

# ***Peltigera* lichen thalli produce highly efficient ice nucleating agents**

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## Abstract

From extracellular freezing to cloud glaciation, the crystallization of water is ubiquitous and shapes life as we know it. Efficient biological ice nucleators (INs) are crucial for survival in cold environments and, when aerosolized, serve as a significant source of atmospheric ice nuclei. Several lichen species have been identified as potent INs capable of inducing freezing at high subzero temperatures. Despite their importance, the abundance and diversity of lichen INs are still not well understood. Here, we investigate ice nucleation activity in the cyanolichen-forming genus *Peltigera* from across a range of ecosystems in the Arctic, the Northwestern United States, and Central and South America. We find strong IN activity in all tested *Peltigera* species, with ice nucleation temperatures above -12°C, and 35% of the samples initiating freezing at temperatures at or above -6.2°C. The *Peltigera* INs in aqueous extract appear resistant to freeze-thaw cycles, suggesting that they can survive dispersal through the atmosphere and thereby potentially influence precipitation patterns. An axenic fungal culture termed L01-tf-B03, from the lichen *Peltigera britannica* JNU22, displayed an ice nucleation temperature of -5.6°C at 1 mg mL<sup>-1</sup> and retained remarkably efficient IN-activity at concentrations as low as 0.1 ng mL<sup>-1</sup>. Our analysis suggests that the INs released from this fungus in culture are 1000 times more efficient than the most potent bacterial INs from *Pseudomonas syringae*. The global distribution of *Peltigera* lichens, in combination with the record-setting IN-activity, emphasizes their potential to act as powerful ice nucleating agents in the atmosphere.

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## 1 Introduction

Ice formation below 0°C is thermodynamically favorable, however the crystallization process is constrained by kinetics. As a result, pure water droplets can be supercooled to temperatures as low as -38°C, below which homogeneous ice nucleation takes place (Koop et al., 2000). In natural systems, water typically freezes in a heterogeneous process facilitated by the presence of particles that serve as ice nucleators (INs). INs of biotic origin are often highly efficient and elevate freezing temperatures from -15°C and above (Maki and Willoughby, 1978; Wilson et al., 2003; Murray et al., 2012). Potent INs are abundant across freeze-tolerant organisms, such as bacteria, fungi, plants, and lichens, and play fundamental roles in their survival (Maki et al., 1974; Kieft and Lindow, 1988; Pouleur et al., 1992; Lundheim, 2002; Fröhlich-Nowoisky et al., 2015; Eufemio et al., 2023).

The most active biological INs described to date are the bacterial plant pathogens, *Pseudomonas syringae*, whose ability to facilitate freezing at exceptionally warm sub-zero temperatures originates from ice nucleating proteins (INPs) located in the cell outer membrane (Govindarajan and Lindow, 1988). *P. syringae* INPs assemble into functional aggregates that are categorized into classes A-C based on freezing temperature

and assembly size. Large protein aggregates associated with class A allow the bacteria to achieve IN-activity close to -1°C, while class C consists of comparatively small INPs active at ~ -7.5°C (Kozloff et al., 1983; Govindarajan and Lindow, 1988; Turner et al., 1990, Renzer et al., 2024).

While there is considerable interest in bacterial INPs, relatively little attention has been paid to lichens, despite several species having been identified as powerful INs that enable ice formation as high as -1.9°C (Kieft and Lindow, 1988; Ashworth and Kieft, 1992; Moffett et al., 2015; Eufemio et al., 2023). Lichen INs are sensitive to protein-degrading treatments, such as high temperatures and urea, implying that, like bacteria, they consist of proteinaceous compounds (Kieft and Ruscetti, 1990). In contrast to most bacterial INPs, lichen INs are cell-free and induce freezing without the need to be anchored in a cell membrane (Kieft and Ruscetti, 1990; Moffett et al., 2015), more similar to what has been observed for several fungi (Fröhlich-Nowoisky et al., 2015; Pummer et al., 2015; Kunert et al., 2019), the Gram-positive bacterium *Lysinibacillus parviboronicapiens* (Failor et al., 2017) and bacteria in the Gram-negative genus *Pantoea*, formerly classified as *Erwinia* (Phelps et al., 1986).

