °C for 1 min, and 72 °C for 1 min, followed by a cycle of 72 °C for 10 min. The PCR products were purified according to the specifications of the PCR products purification kit (E-Z 96 Cycle Pure Kit, Omega, USA), and the purified products were sent to the Beijing Genome Institute Company for DNA sequence analysis.

Phylogenetic tree construction and sequence analysis

The resulting 16S rRNA partial sequences were compared with GenBank entries using Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) to select the reference sequences and obtain a preliminary phylogenetic affiliation for the clones. The nucleotide sequences were checked for chimeric properties using the CHIMERACHECK of RDP (Maidak et al. 2001). The sequences and their GenBank-associated files were downloaded from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). The nucleotide sequences resulting from this study were aligned pair-wise with published bacterial sequences from the GenBank databases using ClustalX-aligned 16S rDNA clone sequences (Neefs et al. 1993). The phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei 1987) in MEGA5 (Tamura et al. 2011).

The phytase sequence assembly was performed using Vector NTI Advance 10.0 software (Invitrogen, Carlsbad, CA, U.S.A.), and the nucleotide sequence was analyzed using the NCBI ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The phytase domain was predicted using SMART (http://smart.embl-heidelberg.de). The DNA and protein sequence similarity tests were carried out using the BLASTn and BLASTp programs, respectively (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple alignments of the protein sequences were conducted using the CLUSTALW program (http://www.ebi.ac.uk/clustalW/). The phylogenetic tree of the multiple microbial phytases was constructed as previously described.

Gene copy number validation by qPCR

The quantitative PCR (qPCR) primer pairs for *Bc-phy* were designed using Primerblast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index). Primers specific for *Bc-phy* were selected according to their alignment in Vector NTI. The PCR amplifications were carried out on aliquots of individual RT reactions containing a gDNA cocktail extracted from the soil samples. The surface soil (0–5 cm) and bottom soil (30–35 cm) samples were collected from the mangrove sediment (22.525° N, 114.007° E) and a nearby tidal flat (22.520° N, 113.999° E) for 4 months (September, December, March, and June). Four replicate amplification reactions containing 500 nM of each primer were conducted for each biological replicate using the StepOnePlus Real-time PCR System (ABi, USA) and the Platinum SYBR Green qPCR Super Mix (Invitrogen, USA), initiated with a 2-min incubation at 95 °C, followed by a cycling regimen of denaturation at 95 °C for 15 s and annealing and extension at 65 °C

for 60 s. Each run was completed with a melting curve analysis to confirm the specificity of amplification and the lack of primer dimers. The amplification efficiency was determined for each amplification reaction using linear regression of efficiency analysis. The number of target molecules was calculated using *Bc-phy*-T plasmid DNA as a quantitative standard (Rutledge and Stewart 2008).

Statistical analysis of the data

The data are presented as the means±standard deviation (SD), with $n=3$. A multiple comparisons test (Tukey's test) was conducted to compare the significant differences between the treatments using the SPSS 13.0 program (SPSS Inc., Chicago, IL, USA), and $p<0.05$ was considered significant.

Nucleotide sequence accession numbers and strain numbers

The partial nucleotide sequences determined in this study were deposited in the GenBank database under the following Accession Nos.: *Bc-Phy* (KF953809), SPC01 (KF889050), SPC02 (KF889051), SPC03-15 (KF945680-92), and SPC16-29 (KF953810-23). Four hundred fifty-four pyrosequencing data were submitted to the NCBI Sequence Read Archive database (SRA) (http://www.ncbi.nlm.nih.gov/Traces/sra/) under accession number SRP045813.

Results

Bacterial community structure of mangrove sediment

The number of bacterial OTUs detected in the bottom soil of the mangrove sediment in September was 4460 at a cutoff of 97 % sequence similarity (Fig. S1). The dominant bacteria in this soil sample were *Chloroflexi*, *Proteobacteria*, and *Firmicutes* (Fig. 1a). The OTUs that could not be assigned to any extant divisions were pooled as "others and unclassified." A thorough investigation at the genus level showed an enrichment trend of two well-defined bacterial groups in the bottom soil; one group was *Lactococcus* (6.05 %), and the other group was *Bacillus* (4.95 %) (Fig. 1b), implying critical roles for these two groups in the soil. The groups that comprised less than 1 % of the population were polled as "others" and accounted for 31.55 % of the population altogether, indicating a high bacterial diversity that is consistent with a Shannon index of 7.57 (Table. S1).

