Cultivable, halotolerant ice nucleating bacteria and fungi in coastal precipitation

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Abstract

Ice nucleating particles (INPs) are a rare subset of aerosol particles that initiate cloud droplet freezing at temperatures above the homogenous freezing point of water (-38 °C). Considering that the ocean covers 70% of the earth's surface and represent a large potential source of INPs, it is imperative that the uncertainties in the identities and emissions of ocean INP become better understood. However, the specific underlying drivers of marine INP emissions and their identities remain largely unknown due to limited observations and the challenge involved in isolating exceptionally rare IN forming particles. By generating nascent sea spray aerosol (SSA) over a range of biological conditions, mesocosm studies show that microbes can contribute to marine INPs. Here, we identify 14 (30%) cultivable halotolerant ice nucleating microbes and fungi among 47 total isolates recovered from precipitation and aerosol samples collected in coastal air in Southern California. IN isolates collected in coastal air were found to nucleate ice from extremely warm to moderate freezing temperatures (-2.3 to -18 °C). Air mass trajectory analyses, and cultivability in marine growth media indicate marine origins of these

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isolates. Further phylogenetic analysis confirmed that at least two of the 14 IN isolates were of marine origin. Moreover, results from cell washing experiments demonstrate that most IN isolates maintained freezing activity in the absence of nutrients and cell growth media. This study provides confirmation of previous studies' findings that implicated microbes as a potential source of marine INPs and additionally demonstrates links between precipitation, marine aerosol and IN microbes.

1 Introduction

Ice nucleating particles (INPs) are rare aerosols (1 in 10^5 or less of total particles in the free troposphere) (Rogers et al., 1998) that induce freezing of cloud droplets at temperatures above the homogenous freezing point of water (-38 °C) and at relative humidities (RH) well below the homogenous freezing RH of aqueous solution droplets. They affect multiple climate-relevant properties of mixed-phase and cold clouds. For example, in-cloud INP distributions can influence the ice-phase partitioning processes that determine a cloud's reflectivity, lifetime and precipitation efficiency (Creamean et al., 2013; DeLeon-Rodriguez et al., 2013; Fröhlich-Nowoisky et al., 2016; Ladino et al., 2016). However, numerical representations of cloud ice processes challenge climate models across all scales (Curry et al., 2000; Furtado and Field, 2017; Kay et al., 2016; Klein et al., 2009; Prenni et al., 2007), and it is believed that the under-characterization of global INP distributions contribute to the relevant uncertainties.

Despite recent evidence that sea spray aerosol (SSA) represents a unique source of INPs (DeMott et al., 2016; McCluskey et al., 2016, 2018a, 2018b), that these INPs can contribute significantly to total INP populations (particularly in remote marine regions where terrestrial aerosols are less abundant) (Burrows et al., 2013; Vergara-Temprado et al., 2017; Vergara-Temprado et al., 2018), and that specific parameterization of marine INPs can influence modelled radiative budgets (Wilson et al., 2015), little is known about the actual entities involved in forming marine INPs. (Schnell and Vali, 1975) were the first to associate phytoplankton blooms with raised ice nucleation activity, in seawater sampled shortly after a bloom in Bedford Basin, Nova Scotia. Recent mesocosm studies have linked SSA ice nucleating (IN) activity specifically to the death phase of phytoplankton blooms. (McCluskey et al., 2017) showed that increases in INP emissions corresponded to increased emissions of heterotrophic bacteria and organic species in SSA, implicating microbes and biomolecules as contributors to marine INP populations. Marine microbes were further linked to INPs in (McCluskey et al., 2018a): subsets of INPs in nascent SSA were found to be heat labile, with sizes greater than 0.2 µm, and INP emissions correlated to increased emissions of cells or cellular material. An IN halotolerant strain of *Pseudomonas fluorescens* was detected in phytoplankton cultures derived from seawater (Fall and Schnell, 1985), and INPs have also been detected in seawater containing marine diatoms, green algae (Alpert et al., 2011; Junge and Swanson, 2007; Ladino et al., 2016; Parker et al., 1985), and sea-ice samples containing marine Antarctic bacteria (Junge and Swanson, 2007; Parker et al., 1985).

While indirect evidence indicates marine microbes and other biogenic entities as possible marine INPs, direct observations of any marine IN entity in the atmosphere (i.e. through isolation and identification in an atmospheric sample) were previously nonexistent. Multiple factors make it difficult to determine INP origin, including the low abundance of INPs and the diversity of aerosols with IN ability (e.g. Kanji et al., 2017). Moreover, it is not always possible to differentiate terrestrial and marine air mass influences within the Marine Boundary Layer (MBL). However, cultivable IN microbes have been isolated from clouds and precipitation for decades (e.g. Sands et al., 1982; Failor et al., 2017; Morris et al., 2008), and the origins of IN isolates can be determined by comparing sequences with reference isolates of known origin. There are several caveats to consider when inferring in-cloud INP concentrations or properties from precipitation samples (Petters and Wright, 2015), including "sweep-out" of additional INPs as the hydrometeor traverses the atmosphere below the cloud (Vali, 1974). However, previous studies have derived estimates of in-cloud INP concentrations and origins from the concentrations and identities of IN microbes from ground-level collections (Christner et al., 2008; Failor et al., 2017a; Joyce et al., 2019; Monteil et al., 2014) by assuming that particles in precipitation originate from the cloud rather than the atmospheric column through which the hydrometeor descended. This assumption is supported by (Vali, 1971), which found that subcloud scavenging of aerosol did not affect INPs observed in precipitation collected at the surface in comparisons of INP spectra from surface samples with samples collected at cloud-base. Furthermore, (Wright et al., 2014) estimated that sweep-out contributed between 1.2 and 14% of INPs suspended in a precipitation sample collected at the surface.