Lichens, including their fungal and prokaryotic communities (Arnold et al., 2009; Hodkinson et al., 2012), can tolerate extreme conditions and survive in environments where other vegetation cannot, and therefore dominate nearly 10% of the earth's surface (Honegger, 2007). Ice nucleation active biological particles derived from lichenized fungi have been detected in the atmosphere, where they are proposed to strongly influence cloud glaciation and trigger precipitation (Zachariassen and Kristiansen, 2000; Henderson-Begg et al., 2009; Moffett et al., 2015). Lichens are mutualistic associations between a fungal symbiont (mycobiont) and a photoautotrophic symbiont (photobiont) (Lutzoni and Miadlikowska, 2009). In approximately 85% of lichenized fungi, the photobiont is a green alga, forming a bimembered association. In about 10% of lichens, the photobiont is exclusively cyanobacterial, forming a different bimembered association. Only 3-4% of lichenized fungi are associated with both photobiont partners, forming trimembered lichens (Honegger, 2007; Nash, 2008; Henskens et al., 2012). Early studies on axenic lichen cultures have identified the mycobiont as IN-active, while the photobiont is comparatively inactive (Kieft and Ahmadjian, 1989).

*Peltigera* lichens are particularly efficient INs, with several species initiating freezing at or above -5°C (Eufemio et al., 2023). *Peltigera* has a global distribution and is one of the most widespread lichen genera (Miadlikowska and Lutzoni, 2000; Martinez et al. 2003; Magain et al., 2017; Magain et al., 2023), making their INs highly relevant to biological and atmospheric processes (Creamean et al., 2021; Moffett et al., 2015). Despite the ecological importance and formidable IN-activity of *Peltigera*, the abundance and efficiency of INs across the genus remain unknown.

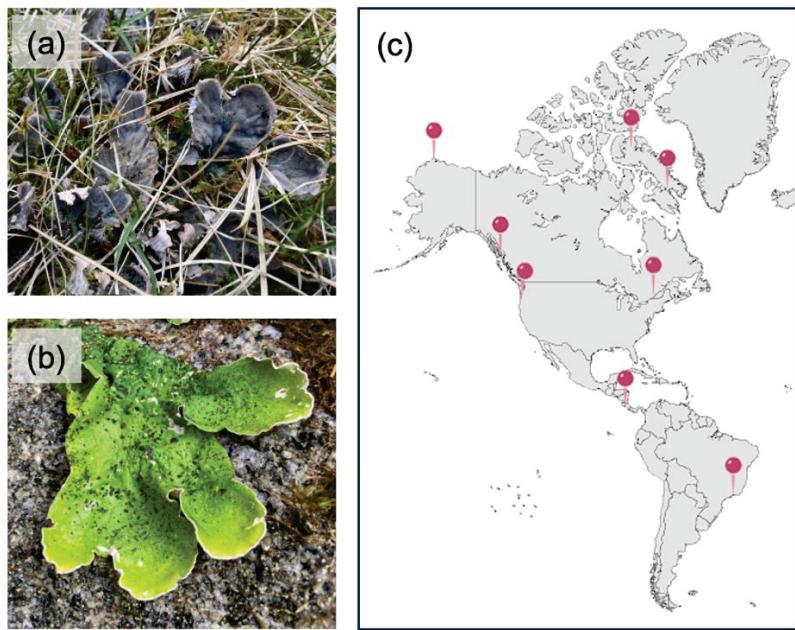
Here, we surveyed *Peltigera* species from across a range of biomes spanning the Arctic, the Northwestern United States, and Central and South America for IN activity. We used a high-throughput twin-

85 plate ice nucleation assay (TINA) to quantify the INs of select thalli and to assess the efficacy of an IN-active culture, L01-tf-B03, isolated from the *Peltigera britannica* JNU22 thallus. We further evaluated the stability of the *Peltigera* INs under freeze-thaw cycles to gain insights into their ability to remain IN-active under environmentally relevant conditions.

