Bioprospecting of Plant Growth Promoting Bacilli and Related Genera Prevalent in Soils of Pristine Sacred Groves: Biochemical and Molecular Approach

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Abstract

*Bacillus* spp. and related genera native to soils of the pristine sacred groves from Meghalaya, India were characterized using biochemical and 16S rRNA gene analysis which revealed dominance of *Bacillus*, *Paenibacillus*, *Lysinibacillus* and *Viridibacillus* in the groves. Biochemical estimation was carried out for *in vitro* testing of plant growth promoting traits present in these isolates. PCR screening were performed for plant growth-promoting related genes involved in the biosynthesis of acid phosphatase (*AcPho*), indolepyruvate decarboxylase (*ipdC*), 1-aminocyclopropane-1-carboxylate deaminase (*accd*) and siderophore biosynthesis protein (*asbA*). 76% of the sacred grove isolates gave an amplified fragment for *AcPho*. Three of the isolates gave an amplified fragment for *Ipdc* gene. Apart from 2 isolates, all the other isolates including the reference strains were positive for the amplification of the *accd* gene indicating their potential to produce ACC deaminase enzyme. 42% of the isolates gave an amplified fragment for *asbA* gene indicating the potential ability of these isolates to produce the catechol type siderophore, petrobactin. Overall findings indicated multiple PGP genetic traits present in these isolates which suggested that these isolates are capable of expressing multiple PGP traits. Phylogenetic and sequence analysis of *accd* and *asbA* genes from the isolates revealed that *asbA* genes from *Paenibacillus tai-chungiensis* SG3 and *Paenibacillus tylophil* SG24 indicated the occurrence of intergeneric horizontal transfer between *Paenibacillus* and *Bacillus*.

Introduction

The state of Meghalaya, covering an area of 22 429 km² and located between 24°47’–26°10’ N and 90°45’–92°47’ E, is one of the species rich area under the mega biodiversity centers [1]. Ethnic people of the region are known to practice an age-old tradition of preserving primary forest patches near their settlements as part of their culture and religious belief. These primary forests called ‘sacred groves’, which are conserved due to the traditional religious beliefs not...
only constitute a major reservoir of the floral and faunal biodiversity, but are also assumed to be treasure troves of novel microorganisms [2].

Plant growth-promoting bacteria (PGPB) have direct stimulation on plant growth by providing plants with fixed nitrogen; soluble phosphates and nutrients; iron sequestered by bacterial siderophores; stimulate plant growth through the production of plant hormones such as indole-3-acetic acid (IAA) and activity of 1-aminocyclopropane-1-carboxylate deaminase (ACCD). Indirectly, these bacteria stimulate plant growth by inhibiting the growth of phytopathogenic microorganisms [3].

The genus *Bacillus* represents one of the most diverse genera in the class bacilli. It includes aerobic and facultatively anaerobic, rod-shaped, gram-positive spore-forming bacteria [4]. 16S rRNA gene sequence analysis has revealed a high level of phylogenetic heterogeneity in this genus, on the basis of which a division into different genera was proposed: *Bacillus*, *Alicyclobacillus*, *Paenibacillus*, *Brevibacillus*, *Aneurinibacillus*, *Virgibacillus*, *Solibacillus* and *Gracilibacillus* [5]. Here the term “*Bacillus* and related genera” is used as an operational term to indicate these organisms.

Genes responsible for PGP traits have been studied and explored intensely in recent years. In case of PGPB, several plant growth-promoting traits of these bacteria are a result of various genetic determinants that are present in their genome. With the advancement of metagenomic studies, information regarding the existence of genetic determinant conferring PGP traits has started to become clearer [6–8]. Several genetic determinants implicated in plant growth-promoting potential were investigated in the present study by considering the genes involved in the biosynthesis of acid phosphatase (*AcPho*; size ~ 734bp), indolepyruvate decarboxylase (*ipdC*; size ~ 1850bp), 1-aminocyclopropane-1-carboxylate deaminase (*accd*; size ~ 850bp) and siderophore biosynthesis protein (*asbA*; size ~ 1750bp) [9].

Mineralization of most organic phosphorus compounds is carried out by phosphatase enzymes and a significant amount of phosphatase activity in soil has been reported [10–13]. Major source of this activity in soil is considered to be of microbial origin [14,15]. Production of phytohormones like auxins is one of the direct mechanisms of enhancing plant growth by PGPB. Auxins like IAA have been shown to be produced by bacteria belonging to the genus *Bacillus* which help in stimulating plant growth [16]. In most bacteria, IAA is synthesized from the precursor tryptophan via the indole-3-pyruvic acid (IPyA) pathway where indole pyruvate decarboxylase (*ipdC*) is one to the most important enzyme in the pathway [17–19]. Another important mechanism by which PGPB stimulates the plant growth is through the activity of the enzyme ACC deaminase which causes lowering of plant ethylene levels resulting in longer roots. It catalyzes the cleavage of ACC, the immediate precursor of ethylene in plants, to α-ketobutyrate and ammonia thereby preventing/reducing the production of plant growth inhibiting levels of ethylene [20–21].

Microbial siderophores may stimulate plant growth directly by increasing the availability of iron in the soil surrounding the roots or indirectly by competitively inhibiting the growth of plant pathogens with less efficient iron-uptake system [22]. Many siderophores are small peptides synthesized by non-ribosomal peptide synthetases, which are multi-modular enzymes that produce peptide products with a particular sequence without an RNA template and can be chemically categorized as catecholates, hydroxamates, or α-hydroxy carboxylates based on their ferric iron ligand-binding functional groups [23]. One of the important siderophores secreted by *Bacillus* spp. is petrobactin, which is a catechol-type siderophore [24,25] and is synthesized via the action of six enzymes, encoded by the *asbABCDEF* gene cluster that includes *asbA, asbB, asbC, asbD, asbE* and *asbF* genes [26].