Identification of phytase-producing bacteria

The phytase-producing bacteria from mangrove sediment were screened using a modified PSM agar medium. A total of 29 colonies that showed growth and a clear zone of hydrolysis on plates were selected and purified. The results (Fig. 2)

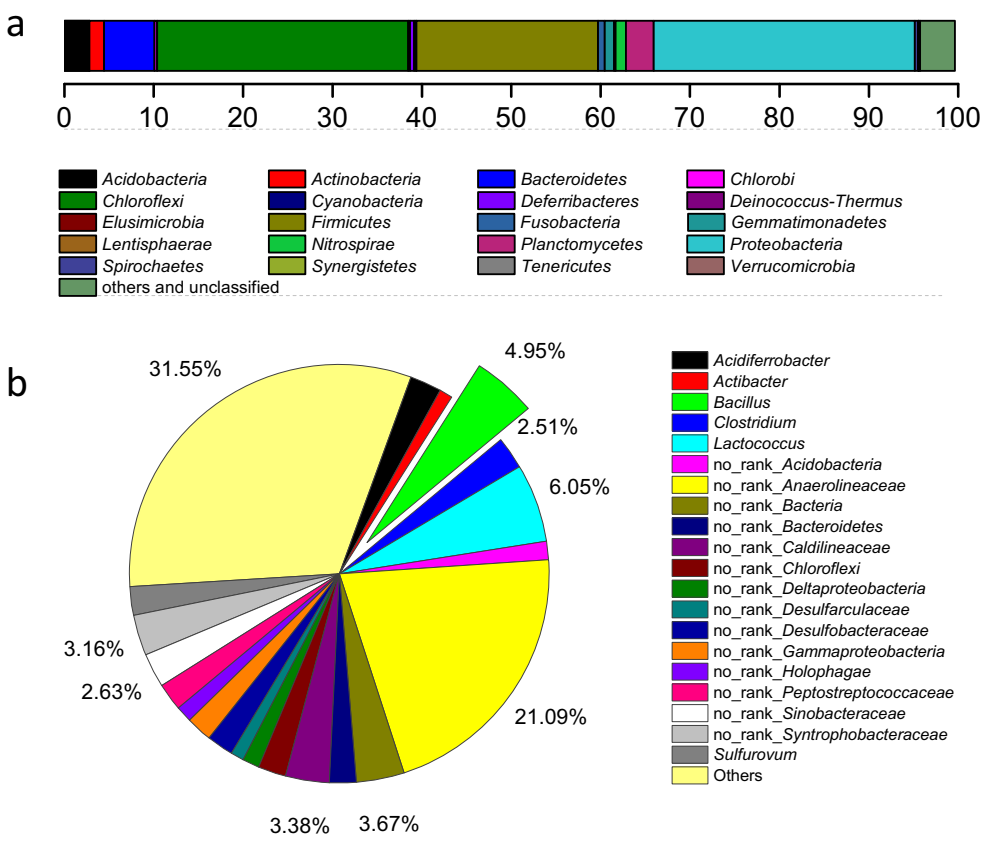

Fig. 1 The bacterial community composition of sediment samples from the Shenzhen Bay mangrove forest at the phylum level (**a**) and genus level (**b**). The percentage of every genus is the average of four replicates. *Others and unclassified* indicates rare OTU genera plus all of the unclassified OTUs

show that the average HD/CD value of the isolates was 2.30 with a range of 1.53 to 4.54.

Phylogenetic tree analyze

With the 29 partial 16S rDNA sequences obtained from this study, 16 complete 16S rDNA sequences of known bacteria were retrieved from the GenBank database to serve as reference sequences. The 16S rDNAs were used to construct the phylogenetic tree as shown in Fig. 3a. The 29 strains of phytase-producing bacteria belonged to seven different groups, including *Bacillaceae*, *Planococcaceae*, *Staphylococcaceae*, *Enterobacteriaceae*, *Halomonadaceae*, *Moraxellaceae*, and *Pseudomonadaceae*. These strains showed close relationships to nine genera, including *Bacillus*, *Lysinibacillus*, *Staphylococcus*, *Enterobacter*, *Klebsiella*, *Proteus*, *Salinicola*, *Psychrobacter*, and *Pseudomonas*. The most prevalent genus among the 29 isolates was *Bacillus*.

Phosphorus solubilization capacity of the isolated strains

All the phytase-producing strains increased the phosphorus concentration of the medium (Fig. 2). Compared to the uninoculated controls, significantly higher ($p<0.05$) phosphorus liberation was observed in the presence of the phytase-producing strains, which ranged between 163.36 and 579.01 mg/L, averaging 279.56 mg/L. The strain SPC09 was demonstrated as having the highest phosphorus-solubilizing ability, reaching a concentration of 579.01 mg/L of phosphorous after 5 days of incubation in the PSM broth. The identical isolate displayed a different phosphorus-solubilizing ability in the liquid medium than when it was cultured on the agar medium.

