The Diversity and Role of Bacterial Ice Nuclei in Rainwater from Mountain Sites in China

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ABSTRACT

Ice nuclei (IN) that catalyze ice nucleation in the atmosphere are considered to be vital to the initiation of ice formation in clouds, which in turn impact precipitation and climate. Some bacterial ice-nucleating particles are presumed to speed up ice formation at relatively warm temperatures (above –10°C), and may thus contribute to the induction of precipitation. In this study, nine rainwater samples were collected from forest ecosystems located in the Changbai, Wuling, and Dinghu mountains in eastern China, and the microbial community compositions were determined. Species of the genus Pseudomonas are considered to be the most efficient ice nucleation-active bacteria; however, Pseudomonas spp. were only observed in two of the rainwater samples from two months (June and September) at the Wuling Mountain site (2% of total Sanger clones sets). The median freezing temperature ($T_{50}$) of crude rain droplets ranged from –11.2°C to –18.6°C based on immersion-mode freezing experiments, and their cumulative IN spectrum revealed a very low or near-zero frequency of bacterial IN at –10°C, which was used as the temperature cutoff to define ice nucleators of biological origin. The $T_{50}$ of the filtrate (< 0.22 µm) was between –16.0°C and –20.8°C. The frequency of IN was higher at –10°C from the particle (≥ 0.22 µm) suspension of rainwater collected in 2013, with an onset freezing temperature of approximately –6°C or warmer, and a $T_{50}$ value from –8.2 to –14.0°C. Moreover, ice nucleation was significantly deactivated by heat treatment (to disrupt the structure of membrane-bound proteins) at –10°C, with an average inhibition of 85%. Our results indicate that bacterial IN are present but play a minor role in ice nucleation. Further studies evaluating the concentration and physical chemistry of bacteria in the atmosphere are needed to confirm these results.

Keywords: Bacterial ice-nuclei; Microbial community composition; Median freezing temperature; Rainwater.

INTRODUCTION

Bioaerosols, as an important component of atmospheric aerosols, are suspensions of airborne particles that contain living organisms or that are released from living organisms (Ariya and Amyot, 2004). These particles are very small, ranging in size from ~10 nm to 100 µm. The intact cellular component is referred to as the primary biological aerosol particle (PBAP), which consists of fragments of plants and animals, bacteria, fungal spores, virus particles, and plant pollen (Matthias-Maser and Jaenicke, 1995; Deguillaume et al., 2008). Biological particles, e.g., bacteria, certain free-living fungi, lichens, pollen, and plankton (Kieft, 1988; Pouleur et al., 1992; Knopf et al., 2011; Pummer et al., 2012), and those of non-biological origin, e.g., mineral dust, salt, soot, and organic compounds, could serve as cloud condensation nuclei (CCN) and ice nuclei (IN) (Christner et al., 2008a; Murray et al., 2012). Therefore, aerosols play an important role in the climate system indirectly (Jacobson, 2001; Breon et al., 2002). Without IN, supercooled cloud droplets would not freeze, or the heterogeneous ice nucleation from the vapor phase at temperatures between 0°C and –38°C could not occur in general (Hoose et al., 2010a; Attard et al., 2012); however, sedimenting ice particles can trigger homogeneous ice formation at colder temperatures. Some bacteria and fungal spores have been found to nucleate at the warmest temperatures among all IN, followed by other biological particles and mineral dust, at temperatures below –10°C (Maki and Willoughby, 1978; Hoose et al., 2010a).

The French meteorologist Soulage (1957) initially identified mold spores in ice crystals. Subsequently, plant pathologists in the early 1970s reported several bacteria that were capable of ice nucleation and could cause frost injury of plants, including Pseudomonas syringae (Maki et al., 1974; Army et al., 1976) and Erwinia herbicola (Lindow et al., 1978). P. syringae was the first bacterium screened for ice nucleation activity (INA) that showed capacity to induce the crystallization of supercooled water at –8°C (Vali, 1971). With the development of new biological technology, the
INA of bacteria has been attributed to an outer membrane-bound protein of around 120–180 kDa in size, which is encoded by a single gene, inaZ, proposed to provide a template for the arrangement of water molecules in crystals (Lindow, 1983; Orser et al., 1985; Wolber et al., 1986). Among the thousands of known bacteria species, at least six species of ice-nucleating bacteria strains have been isolated from the surfaces of plants, all of which are gram-negative, including P. syringae (Maki et al., 1974; Arny et al., 1976), Pseudomonas fluorescens (Obata et al., 1987), Pseudomonas viridiflava (Obata et al., 1989), E. herbicola (Lindow et al., 1978; Hew and Yang, 1992), Erwinia ananas (Hew and Yang, 1992), Erwinia uredovora (Obata et al., 1990), and Xanthomonas campesris (Kim et al., 1987; Hew and Yang, 1992). However, much less is known with respect to the IN nature of fungal species, including those of the genus Fusarium. Some previous studies have indicated that the INA of fungi is also proteinaceous in nature (Hasegawa et al., 1994; Tsukui and Konno, 1994; Humphreys et al., 2001). For example, the urediospores of rust fungi have been identified as effective IN, and the mechanism of their INA has been related to a polysaccharide (Morris et al., 2001). A number of studies have shown that the INA of other biological materials such as pollen, lichen, algae, and plankton, exhibited varying INA in drop-freezing assays (Kieft, 1988; Pummer et al., 2012; Burrows et al., 2013; Hader et al., 2014). For example, Hader et al. (2014) reported that pollen grains initiated freezing at temperatures around –12°C. In China, a total of 250 INA bacteria were isolated from 68 distinct types of vegetation, in which 3 genera and 20 species were identified with potential INA, including Pseudomonas, Erwinia, and Xanthomonas (Sun et al., 1996). Among these, the most widely distributed species were identified as P. syringae and E. ananas (Morris et al., 1992; Sun, 1996).