Horizontal gene transfer (HGT) refers to the movement of genetic material between species other than by descent in which information travels through the generations. HGT is known to
enhance the survivability and proliferation of microbial communities in natural [27, 28]. It has been shown to be prevalent in the prokaryotic and bacterial genome with low frequencies of recombination and is considered to be responsible for generating diversity and adaptability among microorganisms [29]. While the role of HGT has been well documented for the transfer of antibiotic resistance, metal transporters and pathogenic genes in bacteria [30, 31], its contribution to the transfer of PGP related genes is scarce [32]. Phylogenetic incongruency between a gene of interest and a marker gene like 16S rRNA gene is often used as a stand-alone method for detecting HGT during gene evolution [33, 34]. Previously, plant growth promoting bacteria belonging to the genus ‘Bacillus and related genera’ were selectively isolated for exploring for PGP properties [35]. Preliminary screening for PGP traits have also been carried out for these isolates [35]. In the present study, bacterial isolates belonging to bacilli and related genera from the ‘Sacred Grove’ were explored for their plant growth promoting properties using biochemical and molecular approaches. The occurrence of HGT was also examined among the isolates of Bacillus and related genera by using the sequences of 16S rRNA gene and the PGP related genes.

**Materials and Methods**

**Isolation and characterization of *Bacillus* and related genera**

Soil samples were collected and analyzed from five different sacred groves of Meghalaya (24°47′–26°10′ N and 89°45′–92°47′ E) and bacterial isolation was performed as described earlier [1, 35]. For collection of soil samples, no permissions were required for these activities as the field studies did not involve endangered or protected species. The parameters observed for the isolates included microscopic appearance, spore-forming, gram stain, catalase test, oxidase test, and reduction of nitrate to nitrite [36]. *Bacillus* and related genera species were characterized by morphologies and physiology characteristics based on Bergeys’ Manual of Systematic Bacteriology [1, 2, 37].

**Biochemical characterization of PGP traits of the isolates**

**Phosphate solubilization.** Isolates were qualitatively screened for phosphate solubilization on Pikovskaya’s agar plates [19]. The appearance of a transparent halozone around the colony indicated phosphate solubilizing activity of the isolate. Quantitative estimation of tricalcium phosphate {Ca 3 (PO 4) 2} solubilization in broth was carried out using Erlenmeyer flasks (250 ml) containing 100 ml of Pikovskaya’s broth inoculated with 1 ml of bacterial suspension (3 x 10^5 cells/ml). Un-inoculated controls were also used in each case. After 5 days incubation the bacterial culture was centrifuged at 5000 g for 15 min and the supernatant was used to estimate soluble phosphate (P) concentration. Broth P was determined by ammonium molybdate-ascorbic acid method [38]. The experiments were conducted in triplicates and data were expressed as the mean value ± standard error.

**Siderophore production.** Siderophore production was tested qualitatively following the method of Schwyn and Neilands[39]. Plates were incubated at 30°C and observed daily for yellow-orange color formation around each colony for 4 days. Catechol-type siderophores were measured in culture supernatants using Arnow’s assay [40], while hydroxamate siderophores were measured according to Csáky[41]. In the analyses, 2,3-dihydroxybenzoic acid and hydroxylamine hydrochloride, respectively, were used as standards. The experiments were conducted in triplicates and data were expressed as the mean value ± standard error.

**Indole-3-acetic acid (IAA) production.** Indole-3-acetic acid IAA production was analyzed using the method described by Wahyudi et al., where the presence of IAA was determined by the development of pink color[19]. IAA concentration was measured
spectrophotometrically at 520nm and quantified in an IAA standard curve. The experiments were conducted in triplicates and data were expressed as the mean value ± standard error.

1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity. ACC deaminase activity was screened adapting the method of Penrose and Glick [20]. Filter sterilized ACC solution (3mM) was spread over Dworkin and Foster (DF) minimal salts [42] agar plates and allowed to dry and inoculated with bacterial strains. Plates were incubated at 30°C for 3 days. The growth on the plates was checked daily. The ability of a strain to utilize ACC was verified by maintaining the same strain in a control in absence of nitrogen source. Based on the results from the first experiment, quantitative measurement of ACC-deaminase activity was carried out according the method of Penrose and Glick (2003)[20]. This method measures the amount of α-ketobutyrate (α-KB) produced when the enzyme ACC-deaminase cleaves ACC. The amount (mM) of α-KB produced by this reaction was determined by comparing the absorbance at 540 nm of a sample with a standard curve of α-KB. Experiments were conducted in triplicates and data were expressed as the mean value ± standard error.

Screening of PGP related genes. Genomic DNA was extracted using Genomic DNA isolation kit (HiPurA Bacterial and Yeast DNA Purification Spin Kit, HiMedia, India) and amplified using conditions and primer sets specific for different PGP related genes i.e., acid phosphatase (AcPho), indole pyruvate decarboxylase (ipdC), 1-aminocyclopropane-1-carboxylate deaminase (accd) and siderophore biosynthesis protein (asbA) as described by Raddadi et al. [9]. Amplified products were run on 1.2% agarose gels, stained with ethidium bromide and visualized under gel documentation system (UVItec, UK).