While evidence exists for relationships between IN microbes and precipitation in terrestrial systems, studies of the relationship between marine INPs, marine microbes, and precipitation remain quite limited. Here we report the identities and freezing temperatures of 14 cultivable halotolerant IN species derived from coastal precipitation and aerosol samples, two of which were marine in origin. Over the course of 11 precipitation events during an El Niño season, 47 cultivable halotolerant bacteria and fungi were recovered from aerosol and precipitation samples collected in a coastal subtropical climate in southern California. Bacterial and fungal species were isolated, identified, and tested for ice nucleation behavior from 0 to -25 °C using an immersion mode droplet freezing assay technique. Precipitating cloud altitudes and isolate source regions were estimated using the High-Resolution Rapid Refresh atmospheric model (HRRR) and the FLEXible

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PARTicle dispersion model (FLEXPART)(Stohl et al., 1998), respectively. Finally, the effect of media on the observed IN behavior of isolates was investigated through cell washing experiments.

2 Methods

2.1 *Precipitation and Aerosol Sample Collection Methods*

Precipitation and ambient aerosol samples were collected on the Ellen Browning Scripps Memorial Pier at Scripps Institution of Oceanography (SIO) (32.8662 °N, 117.2544 °W) from March 6, 2016 – May 6, 2016. Sampling took place in the surf 8 m above Mean Lower Low Water (MLLW), and samples were only collected during westerly winds. Aerosol samples were collected over 1.5-5 hour periods on polycarbonate filters (45 mm diameter, 0.2 µm pore-size, Whatman® Nuclepore, Chicago, Illinois, USA) placed in open-face Nalgene ® Analytical Filter Units (Waltham, Massachusetts, USA). After collection, aerosol filters were immersed in 12 mL of ultrapure water, and particles were shaken off the filter by hand for 20 minutes. The precipitation samples were collected using a modified Teledyne Isco© Full-Size Portable Sampler (Lincoln, Nebraska, USA), fitted with 24 1-L polypropylene bottles. Prior to sampling, the bottles were immersed in 10 % hydrogen peroxide for 10 minutes, then rinsed three times with ultrapure water. The automated sampler would engage when triggered by precipitation of at least 0.13 cm h^{-1} and would sample using the first of 24 bottles for 30 minutes, and thereafter switch bottles at hourly intervals. Within one to two hours of sample collection, INP concentrations were measured using the SIO-Automated Ice Spectrometer (SIO-AIS) (Beall et al., 2017), an automated offline freezing assay technique for measurement of immersion mode INPs. To decrease the effect of interstitial particle sweep out by falling raindrops on measured INP concentration, precipitation from the first 30 minutes was discarded. Sweep out effects have been estimated to contribute between 1.2 and 14 % to measured concentrations of INP in a precipitation sample (Wright et al., 2014).

The INP measurement technique is described in detail in (Beall *et al.,* 2017*)*. Briefly, the precipitation samples were distributed in microliter aliquots into a clean 96-well disposable polypropylene sample tray. An equal number and volume of aliquots of ultrapure water accompany each sample in the disposable tray as control for contamination from the loading and/or ultrapure water. The sample trays were then inserted into an aluminum block that is cooled until the samples are frozen. Cumulative INP number concentrations per temperature per volume are calculated using the fraction (*f*) of unfrozen wells per given temperature interval:

$$INP = \frac{-ln(f)}{V_d} \qquad \text{Eq. (1)}$$

where V_d is the volume of the sample in each well. For aerosol filter samples, cumulative INP number concentrations are calculated using the ratio of the volume used for resuspension of the particles (V_{re}) to the volume of aerosol sampled (V_A) :

$$INP = \frac{-ln(f) \cdot V_{re}}{V_d \cdot V_A} \qquad \text{Eq. (2)}$$

The fraction of unfrozen wells (f) is adjusted for contamination by subtracting the number of frozen ultrapure water wells per temperature interval from both the total number of unfrozen wells and total wells of the sample. For this study, 30 x 50 µL droplets were deposited into the droplet assay, yielding a detection limit of 0.675 INP mL^{-1} liquid.

Within one to two hours of collection, precipitation and aerosol samples were also inoculated in 5 mL ZoBell growth media (ZoBell, 1947) (5 g peptone, 1 g yeast extract per 1 L of filtered (0.22 µm) autoclaved seawater) and grown under ambient conditions (21 - 24 ˚C). INP concentrations in ZoBell enrichments were measured 1-day post inoculation and for several days thereafter to monitor for sustained IN activity.