Over 30 years ago, Sands et al. (1982) proposed a bioprecipitation cycle in which plant-associated microorganisms are transported to the altitude of clouds as aerosols and incite precipitation via their INA, which was in turn beneficial for their growth on plants; this hypothesis was further developed by Morris and colleagues more recently (Morris et al., 2004; Morris et al., 2008; Morris et al., 2014). INA bacteria have been isolated from rain and snow samples in many areas such as from France and Austria (Morris et al., 2008), the USA (Christner et al., 2008b), England, Canada (Christner et al., 2008a), and Denmark (Santl-Temkiv et al., 2015), at concentrations ranging from ~10^6 to 10^8 L⁻¹. In contrast, Maki and Willoughby (1978) found that no bacteria were active in a limited number (only five) of isolates from rainwater. Ahern et al. (2007) also showed that none of the Pseudomonas isolates collected from cloud water at two mountains in the Hebrides (UK) possessed INA. Similarly, Mortazavi et al. (2008) suggested that the effective ice-nucleating organisms were not frequently detected in snow samples in Canada. Therefore, there are currently many unanswered questions and much controversy with respect to the role of biological IN in precipitation. For example, it remains unclear whether there are adequate amounts of biological IN to effectively trigger precipitation. Recent simulation studies have demonstrated that the contribution of INA bacteria to the precipitation rate may be very small at the global scale, although this does not rule out the existence of substantial regional or local effects (Phillips et al., 2009; Hoose et al., 2010a, b).

To our knowledge, there has been no report of biological (bacterial and fungal) INA in the rainwater of China. Therefore, in this study, we conducted an initial attempt to find effective biological IN (bacteria and fungi) via fall-out with rain from mountain sites in eastern China between 2011 and 2013, in order to estimate the potential contribution of biological IN (bacteria and fungi) to the total IN population and the precipitation processes at the regional or local scales.

**MATERIALS AND METHODS**

**Sampling Site and Method**

Seven rain samples were taken at the Wuling Mountain site in the Xinglong Observing Station of the National Astronomical Observatories, Chinese Academy of Sciences (40°23′N, 117°34′E; 900 m above sea level [a.s.l.]) from August 2011 to September 2013. Two additional rain samples were collected at the Changbaishan Forest Ecosystem Research Station (40°42′N, 127°42′E; 2740 m a.s.l.) on July 2012, and at the Dinghushan Forest Ecosystem Research Station (23°09′N, 112°30′E; 1000 m a.s.l.) on August 2013, respectively. The characteristics of the sampling sites and dates are shown in detail in Table 1. Rain samples were collected in sterilized 3-L beakers equipped with a homemade stainless steel rain collector. The collector was funnel-shaped with a wide outer region (twice the diameter of the beaker) and installed on top of the beaker so that as much rainwater as possible could be collected. The beakers were placed in open areas and at sufficient heights (1.5–2 m) to avoid splashing from plants and the ground, and were rinsed once with the rainwater before being set for collection. It was difficult to meet the required sample volume (~1 L) in one rain event due to the impact of meteorological factors, such as less rainfall and variable wind direction at the sampling site. The rain samples were collected several times in a given month. All samples were immediately stored in a refrigerator (4°C) prior to processing. In some instances, the collected rainwater samples were transitorily stored in a portable incubator. Note that all of the materials used for sampling had been sterilized by autoclaving, and a sterile mask and gloves were worn during sample collection to avoid any potential contamination. The rain samples represent a well-mixed sample of rainwater that was collected within one month on the dates indicated in Table 1.

**Sample Pretreatment**

Crude rain (volume of 0.5 L) was filtered through each of two sterile nitrous cellulose filters with a pore diameter of 0.22 µm (Millipore, Billerica, MA, USA). One of the filters was used for ice nucleation assays, and the other was used for DNA extraction and the associated microbial community composition analyses. The filter was resuspended and agitated with a sterile stir bar for about 5 min with 50 mL
of sterile deionized water in a sterile plastic container. The crude rainwater samples and filtrate were transferred to a sterile plastic container. The mixed-well crude sample was aliquoted (20 mL) into two 10-mL test tubes. One tube was sterilized ultra-pure Milli-Q water (Du et al., 2007). The temperature of the working plate was decreased at an approximately constant rate controlled by a Eurotherm 818P4 temperature controller. The process of the release of latent heat from the freezing droplets was monitored in real-time with a computer and transformed into a voltage signal.