Amplicons of 1-aminocyclopropane-1-carboxylate deaminase (accd) and petrobactin biosynthesis protein (asbA) genes were purified using the QIAquick Gel Extraction Spin Kit (QIAGEN, Germany) and sequenced using the Big Dye Terminator cycle sequencing kit v.3.1 (Applied Biosystems, USA) deploying the standard protocol and an automated Genetic Analyzer ABI 3130XL (Applied Biosystems, USA).

Phylogenetic analyses of 16S rRNA and asbA and accd genes

The 16S rRNA gene sequences of the isolates were searched for their homologue sequences in public domain databases. Basic Local Alignment Search Tool (BLAST) [43] was used to determine the phylogenetic neighbors from the nucleotide database of National Centre for Biotechnology Information (NCBI) and EzTaxon-e (the database of type strains with validly published prokaryotic names available online at http://eztaxon-e.ezbiocloud.net/) [44]. Basic Local Alignment Search Tool (BLAST, sub-program BLASTX) [43] was used to determine the phylogenetic neighbors of asbA genes from NCBI. Molecular Evolutionary Genetics Analysis software (MEGA v4.1) was used for phylogenetic analyses of 16S rRNA, accd and asbA genes. The obtained nucleotide sequences of identified phylogenetic neighbors were assembled and aligned using ClustalW inbuilt in MEGA 4.1 and phylogenetic tree was constructed using Neighbor-Joining method with 1000 bootstrap replications for nodal support [45]. The 16S rRNA gene sequence of Serratia marcescens AJ233431 was taken as an outlier. The G+C content of the sequenced asbA gene was calculated by using Oligo Calculator available at http://mcb.berkeley.edu/labs and was compared to the G+C contents of all other organisms belonging to the same genus.

Results

Isolation and characterization

Twenty six (26) bacterial isolates belonging to Bacillus and related genera were isolated as reported in Lyngwi et al. [35]. DNA sequencing and phylogenetic analysis revealed that the
isolates showed 97–99% similarity to the sequences available in the NCBI GenBank. The 16S rDNA nucleotide partial sequences were submitted to GenBank and accession numbers from JX402416 to JX402441 was obtained for all the 26 sacred grove isolates (Table 1).

Biochemical estimation of plant growth promoting potential

Previous qualitative studies with these isolates showed that they possess certain PGP traits that can be explored for further investigations [35]. Currently in vitro studies were carried out to estimate the PGP properties in these isolates. Phosphorus (P) is major essential macronutrients for biological growth and development. Microorganisms offer a biological rescue system capable of solubilizing the insoluble inorganic phosphorus of soil and make it available to the plants. The ability of some microorganisms to convert insoluble phosphorus to an accessible form, like orthophosphate, is an important trait in a PGPB for increasing plant yields [46,47]. In this study, there was considerable variation among the isolates in the plant growth-promoting

| Isolates | Closest species (sequence similarity %) | AcPho | lpdC | accd | asbA |
|----------|----------------------------------------|-------|------|------|------|
| SG1      | Bacillus thuringiensis (99.80) JX402416 | +     | –    | +    | +    |
| SG2      | Lysinibacillus xylanilyticus (100) JX402417 | –     | –    | –    | –    |
| SG3      | Paenibacillus taihungensis (99.93) JX402418 | –     | –    | +    | +    |
| SG4      | Bacillus marisflavi (99.80) JX402419 | –     | –    | +    | –    |
| SG5      | Bacillus mycoides JX402420 | +     | –    | +    | –    |
| SG6      | Bacillus thuringiensis (98.92) JX402421 | +     | –    | +    | –    |
| SG7      | Lysinibacillus parviboronicapiens (99.12) JX402422 | +     | –    | –    | –    |
| SG8      | Bacillus aryabhattai (99.80) JX402423 | +     | –    | –    | –    |
| SG9      | Bacillus safensis (99.16) JX402424 | –     | –    | +    | –    |
| SG10     | Bacillus cereus (99.66) JX402425 | +     | +    | +    | –    |
| SG11     | Bacillus thuringiensis (99.93) JX402426 | +     | –    | +    | +    |
| SG12     | Bacillus flexus (100) JX402427 | +     | –    | +    | –    |
| SG13     | Bacillus sonorensis (99.50) JX402428 | –     | –    | +    | –    |
| SG14     | Bacillus methylotrophicus (99.65) JX402429 | –     | –    | +    | –    |
| SG15     | Viridibacillus arenosi (99.93) JX402430 | +     | –    | +    | –    |
| SG16     | Bacillus psychrosaccharolyticus (99.65) JX402431 | +     | –    | +    | –    |
| SG17     | Bacillus thuringiensis (99.73) JX402432 | +     | –    | –    | +    |
| SG18     | Bacillus cereus (99.73) JX402433 | +     | –    | +    | –    |
| SG19     | Bacillus weihenstephanensis (99.45) JX402434 | +     | –    | +    | +    |
| SG20     | Bacillus mycoides (100) JX402435 | +     | –    | +    | +    |
| SG21     | Bacillus aryabhattai (99.93) JX402436 | +     | –    | –    | –    |
| SG22     | Bacillus humi (98.30) JX402437 | +     | +    | +    | –    |
| SG23     | Bacillus simplex (97.29) JX402438 | +     | –    | +    | –    |
| SG24     | Paenibacillus tylopii (97.78) JX402439 | +     | –    | –    | +    |
| SG26     | Viridibacillus arvi (99.73) JX402440 | +     | –    | +    | –    |
| SG27     | Bacillus methylotrophicus (99.38) JX402441 | +     | –    | +    | –    |
|          | Bacillus subtilis MTCC 8141 | +     | –    | –    | +    |
|          | Bacillus thuringiensis MTCC 8996 | +     | +    | +    | +    |
|          | Paenibacillus polymyxa MTCC 9489 | +     | +    | +    | +    |
|          | Bacillus cereus MTCC10211 | +     | –    | +    | +    |