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2.2 *Bacterial and fungal isolation and characterization*

Precipitation and SSA microorganisms were cultivated using the ZoBell enrichment described above (ZoBell, 1947) (Fisher Scientific, Houston, Texas, USA). Isolation was performed by successive plating on ZoBell agar (BD Bacto™ Agar, Sparks, MD, USA). Liquid cultures were inoculated from single colonies and grown to late exponential phase. DNA was extracted from liquid cultures of isolates after an overnight lysis with proteinaseK (100 µg mL^{-1}) and lysozyme (5 mg mL^{-1}) (MilliporeSigma, Burlington, Massachusetts, USA) (Boström et al., 2004) using a QIAamp® kit (QIAGEN, Hilden, Germany). 16S V4 ribosomal DNA fragments were amplified using the primers 515F (5′ GTGYCAGCMGCCGCGGTAA 3′) and 926R (5′ CCGYCAATTCMTTTRAGT 3′)(Walters et al., 2015). PCR products were purified using GenElute^{TM} PCR Clean-up kit (MilliporeSigma). 16S fragment DNA sequences were resolved by Sanger sequencing (Retrogen, San Diego, CA). OTUs were determined from 16S sequences using SINA (Pruesse et al., 2012) and individual sequences were inspected using BLAST (https://www.ncbi.nlm.nih.gov/) for further characterization.

To assess for duplicate isolates within the sampling period, 16S sequences were compared. Sequences within the same OTU were adjusted and aligned in DECIPHER(Alignseqs(), AdjustAlignment() with default settings) (Wright, 2015). These sequence alignments were used to generate phylogenetic trees using ClustalW2 (UPGMA)(McWilliam et al., 2013) and visualized with iTOL(Letunic and Bork, 2011). Branch distances were used to evaluate sequence similarity. To facilitate comparisons between organisms assigned to the same OTU, identity assignments including divisions at distances > 0.1 (e.g. 1, 2, 3…) were further subdivided by distances > 0.01 (e.g. 1a, 1b, 1c…). Nonzero distances

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< 0.01 were given sub labels (e.g. 1a1, 1a2…). Zero distances were given identical labels. Distances < 0.01 were determined to be possible duplicates if they were collected during the same sampling period unless the organisms had a different phenotype generally indicated by different pigmentation. Each duplicate was tested for its IN ability, and the results are reported in Table S1 and discussed in the main text. Maximum likelihood phylogenetic trees were computed in MEGA7 (Tamura et al., 2013) after ClustalW alignment with reference sequences (https://www.ncbi.nlm.nih.gov/) .

2.3 *Storm and aerosol source characterization methods*

Cloud altitudes at the time of precipitation sample collection were estimated using the High-Resolution Rapid Refresh model (HRRR). The altitudes and pressure levels of clouds were assumed to be located where RH was > 95-100 % in the model. The specific RH criteria applied to each sampling period are provided in Table S2. HRRR model output was compared with surface RH measurements from the SIO pier weather station during sampling periods, and predicted RH was found to agree with observations with an RMSE of < 10 – 15%, which aligns closely with previously reported RH accuracies over the continental US (Benjamin et al., 2016). Three altitudes of the estimated cloud top, middle and bottom were used as release points of FLEXPART 10-day LaGrangian backward trajectories. Back-trajectories were used to identify potential sources of INPs in the precipitation samples, and to indicate potential sources of land-based contamination in aerosol and precipitation samples due to local wind patterns or land-sea breezes. Satellite composites from the National Weather Service Weather Prediction Center's North American Surface Analysis Products were used for synoptic weather analysis to generally characterize meteorology during each rain event (see Table S3).

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2.4 *Isolate IN activity measurement and controls*

To measure the IN activity of each isolate, liquid cultures were grown to late exponential phase. Growth was monitored by optical density (OD) (590 nm). INP concentrations were measured as described in Sect. 2.1 in liquid cultures and compared to a ZoBell blank as a control. Isolate biomass was estimated from OD measurements using the distribution of OD to biomass conversion factors from (Myers et al., 2013). As Myers et al. (2013) found, in a study of 17 diverse organisms, OD to biomass conversion factors ranged between 0.35 and 0.65 gDW OD^{-1} L^{-1}; we assume that INP g^{-1} biomass may be estimated from OD within a factor of 2. Thus, isolate INP concentrations, and upper and lower limits of 95% confidence intervals were scaled by $\frac{1}{m}$, where m is the mean, minimum or maximum value of the (Myers et al., 2013) biomass conversion factor distribution, respectively (i.e. 0.5, 0.65 and 0.35 gDW OD^{-1} L^{-1}).

To investigate the effect of growth media on IN isolates, a subset of late exponential cultures were washed three times with filtered (0.22 µm) autoclaved seawater (FASW) by successive centrifugation and resuspension. The washing procedure removes everything that is water soluble and whole cells and insoluble molecules pellet upon centrifugation. INP measurements were taken as described and compared to sterile seawater controls (see Fig. S1b and Fig. S1c).