Cloning and sequence analysis of phytase

An entire phytase gene in *B. cereus*, named *Bc-Phy* (KF953809), was obtained and confirmed by sequencing. The ORF of *Bc-Phy* was 1005 bp in length and encoded a 334-amino acid peptide with a calculated molecular mass of 36.29 kDa and a pI of 5.59. No signal peptide of BC-PHY was predicted using the Signal 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/). A BLAST search using the DNA sequence and the deduced amino acid sequence identified the gene/protein as a member of the phytase superfamily. The deduced protein BC-PHY contains a conserved phytase domain and shared the highest identity of 70 % with a 3-phytase (WP_008482552.1) of *Gallaecimonas xiamenensis*. The alignment of the BC-PHY sequence with six known homologues of *Bacillus* from the GenBank showed conserved amino acids of phytase protein in this genus (Fig. 4). Phylogenetic analysis, which compared the phytase of *Bacillus* with other species, showed that BC-PHY was clustered closely with *G. xiamenensis*, *Rheinheimera* sp. *A13L*, *Ferrimonas balearica* DSM 9799, and *Shewanella piezotolerans* WP3, which were identified from the sediment collected from the coast (Fig. 3b).

Production and purification of recombinant BC-PHY

A complete BC-PHY recombinant protein was successfully expressed by 1 mM IPTG at 37 °C for 6 h in vitro. The apparent molecular mass was approximately 38.4 kDa,

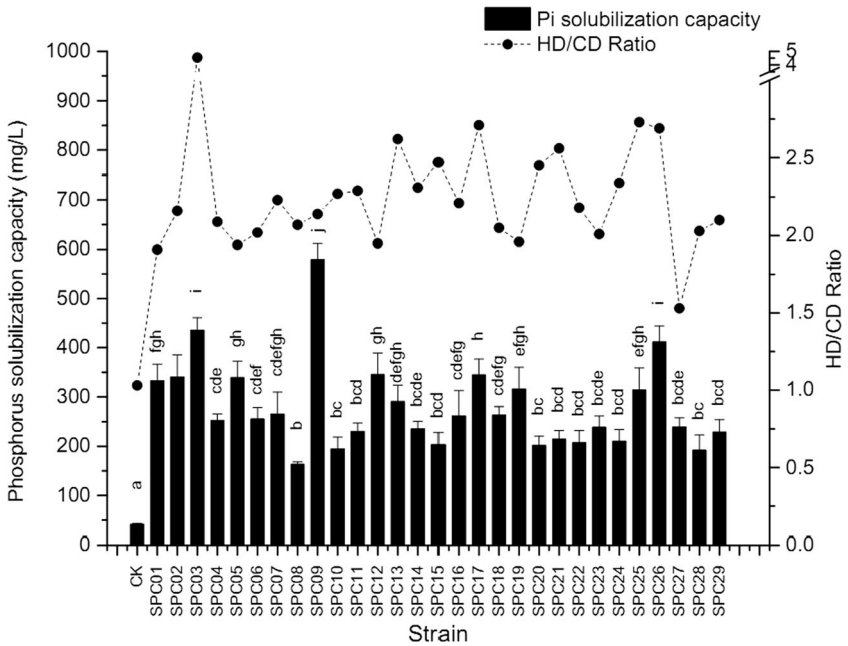

Fig. 2 The identification of the phytase-producing bacteria, determined by the halo diameter and the colony diameter ratio on the PSM agar medium and the phosphorus concentration of the PSM broth. The *values not sharing a common superscript* are significantly different ($p<0.05$)