The median freezing temperature ($T_{50}$) was used for calculations, which represents the temperature at which 50% of the drops froze. The cumulative IN concentration at each temperature was calculated according to the following equation (Vali, 1971):

$$K(T) = \left[ \ln(N_0 - \ln(N(T))) \right]/V$$

where $K(T)$ is the cumulative concentration of IN at temperature $T$, $N_0$ is the number of droplets tested, $N(T)$ is the cumulative number of unfrozen droplets at a given temperature $T$, and $V$ is the volume of the droplet.

### DNA Extraction and Polymerase Chain Reaction (PCR) Amplification

Total microbial DNA was extracted from samples with the Fast DNA spin kit (Bio101, Qbiogene Inc., Carlsbad, CA, USA) according to the manufacturer’s instructions. The 16S rDNA and ITS1-5.8S-ITS2 gene fragments from the extracted total DNA were amplified using PCR (95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min, and a final extension at 72°C for 10 min) with the bacterial 16S rDNA forward primer 27f (5′–AGAGTTTGATCCTGGCTCAG–3′) and reverse primer 1492r (5′–ACGGCTACCTTGTTACGACTT–3′), and the fungal ITS1-5.8S-ITS2 forward primer ITS1f (5′–CTTGTTACTCAGGAGGAACT–3′) and reverse primer ITS4 (5′–TCCTC CGCG TATGATAT–3′), respectively. 

PCRs were performed in a 50-µL mixture containing 5 µL of 10 × PCR Buffer, 4 µL of 2.5 mM dNTPs, 2.0 µL of each primer (10 µM), 0.5 µL of bovine serum albumin (10 mg mL$^{-1}$), 1.0 µL of template DNA, 0.5 µL of fTaq (5 U µL$^{-1}$), and 35 µL of sterile ultrapure water. The PCR products were analyzed by agarose gel electrophoresis (1%, w/v).

### Cloning, Sequencing, and Bioinformatics Analysis

The PCR products were purified using a GeneJET PCR Purification Kit (Thermo Scientific, Waltham, MA, USA) and then ligated into pEASY-Blunt vector (TransGen Biotech, Beijing, China) according to the manufacturer’s instructions. The transformed E. coli JM109 competent cells were selected on LB-ampicillin plates. DNA sequencing was performed by Sangon Biotech (Shanghai, China). The raw sequence data obtained were assembled using SeqMan II (DNASTAR, Madison, WI, USA) and aligned using ClustalW (Thompson et al., 1994). The phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987) using MEGA6. The homology analysis of the obtained sequences was performed using the Basic Local Alignment Search Tool (BLAST) database (Altschul et al., 1990).
Purification Kit (Fermentas, Waltham, MA, USA). PCR-purified products were ligated into a pGEM–T Easy vector, transformed into DH5α Escherichia coli cells following the manufacturer’s instructions (Takara Bio Inc., Tokyo, Japan), and sequenced at Majorbio Bio-Pharm Technology Co. (Shanghai, China). All sequences were checked for chimeric artifacts using the Mallard program (Ashelford et al., 2006). Clones with more than 97% sequence similarity were grouped into the same operational taxonomic unit (OTU), and their representative sequences were compared with those available in the GenBank databases using the Basic Local Alignment Search Tool-nucleotide (BLASTn) through the National Center for Biotechnology Information server. Phylogenetic trees were constructed with the neighbor-joining method using MEGA 4.0 (Tamura et al., 2007). Bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of the tree topologies.

Statistical Analysis
Statistical analyses were carried out with SPSS (version 12.0, SPSS Inc., Chicago, IL, USA) and Origin 8.0 (Origin Lab Corporation, Northampton, MA, USA).

RESULTS AND DISCUSSION
Characterization of Microbial Communities via Sanger Sequencing
A total of 866 bacterial clones and 618 fungal clones were generated across all samples using the Sanger sequencing technique (Tables S1 and S2). Of the 866 clones, 184 unique OTUs were observed (defined as sequences with ≥ 97% sequence similarity). The high level of community coverage (71–94%) indicated that the PCR clone library identified the dominant bacterial communities (Table S1). As shown in Fig. 1, the microbial community structures at the genus level were different among rainwater samples, and the most abundant species were in the genus Burkholderia (21% of total Sanger clones sets), followed by those of the genera Massilia (17%) and Methylobacterium (13%). By contrast, Pseudomonas, the bacterial genus with the highest number of INA species, only accounted for 2% of the bacterial clones, and neither the genus Erwinia nor Xanthomonas, which are also known as IN bacteria, was found in any of the rainwater samples. Pseudomonas appeared in only two samples (MTWL-2013-06 and MTWL-2013-09), accounting for 21.4% and 1.6% of each of the sample clones, respectively. The total number of bacteria in the rainwater was roughly estimated to be ~$10^3$ to $~10^5$ cells mL$^{-1}$, according to previous studies conducted worldwide (Herlihy et al., 1987; Casareto et al., 1996; Sattler et al., 2001; Amato et al., 2005; Amato et al., 2007a).

