A primary assessment of the endophytic bacterial community in a xerophilous moss (*Grimmia montana*) using molecular method and cultivated isolates

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Abstract

Investigating the endophytic bacterial community in special moss species is fundamental to understanding the microbial-plant interactions and discovering the bacteria with stresses tolerance. Thus, the community structure of endophytic bacteria in the xerophilous moss *Grimmia montana* were estimated using a 16S rDNA library and traditional cultivation methods. In total, 212 sequences derived from the 16S rDNA library were used to assess the bacterial diversity. Sequence alignment showed that the endophytes were assigned to 54 genera in 4 phyla (Proteobacteria, Firmicutes, Actinobacteria and Cytophaga/Flexibacter/Bacteroids). Of them, the dominant phyla were Proteobacteria (45.9%) and Firmicutes (27.6%), the most abundant genera included *Acinetobacter*, *Aeromonas*, *Enterobacter*, *Leclercia*, *Microvirga*, *Pseudomonas*, *Rhizobium*, *Planococcus*, *Paenisporosarcina* and *Planomicrobium*. In addition, a total of 14 species belonging to 8 genera in 3 phyla (Proteobacteria, Firmicutes, Actinobacteria) were isolated, *Curtobacterium*, *Massilia*, *Pseudomonas* and *Sphingomonas* were the dominant genera. Although some of the genera isolated were inconsistent with those detected by molecular method, both of two methods proved that many different endophytic bacteria coexist in *G. montana*. According to the potential functional analyses of these bacteria, some species are known to have possible beneficial effects on hosts, but whether this is the case in *G. montana* needs to be confirmed.

Key words: bacterial diversity, endophytes, moss, molecular method, cultivated isolates.

Introduction

In plant-endophyte interactions, plants provide nutrients and residency for the bacteria, while the bacteria in exchange directly or indirectly improve plant growth and health (Mastretta et al., 2006). Once inside the plant, endophytes either reside in specific plant tissues such as the root cortex or the xylem, or colonize the plant systematically by transport through the vascular system or the apoplast (Quadt-Hallmann et al., 1997). Of the nearly 300 000 plant species on earth, each species is host to one or more species of endophytes (Strobel et al., 2004). The complete description of endophytic species has only been enumerated and characterized for a handful of plant species, and the majority of these are common higher plants. Few studies have examined the endophytes of bryophytes, which represent the simplest extant land plants and have been classified by prominent bryologists as “living fossils” (Hornschuh et al., 2002). Consequently, the opportunity to find new and beneficial endophytic microorganisms among the diversity of plants in different ecosystems is considerable.

The mosses, one kind of bryophytes, are a diverse group of land plants that usually colonize habitats with either moist or extremely variable conditions. One of their most important features is their life cycle, which involves alteration between a diploid sporophyte and a dominant free-living haploid gametophyte generation (Opelt and Berg, 2004). Mosses are unique host plants for microorganisms in numerous ways. For example, the small size of mosses results in limited availability of the substratum. In addition, most mosses display an extraordinarily high tolerance to extreme desiccation and can resume normal metabolism very rapidly after rehydration. Hence, successful microbial colonization requires adaptation to these special conditions.
conditions (DoEbbeler, 1997). Analysis of the epiphytes on the gametophyte of Funaria hygrometrica detected numerous bacterial species on the surface of the phylloid. Among these species, two Methylobacterium strains were found to be able to simulate the well-known effect of cytokinin application on bud formation in Funaria protonema and they also promoted the growth of protonemal filaments (Hornschuh et al., 2002). Endophytic methanotrophic bacteria were also found in the hyaline cells and on the stem leaves of Sphagnum mosses; here, they provided carbon for photosynthesis via in situ oxidation of methane to carbon dioxide (Raghoebarsing et al., 2005).

Opelt and Berg (2004) isolated and identified many antagonistic bacteria associated with three moss species (Tortula ruralis, Aulacomnium palustre and Sphagnum rubellum) in the nutrient-poor habitats of the Baltic Sea Coast in Germany. These species belong to nine different genera, among which Burkholderia, Pseudomonas and Serratia were dominant, but the richness and diversity of antagonistic species were moss species-dependent, and the highest number of species with antagonistic activity was isolated from S. rubellum. Another study examined the function and diversity of bacterial species associated with two Sphagnum species (S. fallax and S. magellanicum) that grow in a temperate mire ecosystem. Species belonging to the genus Burkholderia were predominant in Sphagnum species and this genus was possibly involved in antagonism/pathogen defense and nitrogen-fixation. The authors concluded that Sphagnum is a reservoir for powerful and extraordinary antagonists and potentially facultative human pathogens (Opelt et al., 2007). Thus, thorough research on the bacteria associated with other mosses in different niches would be also useful in discovering bacterial resources and helpful in understanding the interactions between mosses and their associated microbes.

Grimmia montana is a xerophilous moss, and has a high tolerance to drought, cold and UV radiation (Yi and Liu, 2007), and can often be found growing in extreme environments. It always lives under extreme desiccation conditions and can resume normal metabolism very rapidly after rehydration. In this paper, our aim is to study the diversity and community structure of its endophytes using 16S rDNA library and culture-dependent approaches, and hope to make a well known on the interactions between endophytes and G. montana and try to find some bacterial resources with the strong tolerance to the stresses.