As ZoBell growth media contained INPs at moderate to cold freezing temperatures (-13 to -25 °C, see Fig. S1a), only isolates exhibiting INPs at significantly higher freezing temperatures (-2.3 to -15 °C) or at significantly higher concentrations than their respective ZoBell growth media sample were considered to be IN. The criterion for significance was chosen to be conservative: a data point along an isolate's measured IN spectrum was considered significant if there was no overlap between the 95 % binomial sampling confidence interval of the given data point (Agresti

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and Coull, 1998) and any ZoBell confidence interval within ± 2.2 °C, the maximum uncertainty in freezing temperature measurement due to heterogeneity in heat transfer rates across the instrument's droplet assay (Beall et al., 2017). This equates to a significance threshold of $p < 0.005$ (Krzywinski and Altman, 2013). The choice of ± 2.2 °C is likely conservative given that in a study of 11 cooling cycles, the average and maximum ΔT observed across the droplet assay when cooling from 0 to -25 °C was 0.38 and 0.98 °C, respectively (and following this study, the addition of a second thermistor under the second sample tray decreased the observed ΔT to within thermistor uncertainty, ± 0.2 °C). The same criterion was applied to isolates washed and suspended in FASW as described above (Figs. S1 b-c). Many isolates were diluted with their respective media (ZoBell or FASW) to decrease opacity such that freezing events could successfully be detected by the camera, so their respective dilution factors were applied to both the INP concentrations measured in the isolate suspension and the INP concentrations measured in the FASW or ZoBell samples for the significance analysis (see Figs. S1 b-c and S2).

3 Results and discussion

3.1 Subtropical coastal storm properties and origins

Aerosol and rain samples were collected from a pier on the coast of La Jolla, CA (32°52'01.4"N 117°15'26.5"W) during an El Niño event spanning 11 precipitation sampling periods March 6 to May 7, 2016. Observations of INPs in precipitation generally fall within bounds of previously reported INP concentrations from precipitation and cloud water samples (Fig. 1, grey shaded region, adapted from Petters and Wright, 2015). AIS measurement uncertainties are represented with 95% binomial sampling intervals (Agresti and Coull, 1998). Observed freezing temperatures ranged from -6.5 to -22.0 °C, with concentrations up to the limit of testing at 10^5 INP

L^{-1} precipitation. Following the assumptions in (Petters and Wright, 2015) to estimate in-cloud INP concentrations from precipitation samples (i.e. condensed water content of 0.4 g m^{-3} air), observations of INP concentrations in fresh precipitation samples are additionally compared to studies of field measurements conducted in marine and coastal environments.

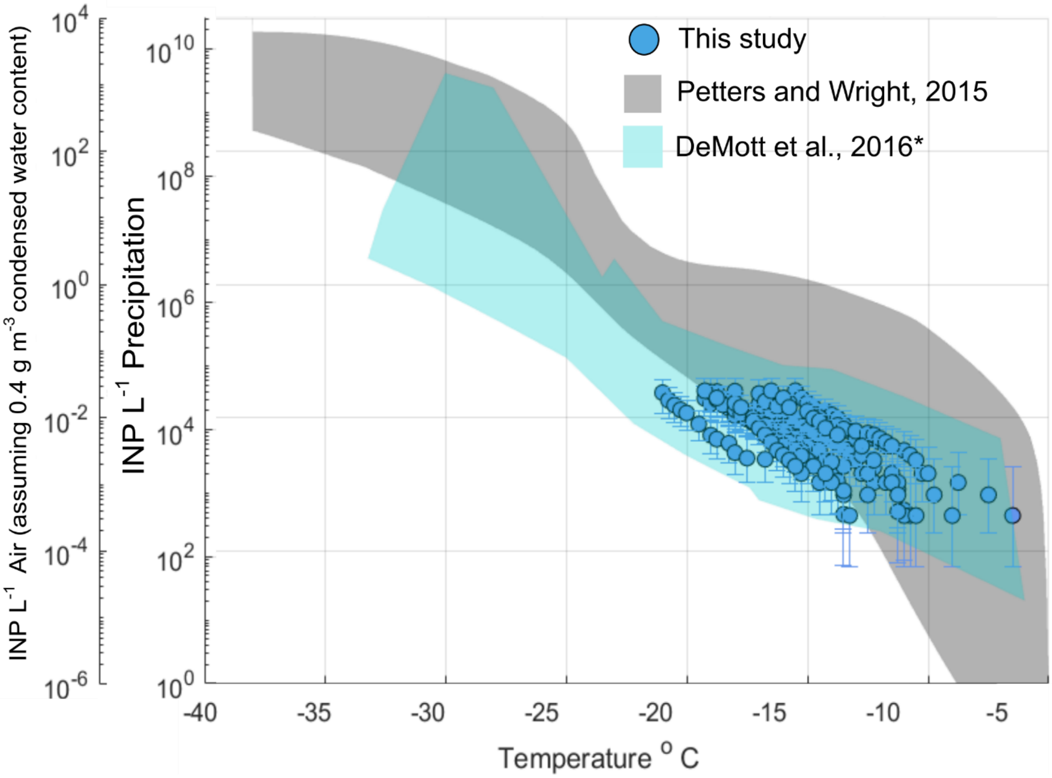

Figure 1. **INP concentrations per liter precipitation and estimated in-cloud INP concentrations per volume of air in 11 precipitation samples collected at Scripps Institution of Oceanography Ellen Browning Scripps Memorial Pier (32.8662 °N, 117.2544 °W, La Jolla, California, USA) between March and May 2016.** Grey shaded region indicates the spectrum of INP concentrations reported in nine previous studies of precipitation and cloud water samples collected from various seasons and locations worldwide, adapted from Fig. 1 in (Petters and Wright, 2015). The blue shaded region represents the composite spectrum of INP concentrations observed in a range of marine and coastal environments including the Caribbean, East Pacific and Bering Sea as well as laboratory-generated nascent sea spray (DeMott et al., 2016).