90 **2 Materials and methods**

**2.1 Sampling**

Lichen thalli of the genus *Peltigera* were obtained in the north-northwestern United States, Canada, Brazil, 95 and Costa Rica (Fig. 1c) between February 2003 and August 2024. *Peltigera* thalli are often large and conspicuous, and the foliose structures protrude from the growth substrate, making it feasible to identify them in the field (Pojar and MacKinnon, 1994). Eleven bimembered (Fig. 1a) and six trimembered (Fig. 1b) lichens were sampled, representing eight *Peltigera* species (Table 1). Four specimens were collected from rock and tree substrates in temperate rainforests in Alaska and Washington, United States, in 2022. Six samples were 100 obtained from the Arctic tundra: Two from Utqiagvik, Alaska, USA, in 2024 and four from Nunavut, Canada, in 2023. Two were collected from boreal forests of Québec, Canada, in 2011. Three lichens were collected in the Atlantic rainforest ecosystem in Minas Gerais, Brazil, in 2012, and two samples were obtained from the Talamanca Mountain Range of Costa Rica in 2003. These locations encompassed a diverse range of 105 biomes across distinct geographic areas (Fig. 1c). The elevations of the collection sites varied from sea level (e.g., in Alaska) to 3400 meters (e.g., in the Talamanca Mountain Range, Costa Rica). Lichen samples were collected based on bioavailability and the accessibility to the sampling locations. Collected specimens were either stored in sterile containers at -18°C or kept at room temperature in a dry state in paper bags.



110 **Figure 1.** Images of (a) bimembered and (b) trimembered *Peltigera* lichens and (c) a map of the sampling locations of the current study.

## 2.2 Isolation of IN-active cultures from *P. britannica* JNU22 thalli

115 Malt/yeast extract medium (MY medium) with pH 5 (Pichler et al., 2021) was prepared by mixing 22 g L<sup>-1</sup> malt yeast extract (Difco<sup>TM</sup> Malt Extract Broth, Fisher Scientific, Massachusetts, United States) and 20 g L<sup>-1</sup> agar (Merck KGaA, Darmstadt, Germany) in water. The medium was sterilized by autoclaving at 121°C for 20 minutes. *P. britannica* JNU22 cultures were cultivated following a procedure adapted from Yoshimura et al. (2002). The lichen thallus was brought to room temperature from -18°C and washed under pure water for 120 10 minutes to remove as many epiphytic micro-organisms as possible. Two methods were employed to isolate the lichen-associated organisms. In the first method, pieces of thallus approximately 1 mm<sup>2</sup> were cut out using a sterilized scalpel blade and placed on MY medium in Petri dishes labeled L01-tf-A and L01-tf-B. The plates were referred to as the 'thallus fragments' (tf). In the second method, three 1 mm<sup>2</sup> thallus pieces were ground to a pulp in 300 µL pure water. This was referred to as a 'thallus slurry' (ts). The slurry was 125 spread on the surface of MY medium in plates labeled L01-ts-A and L01-ts-B and grown for 14 days at room temperature. Both methods produced sufficient growth to allow for subculturing onto new MY medium plates. Sterile tweezers were used to transfer growth from the center of each plate onto clean MY plates and the sub-culturing process was repeated until fourteen pure cultures were obtained. All procedures were carried out under sterile conditions. The pure cultures were grown at room temperature for approximately 7 130 days, then kept at 4°C for up to 8 weeks. Cultures were visually identified as mycelial fungi, yeasts, and

bacteria. Subsequent measurements of ice nucleation activity were conducted to identify IN-active cultures (described in Sect. 2.3 and 2.4).

## 2.3 Purification of aqueous IN extracts

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The *Peltigera* thalli that were stored in a frozen state were prepared for quantitative analysis by washing the thallus in pure water (Millipore Milli-Q® Simplicity 185 Water Purification system, Merck KGaA, Darmstadt, Germany) to minimize contamination from external sources of ice nucleating particles. To ensure that the hydration state of the thalli were consistent, the lichen specimens that were stored in a dehydrated, 140 dormant state were weighed, rehydrated in pure water for 30 minutes, and reweighed prior to washing. Extracts were prepared for ice nucleation assays using a standardized procedure previously described by Eufemio et al. (2023). In short, 2 g of lichen thallus in 5 mL of pure water were ground to a fine pulp. The pulp was centrifuged at 5000 rpm for 10 min and the supernatants were filtered through 0.22  $\mu\text{m}$  pore diameter syringe filters (Millex® Syringe Filter, Merck KGaA, Darmstadt, Germany). The resulting aqueous 145 extracts contained molecules that were both secreted and bound to the cell wall.

Aqueous extracts obtained from pure cultures isolated from *P. britannica* JNU22 were prepared as described by Kunert et al. (2019) with the following modifications. The cultures were collected from the center of each plate and placed into a sterile Eppendorf tube, and the weight of the collected material was determined. A primary suspension was made by suspending 10 mg of harvested material in 1 mL of pure 150 water. The suspensions were vortexed three times at 2700 rpm for 1 min, then filtered through 0.22  $\mu\text{m}$  pore diameter syringe filters. The resulting extracts contained ice nucleators from those cultures.