Material and Methods

Sampling and surface disinfection

Grimmia montana were sampled from the surface of one large stone in Beijing Songshan National Nature Reserve located at an altitude of 890 m, at N: 40°31'00.45" by E:115°49’33.20" on the 19th of April, 2011. About 3 g of plant material, approximately more than one thousand of entire plants was collected after absorbing enough water, and then mixed together and immediately transported to the laboratory for surface disinfection as described previously (Li et al., 2010). The plants were first washed many times with tap water to remove attached substratum. Subsequently, they were immersed in 70% ethanol for 3 min, washed with 15% sodium hypochlorite solution for 10 min, rinsed three times with 70% ethanol for 30 s, and finally washed five times with sterile distilled water. To confirm that the disinfection process was successful, aliquots of the sterile distilled water in the final rinse were used to determine the results of surface disinfection. Bacteria were cultivated by setting 100 µL of the final rinse on R2A and TSA medium plates, and then examining the plates for bacterial growth after incubation at 28 °C for 3 days. Molecular detection of bacterial species was accomplished by 16S rRNA gene PCR detection based on the primers 799f (5’-AACAGGATTAGATACCCG-3’) and 1492r (5’-GTTACCTTGTACGACTT-3’) (Chelius and Triplett, 2001) using the final rinse as template. The 50 µL PCR reaction mixture contained 5 µL of the final rinse, 5 µL 10x Taq reaction buffer (including 1.5 mM MgCl2), 10 pmol of each primer, 200 µM each dNTP, and 1.5 U of Taq DNA polymerase (Takara Co.). After initial denaturation at 94 °C for five minutes, each thermal cycling was as follows: denaturation at 94 °C for one minute, annealing at 53 °C for one minute, and elongation at 72 °C for one minute. At the end of 30 cycles, the final extension step was at 72 °C for 15 min. Products of four parallel PCRs were combined and electrophoretically separated by 1% agarose. Finally, plant samples were determined to be successfully surface disinfected if no bacterium was identified via cultivation and PCR. These plants were used for the subsequent analyses.

DNA extraction and amplification of the bacterial 16S rRNA genes

About 2 g of surface-disinfected G. montana was frozen with liquid nitrogen and ground to a fine powder in a sterilized and precooled mortar. Next, the cetyltrimethylammonium bromide (CTAB) procedure was used to extract total DNA as previously described (Xie et al., 1999). The DNA was resuspended in 150 µL sterile Milli-Q water. The primer pair 799f and 1492r was selected to amplify the 16S rDNA of the endophytic bacteria. The PCR reaction mixture and programs are the same as described above in the section of surface disinfection. We excised the approximately 730 bp band from a 1% agarose gel, following electrophoresis of the DNA, and purified the DNA using the Gel Extraction Kit (Omega Co.), as described by the manufacturer.

Construction of the 16S rDNA clone library
The purified 730 bp PCR products were ligated into the pMD18-T vector (Takara Co.). *Escherichia coli* Top10 competent cells (Tiangen Co.) were transformed with the ligation products and spread onto Luria-Bertani agar plates with ampicillin (100 mg L\(^{-1}\)) for standard blue and white screening (Sambrook *et al*., 1989). Randomly selected colonies were screened directly for inserts by performing colony PCR with primers RV-M (5’-GAGCCGATAAACATTTTACACAGG-3’) and M13-47 (5’-CGCCAGGGTTTTCCCCAGTCACGAC-3’) for the vector (Takara Co.). Two hundred fifty clones containing inserts of the correct size were sequenced using an ABI PRISM 3730 automatic sequencer (Shanghai Sangon Co., Ltd).

**Phylogenetic analysis**

After being trimmed by cutting the vector sequences using the Editseq program in the DNASTar package (Burland, 2000) and removing all the bad sequences as determined by the chimera sequence detection software Mallard 1.02 (www.cardiff.ac.uk/biosi/research/biosoft), all other manually verified nucleotide sequences were submitted to the NCBI GenBank database. Clones of 16S rRNA gene sequences showing 97% similarity or higher were considered to belong to the same phylotype by sequencher 4.8 (Gene Codes, Ann Arbor, MI) and assigned to an Operational Taxonomic Unit (OTU). Sequences of all phylotypes were compared to the NCBI database using BlastN or aligned by the identify analysis of EzTaxon-e (Kim *et al*., 2012). Clones with a 16S rDNA sequence similarity larger than 97% were assigned to the same species; those with > 95% identity were assigned to the same genus; those with < 95% were determined to be uncultured bacterial species. Next, those sequences assigned to uncultured bacteria were aligned using Clustal W (Thompson *et al*., 1994), and tree constructions were done with the MEGA 5 program package (Tamura *et al*., 2011) using the neighbor-joining method (Saitou and Nei, 1987) to infer their classification. Bootstrap analysis was performed with 1,000 replicates.

**Estimation of the size of the clone library**

To estimate the representation of the library, the clone coverage was calculated with the following equation based on the sequencing results: 
\[ C = \left(1 - \frac{n1}{N}\right) \times 100\% \]
where \(n1\) represents the number of phylotypes occurring only once and \(N\) is the number of clones being examined. Diversity of the clone library was investigated using rarefaction analysis. Rarefaction curve was calculated using the Ecosim 7.0 software (Gotelli and Entsminger, 2004).

**Isolation of culturable endophytes and determination of CFU**

To isolate the endophytes from the plants, 1 mL of sterile 0.85% NaCl was added to 0.5 g (fresh weight) of surface disinfected *G. montana* and samples were homogenized in a small sterile mortar. The resultant mixture was serially diluted with sterile 0.85% NaCl and plated onto R2A and TSA media (Difco, Detroit, MI). Plates were incubated for 3 days at 28 °C, after which Colony-Forming Units (CFU) were counted to calculate the average number of colonies per gram of moss. Isolates obtained by plating were purified and stored at -70 °C in sterile broth containing 40% glycerol.

**ARDRA analysis and identification of the isolates by sequencing**

1 uL of the bacterial suspension derived from each isolate was used to amplify the 16S rDNA fragments using the primers 27f and 1492r. The PCR reaction mixture and programs are the same as described above in the section on surface disinfection. The approximately 1490 bp band was excised from a 0.8% agarose gel, and purified using the Gel Extraction Kit (Omega Co.) as described by the manufacturer. Next, the purified products were enzymatically digested with *Hae* III and *Hha* I at 37 °C for 4 h, respectively. According to their electrophoresis pattern on a 1.0% agarose gel, these isolates were classified into different OTUs. Finally, the PCR products of isolates with different OTUs were sequenced using an ABI PRISM 3730 automatic sequencer (Shanghai Sangon Co., Ltd.). After trimming the low quality nucleotides, the sequence similarities were calculated using the EzTaxon-e (Kim *et al*., 2012).