*DeMott et al., 2016 data has been updated with a completed dataset for the ICE-T study, as shown in (Yang et al., 2020).

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Figure 1 shows that atmospheric INP concentration estimates compare with INP concentrations observed in a range of marine and coastal environments, including the Caribbean, East Pacific, and Bering Sea, as well as laboratory-generated nascent sea spray aerosol (DeMott et al., 2016). Observations of INPs in aerosol samples are shown in Fig. S3 and are also comparable with those of DeMott et al. (2016).

The source regions of aerosols present in precipitating clouds were estimated using 10-day FLEXPART back trajectories (Fig. 2). For each of the 11 sampling periods, back trajectories show that the Pacific Ocean from mid to high latitudes was the primary source region to precipitating cloud layers. Periods 5 – 11 may have been additionally influenced by west coast continental sources (particularly periods 6 and 7). 10-day back trajectory simulations for aerosol samples similarly indicated that marine sources dominated (see Fig. S4). Marine aerosols likely originated from regions near the coast (Periods 2, 4-11, A1, A2, A5) or in the mid Pacific Ocean (Periods 1 and 3), where trajectories descended below the marine boundary layer.

Cloud bottom and top altitudes were estimated using the High-Resolution Rapid Refresh model (HRRR), defined by the RH criteria in Table S2. Over the 11 precipitation sampling periods, cloud altitude ranged from 950 – 600 mb, bottom to top, or 500 – 4000 m, with temperatures ranging from 265 – 288 K.

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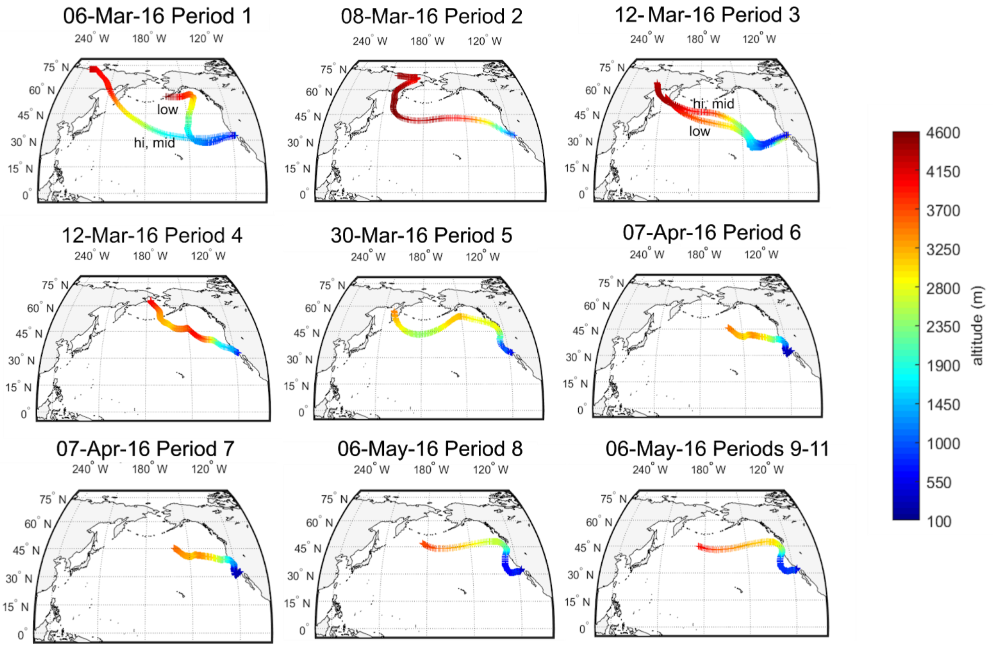

Figure 2. 10-day back-trajectories from cloud base, mid-cloud, and cloud-top during 11 precipitation sampling periods at the SIO Pier (32.8662 °N, 117.2544 °W). FLEXPART back-trajectories were used to estimate potential source regions of INPs to the clouds during precipitation events. Shown are the particle centroids of back-trajectories from three release altitudes within each cloud (see Table S2 for details on altitude selection criteria). If trajectories across the three selected release altitudes differentiated, they are labeled "hi" for cloud top, "mid" for halfway between base and top, and "low" for cloud bottom. Origins of particles in the 10-day simulation are shown to range from 4000 m over Russia to 2500 – 3500 m over the Sea of Okhostk, the Bering Sea, and the north Pacific. FLEXPART results suggest a dominance of marine particle sources to clouds for sampling periods 1-11.