– indicates gene not amplified; + indicates gene amplified

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properties such as the phosphate solubilization and soluble P production by the phosphate solubilizing isolates (Fig 1A). The phosphate solubilization process on Pikovskaya’s agar was observed as solubilization haloes around the colonies. Based on the screening on Pikovskaya’s agar, 18(69%) of the sacred grove isolates were positive for phosphate solubilization. The concentration of soluble P in broth ranged between 73.33–126.23 μg/ml. The highest concentration of soluble P in the broth was detected for Bacillus safensis SG9 (126.23 μg/ml), while the lowest concentration was that of B. thuringiensis SG11 (73.33 μg/ml) (Fig 1A). The ability to solubilize phosphate was not detected in the isolates belonging to the genera Lysinibacillus and Viridibacillus. Another important trait of PGPB, that may directly or indirectly influence the plant growth, is the production of siderophore which is one of the most important attributes for biocontrol mechanisms of PGPB including bacilli groups [25]. All the characterized isolates in the present study showed the production of siderophores. Similar to phosphate solubilization, considerable variations were observed among the isolates in siderophore production and
the quantity produced by the isolates (Fig 1B and 1C). Among all the isolates, the largest halozone for siderophore detection on CAS agar medium was formed by *Bacillus methylotrophicus* SG27 (8 ± 0.5 mm). Quantitative estimation of the amount of siderophore produced by the sacred grove isolates revealed that *B. thuringiensis* SG17 was the highest catechol-type siderophore producer (16.08 µg/ml) (Fig 1B) and *B. safensis* SG9 was the highest producer of hydroxamate-type siderophore (1.01 µg/ml) (Fig 1C). Interestingly, each isolate could produce only one type of siderophore but not both.

IAA (indole-3-acetic acid) is a member of the group of phytohormones and is generally considered the most important native auxin [3]. Among the PGP traits, indolic compounds such as IAA have a positive effect on root growth and morphology, and are thus believed to increase access to more nutrients in the soil [48]. All the studied isolates were found to produce Indole-3-acetic acid (IAA) but the amount of IAA production as determined in the culture supernatant differed among the isolates (Fig 1D). The concentration of IAA produced by the isolates in liquid medium ranged from 2.68–14.48 µg/ml. *Viridibacillus arenosi* SG15 (14.48 µg/ml) was the highest producer of IAA while *Bacillus humi* SG22 (2.68 µg/ml) was the lowest producer of IAA from among the characterized isolates.

Majority of the isolates showed ACC metabolism capacity. By using the ACC metabolism plate assay which is based on the ability of the isolates to use ACC as the sole nitrogen source through the action of ACC deaminase enzyme, it was observed that 20 (77%) of the sacred grove isolates were positive for ACC deaminase activity (Fig 1D). ACC deaminase production by the isolates varied considerably among the isolates. The amount of α-ketobutyrate produced by the isolates from ACC substrate, which indirectly indicated ACC deaminase production and activity, ranged between 0.03–1.42 mM, with *Bacillus methylotrophicus* SG14 (1.42 mM) and *Viridibacillus arvi* SG26 (0.03 mM) being the highest and lowest producer of α-ketobutyrate respectively (Fig 1D).

**Molecular characterization of plant growth promoting genes**

Genes contributing to PGP traits were screened using primers that were described in Raddadi *et al.* [9]. Twenty (76%) of the sacred grove isolates gave an amplified fragment of the expected size for the *AcPho* gene. All the four MTCC reference strains were PCR-positive for this gene (S1A Fig; Table 1). Only 3 of the sacred grove isolates and 2 reference strains gave an amplified fragment of the expected size for the *IpdC* gene (S1C Fig; Table 1). Apart from 2 isolates i.e., *Bacillus aryabhattai* (SG8 & SG21), all the other isolates including the reference strains were positive for *accd* gene (S1B Fig; Table 1). 11 (42%) of the sacred grove isolates and 2 reference strains gave an amplified fragment of the expected size for the *asbA* gene indicating the potential ability of these isolates to produce the catechol type siderophore, petrobactin (S1D Fig; Table 1). Overall in the present study, 2 sacred groves isolates namely, *Bacillus cereus* SG10 and *Bacillus cereus* SG18, and one reference strain *Bacillus thuringiensis* MTCC 8996 gave amplified fragments of the expected size for all the genes screened. On the other hand, 9 sacred groves isolates and 2 reference strains gave amplified fragments for 3 of the total 4 genes that were screened (Table 1). Nucleotide sequences of 1-aminocyclopropane-1-carboxylate deaminase (*accd*) and petrobactin biosynthesis protein (*asbA*) encoding genes have been deposited in NCBI with accession numbers assigned from KF874290 – KF874309 and KF874310 – KF874320 respectively.