## 2.4 Ice nucleation experiments

155 The aqueous extracts of the *Peltigera* thalli and the *P. britannica* JNU22 cultures were tested for ice nucleation activity immediately after purification using a Vali-type set-up (Vali, 1971). 20 droplets (1  $\mu\text{L}$ ) of extract were cooled at  $3^\circ\text{C min}^{-1}$  from  $0^\circ\text{C}$  to  $-20^\circ\text{C}$  on a temperature-controlled aluminum plate (Linkam Scientific Instruments LTD, United Kingdom). The freezing temperature of each droplet was identified based 160 on the optical change in appearance that occurred with freezing. The temperature at which 50% of the droplets froze,  $T_{50}$ , was recorded as a measure of the efficiency of the INs. 1 mg  $\text{mL}^{-1}$  of inactivated *P. syringae* was used as a positive control ( $T_{50}$  of  $-3.5^\circ\text{C}$ ) and water had a  $T_{50}$  of  $-11^\circ\text{C}$ . Aliquots of MY medium were used as negative controls for the ice nucleation assays of the *P. britannica* JNU22 cultures and did not freeze in the investigated temperature interval.

While the Vali-type apparatus was sufficient for initial tests of ice nucleation activity, more robust

165 measurements were needed for quantitative analysis of the extracted INs. High-throughput ice nucleation experiments were performed using TINA, as described by Kunert et al. (2018). The investigated IN extract was serially diluted in 10-fold increments with a liquid-handling station (epMotion ep5073, Eppendorf, Hamburg, Germany). 96 droplets (3  $\mu$ L) per dilution were placed in two 384-well plates, which were cooled at a continuous rate of 1  $^{\circ}$ C min<sup>-1</sup> from 0  $^{\circ}$ C to -30  $^{\circ}$ C. For each experiment, the droplet-freezing temperatures were extracted, and the fraction of frozen droplets at different temperatures was used to calculate the cumulative number of active INs per unit mass of sample ( $N_m$ ) using Vali's equation (Vali, 1971).

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## 2.5 Treatments of aqueous extracts

175 Freeze-thaw cycles were used as a measure of IN stability against temperature fluctuations. Aliquots of aqueous lichen extract were frozen by cooling to -30  $^{\circ}$ C at a rate of 1  $^{\circ}$ C min<sup>-1</sup> and thawed to room temperature up to 6 times over the course of 24 hours. The IN-activity was measured using TINA after each cycle.

## 3 Results

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### 3.1 IN-activity is widespread in *Peltigera* lichens

While several lichen species were previously identified as powerful INs (Kieft and Lindow, 1988; Eufemio et al., 2023), the prevalence of IN-activity within the genus *Peltigera* remains largely unknown. Two assays, 185 a Vali-type droplet freezing assay and TINA, were performed to better evaluate the frequency of IN-activity across *Peltigera* species. Table 1 presents the freezing temperatures of 17 undiluted lichen extracts measured by the Vali-type droplet freezing assay, 9 of which were confirmed by TINA. The initial solutions had a concentration of 0.4 g mL<sup>-1</sup> and were then serially diluted 10-fold. We find that all the *Peltigera* extracts freeze between -4.4  $^{\circ}$ C and -11.2  $^{\circ}$ C. The type of symbiosis, i.e., whether the lichens are trimembered or 190 bimembered, does not appear to have an influence on the ice nucleation activity. Notably, the two most IN-active lichens, the trimembered *P. britannica* JNU22 and bimembered *P. austroamericana* 34529, show only a 0.7  $^{\circ}$ C difference in freezing efficiency. *P. britannica* JNU22 has a TINA  $T_{50}$  value of -5.1  $^{\circ}$ C while *P. austroamericana* 34529 freezes at -4.4  $^{\circ}$ C. Additionally, the ice nucleation activity of lichens does not seem to strongly correlate with the geographic region or climate zone where the samples were collected. For 195 example, TINA measurements reveal that the ice nucleation temperature of *P. aphthosa* PL729, which was obtained from the Canadian Arctic, and *P. neopolydactyla* JNU22, which was collected in the temperate

rainforests of the Northwestern United States, varied by only 0.6°C despite significant ecosystem differences. Because the specimens were collected between 2011 and 2024 and subsequently stored in either a frozen or dehydrated state, we cannot exclude that the age of the sample or the method of storage impacted the ice nucleation efficiency. However, Table 1 provides strong evidence that all tested *Peltigera* lichens contain active INs, and we conclude that ice nucleation activity is a common trait across the genus *Peltigera*.