**Results**

**16S rDNA library analysis of endophytic bacterial community**

Bacterial 16S rDNA fragments were amplified from total DNA that was extracted from surface disinfected *G. montana*, using the primers 799f and 1492r. The amplified DNA displayed only one distinct and one weak band, of approximately 730 bp and 1000 bp, respectively. The sequencing result showed that the 730 bp band represented the bacterial 16S rRNA fragment, while the 1000 bp fragment was mainly derived from the mitochondria of the mosses. Thus, the purified 730 bp PCR products were used to construct a 16S rDNA clone library for the endophytic bacteria.

Of 250 clones, two-hundred and twelve individual sequences were verified. They were determined as 90 phylotypes by sequencher 4.8 and the sequences were deposited in GenBank (Accession No.: JX042330-JX042419). Of them, 48 phylotypes occurring only once, and the calculated coverage of the clone library was 77.4%. The rarefaction curve also showed that the clones detected could reflect the main information of endophytes (Figure 1).

Sequence alignment revealed that 196 individual sequences exhibited > 95% similarity with those of cultivable bacteria. Of these, 90 clones (45.9%) were affiliated with Proteobacteria, 54 clones (27.6%) with Firmicutes, 29
(14.8%) with Actinobacteria, and 23 (11.7%) with Cytophaga/Flavobacterium/Bacteroides (CFB) group. Details of all alignments in the clone library are listed in Table 1.

The sequences attributed to Proteobacteria, which includes alpha, beta and gamma classes, made up the largest fraction of the clone library. Of the 90 clones affiliated with Proteobacteria, 67 clones (or 74.4%) exhibited high similarity to Gammaproteobacteria. The proportion of clones that grouped with the alpha and beta classes was 20% and 5.6%, respectively. However, there were no sequences with > 95% similarity to genera in the delta or epsilon class. The 67 clones of Gammaproteobacteria were related to four orders, bacteria including Pseudomonadales (34 clones), Enterobacteriales (22 clones), Aeromonadales (10 clones) and Xanthomonadales (1 clone). Of these, the dominant genera include: Acinetobacter, Aeromonas, Citrobacter, Enterobacter, Leclercia, Pseudomonas and Psychrobacter; the dominant species were Acinetobacter johnsonii, Acinetobacter junii, Leclercia adecarboxylata, Aeromonas punctata and Enterobacter cancerogenus (Table 1). Alpha-proteobacteria was the second-most abundant subgroup of Proteobacteria in our survey. The 18 clones in this sub-group represented bacteria in four orders (Rhizobiales, Sphingomonadales, Rhodobacterales and Caulobacterales) (Table 1). The dominant genera were Brevundimonas, Microvirga, Rhizobium and Sphingomonas. Of the 5 clones affiliated with Betaproteobacteria, four belonged to bacterial species in Burkholderiales and only one was grouped into Methylophilales. All of them were assigned to different genera, including Bordetella, Comamonas, Methylophilus, Ramlbacter and Variovorax (Table 1).

Among the non-Proteobacteria, 54, 29 and 23 clones exhibited high similarity to bacterial species in the phyla Firmicutes, Actinobacteria and CFB respectively (Table 1). In Firmicutes, 43 clones were closely related to bacteria in Bacillales, 9 clones to Clostridiales and only 2 to Lactobacillales. The dominant genera included Paenibacillus, Planococcus, Planomicrobium, and the most abundant species were Paenibacillus macrurdoensis and Planococcus rifietoensis. Of the 29 clones grouped into Actinomycetales of phylum Actinobacteria, twelve clones were grouped with the Arthrobacter genus, while the others grouped with many other genera including Aeromicrobiium and Ornithinococcus (Table 1). Arthrobacter sulfonivorans was the most common species. In the 23 clones belonging to the CFB phylum, bacteria occurred in four orders, the Sphingobacterales, Cytophagales, Bacteroidales and Flavobacterales. The dominant genera were Adhaeribacter and Segetibacter, and Segetibacter koreensis was the most common species.

Finally, the 16S rDNA sequence of 16 clones, showed < 95% similarity to the previously cultivated bacteria. The phylogenetic analysis showed that these clones exhibited a close relationship with Actinobacteria (4 clones), Alphaproteobacteria (3 clones), Acidobacteria (3 clones), Bacteroidetes (2 clones), Betaproteobacteria (1 clone) and Firmicutes (3 clones) (Figure 2).

Endophytic bacteria communities detected by cultivation method

The isolation result showed that the number of colony-forming units (CFU) as determined for samples grown on R2A medium was higher than the number of CFUs grown on TSA medium. The counts (expressed as g⁻¹ fresh weight) were 2.0*10⁵ and 3.3*10⁴ on R2A and TSA medium, respectively. Totally 49 isolates were sequenced on the basis of 16S rDNA fragments, the ARDRA analysis resulted in the delimitation of 14 OTUs. Based on their 16S rDNA sequences (Genbank no. JX042420 - JX042433), they were assigned to 8 genera in three phyla (Proteobacteria, Actinobacteria and Firmicutes). The strains that were successfully cultivated included some genera in the Proteobacteria (Burkholderia, Massilia, Pseudomonas, Spingomonas, Yersinia), and some genera in Firmicutes and Actinobacteria such as Curtobacterium, Brevibacterium and Streptomyces. The most abundant species were Curtobacterium flaccumfaciens, Massilia brevivalea, Pseudomonas azotoformans and Pseudomonas libanensis (Table 2).

Compared the above bacterial communities with those discovered by 16S rDNA library technique, the cultivated species only involved in three phyla (Firmicutes, Proteobacteria and Actinobacteria) and no bacteria in group CFB was cultivated. The species and genera discovered by cultivation were much less than those detected by molecular method. In addition, some of genera cultivated also could not be found by molecular method, like Curtobacterium, Massilia, Burkholderia and Yersinia.