**Horizontal Gene Transfer**

Occurrence of phylogenetic incongruence between a marker gene like 16S rRNA gene and a gene of interest can be an indicator of horizontal gene transfer. Current studies, compares the
phylogenetic trees constructed using 16S rRNA, accd and asbA genes respectively. BLASTX analysis was performed using the gene sequences of putative accd and asbA amplicons. The accd gene from these isolates shows high sequence similarity percentage with the accd genes of their respective homologs from the same genus and hence incidence of HGT was excluded amongst the isolates. However, out of the 11(42%) isolates that are positive for asbA gene, two isolates which were identified as Paenibacillus sp, i.e. P. taichungiensis SG3 and P. tylopili SG24 showed 98% sequence similarity with the asbA sequence of other genus (i.e. Bacillus thuringiensis) and displayed a poor sequence similarity percentage (<70%) when compared to the corresponding asbA sequence of the same genus (Paenibacillus mucilaginosus KNP414) reported in the GenBank (Table 2).

Phylogenetic analyses using Neighbor-Joining method were performed using the 16S rRNA gene sequence and also the asbA sequence of the 11 isolates (Fig 2). The nine Bacillus isolates clustered together with their corresponding genus in both the trees constructed using 16S rRNA gene sequence and asbA sequence. However, for the two isolates Paenibacillus taichungiensis SG3 and Paenibacillus tylopili SG24, their asbA gene sequences clustered together with the asbA sequence cluster of Bacillus genus unlike when their 16S rRNA gene sequence which clustered together with their corresponding Paenibacillus genus (Fig 2B, Table 2).

Furthermore, when another important criterion for implicating HGT, i.e., moles percent (mol%) of G+C content (atypical sequence composition) [49] was analyzed for the asbA sequences from Paenibacillus taichungiensis SG3 and Paenibacillus tylopili SG24, it was found that their G+C content (~35 mol%) is similar to that of Bacillus thuringiensis whole genome (32~35 mol%) whereas it is clearly lower than the G+C content reported for Paenibacillus taichungiensis (~46.7 mol%) [50]. Based on these criterion for implicating HGT, it can be said that the results obtained on analysis of the asbA sequence of Paenibacillus taichungiensis SG3 and Paenibacillus tylopili SG24, occurrence of HGT between Paenibacillus and Bacillus genus with respect to the siderophore biosynthesis protein (asbA) gene might have occurred during evolution of these co-existing and related groups of bacteria.

**Discussion**

‘Sacred groves’ are pristine habitats that are inhabited by diverse groups of microorganism [35, 51]. Bacterial communities’ structures in these environments are influenced by the existing soil type and vegetation [52]. On the other hand, microbes also affect the soil component which in

| Isolates | Closest match of 16S rRNA with similarity percentage | Closest match of asbA rRNA with similarity percentage |
|---------|-----------------------------------------------------|-----------------------------------------------------|
| SG1     | Bacillus thuringiensis, 99.80%                      | Bacillus thuringiensis, 98%                         |
| SG3     | Paenibacillus taichungiensis, 99.93%               | Bacillus thuringiensis, 98%                        |
| SG5     | Bacillus mycoides, 99.93%                           | Bacillus cereus, 98%                               |
| SG6     | Bacillus thuringiensis, 98.92%                     | Bacillus thuringiensis, 95%                        |
| SG10    | Bacillus cereus, 99.66%                             | Bacillus cereus, 99%                               |
| SG11    | Bacillus thuringiensis, 99.93%                     | Bacillus thuringiensis, 99%                        |
| SG17    | Bacillus thuringiensis, 99.73%                     | Bacillus thuringiensis, 99%                        |
| SG18    | Bacillus cereus, 99.73%                             | Bacillus cereus, 99%                               |
| SG19    | Bacillus weihenstephanensis, 99.45%                | Bacillus cereus, 100%                              |
| SG20    | Bacillus mycoides, 100%                             | Bacillus cereus, 96%                               |
| SG24    | Paenibacillus tylopili, 97.78%                     | Bacillus thuringiensis, 98%                        |

Table 2. Comparative matches for the closest phylogenetic neighbours obtained for the isolates based on profile of 16S rRNA gene and petrobactin biosynthesis protein asbA gene.

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turn directly or indirectly impact the plant growth in that habitat [53]. Soil contains a mixture of both inorganic and organic source of nutrient that are either readily available to the plants or that required to be transformed into soluble form that can be taken up by the plants [54]. PGPB must have the ability to solubilize both mineral and organic nutrients and make it accessible to plants for their growth and development [53, 54]. Macronutrient like phosphorous and nitrogen are utilized by the plants in solubilized form [55]. Phosphate solubilization by PGPB can be achieved through the release of organic acids that chelates the insoluble phosphate through the action of phosphatase enzymes [55]. Eighteen of the isolates (69%) showed phosphate solubilizing properties indicating their ability to produce organic acids and/or phosphatase enzymes capable of converting insoluble phosphate to bioavailable phosphate (eg. orthophosphate). Furthermore, 20 of the isolates showed the presence of acid phosphatase (AcPho) gene which could play a role in phosphate solubilization. Acid phosphatases play a major role in the mineralization of organic phosphorous in soil [56] and the presence of acid phosphatase (AcPho) gene indicate their potential role in mineralization of organic phosphorous in soil. One of the key mechanisms that PGPB use to support plant growth is through the production of phytohormones like auxins such as IAA [55, 57, 58]. All the isolates under study showed their ability to produce IAA even though the level of production varied as evident from the biochemical estimations. In bacteria, two pathways have been characterized for production of IAA: the indole-3-acetamide (IAM) pathway (L-tryptophan → IAM → IAA) and the indole-3-pyruvic acid (IPyA) pathway (L-tryptophan → IPyA → indole-3-acetaldehyde → IAA) [57]. In this study, the Ipdc gene was chosen as the target because it has been reported that beneficial plant associated bacteria frequently synthesized IAA via the IPyA pathway [18]. The Ipdc gene was detected in only 3 of the sacred grove isolates indicating their ability to

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**Fig 2. Phylogenetic incongruency between asbA and 16S rRNA.** The genes encoding (A) 16S rRNA and (B) asbA protein of the isolates were subjected to neighbor-joining analysis. asbA positive isolates predicted to have undergone HGT are connected by dotted lines. Respective accession numbers of gene nucleotide sequences are indicated in bracket.