We observe that the freezing spectra of selected *Peltigera* (Fig. 2) indicate the presence of two distinct activation temperatures, which agrees with prior measurements of lichen INs (Eufemio et al., 2023). *P. britannica* JNU22 consistently has an initial activation temperature of ~ -5°C and a second of -13°C, with a plateau between ~ -7 and -13°C. Similarly, both *P. neckeri* PNW22 and *P. aphthosa* PL729 INs reveal two activation temperatures at ~ -6°C and ~ -11°C. *P. austroamericana* 34529 INs initially induce freezing at ~ -4°C and a slight rise in the freezing spectra at ~ -7°C indicates a second population of active INs. The INs responsible for the two rises in the freezing spectra have been previously assigned as class 1, which are responsible for the initial freezing, and class 2, which refers to INs active at the lower temperature (Eufemio et al., 2023). The differences in activation temperatures across the *Peltigera* species may indicate variations in the macromolecular composition of INs. However, further investigation is needed to decipher the molecular nature of the INs and to identify whether classes 1 and 2 are due to an aggregation mechanism similar to the bacterial INP classes A and C.

All the *Peltigera* INs were found to be susceptible to heat treatments (Table S1 in the Supplement), supporting previous findings that lichen INs are proteinaceous, at least in part (Kieft and Ruscetti, 1990). Some species appear to be highly sensitive to heat, while others are less affected. For example, *P. aphthosa* PL729 activity is substantially lowered from -6.7°C to -9.3°C while *P. membranacea* PNW22 efficiency decreases by only 1.1°C.

**Table 1.** Ice nucleation activity of undiluted *Peltigera* lichen extracts determined using a Vali-type (initial) and high-throughput ice nucleation assay (TINA). Freezing temperatures,  $T_{50}$ , are defined as the temperature at which 50% of the extract droplets are frozen. Extracts labeled N/A were not measured using TINA. Species are arranged according to symbiosis type (tri- or bimembered) and collection location.

<i>Peltigera</i> Species	Symbiosis Type	Collection Location	Initial $T_{50}$ (°C)	TINA $T_{50}$ (°C)
<i>P. britannica</i> JNU22	Trimembered	Alaska, USA	-5.7	-5.1
<i>P. aphthosa</i> BRW1	Trimembered	Alaska, USA	-6.8	N.A
<i>P. aphthosa</i> BRW2	Trimembered	Alaska, USA	-6.9	N/A

