Geographic, seasonal, and precipitation chemistry influence on the abundance and activity of biological ice nucleators in rain and snow

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Biological ice nucleators (IN) function as catalysts for freezing at relatively warm temperatures (warmer than −10 °C). We examined the concentration (per volume of liquid) and nature of IN in precipitation collected from Montana and Louisiana, the Alps and Pyrenees (France), Ross Island (Antarctica), and Yukon (Canada). The temperature of detectable ice-nucleating activity for more than half of the samples was ≈ −5 °C based on immersion freezing testing. Digestion of the samples with lysozyme (i.e., to hydrolyze bacterial cell walls) led to reductions in the frequency of freezing (0–100%); heat treatment greatly reduced (95% average) or completely eliminated ice nucleation at the measured conditions in every sample. These behaviors were consistent with the activity being bacterial and/or proteinaceous in origin. Statistical analysis revealed seasonal similarities between warm-temperature ice-nucleating activities in snow samples collected over 7 months in Montana. Multiple regression was used to construct models with biogeochemical data [major ions, total organic carbon (TOC), particle, and cell concentration] that were accurate in predicting the concentration of microbial cells and biological IN in precipitation based on the concentration of TOC, Ca2+, and NH4+, or TOC, cells, Ca2+, NH4+, K+, PO43−, SO42−, Cl−, and HCO3−. Our results indicate that biological IN are ubiquitous in precipitation and that for some geographic locations the activity and concentration of these particles is related to the season and precipitation chemistry. Thus, our research suggests that biological IN are widespread in the atmosphere and may affect meteorological processes that lead to precipitation.

A t subzero temperatures warmer than −40 °C, aerosol particles in clouds initiate freezing through the heterogeneous nucleation of ice directly from water vapor or by freezing droplets via several mechanisms: deposition, condensation, contact, and immersion freezing (1). These processes lead to ice formation in clouds that can trigger precipitation. A diverse range of natural and anthropogenic particles, referred to as ice-forming nuclei or ice nucleators (IN), are capable of initiating the ice phase (2). The maximum temperature at which an IN can initiate freezing is specific to that nucleator, but they function similarly by providing templates for the aggregation of individual water molecules in the configuration of an ice embryo, resulting in a subsequent phase change and the cascade of crystal formation (3). Consequently, knowledge of the nature and sources of IN in the atmosphere is important for understanding the meteorological processes responsible for precipitation. The most active naturally occurring IN are biological in origin and have the capacity to catalyze freezing at temperatures near −2 °C (4). The most widespread and well-studied biological aerosols with ice-nucleating activity are comprised of certain species of plant-associated bacteria (Pseudomonas syringae, Pseudomonas viridiflava, Pseudomonas fluorescens, Pandoea agglomerans, and Xanthomonas campestris), but also fungi (e.g., Fusarium avenaceum), algae such as Chlorella minutissima, and birch pollen (5). P. syringae (6–8) and F. avenaceum (7) in particular have been detected in atmospheric aerosols and clouds. Ice-nucleating strains of P. syringae possess a 120- to 180-kDa ice nucleation active protein in their outer membrane comprised of contiguous repeats of a consensus octapeptide; the protein binds water molecules in an ordered arrangement, providing a nucleating template that enhances ice crystal formation (9).

Based on reports of ice-nucleating bacteria at altitudes of several kilometers (6, 10) and the warm temperatures at which they function as ice nuclei (−2 °C to −7 °C; ref. 5), biological IN may play a role in the precipitation cycle. Very large numbers of microorganisms inhabit leaf surfaces globally (1024 to 1026 cells; ref. 6), deciduous plants harbor large populations of ice-nucleating bacteria on their leaves (∼105 ice-nucleating bacteria cm−2; ref. 12), and various species of ice-nucleating bacteria or biological IN in general have been detected in phytoplankton-rich marine waters (13), snow (14, 15), and rain (15, 16). Although biological particles represent a significant component of atmospheric aerosols (17), few data have been available on the concentration of airborne biological ice nuclei. Therefore, the abundance and distribution of the most active IN in the atmosphere (i.e., biological IN) are described in only a very limited way and their meteorological role is unknown.