Discussion

In this study, we provide a thorough description of the endophytic bacterial community of G. montana, using a combined approach of molecular methods and cultivation-dependent techniques. G. montana individuals were sampled from stone surfaces poor in nutrient availability and subject to strong stresses, such as a wide range of temperatures and extreme drought conditions. As far as we know, ours is the first description to date of the endophytic community of a xerophilous moss species in the Grimmiaaceae.

Bacterial species detected by 16S rDNA library technique belong to 4 phyla and 54 genera, with a high proportion of Gammaproteobacteria, Firmicutes and Actinobacteria. Isolates from R2A and TSA media also discovered species in these groups, no bacteria in phylum CFB was cultivated. Although some of the genera discovered by these two methods were inconsistent, it reflected that using the combination of 16S rDNA library and cultivated method would be helpful to discover the bacterial in-
formation completely. Both of them proved that many different species coexisted in this small host (G. montana).

Compared to published accounts of bacterial communities associated with other moss species growing in peat bog, such as Sphagnum, our study revealed the different endophytes inhabiting the tissue of G. montana. In previous studies, Serratia and Pseudomonas of the Gammaproteobacteria, Burkholderia of the beta subgroup, Methylocella and Methylocapsa of the alpha subgroup (Raghoebarsing et al., 2005) and Staphylococcus of the Firmicutes (Opelt et al., 2007) were reported to be associated with Sphagnum species. In this survey, of the Gammaproteobacteria subgroup, Acinetobacter, Leclercia and Aeromonas were the dominant genera. Rhizobium of the Alphaproteobacteria, Massilia, Burkholderia and five of other genera of beta-proteobacteria were also detected. In addition, there were also a high proportion of Gram positive bacteria detected in our library. Of them, clones assigned to Firmicutes comprised 25.5% of the total. Planococcus, Paenisporosarcina, Planomicrobium and Bacillus were the dominant genera; while Arthrobacter and Curtobacterium of Actinobacteria were also abundant. The inconsistent endophytic bacterial community in G. montana and Sphagnum species proved that plant species and niches could cooperatively shape the structure of endophytic bacterial communities (Berg and Smalla, 2009).

Analyzing the function of those bacteria dominated in G. montana would be helpful to understand the interactions between endophytes and hosts. Of gammaproteobacteria class, the dominant species Acinetobacter johnsonii has been reported to produce alkaline and low-temperature lipase (Wang et al., 2011a); Acinetobacter junii was considered to be a kind of cellulosytic bacterium that can produce xylanase, cellulose and pectinase (Lo et al., 2010; Zhai et al., 2010) and also could remove (via accumulation) phosphate from synthetic wastewater (Hrenovic et al., 2010); Leclercia adecarboxylata could degrade two and three benzene-ring polycyclic aromatic hydrocarbon compounds (Sarma et al., 2004; Sarma et al., 2010); Aeromonas veronii and Aeromonas punctata subsp. caviae, could produce enzymes such as the amino acid racemase, and xylanase (Cao et al., 2007; Cruz et al., 2008; Silver et al., 2011). As with the Sphagnum bacterial communities, Pseudomonas was also the dominant genus in our study. The isolated species Pseudomonas azotoformans (Komedal et al., 2004; Nie et al., 2011) could degrade Cyhalofop-butyl, while Pseudomonas libanensis could produce the biosurfactant viscosin (Dabboussi et al., 1999; Saini et al., 2008). Rhizobium pusense of the Alphaproteobacteria was first isolated from the rhizosphere of chickpea plants and considered to be a non-symbiotic rhizobium. In our survey utilizing a 16S rDNA library, five clones of Rhizobium pusense were detected, indicating that this species could be in symbiosis with G. montana.

Of bacteria assigned to Firmicutes, Planococcus riftiensis and Paenisporosarcina macmurdensis were the dominant species, which have ever been previously isolated from algal or cyanobacterial mats in sulfurous springs (Reddy et al., 2003; Romano et al., 2003). Four Planomicrobium species were also found, which have been previously isolated from coastal sediments (Dai et al., 2005), seafood jeotgal (Yoon et al., 2001) and glaciers (Zhang et al., 2009a); they were considered as the cold tolerant bacteria (Yang et al., 2011; Zhang et al., 2009a). In addition, Bacillus simplex was isolated by cultivation, which was ever provided to have strong antioxidant activity (Wang et al., 2011b). Among the Actinobacteria, Arthrobacter sulfonivorans could produce membrane-associated dimethylsulfone- and dimethylsulfoxide-reductases (Borodina et al., 2002); Arthrobacter agilis could release N,N-dimethyl-hexadecanamine (dimethylhexadecylamine) to directly affect plant morphogenesis (Fong et al., 2001; Velazquez-Becerra et al., 2011) and could contribute to membrane stabilization in response to thermal and salt stress by increasing carotenoid accumulation (Fong et al., 2001); Curtobacterium was a dominant genus discovered in the cultures, and Curtobacterium flaccumfaciens, as the most dominant species in this group, also was known to reduce symptoms caused by Xylella fastidiosa in Catharanthus roseus (Lacava et al., 2007); the

Figure 1 - Rarefaction curve for the endophytic bacterial 16S rDNA clone library of Grimmia montana.
cultivable *Streptomyces griseoplanus* could produce antcapsin and Erythromycin-a, and might probably help to resist pathogens in the host (Boeck *et al.*, 1971; Thompson *et al.*, 1971).

The dominant species *Segeribacter koreensis* from CFB phylum was first isolated from ginseng fields in South Korea (An *et al.*, 2007), while *Adhaeribacter tereus* and *Adhaeribacter aquaticus* were ever isolated from soil (Zhang *et al.*, 2009b) and water biofilms (Rickard *et al.*, 2005), respectively. This is the first time that these species have been found as endophytes, and their possible functions remain unclear.