The fungal Sanger data set consisted of 618 clones generated from all samples. Of the 618 fungal clones, 165 OTUs were identified at the 97% sequence similarity level, and the community coverage (45–95%) indicated that the 618 clones captured a major fraction of the fungal community (Table S2). The majority of the sequences were identified as Taphrina and Cryptococcus, representing 21% and 20% of the fungal clones in the rainwater, respectively. The genus Fusarium, which is known as biological IN (Pouleur et al., 1992; Richard et al., 1996), was not found in any of our rain samples. Fig. 2 provides further details on the composition of the fungal communities across individual samples.

Determination of INA with Drop-Freezing Assays
Once the first ice crystal is initiated by IN in the clouds (at temperatures between 0 and −38°C), the number of ice particles can rapidly grow by the Hallett–Mossop process in the temperature range of −3°C to −8°C, and some small

Fig. 1. Composition of the bacteria communities as determined using Sanger sequencing.
ice crystals can become enlarged through the Wegener–Bergeron–Findeisen process to consequently stimulate precipitation formation (Hoose et al., 2010b; Morris et al., 2014). These precipitation events in turn lead to scavenging processes, including nucleation scavenging and in-cloud and below-cloud impaction scavenging, allowing for some particles that are present in air, whether interstitial aerosol or not, to be collected through a wet deposition pathway. Nine sequences derived from clones of bacteria collected from rainwater samples at the Wuling mountain sites showed high similarity with *Pseudomonas*-like bacteria species. This suggested that some bacteria from the rainwater samples potentially possess INA. In this study, the contribution of bacterial ice-nucleating particles to the total IN population in the atmosphere was extrapolated based on the contribution in the rainwater (Vali, 1971; Christner et al., 2008a, b).

Table 2 shows the median freezing temperatures ($T_{50}$) of nine rain samples taken at three mountain sites from August 2011 to August 2013 in eastern China. The $T_{50}$ at each site was: $–15.8°C$ (MTWL-2011-08), $–16.2°C$ (MTWL-2012-07), $–18.6°C$ (MTWL-2012-09), $–14.6°C$ (MTWL-2013-06), $–15.2°C$ (MTWL-2013-07), $–18.0°C$ (MTWL-2013-08), $–18.4°C$ (MTWL-2013-09), $–11.2°C$ (MTCB-2012-07), and $–15.4°C$ (MTDH-2013-08). Feng et al. (2002) reported that the $T_{50}$ values of rainwater determined with a drop-freezing assay (200 droplets of 10 µL each) were $–18.2°C$, $–18.7°C$, and $–11.2°C$ collected in March 1996, July 1996, and April 1997, respectively. Fig. 3 shows the cumulative IN concentration spectra in the nine crude rainwater samples. The onset freezing temperature was between $–4.2°C$ and $–10.2°C$ due to the presence of 1–40 IN mL$–1$ (Fig. 3, Table 3), and most of the samples nucleated at temperatures below $–10°C$, which is commonly used as a cutoff to define ice nucleators of biological origin (Christner et al., 2008a; Bowers et al., 2009); the peaks of freezing temperatures centered around $–13°C$, $–16°C$, and $–18°C$. At $–10°C$, the total IN concentrations of rainwater ranged from 1 to 50 IN mL$–1$ (Table 3), which was lower than that reported by Joly et al. (2014) at $–8–150$ IN mL$–1$, using a similar method.

Bacterial IN appear to be very efficient immersion-freezing nuclei owing to the outer membrane proteins or proteinaceous compounds (Christner et al., 2008b). Indeed, the initial freezing temperature of *PS* (10$^8$ cells mL$–1$) was around $–2.5°C$, which is similar to the previously reported value (Du et al., 2015). Since biological IN would be inactivated by heat treatment, whereas IN of non-biological origin would not. Accordingly, the reduction in IN concentration following heat treatment could be used to represent the total pool of bacterial IN in a sample. Previous studies have found that ice nucleation could be deactivated by heat treatment (95°C for 10 min) by an average of 69–100% in precipitation samples collected from locations worldwide (Christner et al., 2008a, b). In our study, heat treatment led to complete ice nucleation elimination at temperatures above $–10°C$ in the ice-nucleating bacteria *PS* (10$^8$ cells mL$–1$) (Fig. 3). A significant difference of IN concentration was observed at the temperature range of $–10$ to $–15°C$ (4–133 IN mL$–1$) and temperatures higher than $–10°C$ (0–51 IN mL$–1$) (Table 4). Therefore, we next examined the effect of heat treatment, which could disrupt the structure of proteinaceous IN, on the median freezing temperature and cumulative nucleus concentrations. Compared to crude rainwater, heat treatment eliminated IN concentration by 50–100% at temperatures above $–10°C$ and by 1–80%
Table 2. The median freezing temperature ($T_{50}$) of rainwater samples collected at Changbai Mountain, Wuling Mountain, and Dinghu Mountain in eastern China under different treatments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Crude</th>
<th>Crude*</th>
<th>Particle suspensions</th>
<th>Particle suspensions*</th>
<th>Filtrate</th>
<th>Filtrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTWL-2011-08</td>
<td>–15.8</td>
<td></td>
<td>–16.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTWL-2012-07</td>
<td>–16.2</td>
<td></td>
<td>–18.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTWL-2012-09</td>
<td>–18.6</td>
<td></td>
<td>–20.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTWL-2013-06</td>
<td>–14.6</td>
<td>–17.0</td>
<td>–8.2</td>
<td>–14.0</td>
<td>–18.4</td>
<td>–18.9</td>
</tr>
<tr>
<td>MTWL-2013-07</td>
<td>–15.2</td>
<td>–17.6</td>
<td>–9.4</td>
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<td>–17.8</td>
</tr>
<tr>
<td>MTWL-2013-08</td>
<td>–18.0</td>
<td>–19.0</td>
<td>–18.6</td>
<td>–19.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTWL-2013-09</td>
<td>–18.4</td>
<td>–18.8</td>
<td>–18.4</td>
<td>–19.2</td>
<td></td>
<td></td>
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<td></td>
<td>–16.6</td>
<td></td>
<td></td>
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<tr>
<td>MTDH-2013-08</td>
<td>–15.4</td>
<td>–17.2</td>
<td>–13.2</td>
<td>–19.4</td>
<td>–19.6</td>
<td></td>
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<tr>
<td>PS</td>
<td>–4.0</td>
<td>–15.8</td>
<td>–20.2</td>
<td>–20.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes: The superscript * denotes each sample exposed to heat treatment.