Our previous work on snowfall collected from a variety of mid- and high-latitude locations indicated that biological IN are ubiquitous in precipitation from a range of global locations and represent the most active IN at temperatures warmer than −10 °C (14). Here, we present results from a comprehensive analysis of biological IN in snow and rain and use statistical methods to examine correlations between the microbiological and biogeochemical data. A primary objective of this study was to elucidate biogeochemical markers of the presence of biological IN to provide information on conditions that favor their distribution in the atmosphere and predict other contexts where they would be abundant. We show that for some geographical locations the concentration (i.e., per rain or snow water equivalent volume) and activity of biological IN is correlated with the biogeochemistry of the precipitation and season of deposition.

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The cumulative ice nuclei spectra for particles in snow and rain. A) The cumulative spectra of ice nuclei in snow (4 ice nuclei L⁻¹) and in rain (8–230 ice nuclei L⁻¹) were reconstituted in deionized water at temperatures between −5 °C and −9 °C. There were clearly 2 outlying samples (August 17 and October 4) that contained significantly higher ice nucleus concentrations at −9 °C (480 and 510 ice nuclei L⁻¹, respectively) than the other rainfalls analyzed. However, the highest measurable ice nucleus concentration was observed at −11 °C (660 ice nuclei L⁻¹) from a sample collected on August 28 in which the ice nuclei concentration increased nearly 10-fold between −10 °C and −11 °C (Fig. 1B). When the cumulative ice nucleus concentration at temperatures warmer than −7 °C in snow from various locations (Fig. 1A) is compared with that in rain from Louisiana (Fig. 1B), the range of ice nuclei concentrations observed was similar (1–150 ice nuclei L⁻¹ in snow; 8–230 ice nuclei L⁻¹ in rain).

Biological Ice Nuclei in Precipitation. Most known biological ice nuclei are efficient at catalyzing freezing because of the presence of proteinaceous IN (5). Therefore, the concentration of ice nuclei that were sensitive to heat (i.e., to disrupt the structure of proteinaceous IN) was inferred to represent the total pool of biological ice nuclei in a sample (hereafter referred to as the total biological ice nuclei fraction). Furthermore, few other naturally occurring particles in the atmosphere have nucleating temperatures as warm as those of biological ice nuclei and would not be expected to have any noticeable change in activity, caused by the heat treatment, at the temperatures of our freezing tests. Although the coefficient of variation was high for data collected at a given geographical location (0.6–0.8), snow samples from France and Montana contained a similar average concentration of biological ice nuclei active at −9 °C (55 and 54 ice nuclei L⁻¹, respectively) compared with higher values for Louisiana rain (110 ice nuclei L⁻¹; calculated excluding data outliers from August 17 and October 4; Fig. 1B) [supporting information (SI) Fig. S1], but these differences were statistically significant only between the Montana and Louisiana samples (P = 0.04).