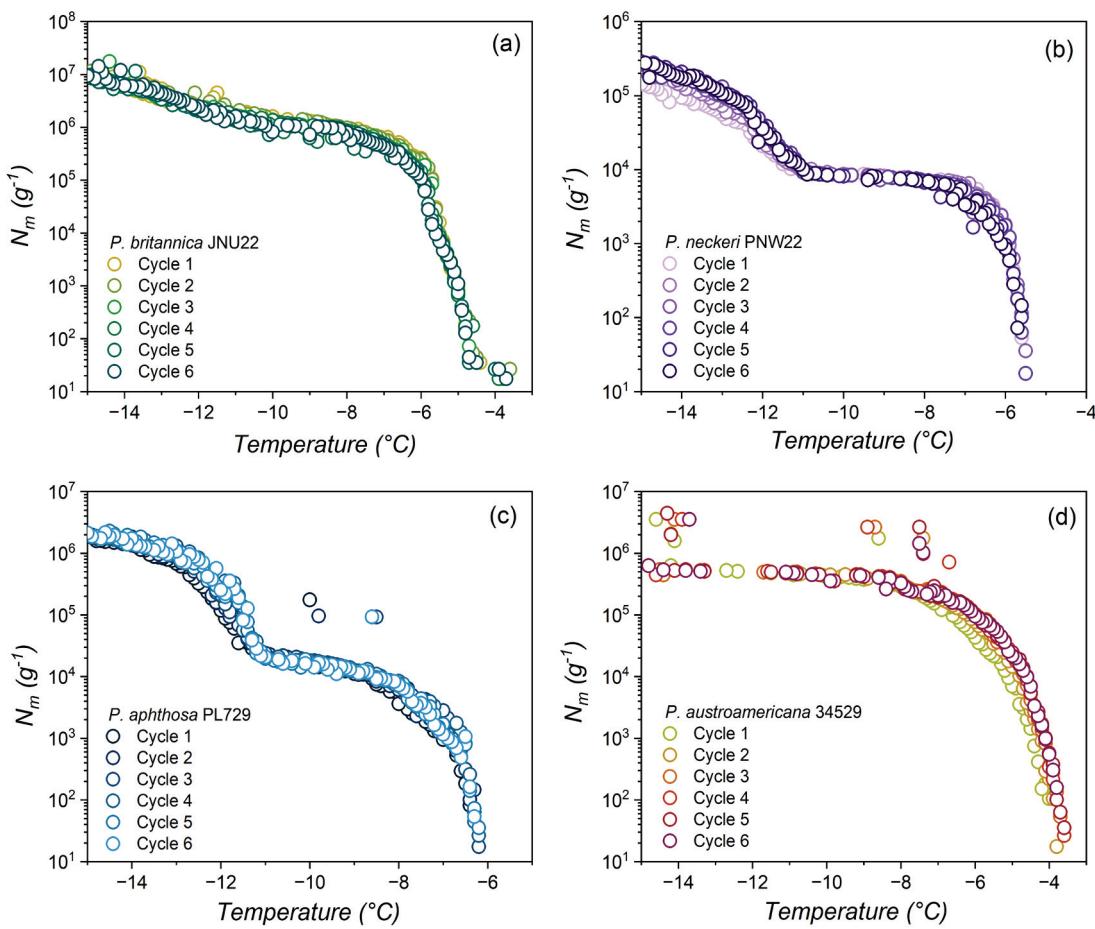
<i>P. aphthosa</i> PL729	Trimembered	Nunavut, Canada	-6.7	-6.7
<i>P. aphthosa</i> PL708	Trimembered	Nunavut, Canada	-6.8	N/A
<i>P. aphthosa</i> P5057	Trimembered	Quebec, Canada	-8.3	-11.2
<i>P. neopolydactyla</i> JNU22	Bimembered	Alaska, USA	-6.3	-6.1
<i>P. membranacea</i> PNW22	Bimembered	Washington, USA	-7.3	-5.8
<i>P. neckeri</i> PNW22	Bimembered	Washington, USA	-7.5	-5.9
<i>P. malacea</i> PL744	Bimembered	Nunavut, Canada	-6.8	-6.2
<i>P. neckeri</i> PL713	Bimembered	Nunavut, Canada	-6.6	-7.1
<i>P. neopolydactyla</i> P0309	Bimembered	Quebec, Canada	-6.9	N/A
<i>P. austroamericana</i> 34390	Bimembered	Brazil	-9.3	N/A
<i>P. austroamericana</i> 34529	Bimembered	Brazil	-4.9	-4.4
<i>P. dolichorrhiza</i> 34433	Bimembered	Brazil	-8.1	N/A
<i>P. dolichorrhiza</i> CR-8	Bimembered	Costa Rica	-8.8	N/A
<i>P. dolichorrhiza</i> CR-4	Bimembered	Costa Rica	-8.7	N/A

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### 3.2 *Peltigera* INs retain activity with freeze-thaw cycles

Several lichen species outside of the genus *Peltigera* have been found to maintain freezing efficiency after exposure to conditions associated with high altitudes, such as rapidly changing temperatures (Eufemio et al.,

230 2023). Figure 2 displays the effects of consecutive freeze-thaw cycles on aqueous extracts of *P. britannica* JNU22, *P. neckeri* PNW22, *P. aphthosa* PL729, and *P. austroamericana* 34529, which were collected in Alaska and Washington, USA, northern Canada, and Brazil, respectively. The freezing spectra show that the lichens retain ice nucleation across six cycles, independent of the geographic region of origin. Across all cycles, the cumulative number of INs remains nearly constant. The negligible impacts of consecutive freeze-  
235 thaw cycles on the ice nucleation efficiency highlight the stability of *Peltigera* INs and emphasize both their likelihood to survive the aerosolization process and their capacity to act as ice nucleating agents in the atmosphere.



240 **Figure 2.** Effects of freeze-thaw cycles on *Peltigera* lichen ice nucleation activity. Shown are the cumulative number of INs per unit mass ( $N_m$ ) of (a) *P. britannica* JNU22, (b) *P. neckeri* PNW22, (c) *P. aphthosa* PL729 and (d) *P. austroamericana* 34529 across 6 consecutive cycles.