Fig. 3. Cumulative ice nuclei spectrum of rainwater samples collected from Changbai Mountain, Wuling Mountain and Dinghu Mountain under different treatments. The superscript * refers to each sample exposed to heat treatment. The lowest IN concentration was 1 IN mL$^{-1}$ detected at –2.5°C in P-suspension.

at temperatures between –10°C and –15°C (Fig. 3, Table 4). This result represents an overall decrease in $T_{50}$ of around 0.4–2.4°C in all rainwater samples collected in 2013 (Table 2). The only materials known to nucleate ice above –15°C are biological, whereas soot and mineral dusts predominantly nucleate ice below –15°C (Schaller and Fukuta, 1979; Bowers et al., 2009; Murray et al., 2012). Christner et al. (2008a) noted that other naturally occurring particles in the atmosphere did not show any noticeable change in activity under heat treatment. In our study, heating for 10 min at 100°C induced a high proportion (1–100%) of IN elimination at temperatures higher than –15°C, and the onset freezing temperature decreased by 0.2–4°C (Table 4), whereas the heat treatment led to a decrease of around 0.4–2.4°C in $T_{50}$. Thus, we could conclude that the rainwater sample at the Wuling Mountain site may contain bacterial IN that play a minor role in ice nucleation.

Certain INA bacteria are commonly found on plants and are typically rod-shaped with an approximate size of 1–3 µm in length and 0.3–0.5 µm in width (Morris et al., 2004). The filter used in this study could eliminate intact cells with sizes > 0.22 µm. As a control experiment, filtration led to the complete elimination of the ice-nucleating bacterium PS ($10^8$ cells mL$^{-1}$) (Fig. 3). On the other hand, filtration completely decreased ice nucleation at temperatures above –10°C, and filtration caused a 24–92% decrease in the
Table 3. Total ice nuclei (IN) concentrations and decreased proportions of crude rainwater under filtration treatment at –5°C, –10°C, and –15°C.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Onset freezing temperature (°C)</th>
<th>Onset freezing temperature after filtration treatment (°C)</th>
<th>Decrease of onset freezing temperature by filtration treatment (°C)</th>
<th>IN mL⁻¹ [total (% filtration-sensitive)]</th>
<th>Temperature (°C)</th>
<th>–5</th>
<th>–10</th>
<th>–15</th>
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<tr>
<td>MTWL-2011-08</td>
<td>–5.4</td>
<td>–10.2</td>
<td>4.8</td>
<td>0(-)</td>
<td>40(100)</td>
<td>407(92)</td>
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<td>MTWL-2012-07</td>
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<td>–10.2</td>
<td>2.8</td>
<td>0(-)</td>
<td>1(100)</td>
<td>25(50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTWL-2012-09</td>
<td>–8.6</td>
<td>–10.4</td>
<td>1.8</td>
<td>0(-)</td>
<td>1(100)</td>
<td>5(33)</td>
<td></td>
<td></td>
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<tr>
<td>MTWL-2013-06</td>
<td>–4.8</td>
<td>–10.8</td>
<td>6</td>
<td>1(1)</td>
<td>22(100)</td>
<td>92(86)</td>
<td></td>
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<tr>
<td>MTWL-2013-07</td>
<td>–4.2</td>
<td>–10</td>
<td>5.8</td>
<td>2(-)</td>
<td>5(99)</td>
<td>133(88)</td>
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<tr>
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<td>–10</td>
<td>–10.2</td>
<td>0.2</td>
<td>0(-)</td>
<td>0(-)</td>
<td>4(76)</td>
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<td>MTWL-2013-09</td>
<td>–10.2</td>
<td>–11</td>
<td>0.2</td>
<td>0(-)</td>
<td>0(-)</td>
<td>6(24)</td>
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<td>MTCB-2012-07</td>
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<td>4.8</td>
<td>0(-)</td>
<td>40(100)</td>
<td>407(92)</td>
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<td>MTDLH-2013-08</td>
<td>–9.2</td>
<td>–10.2</td>
<td>1</td>
<td>0(-)</td>
<td>1(100)</td>
<td>61(89)</td>
<td></td>
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<tr>
<td>PS</td>
<td>–3</td>
<td>–15.6</td>
<td>12.6</td>
<td>320(100)</td>