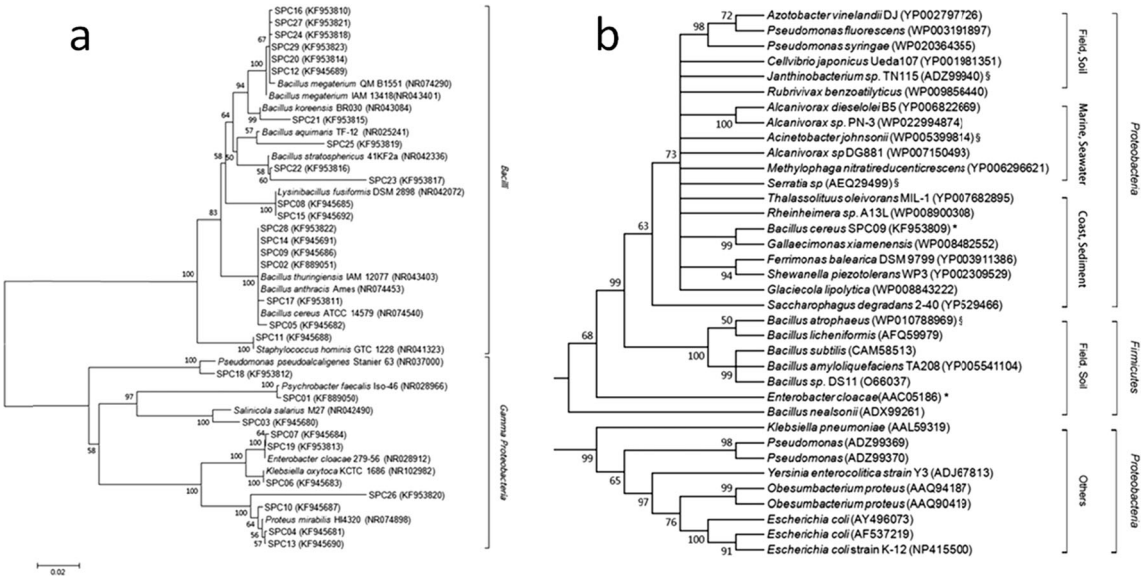

Fig. 3 Phylogenetic analysis. **a** Phylogenetic tree of the partial 16S rDNA sampled from sediment. The differences between the sequences are indicated by the sum of the horizontal lengths, and the *scale bar* is in fixed nucleotide substitutions per sequence position. **b** The phytase phylogeny analysis of BC-PHY with its homologues of other bacterial species from GenBank (accession numbers in *parentheses*). The evolutionary history of these phytase proteins was inferred using the neighbor-joining method, based on the amino acid sequence. The cutoff value for the condensed tree is 50 %. The bootstrap consensus tree was inferred using 1000 replicates. Exception to the clustered group of the indicating isolation source (§). Exception to the clustered group of the indicating phylum (*asterisk*)

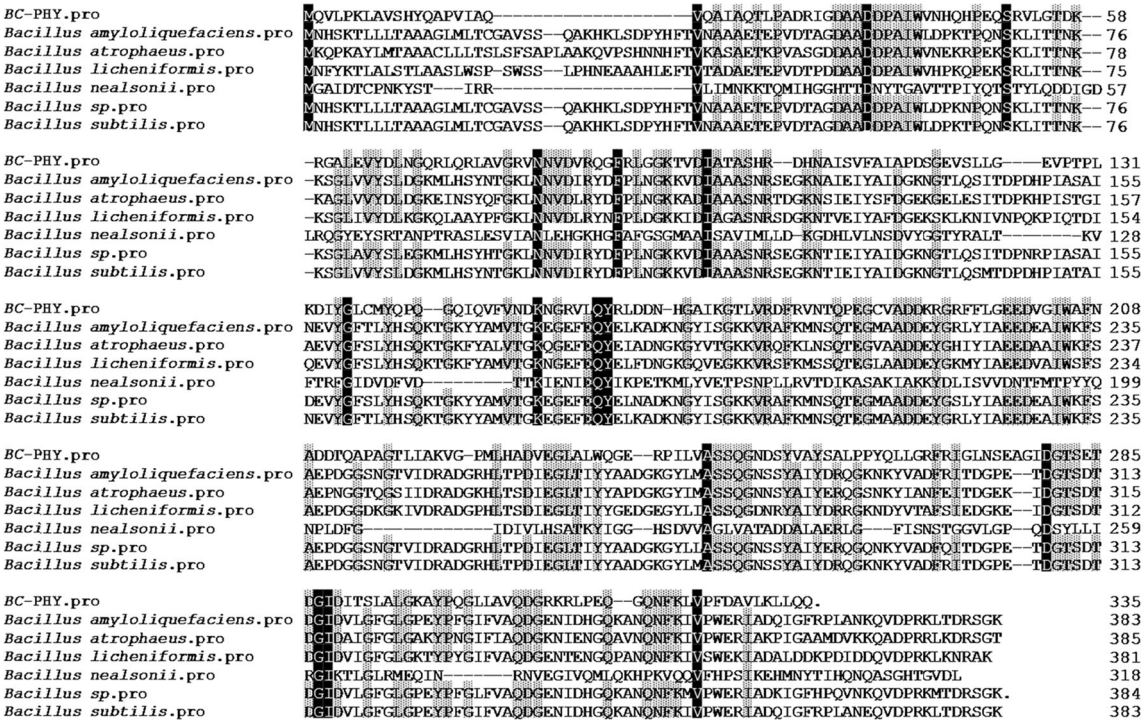

Fig. 4 The alignment of BC-PHY with the phytase homologues in other *Bacillus* bacteria. In addition to BC-PHY, *Bacillus atrophaeus* (WP_010788969), *Bacillus amyloliquefaciens* TA208 (YP_005541104), *Bacillus licheniformis* (AFQ59979), *Bacillus nealsonii* (ADX99261), *Bacillus* sp. DS11 (O66037), and *Bacillus subtilis* (CAM58513) are aligned. The *numbers on the right* indicate the positions of the amino acid residues. The consensus residues that are identical in all the sequences are indicated with *white letters on a black background*. The consensus residues that are identical in a majority of the sequences (≥6) are indicated by a *gray background*

matching the predicted molecular weight that was calculated from the deduced amino acid sequence of BC-PHY fused with a 6× His-tag. To confirm that this is a recombinant protein of BC-PHY, the protein was purified to electrophoretic homogeneity by Ni^{2+}–NTA affinity chromatography and migrated as a single band on SDS-PAGE with a molecular mass of 38.4 kDa (Fig. 5a).