3.2 Bacterial and fungal taxonomy

Cultivable bacteria and fungi were enriched from rain and aerosol samples in marine bacterial growth media, and strains were further isolated on marine agar. This resulted in 34 isolates from rain samples, and 13 isolates from aerosol samples with 29 unique operational taxonomic units (OTUs) as determined by > 97 % sequence identity of 16S sequences (Table

S1). Many of the isolates derived from rain and aerosol were highly pigmented, as observed in other studies (Delort et al., 2017; Fahlgren et al., 2010, 2015; Hwang and Cho, 2011; Tong and Lighthart, 1997), presumably aiding their survival under high *uv* exposure (Fig. S5). This pigmentation was especially prevalent in rain samples. The higher number of precipitation-derived isolates compared to aerosol is likely the result of lower aerosol bacterial and fungal loads during rain events. INP concentration decreases in aerosol during precipitation events support this conclusion. For 3 of the 11 precipitation events featured in this study (see Fig. S6), INP concentrations in aerosol were measured immediately before, during, and after precipitation events. In each of the three events, INP concentrations in aerosol decreased below detection levels during precipitation and increased again soon after the end of the precipitation event (in under 24 hours), though not beyond concentrations observed prior to the precipitation event. Interestingly, these features (i.e. the observed decreased INP concentrations during precipitation events and absence of increased INP concentrations within 24 hours of precipitation events) are in opposition to multiple studies of INP concentrations during and after rainfall events in terrestrial systems (Bigg, 1958; Conen et al., 2017; Huffman et al., 2013; Prenni et al., 2013). Additionally, (Levin et al., 2019) observed an increase in INP concentrations after precipitation events in a coastal environment, though this increase may have been related to a shift from marine to terrestrial aerosol sources as indicated by the back trajectories. Thus, results in this study indicate that the positive feedbacks between rainfall and surface INP emissions observed in terrestrial systems (Bigg et al., 2015; Morris et al., 2017) may not always apply to marine environments.

The taxonomy of the aerosol and rain isolates show higher diversity in the precipitation samples (Fig. S7 and Table S1), which may be due to artificial biases from low aerosol isolate recovery or sweep out of interstitial particles during raindrop descent. The rain samples had a high proportion of Actinobacteria, whereas in aerosol, Firmicutes and Proteobacteria were more dominant.

The microbes isolated in our study are closely related to microbial communities described in other studies, despite being isolated from a warmer climate (Bowers et al., 2009; Fröhlich-Nowoisky et al., 2016; Santl-Temkiv et al., 2015; Vaïtilingom et al., 2012). As (Michaud *et al.*, 2018) showed, Actinobacteria, as well as select Proteobacteria and Firmicutes, have an increased ability to aerosolize from seawater, and so SSA emissions may also explain their presence here. Two isolates (one from rain and one from aerosol, 3.5% of total isolates) are related to *Pantoea* sp., strains of which are known to possess IN proteins (e.g., Hill et al., 2014). *Pantoea* sp. and *Psychrobacter* sp. were the only bacterial taxa identified previously known to possess ice nucleation activity (Hill et al., 2014; Ponder et al., 2005). However, both *Psychrobacter sp*. and *Idiomarina* sp. have been shown to be capable of inhibiting ice recrystallization, possibly through the production of antifreeze proteins (AFPs) which can both inhibit freezing at moderate temperatures and serve as INPs at colder temperatures (Wilson and Walker, 2010).

The phylogenetic relationships between isolates and reference sequences (Fig. 3) indicate that marine origin is highly likely for two of the 14 IN isolates, *Idiomarina* sp. and *Psychrobacter* sp. 1c2, both of which were derived from coastal aerosol. Additionally, considering the aerosol transport simulation data (Fig. 2), the evidence of marine influence in precipitation INP spectra (Fig. 1), and the use of marine growth media, multiple other IN isolates derived from the precipitation samples are also possibly marine. Furthermore, other IN isolates from precipitation samples cluster closely with marine reference sequences. For example, *Pantoea* sp.1a and *Brevibacterium* sp. show high similarity to reference sequences derived from

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marine environments (Fig. 3 and S8). However, several of the species identified in this study are likely more ubiquitous, and closely related to reference isolates found in terrestrial and freshwater systems (Bowers et al., 2009; Fröhlich-Nowoisky et al., 2016; Santl-Temkiv et al., 2015; Vaïtilingom et al., 2012), including two of the IN isolates, *Psychrobacter* sp. 1b2 and *Paenbacillus* sp. 1.