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Table 4. Total ice nuclei (IN) concentration and decreased proportion of IN of solutions (crude rainwater, filtrate and particle suspensions) under heat treatment at –5°C, –10°C, and –15°C.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Onset freezing temperature (°C)</th>
<th>Onset freezing temperature after heat treatment (°C)</th>
<th>Decrease of onset freezing temperature by heat treatment (°C)</th>
<th>IN mL⁻¹ [total (% heat-sensitive)]</th>
<th>Temperature (°C)</th>
<th>–5</th>
<th>–10</th>
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<tbody>
<tr>
<td>crude rainwater</td>
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<tr>
<td>MTWL-2013-06</td>
<td>–4.8</td>
<td>–8.8</td>
<td>4</td>
<td>1(-)</td>
<td>22(96)</td>
<td>92(70)</td>
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<td>MTWL-2013-07</td>
<td>–4.2</td>
<td>–11.2</td>
<td>7</td>
<td>2(-)</td>
<td>51(100)</td>
<td>133(80)</td>
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<tr>
<td>MTWL-2013-08</td>
<td>–10</td>
<td>–10.2</td>
<td>0.2</td>
<td>0(-)</td>
<td>0(-)</td>
<td>4(60)</td>
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<tr>
<td>MTWL-2013-09</td>
<td>–10.2</td>
<td>–11</td>
<td>0.2</td>
<td>0(-)</td>
<td>0(-)</td>
<td>6(1)</td>
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<tr>
<td>MTDLH-2013-08</td>
<td>–9.2</td>
<td>–9.4</td>
<td>0.2</td>
<td>0(-)</td>
<td>1(50)</td>
<td>61(54)</td>
<td></td>
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<td>filtrate</td>
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</tr>
<tr>
<td>MTWL-2013-06</td>
<td>–10.8</td>
<td>–11.0</td>
<td>0.2</td>
<td>0(-)</td>
<td>0(-)</td>
<td>12(58)</td>
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<td>MTWL-2013-07</td>
<td>–11.2</td>
<td>–11.8</td>
<td>0.6</td>
<td>0(-)</td>
<td>0(-)</td>
<td>16(31)</td>
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<td>MTWL-2013-08</td>
<td>–11.8</td>
<td>–12</td>
<td>0.2</td>
<td>0(-)</td>
<td>0(-)</td>
<td>10(87)</td>
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<tr>
<td>MTWL-2013-09</td>
<td>–11</td>
<td>–11.8</td>
<td>0.8</td>
<td>0(-)</td>
<td>0(-)</td>
<td>4(40)</td>
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<td>MTDLH-2013-08</td>
<td>–10.2</td>
<td>–10.8</td>
<td>0.6</td>
<td>0(-)</td>
<td>0(-)</td>
<td>7(1)</td>
<td></td>
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<tr>
<td>particle suspensions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MTWL-2013-06</td>
<td>–4.2</td>
<td>–7</td>
<td>2.8</td>
<td>1(-)</td>
<td>338(99)</td>
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<tr>
<td>MTWL-2013-07</td>
<td>–4.0</td>
<td>–7.4</td>
<td>3.4</td>
<td>2(100)</td>
<td>124(100)</td>
<td>545(91)</td>
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<tr>
<td>MTWL-2013-09</td>
<td>–6.6</td>
<td>–7.4</td>
<td>0.8</td>
<td>0(-)</td>
<td>15(92)</td>
<td>116(84)</td>
<td></td>
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</tr>
<tr>
<td>MTDLH-2013-08</td>
<td>–6</td>
<td>–8.6</td>
<td>2.6</td>
<td>0(-)</td>
<td>4(50)</td>
<td>179(19)</td>
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abundance of IN between –10°C and –15°C (Table 3). As a result of filtration treatment, the T₅₀ of the filtrate was between –16.0°C and –20.8°C, and decreased by around 1.0–5.2°C compared with that of crude rainwater (Table 2). The onset freezing temperature decreased by about 0.2–5.8°C, which was higher than the temperature difference caused by heating (0.2–4°C), and no freeze events occurred at temperatures higher than –10°C (Tables 3 and 4). This was likely due to the fact that some non-biological particles such as mineral dust might carry biogenic and small biological particles, e.g., fungi and pollen (Després et al., 2012). This further confirmed that bacterial IN in the rainwater samples had little effect on ice nucleation. Compared to filtration, heating treatment caused a 1–87% decrease in the abundance of IN between –10°C and –15°C, and decreased the onset freezing temperature by 0.2–0.6°C and T₅₀ by around 0.2–0.8°C (Table 4), which suggested that some small biological particles (< 0.22 μm) were likely present in the filtrate but also had little effect on ice nucleation.