### 3.3 Isolation of IN-active lichen-associated organisms

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Besides the primary mycobiont and photobiont partners, lichens are host to a diverse community of additional micro-organisms, including fungi and bacteria (Arnold et al., 2009; Hodkinson et al., 2012). We surveyed *P. britannica* JNU22-associated biota for ice nucleation activity to investigate the relative contributions of each lichen-associated organism to the lichen freezing efficiency. Fourteen pure cultures were isolated from the 250 thallus of *P. britannica* JNU22 and identified as fungi (including molds and yeasts) and bacteria based on the morphology and microscopic images. Table S2 presents the freezing temperatures of the aqueous extracts containing INs of each culture as measured by the Vali-type droplet freezing assay. Initial screenings reveal substantial variations in the ice nucleation efficiency of the cultures. The aqueous extract containing INs from the most active culture, labeled L01-tf-B03, induced freezing at -5.2°C. Based on the fast growth rate and presence of mycelial-like growth, we classified L01-tf-B03 as a lichen-associated fungus. It is notoriously 255

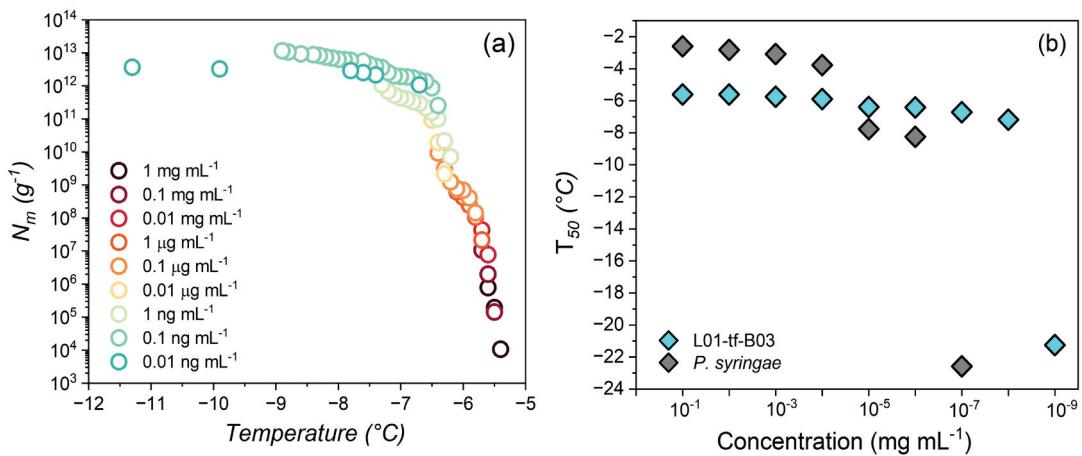
difficult to isolate mycobionts (Cornejo et al., 2015), which are very slow growing, and in the case of *Peltigera* has never been successful. The least active culture, L01-tf-B01, appeared to be a mold that did not freeze until -9.6°C.

260 **3.4 The isolate L01-tf-B03 culture is the most efficient ice nucleator**

Figure 3a displays the results of TINA measurements of an aqueous extract of INs from the mycelial surfaces of L01-tf-B03. The initial concentration was 1 mg mL<sup>-1</sup> and a complete dilution series was performed. The freezing spectra of L01-tf-B03 confirm the potency of the culture as determined by the Vali-type assay (Table 265 S2 in the Supplement). We observe two rises in the spectra of L01-tf-B03 at ~ -5.6°C and ~ -6.5°C.

Figure 3b shows that at 1 mg mL<sup>-1</sup>, the  $T_{50}$  value of L01-tf-B03 is approximately -5.6°C. Impressively, the extract retains strong IN-activity as low as 1 µg mL<sup>-1</sup>, with the  $T_{50}$  decreasing to only ~ 5.8°C. A minor decrease in IN-activity occurs at a concentration of 0.1 µg mL<sup>-1</sup>, at which the  $T_{50}$  lowers from -5.8°C to ~ -6.3°C. However, it is not until the extract is diluted to below 0.1 ng mL<sup>-1</sup> that significant impacts on the IN-270 activity are observed. 0.1 ng mL<sup>-1</sup> corresponds to a  $T_{50}$  of ~ -7.2°C, after which the  $T_{50}$  values are dramatically lowered and are similar to pure water ( $T_{50}$  ~ -23.5°C). The efficiency of L01-tf-B03 is evident in comparison to the live bacteria from the strain *P. syringae* Cit7 (Renzer et al., 2024), which was subjected to the same dilution series as the lichen culture. While *P. syringae* INs are highly active at 1 mg mL<sup>-1</sup>, with a  $T_{50}$  of ~ -2°C, the potency is rapidly lost with subsequent dilutions. At a concentration of 0.01 mg mL<sup>-1</sup>, the  $T_{50}$  is 275 lowered to ~ -3.1°C, and at 0.1 µg mL<sup>-1</sup> the IN-activity decreases further to -7.7°C. The large decrease of over 4°C in bacterial freezing efficiency is in striking contrast to L01-tf-B03, for which the IN-activity is reduced by less than 1°C at the same concentration. At 1 ng mL<sup>-1</sup>, the IN-activity of *P. syringae* is abolished and the  $T_{50}$  values are comparable to pure water. At the lowest measured dilution, L01-tf-B03 contains a significantly larger cumulative number of active INs than *P. syringae* (details can be found in Fig. S1 in the 280 Supplement).