Heat treatment completely eliminated ice nucleation at temperatures ≥ −9 °C in 69–100% of the snow and rain samples. Lysozyme catalyzes the hydrolysis of peptidoglycan molecules in bacterial cell walls thereby destabilizing their ability to maintain tertiary structure of the IN proteins, which is important for their activity as IN. Lysozyme-sensitive bacteria represented the majority of ice nuclei at ≥ −9 °C in 64%, 50%, and 25% of the samples from France, Montana, and Louisiana, respectively (Fig. 2). Although there was little variation between samples and locations in the fraction of total ice nuclei active at ≥ −9 °C that were sensitive to heat (>92%), there were substantial differences in the percentage of ice nuclei that were susceptible to lysozyme (0–100%) and maximum temperature of ice-nucleating activity (−4 °C to −7 °C) (Fig. 2 and Fig. S1).
average proportion of heat-sensitive ice nuclei that were inactivated by lysozyme was slightly higher in samples from France (63%) compared with those from Montana (51%) and Louisiana (36%). At temperatures between $-10^\circ C$ and $-12^\circ C$, the average percentage of ice nuclei that were heat-sensitive (63–83%) and lysozyme-sensitive (24–30%) in Louisiana rain generally decreased with temperature (Fig. 2B). Despite an overall decreasing trend with temperature, the highest concentrations of biological (630 ice nuclei L$^{-1}$ at $-11^\circ C$, 95% of the total; August 28) and bacterial (290 ice nuclei L$^{-1}$ at $-12^\circ C$, 73% of the total; September 12) ice nuclei were observed at temperatures below $-10^\circ C$ in Louisiana rain samples (Fig. 2B and Fig. S1B). Two fresh snow samples were analyzed from Antarctica and Yukon, and all of the ice nuclei active at $\geq -9^\circ C$ were susceptible to the heat treatment, whereas 0–100% and 35–56%, respectively, of these ice nuclei were destroyed by lysozyme (data not shown). When the precipitation data from the various global locations are compiled, the average percent of ice nuclei active at $\geq -9^\circ C$ that were inactivated by lysozyme and the heat treatment was 42% and 95%, respectively.

There were certain precipitation events in the Montana (BB, October 4, December 29, and February 24; YC, February 24; and BS, April 6), France (VdL, December 29; SAT, January 26; LC, March 10), and Louisiana (August 28 and September 12) samples in which bacteria dominated the fraction of ice nuclei active at all temperatures warmer than $-9^\circ C$ (52–100% of the total; Fig. 2). Three samples from Montana (BB, November 4 and January 15; YC, December 29) contained 4–110 biological ice nuclei L$^{-1}$ that were active at $\geq -9^\circ C$ (Fig. S1A), but none of these ice nuclei were sensitive to lysozyme. There were 2 dates in the Montana snow record (December 29 and February 24) where samples were collected at different sites 80 km apart (Fig. 2A), and there were significant differences in the concentration of all classes of ice nuclei between sites. In particular, 95% confidence interval estimates of the cumulative concentrations at $7^\circ C$ (BB and YC sites on December 29 are [69.1, 281.8] and [24.5, 59.3], respectively; [72.8, 285.7] and [1.6, 15.8] for February 24, respectively) do not overlap, providing strong evidence that the cumulative concentrations at the two sites were significantly different.

**Cluster Analysis of Montana Snowfall.** To investigate similarities between snow events in terms of the ice nuclei active at $\geq -9^\circ C$, cluster analysis was used to organize the snow events into groups having similar freeze profiles (Fig. 3). The cumulative ice nuclei concentration at a given temperature is a monotone function of the probability of being frozen at that temperature. This finding implies that snow events that are similar with respect to the

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**Fig. 2.** The percentage of ice nuclei at decreasing temperature that were susceptible to heat treatment and lysozyme digestion. (A) Snow from Montana and France. (B) Rain from Louisiana. See Fig. 1 legend for site designations.
cumulative ice nuclei concentration will be similar with respect to their freeze profiles as well, so that assessments of similarity based on these two quantities will be essentially equivalent. This idea was verified empirically by comparing curves generated from the freeze probabilities (data not shown) with those of the estimated cumulative ice nuclei spectra (Fig. 1), which showed that the shapes of the freeze profiles matched those of the ice nuclei content in the corresponding snow samples.

The single-linkage, complete-linkage, average-linkage, and Ward’s method produced dendrograms with slightly different topologies (Fig. 3 and Fig. S2). That these methods have produced somewhat different results is not unusual or unexpected. Because the four methods apply different metrics, they use different aspects of the data in forming clusters, and consequently, can produce different results in general. However, all four methods agree in several ways: (i) the October and November snow events from the BB site appear to be different from the other observations and are grouped separately; (ii) the BB December 29 and February 24 snow events are always clustered and are not similar to samples collected on the same date from the YC site; and (iii) snow events from the BB site were always grouped separately from those collected from sites that are <10 km apart (i.e., the BS, YC, and MB sites).