The particle suspensions of rain samples collected in June and July at the Wuling Mountain site showed concentrations that were orders of magnitude larger than those of other samples above their freezing temperature ranges (Fig. 4). For example, the concentration of INA at ≥ –10°C in suspensions collected in June (338 IN mL⁻¹) was 22-times higher than that of suspensions collected in September (15 IN mL⁻¹) (Table 4). The IN concentrations of particle suspensions from MTWL-2013-09 were 15 and 116 IN mL⁻¹ at –10°C and –15°C, respectively, which was in agreement with that of snow samples analyzed with similar methods by
Fig. 4. Cumulative ice nuclei spectrum of particle suspension collected from Wuling Mountain and Dinghu Mountain. The superscript * refers to each sample exposed to heat treatment.

Mortazavi et al. (2008) (10 and 100 IN mL$^{-1}$, respectively). The $T_{50}$ values of the particle suspensions of rain samples collected at the Wuling Mountain site were $-8.2^\circ$C (MTWL-2013-06), $-9.4^\circ$C (MTWL-2013-07), and $-14.0^\circ$C (MTWL-2013-09), respectively. The $T_{50}$ value of the particle suspension of rain samples collected at the Dinghu Mountain site was $-13.2^\circ$C. Moreover, these four particle suspensions contained high concentrations of IN that were active at warmer temperatures, especially the sample collected in June at Wuling Mountain. Indeed, the spectrum in Fig. 4 shows that all of the aliquots of the particles concentrated from the rainwater samples collected in June at Wuling Mountain were almost completely frozen at $-10^\circ$C. The percentages of IN at temperatures warmer than $-15^\circ$C in samples MTWL-2013-06, MTWL-2013-07, and MTWL-2013-09 were greatly reduced after heat treatment, by 99%, 100%, and 92%, respectively. Their $T_{50}$ values also significantly decreased by $\sim3.6-6.4^\circ$C; however, in the MTDH-2013-08 particle suspension, the $T_{50}$ decreased only slightly, by $\sim0.6^\circ$C. The percentages of IN in samples MTDH-2013-08 were greatly reduced by 50% after heating. The difference in the $T_{50}$ in the two particle suspensions (MTWL-2013-06 and MTWL-2013-09) might be ascribed to the $Pseudomonas$-like bacteria species that were identified in rainwater samples collected in the Wuling Mountain sites. The onset freezing temperature of the particle suspensions was $-6.6^\circ$C or warmer (Fig. 4, Table 4). Yankofsky et al. (1981) categorized INA cells into three main types, types I, II, and III, with freezing temperature ranges of $-2^\circ$C to $-4^\circ$C, $-5^\circ$C to $-7^\circ$C, and $-8^\circ$C to $-10^\circ$C, respectively. However, heating invariably decreased the onset freezing temperature by 0.8 to 3.4$^\circ$C. Therefore, the difference in the cumulative ice nuclei concentration and $T_{50}$ of the particle suspensions could indicate the presence of bacterial IN. However, these bacterial IN did not seem to show the INA expected.

**Phylogenetic Analyses**

Sequences (201306a, 201306b, 201306c, 201306d, 201306e, 201306f, 201306g, 201306h, and 201309) identified as belonging to the genus $Pseudomonas$, some of which have previously been reported to be ice nucleators, were only present in two rain samples (MTWL-2013-06 and MTWL-2013-09). Furthermore, a close phylogenetic relationship among the 16S rDNA gene sequences of the identified $Pseudomonas$ strains and many of the verified ice-nucleating $Pseudomonas$ species was confirmed, including $P. syringae$ (Amato et al., 2007b; Vaïtilingom et al., 2012), $P. fluorescens$ (Vaïtilingom et al., 2012), $P. graminis$ (Amato et al., 2007b; Vaïtilingom et al., 2012), and other $Pseudomonas$ spp. (Fig. 5). The sequence 201306g fell into the branch closely related to $P. graminis$ 38b-9 (accession number JF706541.1), $P. graminis$ 32b-66 (HQ256863.1), $P. graminis$ 32b-60 (HQ256858.1), $P. graminis$ 32b-55 (HQ256853.1), and $P. graminis$ 13b-3 (DQ512786.1). The sequence 201306c was closely related to $P. viridiflava$ 14b-14 (DQ512797.1) with 97% identity. Sequence 201309
showed 97% identity with four *Pseudomonas* spp. Overall, the 16S rDNA-based phylogeny showed that sequences 201306b, 201306d, and 201306h formed a new branch. Sequences 201306e, 201306a, and 201306f fell into a branch closely related to *P. reactans* 47b-7 (JN176591.1). *Pseudomonas*-like bacteria species occurred mainly in rainwater samples collected from Wuling Mountain, where the annual precipitation is relatively lower than at other sites. By contrast, no *Pseudomonas*-like bacteria were detected in Changbai Mountain samples, where the annual precipitation is high, and the population of *Pseudomonas*-like bacteria was also scarce in rainwater samples collected in Dinghu Mountain. This difference can be attributed to the different sampling frequencies at different sites, and that *Pseudomonas* is not generally present in precipitation-heavy months. The mean rainfall in August at the Dinghu
Mountain site and in July at the Changbai Mountain site accounted for 37% and 38% of the annual rainfall, respectively, whereas the proportion of total rainfall in July and August at the Wuling Mountain site was 54%.