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synthesize IAA via the IPyA pathway and their potential as beneficial plant associated bacteria. Interestingly, most of the isolates that responded positively to the Salkowski’s test for IAA production (data not shown) did not give amplification for the ipdC gene. This could possibly be that the IAA production in these isolates is via the indole-3-acetamide (IAM) pathway and not the IPyA pathway [59].

Plant tends to synthesize ethylene in response to stress conditions and if present in high concentrations, can lead to growth inhibition and death [60]. ACC deaminases have been shown to protect plants from the deleterious effects of various biotic and abiotic stresses by lowering the inhibiting levels of ethylene in plants [61–65]. This enzyme catalyzes the breakdown of ACC which is the precursor of ethylene into ammonia and \( \alpha \)-ketobutyrate [66]. Seventy seven percent of the isolates were able to metabolize ACC when used as sole nitrogen source indicating the presence of ACC deaminase enzyme. Moreover, almost all sacred grove isolates, except two isolates of *Bacillus aryabhattai* (SG8 & SG21), were positive for the amplification of the accd gene. PGPB expressing ACC deaminase can help in preventing the buildup of ethylene through an intricate and well regulated mechanism that balances the concentration of ethylene and its precursor (ACC) with minimal damage to the plants [21]. This high frequency presence of the accd gene could also be explained by considering that this enzyme could be implicated in the deamination of substrates other than ACC as was found for the ACCD enzymes from *Pseudomonas putida* UW4 [67] and *Pyrococcus horikoshii* [68].

Overall, 2 sacred groves isolates viz. *Bacillus cereus* SG10 and *Bacillus cereus* SG18, and one reference strain *Bacillus thuringiensis* MTCC 8996 gave amplified fragments of the expected size for all the PGP genes screened. On the other hand, 9 sacred groves isolates and 2 reference strains gave amplified fragments for 3 of the 4 genes that were screened. This indicated multiple PGP genetic traits that are present in these isolates which suggested that these isolates are capable of expressing traits that are important in biofertilization, biostimulation, bioprotection and biocontrol activities.

In the present study, the possible occurrence of HGT with respect to the siderophore biosynthesis protein (asbA) gene was observed among the two sacred groves isolates *Paenibacillus taihongiensis* SG3 and *Paenibacillus tylopili* SG24. The frequencies of HGT between closely related genera are comparatively higher [29] although with very low detection even at molecular level [69]. The high sequence similarity (98%) and almost similar G+C content (~35 mol%) between the asbA sequences of these two isolates with that of *Bacillus thuringiensis* instead of *Paenibacillus* sp. suggests that HGT might have occurred from *Bacillus* sp. to the *Paenibacillus* sp. since HGT creates an unusually high degree of similarity between the donor and the recipient strains for the character in question [70]. This was further supported by the phylogenetic incongruence that was observed in these two isolates. Although, the contribution of HGT to the transfer of PGP related genes is being reported with respect to ACC deaminase gene [7,32], this study reports for the first time the possible occurrence of HGT among bacilli isolated from sacred grove with respect to the siderophore biosynthesis protein (asbA) gene. The current study showed that bacterial isolates belonging to ‘*Bacillus* and related genera’ possess promising plant growth promoting properties that can be further explored for agrobiotechnological applications.

**Supporting Information**

S1 Fig. PCR products on agarose gel showing. A) AcPho gene; B) ipdC gene; C) accd gene; D) asbAgene.

(TIF)
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Author Contributions

Conceived and designed the experiments: NAL MN SRJ. Performed the experiments: NAL MN. Analyzed the data: NAL MN DK SRJ. Contributed reagents/materials/analysis tools: NAL MN DK SRJ. Wrote the paper: NAL MN DK SRJ.