Statistical Modeling of the Cell and Biological Ice Nuclei Concentrations in Snow. The major ion, total organic carbon (TOC), particle, cell (Table S1), and biological ice nuclei (where applicable) concentration data from the Montana snow record (Fig. S14) were used to construct multiple regression models in SAS. Because of the small sample size (n = 10) and the comparatively large number of explanatory variables (P = 14 or 15), model selection procedures that consider subsets of the explanatory variables had to be used. We used several model selection methods in SAS to identify potential models, and the final models were selected based on their parsimony, overall fit, stability of parameter estimates, adherence to model assumptions such as normality of the errors, and homoscedasticity (constant variance).

There was a strong correlation (R² = 0.8630) between the measured cell concentration and that predicted from a model based on the concentration of TOC, NH₄⁺, and Ca²⁺, and the fit of the model was improved (R² = 0.9655) when site was included as a class variable (Fig. S3). The relative importance of these variables can be determined by comparing P values from the parameter estimates, which indicated that TOC was the most significant (P = 0.0053) and Ca²⁺ (P = 0.0517) was the least significant variable for predicting cell concentration. At −7 °C, −6 °C, and −5 °C, there was a strong correlation (Fig. S3) between the abundance of biological ice nuclei and the concentration of Cl⁻, SO₄²⁻, and TOC, SO₄²⁻, HCO₃⁻, cells, and, NH₄⁺, and NH₃⁻, PO₄³⁻, K⁺, and Ca²⁺, respectively, which are listed as variables of higher to lower significance based on their P value ranking.

Discussion

More than half of the rain and snow samples contained ice nuclei active at ≈ −5 °C, and all were active at temperatures warmer than −10 °C (Fig. 1). Based on their thermal sensitivity (Fig. 2), the vast majority (95%) of ice nuclei active at temperatures warmer than −10 °C are inferred to be proteinaceous, and therefore, biological in origin. There were samples where 100% of the ice nuclei at a given temperature were inactivated by lysozyme, which hydrolyzes the peptidoglycan backbone of bacterial cell walls, but on average, less than half (42%) were susceptible to digestion by this enzyme. If all bacteria in our samples were sensitive to lysozyme, then our results suggest that the majority of biological ice nuclei we analyzed originated from other sources (e.g., plants, fungi, and/or archaea). However, many bacteria are resistant to lysozyme and their susceptibility largely depends on the cells’ physiological state. Hence, the fraction of ice nuclei we report as originating from bacteria should be viewed as a conservative estimate and deciphering the specific nature of these biological particles requires further experimentation.

Plant canopies and decaying vegetation are thought to represent significant terrestrial sources of atmospheric biological ice nuclei (8, 12, 19, 20), and consistent with this idea, the lowest concentrations of biological ice nuclei observed were in snow from Antarctica. The snow samples examined from Montana and France were collected during months when local deciduous plants were defoliated and leaf litter was buried beneath a seasonal snow pack. However, lower elevation plains and pasture lands in France and the western United States are snow-free and have exposed vegetation for much of the winter. Although coniferous vegetation existed near all of the sampling sites in Montana, previous studies have shown that these plant species harbor ice-nucleating bacteria at concentrations that are “very low or below detection” (12). Morris et al. (15) recovered pathogenic ice nucleating strains of P. syringae from 3 of the 7 snow samples we analyzed from France. Although we cannot determine what portion of the P. syringae cells in the snow were ice nucleating active at the time of deposition, these findings suggest that this phytopathogen may represent a significant fraction of the biological ice nuclei in precipitation at some locations and under certain environmental conditions.