Sequences identified as *Pseudomonas* were only present in MTWL-2013-06 and MTWL-2013-09, accounting for 21.4% and 1.6% of the sample clones in each month, respectively. However, the total clones identified by Sanger sequencing in these two months represented merely 5% of the total Sanger clone sets of rainwater samples in Wuling Mountain in 2013. Moreover, it is well known that most strains of the *Pseudomonas* genus do not have INA. Even among the ice nucleation-active strains such as *P. syringae*, the fraction of ice-nucleating cells varies significantly (Hirano and Upper, 1995; Després et al., 2012). Ahern et al. (2007) found that isolates from cloud and rainwater were characterized as *Pseudomonas* but did not cause ice nucleation. This could be one reason that some sequences were closely related to verified ice-nucleating *Pseudomonas* species but did not show the expected efficient INA. However, due to the limitation of our study design and methods, another reason can’t be ruled out that concentration of *Pseudomonas* was relatively low. Further studies comprehensively evaluating the concentration of bacteria in this context are needed.

Considering the results of the drop-freezing testing and phylogenetic analyses together, our results suggest that bacterial IN are present in rain samples in the mountain sites, although their contribution to ice nucleation is very small. This conclusion is in line with a previous report by Bowers et al. (2009), in which the changes in the number of INA at −10°C were not associated with changes in the relative abundances of the most commonly studied ice-nucleating bacteria. Diehl and Wurzler (2010) employed an adiabatic air parcel model to investigate the effect of bacteria acting as immersion IN, and noted that bacterial IN would have to be enriched by at least $10^4$ times the reported concentrations in cloud and snow samples (Amato et al., 2005; Christner et al., 2008a) in order to have an impact equivalent to mineral dust IN.

CONCLUSIONS

In the atmosphere, PBAPs have been shown to play important roles in atmospheric chemistry and physics, but their role in ice formation remains poorly understood. Both non-biological and biological particles could accumulate by scrubbing, including in-cloud and below-cloud scavenging, as precipitation droplets fall. Therefore, biological IN in the cloud water or some PBAPs in the atmosphere could ultimately become deposited in rainwater. Owing to the limitations of the present study, we only investigated the diversity and role of bacterial IN. The roles of other biological components of IN were not estimated, such as pollen. Nevertheless, our results identified the bacteria genus *Pseudomonas* (2% of the total Sanger clones sets) in samples from rainwater collected over nine months from diverse mountain sites, whereas the genera *Erwinia* and *Xanthomonas*, which are known as bacterial IN, and the genus *Fusarium*, which is a known fungal IN, were not detected in the present study. The effects of different treatments (heat and filtration) on the median freezing temperature ($T_{05}$) and cumulative IN concentration by drop-freezing assays suggested that bacterial IN were present in rain samples in the mountain sites but play a minor role in ice nucleation. These results suggest that the bacterial IN are dispersed in the atmosphere and in clouds, but their overall contribution to heterogeneous ice nucleation in mixed-phase clouds may not be significant at the local regional scale.

All currently known INA microorganisms are now amenable to culture-dependent methods, and several other INA microbes likely remain undiscovered given that the number of cultivated microbes identified does not reflect the actual diversity of microbial communities. On the other hand, research on ice nucleation in the atmosphere, conducted to date, has been based on cloud chamber-like experiments and individual-drop isolation techniques. For example, the droplet freezing assay deviates from the actual natural process because the droplet size is significantly larger than the actual size of a cloud droplet, and because the single-freezing mode (immersion freezing) was employed, which can result in inaccurate calculation of the cumulative nucleus concentration (Mortazavi et al., 2008; Knopf and Alpert, 2013). Nonetheless, the drop-freezing assay is widely adopted in many studies because it can provide a relatively direct method for detection of a nucleation event (e.g., Feng et al., 2002; Christner et al., 2008a, b; Du and Ariya, 2008; Attard et al., 2012; Morris et al., 2013; Du et al., 2015). It is difficult to reproduce atmospheric conditions in the laboratory for evaluating the importance of bioaerosols in the atmospheric process resulting in precipitation. Because these microorganisms are not geographically restricted, and meteorological conditions influence biological ice nucleation, more information about the concentration and physical chemistry of bioaerosols is required, which should be combined with atmospheric modeling approaches to best evaluate their common climatic importance. Therefore, research in this field needs to be further developed in order to accurately determine the effects of biological IN on cloud droplets and their contribution to the formation of precipitation.

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SUPPLEMENTARY MATERIALS

Supplementary data associated with this article can be found in the online version at http://www.aaqr.org.

REFERENCES


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