Optimal pH and temperature of phytase activity

The activity of BC-PHY was determined at different pH and temperature values, as described in the "Materials and methods" section. The enzyme was found to be active at 37 °C between pH 3.0 and 9.0, with the peak activity within the pH range of 6.5–7.0; more than 40 % of the maximal activity was displayed from pH 5.5 to 8.5 (Fig. 5b).

The enzyme exhibited activity at temperatures ranging from 30 to 80 °C, whereas a rapid decline of the phytase activity above the optimal temperature was observed at four pH values; similarly, the activity rapidly increased immediately below 30 °C. An optimum activity was observed at 60 °C and pH 6.5, and more than 40 % of the maximal activity was maintained at temperatures between 50 and 65 °C (Fig. 5c).

Copy number of *Bc-phy* in mangrove sediment

The standard curves and dissociation curves indicated an amplification efficiency of 107 % and the specificity presenting of a single amplicon respectively of the primer set which was used in qPCR reactions (S2). The abundance of bacteria containing the *Bc-phy* gene, as determined using qPCR, was generally higher in the mangrove forest than in the tidal flat and in surface soil compared to the bottom soil. In the sediment of the mangrove forest, the amount of responding bacteria increased nearly fourfold in the bottom soil and fivefold in surface soil from September to June of the following year. In June, the copy numbers of *Bc-phy* reached peak values of 20,145.37 and 42,511.66 per 5 ng of soil DNA in the bottom and surface sediments, respectively (Fig. 6).