Despite substantial differences in local ecosystems, sources of moisture, and air mass trajectory, the concentrations of ice nuclei active at ≈ −7 °C in midlatitude snow (3–150 ice nuclei L⁻¹) and Louisiana rain (8–230 ice nuclei L⁻¹) were remarkably similar (Fig. 1). These values are comparable to the range of ice nuclei concentrations reported in various rain samples from Alberta, Canada (≈20–600 ice nuclei L⁻¹) based visually on data in figures 2 and 3 of ref. 21. The highest ice nuclei concentrations reported by Vali (21) corresponded to “the first period of high intensity rain,” which were significantly higher than those observed for “continuous-type rain.” Because we did not sample temporally, our data represent a running count of all ice nuclei deposited per total volume of material collected for each precipitation event. There were two rain samples (August 17 and October 4) that contained substantially higher concentrations of ice nuclei than all others analyzed (Fig. 1B). These highly active samples were collected during brief showers and are consistent.
with the observations of Vali (21). This result may be caused by elevated concentrations of ice nuclei present in the troposphere where the precipitation formed or could be a consequence of atmospheric washout, whereby the airborne particles were concentrated in the small rain volumes collected during these transitory precipitation events.

Based on the known sources of aerosols in the atmosphere, it was reasonable to assume a priori that seasonal climatic and vegetation differences affect the distribution of biological and chemical species in the atmosphere. Therefore, statistical methods were used to elucidate seasonal relationships in the immersion freezing activity of biological ice nuclei and examine correlations between their concentration and the chemistry of the precipitation. We used cluster analysis to examine 7 months of Montana snow data, which revealed a seasonal pattern between snowfalls with respect to their ice-nucleating activities (Fig. 3). The ice-nucleating activity of snow collected in October and November appeared to have unique characteristics, and snows at each site from December to April were similar. One potential explanation for the seasonal grouping of observations relates to substantial vegetation changes in the northwest United States from fall to winter and winter to early spring. For example, fall harvest of cereal grains and subsequent growth of winter wheat may represent a source of airborne bacteria. Populations of plant epiphytic bacteria in the Pacific Northwest, including P. syringae, vary greatly seasonally, with numbers increasing because of rain and subsequent plant growth in the spring (22).

Factors influencing the concentration of biological particles in clouds and the atmosphere include the ecosystems that contribute the particles, local topography, climatic conditions, season, wet scavenging, and cloud water chemistry (1, 23–27). Multiple regression analysis of the physical, microbiological, and biogeochemical data produced relatively simple models that fit the observed data well. Although the Montana sampling sites were located only ~80 km apart, when site was included as a variable, the fit of the model was substantially improved, which suggests that the airborne biological particle concentration and snow chemistry were not homogeneous between the sites, which is also supported by the significant variation observed in the concentration of all classes of ice nuclei in snow deposited on the same date at the two sites (December 29 and February 24; Fig. 2A and Fig. S1A) and cluster analysis (Fig. 3 and Fig. S2). Not surprisingly, differences in the history of the air masses from which the particles originated, heterogeneity in the chemical species distributed in the atmosphere, and/or local microclimatic factors appear to play an important role in the concentration of the cells coprecipitated with snow.

There was a negative correlation between cell density and the concentration of NH4+ and TOC in Montana snowfall. Microbial activity in natural and agricultural soils is a source of NH3 gas to the atmosphere, where it is subsequently converted to NH4+ (28, 29). Sources of atmospheric TOC include vegetation, soils, photochemical reactions, and the combustion of fossil fuels and biomass (30–32). Recently it has been shown that suitable conditions and nutrient concentrations exist in cloud water to support bacterial reproduction (33, 34). At 0°C, Sattler et al. (33) estimate that bacterial biomass in cloud droplets could increase 20% per day. Although we do not know the physiological status of the microorganisms deposited in the snow or their metabolic capabilities under atmospheric conditions, heterotrophic growth via the assimilation of carbon and nitrogen provides one plausible explanation for our results given the negative regression relationship between both TOC and NH4+ and the cell concentration.