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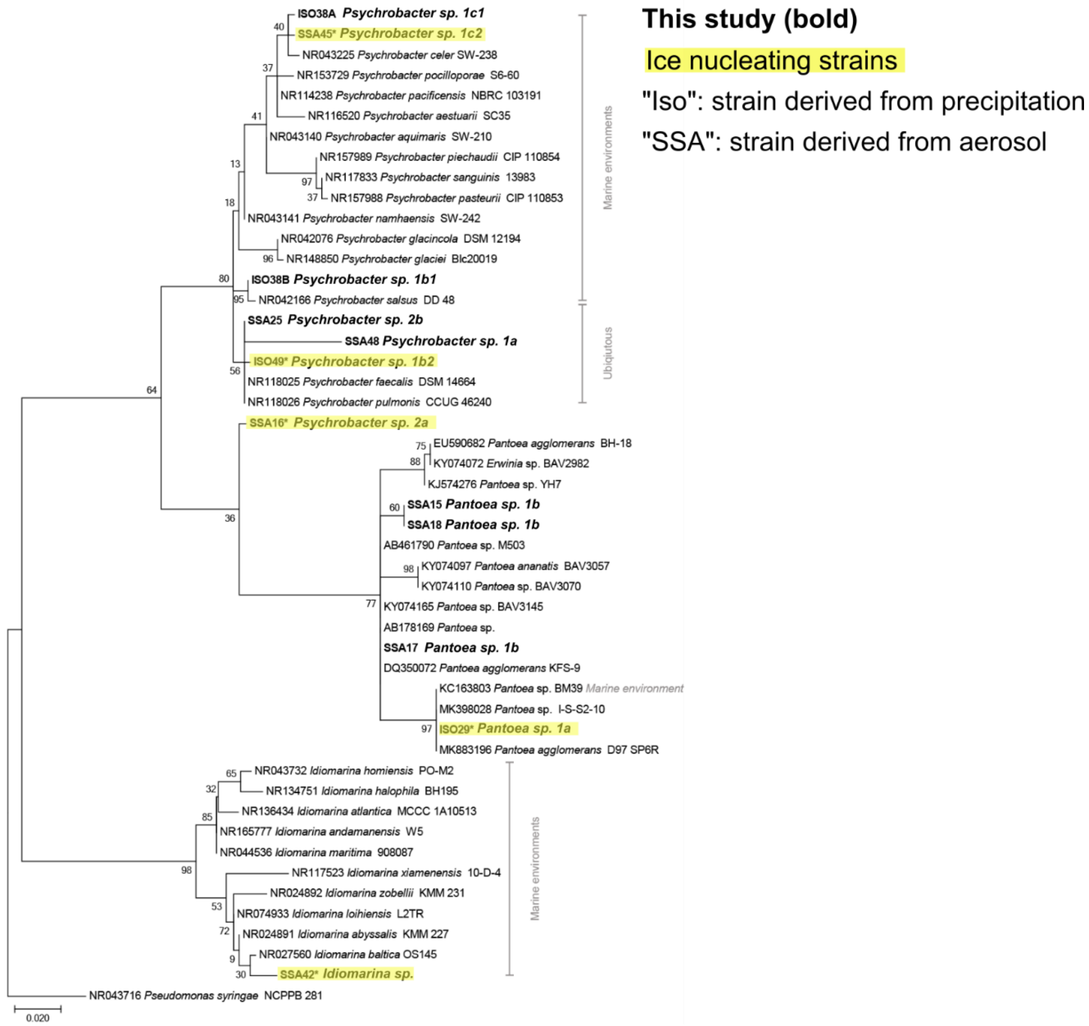

Figure 3. Phylogenetic relationships of isolates (in bold) related to Gamma-proteobacteria reference sequences. The environmental source of the reference sequences (based on NCBI metadata) is indicated in grey. Isolates with ice nucleating properties are shaded in yellow; bootstrap values (n=500) are indicated at nodes; scale bar represents changes per positions.

3.3 Ice Nucleating Properties of Rain and SSA isolates

Of the 47 total isolates derived from precipitation and aerosol samples, 14 were found to be significantly ice nucleating according to the selection criterion described in Methods Sect. 2.4.

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Within the technique's temperature and detection limit of 0.675 INP mL^{-1} liquid between 0 and -25 °C, 11 precipitation isolates exhibited freezing temperatures between -2.3 and -24.3 °C, and 3 aerosol isolates exhibited freezing temperatures between -14.0 and -24.5 °C (Table 1). Prior to this study, *Lysinibacillus* sp. was the only known gram-positive species found to be capable of ice nucleation (Failor et al., 2017a). Yet several IN isolates identified in this study are also gram-positive, including isolates of *Brevibacterium* sp., *Paenbacillus* sp., *Planococcus* sp., *Bacillus* sp., *Arthrobacter* sp., and *Cellulosimicrobium* sp.

Table 1. Identities of 14 cultivable, halotolerant IN bacteria derived from aerosol or precipitation samples (see Table S2 for precipitation and aerosol sample details).

IsoID	Isolate	IN Onset Temperature °C	Precipitation or Aerosol Sample Number
Iso2	*Cryptococcus* sp. 1	-9.3	1
Iso10B	*Paenibacillus* sp. 1	-14.8	2
Iso8	*Brevibacterium* sp. 1b	-2.3	4
Iso32B	*Planocococcus* sp. 1	-12.3	7
Iso29	*Pantoea* sp. 1a	-17	8
Iso31	*Bacillus* sp.1a1	-14.5	8
Iso21	*Cellulosimicrobium* sp. 1a1	-14	9
Iso23	Unknown *Arthrobacter*	-13.3	9
Iso24A	*Metschikowia* sp.	-16.5	9
Iso27	*Cellulosimicrobium* sp. 1a3	-14.8	10
Iso49	*Psychrobacter* sp. 1b2	-13.8	11
SSA42	*Idiomarina* sp.	-14.3	A1
SSA16	*Psychrobacter* sp. 1c2	-17.5	A2
SSA45	*Psychrobacter* sp. 2b	-14	A5