In conclusion, the most important findings of this study were: (1) a high endophytic bacterial diversity and complex community structure were found associated with *G. montana*, using a combination of molecular and cultivation techniques; (2) community structure differed from that of endophytic communities of *Sphagnum* mosses, especially in the abundance of Actinobacteria and Firmicutes (higher in *G. montana*); and (3) Some bacterial species found endophytically in *G. montana* are known to have possible beneficial effects on plants, but whether this is the case in *G. montana* is not proven. Thus, in order to improve our understanding of the concrete mechanisms through which endophytic bacteria (such as those of *G. montana*) adapt to extreme environments and discover new bacterial resources, further work needs to be done in the future.

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References

An DS, Lee HG, Im WT, Liu OM, Lee ST (2007) *Segeribacter koreensis* gen. nov., sp nov., a novel member of the phylum Bacteroidetes, isolated from the soil of a ginseng field in South Korea. Int J Syst Evol Microbiol 57:1828-1833.

Berg G, Smalla K (2009) Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS Microbiol Ecol 68:1-13.

Boeck LD, Christy KL, Shah R (1971) Production of Antcapsin by *Streptomyces griseoplanus*. Appl Microbiol 21:1075-1079.

Borodina E, Kelly DP, Schumann P, Rainey FA, Ward-Rainey NL, Wood AP (2002) Enzymes of dimethylsulfone metabolism and the phylogenetic characterization of the facultative methylotrophs *Arthrobacter sulfitivorans* sp. nov., *Arthrobacter methylotrophus* sp. nov. and *Hyphomicrobiurn sulfitivorans* sp. nov. Arch Microbiol 177:173-183.

Burland TG (2000) DNASTAR’s Lasergene sequence analysis software. Methods Mol Biol 132:71-91.

Cao HP, Yang XL, Wang YH, Li YY (2007) Isolation and growth characteristics of pathogenic *Aeromonas punctata* caviae from Sturgeons. Chinese J Zool 42:1-6.

Chelius M, Tripplett E (2001) The diversity of archaea and bacteria in association with the roots of *Zea mays* L. Microbiol Ecol 41:252-263.

Cruz A, Caetano T, Suzuki S, Mendo S (2008) *Aeromonas veronii*, a tributyltin (TBT)-degrading bacterium isolated from an estuarine environment, *Rio de Aveiro* in Portugal (vol 64, pg 639, 2007). Mar Environ Res 66:309-309.

Dabbousi F, Hamze M, E1omari M, Vherille S, Baida N, Izard D, Leclerc H (1999) Pseudomonas libanensis sp. nov., a new species isolated from Lebanese spring waters. Int J Syst Bacteriol 49:1091-1101.

Dai X, Wang YN, Wang BJ, Liu SJ, Zhou YG (2005) *Planomicrobiurn chinense* sp. nov., isolated from coastal sediment, and transfer of *Planococcus psychrophilus* and *Planococcus alkanaclasticus* to *Planomicrobiurn as* *Planomicrobiurn psychrophilum* comb. nov. and *Planomicrobiurn alkanaclastic* comb. nov. Int J Syst Evol Microbiol 55:699-702.

Deébbeler P (1997) Biodiversity of bryophilous ascomycetes. Biodivers Conserv 6:721-738.

Fong NJC, Burgess ML, Barrow KD, Glenn DR (2001) Carbonoid accumulation in the psychrotrophic bacterium *Arthrobacter agilis* in response to thermal and salt stress. Appl Microbiol Biot 56:750-756.

Gotelli NJ and Entsminger GL (2004) EcoSim: Null models software for ecology. Version 7. Acquired Intelligence Inc. & Kesey-Bear. Jericho, VT 05465. Available at: http://garyentsminger.com/ecosim/index.htm.

Hornschuh M, Grotha R, Kutscher U (2002) Epiphytic bacteria associated with the bryophyte *Funaria hygrometrica*: Effects of methylbacterium strains on protonema development. Plant Biology 4:682-687.

Hrenovic J, Tibiljas D, Ivankovic T, Kovacevic D, Sekovanic L (2010) Sepiolite as carrier of the phosphate-accumulating bacteria *Acinetobacter junii*. Appl Clay Sci 50:582-587.

Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH, Yi H *et al.* (2012) Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylogenotypes that represent uncultured species. Int J Syst Evol Microbiol 62:716-721.

Komeda H, Harada H, Washika S, Sakamoto T, Ueda M, Asano Y (2004) S-Stereoselective piperazine-2-tert-butylcarboxamide hydrolase from *Pseudomonas azotoformans* IAM 1603 is a novel L-amino acid amidase. Eur J Biochem 271:1465-1475.

Lacava PT, Li W, Araujo WL, Azevedo JL, Hartung JS (2007) *Curtobacterium flaccumfaciens* pv. *Papulosum* reduced symptoms caused by *Xylella fastidiosa* in *Catharanthus roseus*. J Microbiol 45:388-393.

Li YH, Zhu JN, Zhai ZH, Zhang QA (2010) Endophytic bacterial diversity in roots of *Phragmites australis* in constructed Beijing Cuihu Wetland (China). FEMS Microbiol Lett 309:84-93.

Lo YC, Lu WC, Chen CY, Chen WM, Chang JS (2010) Characterization and high-level production of xylanase from an in-
digeneous cellulolytic bacterium *Acinetobacter junii* F6-02 from southern Taiwan soil. Biochem Eng J 53:77-84.

Mastretta C, Barac T, Vangronsveld J, Newman L, Taghavi S, Van der Lelie D (2006) Endophytic bacteria and their potential application to improve the phytoremediation of contaminated environments. Biotechnol Genet Eng 23:175-207.

Nie ZJ, Hang BJ, Cai S, Xie XT, He J, Li SP (2011) Degradation of cyhalofop-butyl (CyB) by *Pseudomonas azotoformans* strain QDZ-1 and cloning of a novel gene encoding CyB-hydrolyzing esterase. J Agr Food Chem 59:6040-6046.

Opelt K, Berg C, Berg G (2007) The bryophyte genus *Sphagnum* is a reservoir for powerful and extraordinary antagonists and potentially facultative human pathogens. FEMS Microbiol Ecol 61:38-53.

Opelt K, Berg G (2004) Diversity and antagonistic potential of bacteria associated with bryophytes from nutrient-poor habitats of the Baltic Sea coast. Appl Environ Microb 70:6569-6579.