References

1. Lyngwi NA, Koijam K, Sharma D, Joshi SR. Cultivable bacterial diversity along the altitudinal zonation and vegetation range of tropical Eastern Himalaya, Rev Biol Trop. 2013; 61:467–490. PMID: 23894996
2. Bhagobaty RK, Joshi SR. Fungal endophytes of five medicinal plants prevalent in the traditionally preserved ‘Sacred forests’ of Meghalaya, India. Forest Sci Technol. 2011; 7:151–154.
3. Glick BR, Patten CL, Holguin G, Penrose DM. Biochemical and genetic mechanisms used by plant growth promoting bacteria, London, pp. 45–85. Imperial College Press, 1999.
4. Prescott LM, Harley JP, Klein DA. Microbiology, pp.34. McGraw-Hill, New York, NY. 2002. PMID: 12435277
5. Joung KB, Cote JC. Evaluation of ribosomal RNA gene restriction patterns for the classification of Bacillus species and related genera. J Appl Microbiol. 2002; 92:97–108. PMID: 11849333
6. Chen XH, Kourmoutsi A, Scholz R, Eisenreich A, Schneider K, Heinemeyer I et al. Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium Bacillus amyloliquefaciens FZB42. Nat Biotechnol. 2007; 25:1007–1014. PMID: 17704766
7. Bruto M, Prigent-Combaret C, Muller D Moenne-Loccoz Y. Analysis of genes contributing to plant-beneficial functions in Plant Growth-Promoting Rhizobacteria and related Proteobacteria. Sci Rep. 2014; 4:6261, doi: 10.1038/srep06261 PMID: 25179219
8. Seshadri R, Reeve WG, Ardley JK, Tennessen K, Woyke T, Kyrpides NC, Ivanova NN. Discovery of Novel Plant Interaction Determinants from the Genomes of 163 Root Nodule Bacteria. 2015; Sci Rep 5:16825; doi:10.1038/srep16825 PMID: 26584898
9. Raddadi N, Cherif A, Boudabous A, Daffonchio D. Screening of plant growth-promoting traits of Bacillus thuringiensis. Ann Microbiol. 2008; 58: 47–52.
10. Lynch JM. Microbial metabolites. In: Lynch JM. editor. The Rhizosphere, John Wiley and Sons Ltd, Chichester; 1990.pp. 177–206
11. El-Sawah MMA, Hauka FIA, El-Rafey HH. Study on some enzymes cleaving phosphorus from organic substrates in soil. J Agri Sci. 1993; 18:2775–2785.
12. Bishop ML, Chang AC, Lee RWK. Enzymatic mineralization of organic phosphorus in a volcanic soil in Chile. Soil Sci. 1994; 157:238–243.
13. Feller C, Frossard E, Brossard M. Phosphatase activity in low activity tropical clay soils. Distribution in the various particle size fractions. Can J Soil Sci. 1994; 74:121–129.
14. Garcia C, Fernandez T, Costa F, Cerranti B, Macciandaro G. Kinetics of phosphatase activity in organic wastes. Soil Biol and Biochem. 1992; 25:361–365.
15. Xu JG, Johnson RL (1995) Root growth, microbial activity and phosphatase activity in oil-contaminated, remediated and uncontaminated soils planted to barley and field pea. Plant and Soil, 173, 3–10.
16. Patten C, Glick BR. Bacterial biosynthesis of indole-3-acetic acid. Can J Microbiol. 1996; 42:207–220 PMID: 868227
17. Ivanova N, Sorokin A, Anderson I, Galleron N, Candelon B, Kapatral V, et al. Genome sequence of Bacillus cereus and comparative analysis with Bacillus anthracis. Nature. 2003; 423:87–91. PMID: 12721630
18. Schultz A, Golbik R, Tittman K, Svergun DI, Koch MHJ, Hubner G, Konig S. Studies on structure-function relationships of indolepyruvate decarboxylase from Enterobacter cloacae, a key enzyme of the indole acetic acid pathway. Eur J Biochem. 2003; 270:2322–2331. PMID: 12752452
19. Wahyudi AT, Astuti RP, Widayawati A, Meryandini A, Nawangsih AA. Characterization of Bacillus sp. strains isolated from rhizosphere of soybean plants for their use as potential plant growth for promoting rhizobacteria. J Microbiol Antimicrob. 2011; 3:34–40.
20. Penrose DM, Glick BR. Methods for isolating and characterizing ACC deaminase containing plant growth-promoting rhizobacteria. Physiol Plant. 2003; 118:10–15. PMID: 12702008

21. Glick BR. Bacteria with ACC deaminase can promote plant growth and help to feed the world. Microbiol Res. 2014; 169:30–39 doi: 10.1016/j.mires.2013.09.009 PMID: 24095256

22. Marek-Kozaczuk M, Deryto M, Skorupska A. Tn5 insertion mutants of Pseudomonas sp 267 defective siderophore production and their effect on clover (Trifolium pratense) nodulated with Rhizobium leguminosarum bv trifolii. Plant Soil. 1996; 179: 269–274.

23. Crossa JH, Walsh CT. Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. Microbiol Mol Biol Rev. 2002; 6:223–249.

24. Koppisch AT, Dhungana S, Hill KK, Boukhalfa H, Heine HS, Colip LA, et al. Petrobactin is produced by both pathogenic and nonpathogenic isolates of the Bacillus cereus group of bacteria. BioMetals. 2008; 21:581–589. doi: 10.1007/s10534-008-9144-9 PMID: 18459058

25. Wilson MK, Abergel PJ, Arceneaux JE, Raymond KN, Byers BR. Temporal production of the two Bacillus anthracis siderophores, petrobactin and bacillibactin. Biometals. 2010; 23:129–134. doi: 10.1007/s10534-009-9272-x PMID: 19816776

26. Lee JY, Janes BK, Passalacqua KD, Pfleger BF, Bergman NH, Liu H, et al. Biosynthetic analysis of the petrobactin siderophore pathway from Bacillus anthracis. J Bacteriol. 2007; 189: 1698–1710. PMID: 17189355

27. Beiko RG, Harlow TJ, Ragan MA. Highways of gene sharing in prokaryotes. Proc Natl Acad Sci U S A. 2005; 102:14332–14337. PMID: 16176988

28. Gogarten JP, Townsend JP. Horizontal gene transfer, genome innovation and evolution. Nat Rev Microbiol. 2005; 3:679–687. PMID: 16138096

29. Gogarten JP, Doolittle WF. Lawrence JG. Prokaryotic evolution in light of gene transfer. Mol Biol Evol. 2002; 19: 2226–2238. doi: 10.1093/molbev/004046 PMID: 12446813

30. Hacker J, Kaper JB. Pathogenicity islands and the evolution of microbes. Annu Rev Microbiol. 2000; 54:641–79. PMID: 11018140

31. Nongkhlaw M, Kumar R, Acharya C, Joshi SR (2012) Occurrence of horizontal gene transfer of P(IB)-type ATPase genes among bacteria isolated from the uranium rich deposit of Domiasiat in North East India. PLoS One. 7, e48199. doi:10.1371/journal.pone.0048199 PMID: 23133569