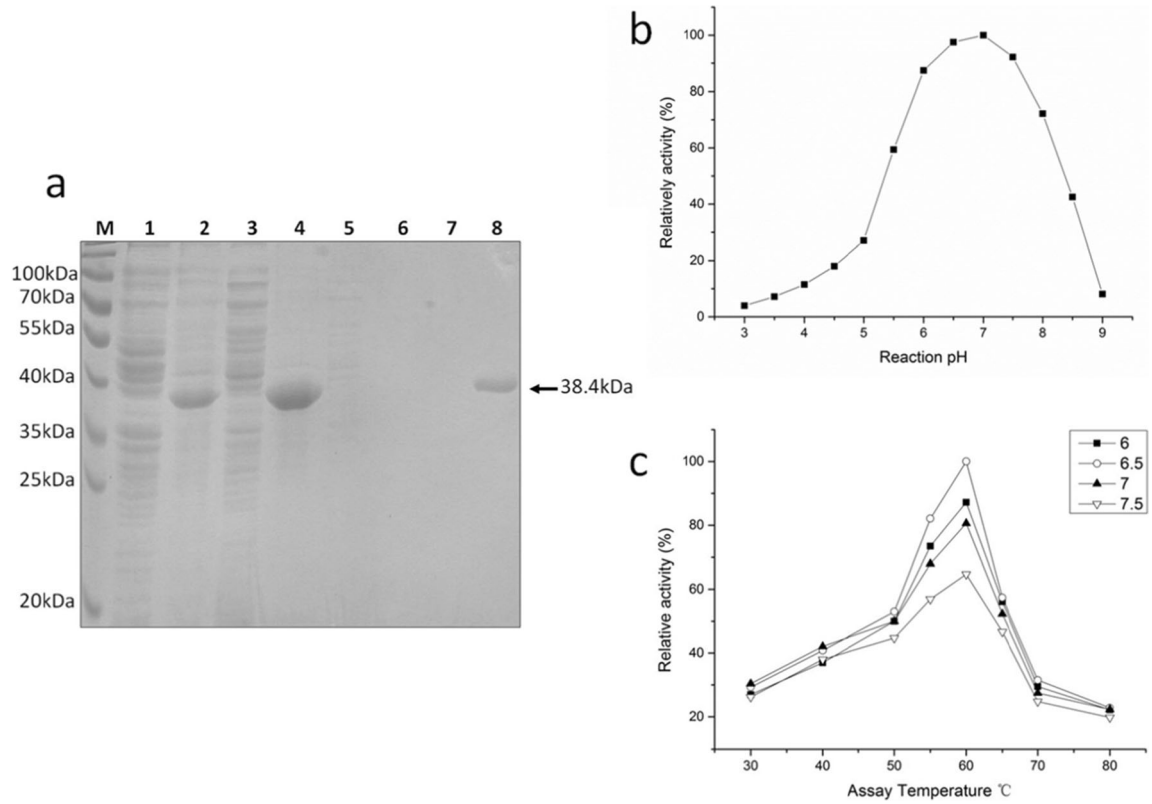

Fig. 5 Characterization of BC-PHY protein. **a** The SDS-PAGE gel electrophoresis analysis of the expression and purification of the recombinant BC-PHY protein in a bacterial expression system. The recombinant BC-PHY protein fused with a 6× His tag was 38.4 kDa. Molecular mass of the standard protein (*lane M*). The control proteins expressed in the pPROEX HTb vector alone (*lane 1*). The total protein of pPRO-BC-PHY (*lane 2*). The soluble fraction (*lane 3*). The insoluble fraction (*lane 4*). The effluents of each step during the purification (*lane 5–7*). The purified BC-PHY protein (*lane 8*). **b** The effect of pH on phytase activity in defined buffers at 37 °C. The phytase activities are expressed as the relative activity. The enzyme assays were run in triplicate, and the standard error was below 0.04. **c** The effect of temperature on the phytase activity in the defined buffers at different pH values. The phytase activities are expressed as the relative activity (compared to highest activity corresponding to the 100 % value). The enzyme assays were run in triplicate, and the standard error was below 0.04

Discussion

Nitrogen and phosphorus have been repeatedly implicated as the nutrients most likely to limit growth in mangroves. In contrast with the temperate zone, in which coastal ecosystems have been experimentally shown to be nitrogen-limited, the few tropical and subtropical mangrove wetlands have been validated as primarily phosphorus-limited. The total phosphorus levels contained in the mangrove sediment and seawater of Shenzhen Bay were determined to be 199.7 mg/kg and 0.55 mg/L, respectively (data not shown), which are lower than those of other wetlands in China (Zheng et al. 2003; Zhou et al. 2007). These low numbers highlight the importance of studying the complex interactions among the biotic factors, especially the bacterial communities, that control the availability of phosphorus in the mangrove forest in this coastal area. The microbial community in the mangrove sediment is significantly affected by bio-geographical, anthropological, and ecological influences, and the diversity of the bacteria found reflects the nutrient transformation, biogeochemical cycling, and pollutant removal functions that are important in this particular ecosystem (Ghosh et al. 2010). However, there is a lack of information on bacterial community structure in mangrove sediments of diverse depth under the impact of vegetation coverage. In this study, a metagenome dataset using 454 technology for DNA sequencing based on the bacteria in the rhizosphere soil was generated. Based on the results presented in Fig. 1, the major bacterial groups in the Shenzhen Bay mangrove sediments are *Chloroflexi*, *Proteobacteria*, and *Firmicutes*. The proportion of *Firmicutes* was 20.30 %, which was four times higher than that (4 %) of surface sediment samples of the same mangrove swamp, as previously described (Liang et al. 2007). By sampling in the same mangrove forest, Liang et al. aimed to demonstrate the microbial diversity in very surface soil (within 0–2 cm zone) but did not focus on rhizosphere soil. In the mangrove sediment environment of Sao Paulo State, Brazil, the metagenomic data exhibited an abundance of *Proteobacteria* (47.1–56.3 %), *Firmicutes* (10.5–13.8 %), *Actinobacteria* (5.4–12.2 %), and *Bacteroidetes* (3.8–11.8 %) (Andreote et al. 2012). Analysis of mangrove sediment samples in Guanabara Bay (Rio de Janeiro, Brazil) by means of denaturing gradient gel electrophoresis (DGGE) revealed dominant ribotypes related to *Alteromonadales*, *Burkholderiales*, *Pseudomonadales*, *Rhodobacterales*, and *Rhodocyclales*, all of which belong to *Proteobacteria* (Marcial Gomes et al. 2008). The differences among these datasets emphasized diverse microbial communities at distinct soil depths and locations. In the phylum of *Firmicutes*, *Bacillus* is a beneficial group of bacteria that have a growth-promoting role and a protective effect against microorganism diseases (Abo-Elnaga 2006), accounting for 4.9 % of the bacteria in the rhizosphere soil of the mangrove forest in the present study. These results suggest that the sediment soil was a natural rich source of phosphorus-solubilizing bacteria. Our purpose is to isolate a culturable bacterium to help increase the content of inorganic phosphorus in mangrove sediments under a pH value of approximately 6. PSM medium was chosen for the screening, and phytate was autoclaved separately to avoid dissolving phytate in the medium during sterilization (Yoon et al. 1996). In this strategy of screening, the influence of organic acids produced by bacteria could be ignored because the solubility of phytate complexes is very low even at pH 3~4; released inorganic phosphorus from dead cells caused by a