Zhai QM, Xue WW, Xue YC, Zheng LJ (2010) Optimizations of fermentation for pectinase production with *Acinetobacter junii* FM208850. Biotechnology 20:65-69. (in Chinese)

Quadt-Hallmann A, Benhamou N, Kloepper JW (1997) Bacterial endophytes in cotton: Mechanisms of entering the plant. Can J Microbiol 43:577-582.

Raghoebarsing AA, Smolders AJP, Schmid MC, Rijpstra WIC, Wolters-Arts M, Derksen J, Jetten MSW, Schouten S, Damste JSS, Lamers LPM *et al.* (2005) Methanotrophic symbionts provide carbon for photosynthesis in peat bogs. Nature 436:1153-1156.

Reddy GSN, Matsumoto GI, Shivaji S (2003) *Sporosarcina macmurdensis* sp. nov., from a cyanobacterial mat sample from a pond in the McMurdo Dry Valleys, Antarctica. Int J Syst Evol Microbiol 53:1363-1367.

Rickard AH, Stead AT, O’May GA, Lindsay S, Banner M, Handleys PS, Gilbert P (2005) *Adhaeribacter aquaticus* gen. nov., sp. nov., a Gram-negative isolate from a potable water biofilm. Int J Syst Evol Microbiol 55:821-829.

Romano I, Giordano A, Lama L, Nicolaus B, Gambacorta A (2003) Planococcus rifietensis sp. nov., isolated from alg mat collected from a sulfurous spring in Campania (Italy). Syst Appl Microbiol 26:357-366.

Saini HS, Barragan-Huerta BE, Lebron-Paler A, Pemberton JE, Vazquez RR, Burns AM, Marron MT, Seliga CJ, Gunatilaka AAL, Maier RM (2008) Efficient purification of the biosurfactant viscosin from *Pseudomonas libanensis* strain M9-3 and its physicochemical and biological properties. J Nat Prod 71:1011-1015.

Saitou N, Nei M (1987) The neighbor-joining method - a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: A laboratory manual*. 2nd edition edn: Cold Spring Harbor Laboratory Press.

Sarma PM, Bhattacharya D, Krishnan S, Lal BW (2004) Degradation of polycyclic aromatic hydrocarbons by a newly discovered enteric bacterium, *Leclercia decacarboxylata*. Appl Environ Microb 70:3163-3166.

Sarma PM, Duraja P, Deshpande S, Lal B (2010) Degradation of pyrene by an enteric bacterium, *Leclercia decacarboxylata* PS4040. Biodegradation 21:59-69.

Silver AC, Williams D, Faucher J, Horneman AJ, Gogarten JP, Graf J (2011) Complex evolutionary history of the