Overall, our findings suggest that L01-tf-B03 is 1000 times more efficient than the INs from *P. syringae*, given that the total decay of *P. syringae* IN-activity occurs at a concentration of 1 ng mL<sup>-1</sup> while L01-tf-B03 activity is not fully eliminated until 0.01 ng mL<sup>-1</sup>. It is worth noting that the IN-activity of *P. syringae* was measured based on the mass of the bacterial cells, whereas for L01-tf-B03, the measurements 285 were based on the mass of the INs released from the mycelium. To the best of our knowledge, the ability of L01-tf-B03 to retain IN-activity at exceptionally low concentrations makes it the most efficient documented IN to date. We aim to confirm the genetic identity of L01-tf-B03 in future studies.



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**Figure 3.** (a) Freezing experiment of aqueous extract containing INs ( $1 \text{ mg mL}^{-1}$  to  $0.01 \text{ ng mL}^{-1}$ ) from L01-tf-B03. Shown are the cumulative number of INs per unit mass ( $N_m$ ) of L01-tf-B03. (b) Dilution effects on the IN-activity of L01-tf-B03 (cyan) aqueous extract and *P. syringae* in water (gray). *P. syringae* INs are inactive at concentrations below  $1 \text{ ng mL}^{-1}$  and are not shown.

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#### 4 Conclusions

Ice nucleation activity of *Peltigera* lichens was investigated in a Pan-American survey across tropical to 300 arctic environments. All the lichens were IN-active above  $-12^{\circ}\text{C}$  and 35% initiated freezing at or above  $-6.2^{\circ}\text{C}$ . We find no relationship between the symbiosis type and freezing efficiency. Further, there is no apparent correlation between biogeographic patterns and lichen IN-activity. Our findings illustrate that 305 efficient ice nucleation may be a common trait across *Peltigera*. A concentration series of select samples exposed to repetitive freeze-thaw revealed that *Peltigera* INs maintain activity under experimental conditions that serve as a proxy for high altitudes. Given the stability and the global abundance of *Peltigera*, the lichen INs are likely prevalent in airborne fungal communities and may have considerable influence on atmospheric processes (Zachariassen and Kristiansen, 2000; Henderson-Begg et al., 2009; Moffett et al., 2015; Womack et al., 2015).

The *P. britannica* JNU22 isolate, L01-tf-B03, was identified as a lichen-associated fungus. These 310 findings are consistent with previous measurements of mycobiont IN-activity by Kieft and Ahmadjian (1989), in which the fungal cultures were found to produce more efficient INs than the photobionts. L01-tf-B03 induced warm sub-zero ice formation at  $-5.6^{\circ}\text{C}$  at  $1 \text{ mg mL}^{-1}$  and retained IN-activity of  $\sim -7.2^{\circ}\text{C}$  at concentrations as low as  $0.1 \text{ ng mL}^{-1}$ . Our results demonstrate the efficiency of L01-tf-B03 as compared to

315 *P. syringae* and show that the INs released from this fungal culture are nearly 1000 times more efficient than  
the most potent bacterial cell-anchored INs classified to date.

*Data availability.* All data are available from the corresponding author upon request.

320 *Author Contributions.* KM and RJE designed the experiments. TLS, JM, and FL provided lichen samples  
and identified lichen. RJE and GR performed the experiments. MR, BAV, JM, and FL provided guidance on  
culture growth and lichen ecology. RJE, KM, GR, BAV and FL discussed the results. RJE and KM wrote the  
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*Competing interests.* The authors declare that they have no conflict of interest.

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