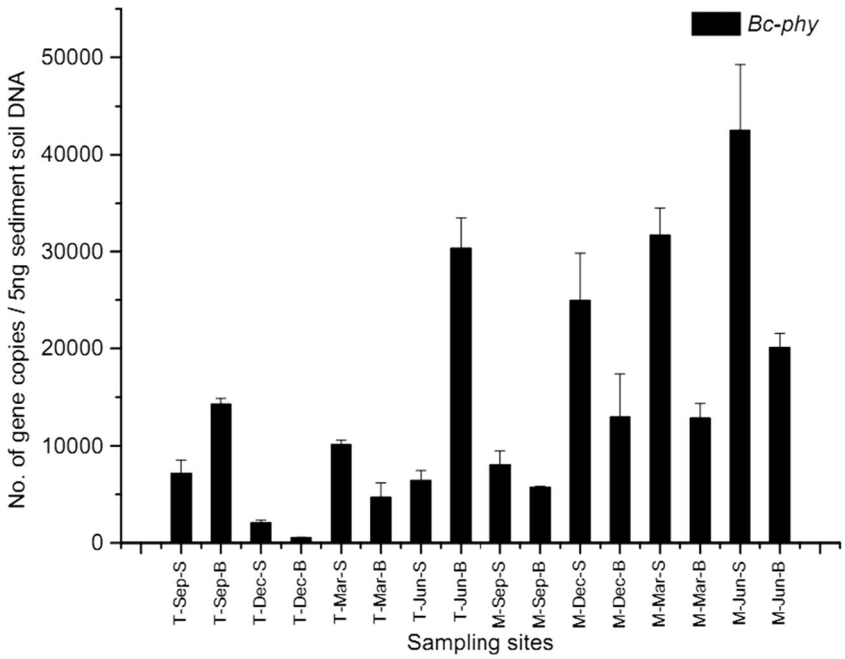

Fig. 6 The absolutely quantitative RT-PCR analysis of the gene copies of *Bc-phy*. The numbers of gene copies per 5 ng of soil DNA, extracted from the bottom and surface soil samples from tidal flat and mangrove forest at four different time points, were measured by qPCR. Each value is the mean of three technical replicates for each of three biological replicates. *T* tidal flat, *M* mangrove forest, *Sep* September, *Dec* December, *Mar* March, *Jun* June, *B* bottom, *S* surface

long-term culture could be accounted for because it is also suitable for plant uptake (Cheryan and Rackis 1980). According to the phosphorus liberation screening in this study, 16 of the 29 selected bacterial strains belong to the genus *Bacillus* (Fig. 3a). The most efficient of the positive strains, SPC09, performed the greatest phosphorus solubilization, at 579.01 mg/L, and was verified as *B. cereus* (Fig. 2). These results indicated that *Bacillus* plays an irreplaceable role during phosphorus cycling in mangrove ecosystems.

Bacillus might play a role in the long-term promotion of plant growth by producing endospores under stressful environmental conditions and by secreting large quantities of enzymes. Bacillus had been widely studied for their ability to solubilize phytate and for their potential use as biofertilizers in agriculture (Richardson and Simpson 2011). Phytases, as critical components of the phosphorous cycle, have been characterized using gene cloning and enzymatic activity (Drakakaki et al. 2005; Guerrero-Olazarán et al. 2010; Kerovuo et al. 1998). Several phytase classes are identified: histidine acid phosphatase, beta-propeller phytases (BPP), cysteine phosphatase, and purple acid phosphatase (Mullaney et al. 2007). Until now, 204 records of a total of 265 bacterial phytase proteins were assigned to the *Bacillus* genus in the database (http://www.ncbi.nlm.nih.gov/protein/?term=bacillus+phytase). Various *Bacillus* were known to possess BPP, which were effective for the dephosphorylation or mineralization of phytates (Hill et al. 2007). Beta-propeller phytases existed in plants, soil, and aquatic bacteria, suggesting that they might play a major in the phytate cycling in both soil and aquatic environments (Jorquera et al. 2008). BPPs, which have been isolated mainly from *Bacillus sp*., have high thermo stability and an optimal pH range of 6.0–8.0, which enabled them to tolerate the neutral digestive tracts of animals and the steam or high temperatures of pelleting during industrial applications (Oh et al. 2004).

Compared to the commercialized fungal phytases used in industrial production, *Bacillus* phytases could serve as an alternative because of their high thermal stability, pH profile, proteolysis resistance, and far greater specificity to phytates. Effective phosphate compounds other than phytate are not hydrolyzed by the *Bacillus* phytases and remain available for plant uptake, particularly in nutrient-limited environments such as mangrove forests (Oh et al. 2004; Yu and Chen 2013). The heterologously recombinant *Bacillus* phytase has been studied by several teams and was shown to exhibit low production levels and high protease activity (Guerrero-Olazarán et al. 2010; Kerovuo et al. 1998; Rao et al. 2008); most of the enzymes originated from *B. subtilis*, and none were from *B. cereus*. However, the alignment analysis reported in this study confirmed that the phytase from *B. cereus* SPC09 did not have a conserved RHGXRXP motif of beta-propeller phytase (Ullah and Wodzinski 1996) and had a low similarity to other known *Bacillus* phytases (Fig. 4). BC-PHY contains 11.67 % of acid residues and 38.03 % of hydrophobic residues, higher levels than those of other β-propeller phytases. Though according to the 3D modeling result, BC-PHY was presumed to have a β-propeller shape, there is no proof supporting a solid classification of this protein. It was therefore necessary to determine the activity and validate the optimal conditions of this novel phytase. The recombinant BC-PHY in this study showed phytate-hydrolyzing activity that was dramatically enhanced between pH 6.0 and 7.5 (Fig. 5b). Because the enzymatic activity was influenced by environmental pH and temperature, a pH-temperature-activity measurement was performed on BC-PHY to confirm the dynamic curve (Fig. 5c). These results illustrate that, although the gene we cloned from *B. cereus* SPC09 in mangrove sediment shared no conserved domain and low similarity to that of other *Bacillus* strains, it still functioned as a phytase of the highest activity at pH 6.5 and 60 °C. Further mutation studies and crystal structure analysis will be carried out to elucidate the basic mechanism of BC-PHY and its interaction with other proteins.