Figure 2 - 16S rDNA–based dendrogram showing the phylogenetic relationships of uncultured endophytic bacterial clones from *Grimmia montana*. Phylogeny was inferred using a neighbor-joining analysis and trees were generated using MEGA5 software. Numbers in parentheses represent the sequence accession numbers in GenBank. Numbers in square brackets indicate the number of clones out of the total clones. Numbers at branch points indicate bootstrap values. The scale bar represents a 2% estimated difference in nucleotide sequence.
| Group                   | Number of clones | The closest match                       | Accession No. | Sequence similarity% |
|------------------------|------------------|-----------------------------------------|---------------|----------------------|
| Gammaproteobacteria    | 67               | *Acinetobacter johnsonii* DSM 6963(T)   | X81663        | 100                  |
|                        | 17               | *Acinetobacter guillouiae* ATCC 11171(T)| X81659        | 99                   |
|                        | 5                | *Acinetobacter junii* LMG 998(T)        | AM410704      | 100                  |
|                        | 11               | *Leclercia decarboxylata* GTC 1267(T)   | AB273740      | 100                  |
|                        | 5                | *Aeromonas punctata* subsp. *caviae* ATCC 15468(T) | X74674 | 100                  |
|                        | 5                | *Aeromonas veronii* ATCC 35624(T)       | X60414        | 100                  |
|                        | 5                | *Enterobacter cancerogenus* LMG 2693(T) | Z96078        | 99                   |
|                        | 4                | *Pseudomonas balearica* SP1402(T)       | U26418        | 100                  |
|                        | 1                | *Pseudomonas knackmussii* B13(T)        | AF039489      | 100                  |
|                        | 2                | *Psychrobacter palmonis* CECT 5989(T)   | AJ437696      | 100                  |
|                        | 4                | *Citrobacter murliniae* CDC 2970-59(T)  | AF025369      | 100                  |
|                        | 2                | *Pectobacterium wasabiae* ATCC 43316(T) | U80199       | 97                   |
|                        | 1                | *Arenimonas composti* TR7-09(T)          | AM229324      | 97                   |
|                        | 1                | *Enhydrobacter aerosaccus* LMG 21877(T)| AJ550856      | 99                   |
| Alphaproteobacteria    | 18               | *Rhizobium pusense* NRCPB10(T)          | FJ969841      | 100                  |
|                        | 5                | *Brevundimonas vesicularis* LMG 2350(T) | AJ227780      | 100                  |
|                        | 2                | *Microvirga aerophila* 5420S-12(T)      | GQ421848      | 95                   |
|                        | 2                | *Microvirga subterranea* DSM 14364(T)   | FR733708      | 97                   |
|                        | 1                | *Microvirga flocculans* TFB(T)          | AB098515      | 98                   |
|                        | 1                | *Altererythrobacter ishigakiensis* JGCCMB0017(T) | AB363004 | 97                   |
|                        | 1                | *Methylobacterium brachiatum* B0021(T)  | AB175649      | 100                  |
|                        | 1                | *Paracoccus stylophorae* KTW-16(T)      | GQ281379      | 98                   |
|                        | 1                | *Rhodovulum euryhalinum* DSM 4868(T)    | D16426        | 97                   |
|                        | 1                | *Sphingomonas koreensis* JSS26(T)       | AF131296      | 98                   |
|                        | 1                | *Sphingomonas molluscum* KMM 3882(T)    | AB248285      | 97                   |
| Betaproteobacteria     | 5                |                                         |               |                      |
|                        | 1                | *Bordetella avium* avium197N            | AM167904      | 99                   |
|                        | 1                | *Comamonas terrigena* LMG 1253(T)       | AJ430342      | 100                  |
|                        | 1                | *Methylphilus flavus* Ship(T)           | FJ872108      | 100                  |
|                        | 1                | *Ramlibacter henchirensis* TMB834(T)    | AF439400      | 97                   |
|                        | 1                | *Variorovax dokdonensis* DS-43(T)       | DQ178978      | 99                   |
| Firmicutes             | 54               |                                         |               |                      |
|                        | 12               | *Planococcus rifietensis* M8(T)         | A3493659      | 100                  |
|                        | 2                | *Planococcus donghaensis* IH1(T)        | EF079063      | 97                   |
|                        | 1                | *Planococcus citreus* NCIMB 1493(T)     | X62172        | 99                   |
|                        | 1                | *Planococcus maritimus* T9-9(T)         | A5F00007      | 100                  |
|                        | 13               | *Paenibacillus macruridensis* CMS 21w(T)| A5J14408      | 100                  |
|                        | 3                | *Planomicrobiurn koreense* JG07(T)      | AF144750      | 100                  |
|                        | 2                | *Planomicrobiurn glaciei* 423(T)        | EU036220      | 100                  |
|                        | 3                | *Planomicrobiurn chinense* DX3-12(T)    | A3697862      | 100                  |
|                        | 1                | *Planomicrobiurn oceanokoites* IFO 12536(T) | D55729 | 99                   |
|                        | 2                | *Anaerotruncus colihominis* DSM 17241(T)| ABGD02000032 | 95                   |
|                        | 2                | *Bacillus vallismortis* DSM 11031(T)    | AB021198      | 100                  |
|                        | 2                | *Pseudoflavonifractor capillosus* ATCC 29799(T) | AAXG02000048 | 98                   |
| Group                      | Number of clones | The closest match                                | Accession No. | Sequence similarity% |
|---------------------------|------------------|--------------------------------------------------|---------------|----------------------|
|                           |                  | *Robinsoniella peoriensis* PPC31(T)               | AF445285      | 96                   |
|                           | 2                | *Staphylococcus hominis* subsp. *hominis* DSM 20328(T) | X66101       | 100                  |
|                           | 1                | *Alkalibacterium kapii* T22-1-2(T)                | AB294171      | 98                   |
|                           | 1                | *Atoptipes suilococalis* PPC79(T)                 | AF445248      | 95                   |
|                           | 1                | *Finegoldia magna* CCUG 17636(T)                 | AF542227      | 100                  |
|                           | 1                | *Paenibacillus agaridevorans* DSM 1355(T)         | AJ345023      | 98                   |
|                           | 2                | *Roseburia intestinalis* L1-82(T)                 | AJ312385      | 95                   |
| Actinobacteria            | 29               |                                                  |               |                      |
|                           | 5                | *Arthrobacter sulfonivorans* ALL(T)               | AF235091      | 99                   |
|                           | 3                | *Arthrobacter agilis* DSM 20550(T)               | X80748        | 100                  |
|                           | 2                | *Arthrobacter bergerei* CIP 108036(T)            | AJ609630      | 100                  |
|                           | 3                | *Arthrobacter sulfures* DSM 20167(T)             | X83409        | 100                  |
|                           | 3                | *Ornithinococcus hortensis* KHI 0125(T)          | Y17869        | 98                   |
|                           | 2                | *Aeromonibacter erythreum* NRRL B-3381(T)        | AF005021      | 99                   |
|                           | 2                | *Corynebacterium lipophiloflavum* DSM 4429(T)     | ACHJ01000075  | 100                  |
|                           | 1                | *Agrococcus jenensis* DSM 9580(T)                | X92492        | 100                  |
|                           | 1                | *Cellulomonas aerilata* 54205-23(T)              | EU560979      | 100                  |
|                           | 1                | *Geodermatophilus obscurus* DSM 43160(T)         | CP001867      | 99                   |
|                           | 1                | *Microbacterium panaciera* Geoil 954(T)          | AB271051      | 97                   |
|                           | 1                | *Nocardia islandicus* MSL 26(T)                  | EF466123      | 98                   |
|                           | 1                | *Sporichthya brevicatena* IFO 16195(T)           | AB006164      | 95                   |
|                           | 1                | *Streptomyces resistomycificus* NBRC 12814(T)    | AB184166      | 100                  |
|                           | 1                | *Tessaracoccus profund* CB31(T)                  | FJ228690      | 98                   |
|                           | 1                | *Yonghaparkia alkaliphila* KSL-113(T)            | DQ256087      | 100                  |
| Cytophaga/                |                  |                                                  |               |                      |
| Flavobacterium/           |                  |                                                  |               |                      |
| Bacteroides               | 23               |                                                  |               |                      |
|                           | 6                | *Segetibacter koreensis* Geoil 664(T)             | AB267478      | 98                   |
|                           | 3                | *Segetibacter aerophilus* 6424S-61(T)             | GQ421847      | 97                   |
|                           | 2                | *Adhaeribacter terreus* DNG6(T)                  | EU682684      | 99                   |
|                           | 1                | *Adhaeribacter aquaticus* MBGR1.5(T)             | AJ626894      | 97                   |
|                           | 1                | *Adhaeribacter terreus* DNG6(T)                  | EU682684      | 95                   |
|                           | 2                | *Bacteroides nordii* WAL 11050(T)                | AY608697      | 95                   |
|                           | 2                | *Dysgonomonas mossii* DSM 22836(T)               | ADLW01000023  | 95                   |
|                           | 1                | *Aequorivita sublithincola* 9-3(T)               | AF170749      | 97                   |
|                           | 1                | *Cloacibacterium normanense* CCUG 46293(T)       | AJ575430      | 99                   |
|                           | 1                | *Flavobacterium swingsii* WB 2.3-68(T)           | AM934651      | 96                   |
|                           | 1                | *Ohtaekwangia koreensis* 3B-2(T)                 | GU117702      | 95                   |
|                           | 1                | *Parasegetibacter huojiensis* RHYL-37(T)         | EU877263      | 97                   |
|                           | 1                | *Rhodocytophaga aerolata* 5416T-29(T)            | EU004198      | 98                   |
| Uncultured bacteria       | 16               |                                                  |               |                      |
|                           | 3                | Uncultured bacterium                             | EU289421      | 99                   |
|                           | 2                | Uncultured bacterium                             | JF429066      | 98                   |
|                           | 2                | Uncultured bacterium                             | EF0166801     | 98                   |
|                           | 1                | Uncultured bacterium                             | FJ764201      | 98                   |
|                           | 1                | Uncultured bacterium                             | EU979093      | 98                   |
|                           | 1                | Uncultured bacterium                             | HQ597451      | 98                   |
|                           | 1                | Uncultured bacterium                             | JN038624      | 98                   |
|                           | 1                | Uncultured bacterium                             | EF445161      | 92                   |
**Aeromonas veronii** group revealed by host interaction and DNA sequence data. Plos One 6.