Known biological IN from bacteria, fungi, plants, lichens, and invertebrates catalyze ice formation at temperatures ranging from ~2 to ~7°C (5), and even within a clonal bacterial population, different temperature classes of ice nuclei can simultaneously exist (35). The expression of ice nuclei has been shown to be tightly regulated by the nature of the carbon source, presence of nutrients (N, P, S, and Fe), and temperature (36). At each temperature modeled, nutrients such as NH4+, PO43−, and TOC were identified as statistically significant predictors for the concentration of biological ice nuclei. This observation may be related to the fact that expression of the ice nucleation phenotype depends on certain environmental factors (36). Although some marine environments appear to be sources of atmospheric ice nuclei (13, 37), there was a negative correlation between Cl− and the biological ice nuclei concentration at ~7°C, implying that marine aerosols were not a source of these particles in the Montana snow samples at this temperature and may have been a source of inhibition. There was a negative regression relationship between the concentration of K+ and biological ice nuclei at −5°C, and although there is a weak positive correlation between the concentration of K+ and biological ice nuclei at −5°C, and although there is a weak positive correlation between the concentration of K+ and biological ice nuclei at −5°C, and although there is a weak positive correlation between the concentration of K+ and biological ice nuclei at −5°C, and although there is a weak positive correlation between the concentration of K+ and biological ice nuclei at −5°C, there was a negative correlation between the concentration of Ca2+ and biological ice nuclei at −5°C, and there was a positive correlation between the Ca2+ and the cell and biological ice nuclei concentrations at −5°C. Soils have been shown to contain large concentrations of highly-active ice nuclei (39), and soil-derived particles transported through the atmosphere are likely to harbor assemblages of attached microorganisms (33). Elevated microbial cell concentrations have also been shown to be correlated with high dust content in ice cores (40, 41).

Conclusion

The cumulative concentration of biological ice nuclei active at temperatures warmer than −10°C in precipitation collected from diverse worldwide locations ranged from 4 to 490 ice nuclei L−1 of rain or snow water equivalent. Based on their distribution in the atmosphere, biological ice nuclei are likely to encounter the appropriate conditions to affect atmospheric processes leading to precipitation. The meteorological role of biological ice nuclei could be particularly relevant at cloud temperatures that limit the action of other ice nuclei, but clearly, additional experimentation in atmospheric physics and modeling is required to assess their climatic importance. In addition to the potential role that cells may play in meteorological processes, microbiological studies of the atmosphere have broader implications for understanding the distribution and evolution of microbial life. Unlike animals, microorganisms are not geographically restricted and many species have been shown to occur globally (e.g., ref. 42); however, very little is known about modes of microbial dispersal on a planetary scale. The concept that biologically-catalyzed ice formation in the atmosphere could serve as a microbial dissemination strategy warrants more detailed investigation.

A key point regarding our data is that we infer that 95% of all ice nuclei active at temperatures warmer than −10°C in snow
and rain were biological particles and >40% were confirmed to be bacterial in origin. However, the data available do not allow us to identify the specific nature of the majority of ice-nucleating biological particles that were detected. Statistical analysis revealed significant correlations between select biogeochemical variables and the cell and biological ice nuclei concentration in the precipitation. Based on the known characteristics of these aerosols and similarities between observations from the same season, our results lend support to the idea that terrestrial vegetation and soils are an important source of the biological ice nuclei distributed in the atmosphere. Although it is not possible to accurately back-calculate the abundance of ice nuclei in clouds based on concentrations in precipitation, our results imply that biological ice nuclei are widespread in the troposphere and underscore the need for quantitative data to gauge their influence on climate.

Materials and Methods

The particles >0.2 μm in precipitation samples were collected and reconstituted in deionized water followed by immersion freezing testing and successful treatment with lysozyme (3 mg mL⁻¹; 4 °C for 72 h) and heat (95 °C for 10 min). Profiles of freezing were generated at 1 °C increments between -2 °C and -13 °C for aliquots of this material and used to estimate the total, lysozyme-sensitive, and heat-sensitive cumulative IN concentration per volume of sample (14). Data from the Montana snow samples, together with concomitant measurements of the major ion (Metromax Peak Ion Chromatograph), organic carbon (Dohrmann DC80), cell, and particle (Microcyte flow cytometer) concentrations, were analyzed statistically with SAS (www.sas.com). Cluster and multiple regression analyses were used to examine similarity between freeze characteristics in individual precipitation events and identify the most significant predictors of the biological IN and cell concentration, respectively. Explicit detail on our methodology is provided in SI Text.

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