Phytase is a widespread gene among diverse species that appears to have evolved and to sometimes share low identity within a genus. Based on the alignment report, a low identity of phytase proteins among *Bacillus* could be observed compared with BC-PHY, whereas an annotated phytase of *Bacillus nealsonii* displayed the highest diversity from the others (Fig. 4). Compared with that of other species reported previously, BC-PHY exhibited the highest identity (70 %), with a putative 3-phytase from the genomic sequence (GenBank Assemble ID GCA_000299915.1) of *G. xiamenensis* type strain 3-C-1 at the amino acid level (Lai et al. 2012). *G. xiamenensis* was isolated from a crude-oil-degrading consortium produced by the enrichment of a sample of surface seawater collected near Xiamen Island in China (Wang et al. 2013). However, there were no subsequent experimental figures to indicate that the putative sequence of this species was functionally a phytase, which emphasized the necessity of validating the activity of these phytases. To determine whether the environmental factors of a certain microorganism had greater effects on its phytase evolution than the individual origin, a phylogenetic tree was constructed including known phytases of the genus *Bacillus* as well as that of other species that shared >50 % identity with BC-PHY (Fig. 3b). Not all of the phytase proteins were found to be closely related to another within a genus, including *Bacillus* and *Pseudomonas*. The bacteria isolated from similar sources had a closer relationship with each other. BC-PHY from Shenzhen Bay and *G. xiamenensis* phytase from Xiamen Island were both retrieved from coastal areas and shared the greatest similarity among all the candidates, although these two strains were of *Firmicutes* and *Proteobacteria*, respectively. It has been previously reported that the concentrations of organic pollutants in the two locations are relatively similar: PAHs ranged from 237 to 726 ng/g dw and PCBs ranged from 2.9 to 4.7 ng/g dw in Shenzhen,

while PAHs ranged 171–223 ng/g dw and PCBs were approximately 4.4 ng/g dw in Xiamen (Vane et al. 2009). This finding supports the hypothesis that environmental conditions impose the major stress on the evolution of the hypermutated phytase genes: The strain with a demanded mutation was selected and maintained in a particular ecosystem to play its role in phosphorus solubilization.

Moreover, despite sampling within the area of Shenzhen Bay, the portion of *Bc-phy* gene varied among different sampling sites or depths of sediment soil (Fig. 6). A previous study showed that complex environmental conditions, including habitat type, habitat heterogeneity, primary productivity, disturbance, and biogeographical patterns, might allow high microbial diversity in an area (Horner-Devine et al. 2004). Bacteria and plants are interdependent in the mangrove environment; plants transport nutrients and oxygen to the soil through the root system, whereas bacteria promote plant growth by nitrogen fixation, phosphorus solubilization, and indole-3-acetic acid production (Castagno et al. 2011). Detected by qPCR, the copy number of *Bc-phy* was (i) higher in the surface sediment than in the bottom sediment and (ii) higher in mangrove sediment, and the differences between the surface and bottom sediments are more regular than those in the tidal flat. These results imply that the bacteria with *Bc-phy* might rely on the nutrient inputs from the air, tides, litter, and benthic animal carcasses and correlate positively with the plant growth rate. The expression of similar and native *Bc-phy* genes contained in unknown indigenous bacteria might improve the ability of plants to utilize accumulated forms of soil.

In conclusion, the results reported in this study provide fundamental insights into the structure and diversity of the major phosphorus-solubilizing bacterial populations in mangrove sediments and reveal the cloning and function of a novel phytase gene of *Bacillus*. This phytase had a certain activity and played an important role in the particular mangrove ecosystems that were in poor phosphorus condition. Accordingly, *Bc-phy* should be defined as a functional gene that participated in promoting plant growth. We propose the use of the phytase gene as a proto-probe for a thorough future investigation of the underlying microbial groups involved in the sediment phosphorus-solubilizing process, which is valuable for developing engineered bacteria.

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Conflict of interest The authors declare that they have no conflict of interest.

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