32. Holt JG. Bergey's Manual of Systematic Bacteriology. Baltimore. 9th ed, Williams and Wilkins; 1994.

33. Collins CH, Lyne PM, Grange JM, Falkinham JO. Microbiological methods, 4th ed. New York, NY., pp.98–99. Oxford University Press Inc.2001

34. Holt JG, Bergey's Manual of Systematic Bacteriology. Baltimore. 9th ed., Williams and Wilkins;1994.

35. Knudsen D and Beegle D. Recommended phosphorous tests. In: Dahnke W.C. (ed.), Recommended chemical soil tests procedures for the North Central Region, Bull North Dakota Agric Exp Stn, North Dakota, USA, 1988. pp. 12–15.

36. Schwyn B and Neilands JB. Universal chemical assay for the detection and determination of siderophores. Anal Biochem. 1987; 160: 47–56. PMID: 2952030

37. Arnow LE. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine tyrosine mixtures. J Biol Chem. (1937); 118: 531–537.

38. Dworkin M, Foster J. Experiments with some microorganisms which utilize ethane and hydrogen. J Bacteriol.1958; 75:592–601. PMID: 13538930

39. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acids Res. 1997; 25:3389–3402. PMID: 9254694

40. Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, et al. Introducing EzTaxon-e: a prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol. 2012; 62:716–721. doi: 10.1099/ijs.0.038075-0 PMID: 22140171
45. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 2007; 24:1596–1599. PMID: 17488738

46. Chen YP, Rekha PD, Arun AB, Shen FT, Lai WA, Young CC. Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. Appl Soil Ecol. 2006; 34: 33–41.

47. Rodriguez H, Fraga R, Gonzalez T, Bashan Y. Genetics of phosphate solubilization and its potential application for improving plant growth-promoting bacteria. Plant Soil. 2006; 287: 15–21.

48. Vessey JK. Plant growth-promoting rhizobacteria as biofertilizers. Plant Soil. 2003; 255:571–586.

49. Muto A, Osawa S. The guanine and cytosine content of genomic DNA and bacterial evolution. Proc Natl Acad Sci U S A. 1987; 84:166–169. PMID: 3467347

50. Lee JC, Yoon KH. Paenibacillus woosongensis sp nov., a xylanolytic bacterium isolated from forest soil. Int J Syst Evol Microbiol. 2008; 58: 612–616. doi: 10.1099/ijs.0.65350-0 PMID: 18319465

51. Kayang H. Soil microbial population numbers in sacred grove forest of Meghalaya, Northeast India. Asian J Microbial Biotech Env Sc. 2006; 8: 521–526.

52. Carney KM, Matson PA. The Influence of Tropical Plant Diversity and Composition on Soil Microbial Communities. Microbial Ecology. 2006; 52:226–238. PMID: 16897297

53. Lugtenberg B, Kamilova F. Plant-growth-promoting rhizobacteria. Annu Rev Microbiol. 2009; 63:541–556. doi:10.1146/annurev.micro.62.081307.162918 PMID: 19575558

54. Paul EA, Clark FE. Soil Microbiology and Biochemistry. Academic Press, San Diego, California 340.1997.

55. Gamelaro E, Glick BR. Mechanisms Used by Plant Growth-Promoting Bacteria.In: Bacteria in Agrobiology: Plant Nutrient Management. Maheshwari DK ed. Springer. 2011, pp 17–46.

56. Rodriguez H, and Fraga R. Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol Adv. 1999; 17: 319–339. PMID: 14538133

57. Brown ME. Seed and root bacterization. Annu Rev Phytopathol. 1974; 12: 181–197.

58. Patten C, Glick BR. Bacterial biosynthesis of indole-3-acetic acid. Can J Microbiol. 1996. 42: 207–220. PMID: 8688227

59. Kawaguchi M, Syono K. The excessive production of indole-3-acetic acid and its significance in studies of the biosynthesis of this regulator of plant growth and development. Plant Cell Physiol. 1996; 37:1043–1048. PMID: 9032962

60. Abeles FB, Morgan PW, Saltveit ME Jr. Ethylene in plant biology. 2nd ed. New York Academic Press;1992.

61. Wang C, Knill E, Glick BR, Defago G. Effect of transferring 1-aminocyclopropoane-1-carboxylic acid (ACC) deaminase genes into Pseudomonas fluorescens strain CH40 and its gacA derivative CHA96 on their growth-promoting and disease-suppressive capacities. Can J Microbiol. 2000; 46:898–907. PMID: 11068676

62. Gricikko VP, Glick BR. Amelioration of flooding stress by ACC deaminase-containing plant growth-promoting bacteria. Plant Physiol Biochem. 2001; 39:11–17.

63. Mayak S, Tirosh T, Glick BR, Saltveit ME Jr. Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (Brassica juncea L. Czern.). Soil Biol Biochem. 2005; 37:241–250.

64. Honma M, Shimomura T. Metabolism of 1-aminocyclopropene-1-carboxylic acid. Agric Biol Chem. 1978; 43:1825–31.

65. Eisen JA. Horizontal gene transfer among microbial genomes: new insights from complete genome analysis. Curr Opin Genet Dev. 2000; 10: 606–611. doi: 10.1016/s0959-437x(00)00143-x PMID: 11088009

66. Ochman H, Lawrence JG, Groisman E. Lateral gene transfer and the nature of bacterial innovation. Nature. 2000; 405: 299–304. PMID: 10830951