Strobel G, Daisy B, Castillo U, Harper J (2004) Natural products from endophytic microorganisms. J Nat Prod 67:257-268.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731-2739.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673-4680.

Thompson RM, Strong FM (1971) Identification of erythromycin-a in cultures of *Streptomyces griseoplanus*. Biochem Bioph Res Co 43:213-216.

Velazquez-Becerra C, Iveth Macias-Rodriguez L, Lopez-Bucio J, Altamirano-Hernandez J, Flores-Cortez I, Valencia-Cantero E (2011) A volatile organic compound analysis from *Arthrobacter agilis* identifies dimethylhexadecylamine, an amino-containing lipid modulating bacterial growth and *Medicago sativa* morphogenesis in vitro. Plant Soil 339:329-340.

Wang HK, Shao J, Wei YJ, Zhang L, Qi W (2011) A novel low-temperature alkaline lipase from *Acinetobacter johnsonii* LP28 suitable for detergent formulation. Food Technol Biotech 49:96-102.

Wang ZR, Sheng JP, Tian XL, Wu TT, Liu WZ, Shen L (2011) The in vitro antioxidant properties of *Bacillus simplex* XI-25 isolated from sand biological soil crusts. Afr J Microbiol Res 5:4780-4786.

Xie Z, Ge S, Hong D (1999) Preparation of DNA from silica gel dried mini-amount of leaves of *Oryza rufipogon* for RAPD study and total DNA bank construction. Acta Bot Sin 41:802-807.

Yang X, Chen X, Xu X, Zeng R (2011) Cold-adaptive alkaline protease from the psychrophilic *Planomicrobium* sp. 547: enzyme characterization and gene cloning. Adv Polar Sci 22:49-54.

Yi YJ, Liu JY (2007) Photochemical analysis of PSII in response to dehydration and rehydration in moss *Grimmia pilifer* P. Beauv Acta Ecol Sin 27:5238-5244. (in Chinese)

Yoon JH, Kang SS, Lee KC, Lee ES, Kho YH, Kang KH, Park YH (2001) *Planomicrobium koreense* gen. nov., sp. nov., a bacterium isolated from the Korean traditional fermented seafood jeotgal, and transfer of *Planococcus okeanokoites*

### Table 2 - The cultivable endophytic bacteria isolated from *Grimmia montana*.

| Group            | No. of isolates | The closest match                      | Accession No. | Sequence similarity % |
|------------------|-----------------|----------------------------------------|---------------|----------------------|
| Gammaproteobacteria | 21              | *Pseudomonas azotoformans* IAM1603(T)  | D84009        | 99.7                 |
|                  | 11              | *Pseudomonas libanensis* CIP 105460(T) | AF057645      | 99.5                 |
|                  | 7               | *Pseudomonas graminis* DSM 11363(T)    | Y11150        | 99.9                 |
|                  | 1               | *Pseudomonas koreensis* Ps9-14 (T)     | AF468452      | 99.9                 |
|                  | 1               | *Yersinia intermedia* ATCC 29909(T)    | AF366380      | 99.4                 |
| Alphaproteobacteria | 5              | *Sphingomonas aquatilis* JSS7(T)       | AF131295      | 98.8                 |
|                  | 2               | *Sphingomonas azotifigens* NBRC 15497(T) | AB217471     | 99.9                 |
|                  | 2               | *Sphingomonas melonis* DAPP-PG 224(T)  | AB055863      | 98.7                 |
| Betaproteobacteria | 12             | *Burkholderia glathei* ATCC 29195(T)   | Y17052        | 97.1                 |
|                  | 11             | *Massilia brevitatea* byr23-80(T)     | EFS46777      | 97.9                 |
| Actinobacteria   | 9              | *Curtobacterium flaccumfaciens* LMG 3645(T) | AJ312209     | 100                  |
|                  | 2              | *Curtobacterium herbarum* P 420/07(T)  | AJ310413      | 99.3                 |
|                  | 5              | *Streptomyces griseolus* AS 4.1868(T) | AY999894      | 99.9                 |
| Firmicutes       | 2              | *Bacillus simplex* NBRC 15720 (T)      | AB363738      | 99.9                 |
(Nakagawa et al., 1996) and Planococcus mcmeekinii (Junge et al., 1998) to the genus Planomicrobium. Int J Syst Evol Microbiol 51:1511-1520.
Zhang DC, Liu HC, Xin YH, Yu Y, Zhou PJ, Zhou YG (2009) Planomicrobium glaciei sp. nov., a psychrotolerant bacterium isolated from a glacier. Int J Syst Evol Microbiol 59:1387-1390.
Zhang JY, Liu XY, Liu SJ (2009) Adhaeribacter terreus sp. nov., isolated from forest soil. Int J Syst Evol Microbiol 59:1595-1598.

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