Isolate INP spectra are shown in Fig. 4, normalized to biomass, n_m g^{-1} (see Sect. 2.4 for details on biomass estimates). Also plotted in Fig. 4 are observations of a variety of marine and terrestrial bioaerosols from prior studies, including pollens, fungi, lichens, plankton, leaf litter and soil dusts (Conen et al., 2011; Conen and Yakutin, 2018; Després et al., 2012; Fröhlich-

Nowoisky et al., 2015; Kunert et al., 2019; O'Sullivan et al., 2015; Wex et al., 2015). Results show that with the exception of *Brevibacterium* sp., isolates from this study are generally less efficient than most terrestrial IN biological particles, with lower concentrations and activation temperatures. Concentrations of INP per mL in ZoBell suspension are additionally shown in Fig. S10.

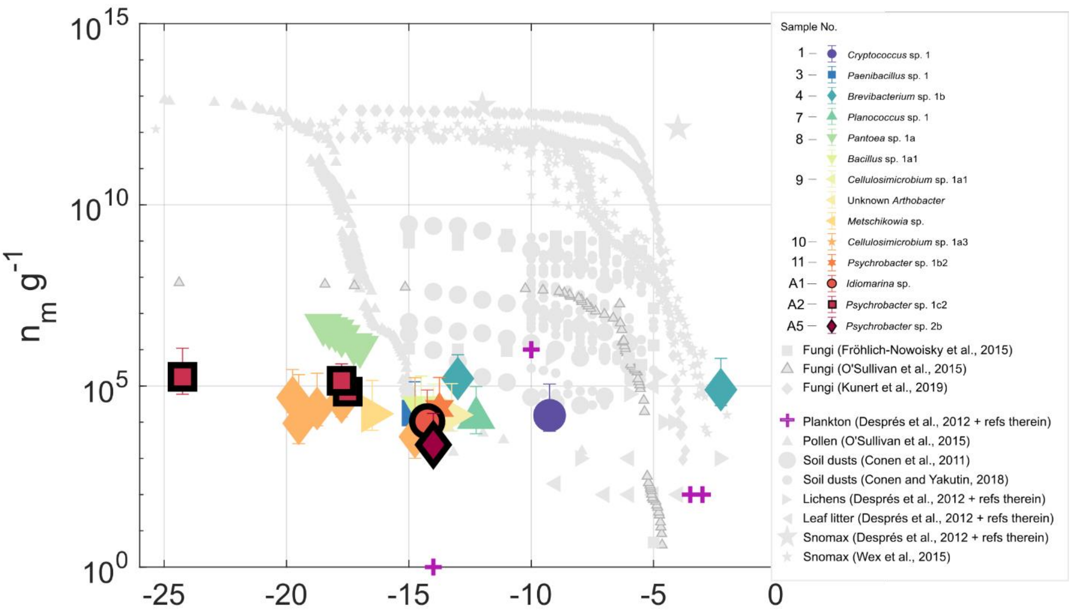

Figure 4. INP concentrations (g^{-1} biomass) for 14 halotolerant isolates derived from precipitation and aerosol samples. Also shown are INP observations of various biological particles from published studies. Sample numbers in the legend indicate the precipitation or aerosol sample from which the isolate was derived (see Table S3). Datapoints corresponding to isolates from aerosol are outlined in black. Error bars indicate 95% confidence intervals and uncertainty associated with biomass estimate (see Sect. 3.3 for details). Only freezing activity that was significantly enhanced ($p < 0.005$) above ZoBell growth media is shown. Results show that with the exception of *Brevibacterium* sp., isolates are generally less efficient ice nucleators than most biological INPs of terrestrial origin.

To examine the IN properties of unique strains within samples, multiple sequence alignment of the 16S sequences was used to identity and remove duplicates. The relationship

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between 16S sequences of isolates within their OTUs is shown in Fig. S11. Ice nucleating precipitation and aerosol isolates exhibit moderate IN freezing temperatures (< -10 °C) (Fig. 4), with the exception of two warm freezing isolates: a fungal isolate from sampling period 1, *Cryptococcus* sp., which triggered freezing at -9.3 °C, and a bacterial isolate from sampling period 4, *Brevibacterium* sp., at an exceptionally warm freezing temperature of -2.3 °C. The freezing temperatures of all but *Brevibacterium* sp. 1b overlap with previously reported freezing temperatures of INPs produced from fresh SSA (-7 to -33 °C), and, in particular, with the freezing temperatures shown to be likely associated with microbes or cellular material in SSA (-8 to -22 °C). (DeMott et al., 2016; McCluskey et al., 2017). Isolate freezing temperatures also overlap with INP freezing temperatures in samples of Arctic marine sea surface microlayer (Irish et al., 2017; Wilson et al., 2015).

Considering that only IN microbes of continental origins, such as *Pseudomonas syringae*, have been reported with freezing temperatures as high as -2 or -3 °C (e.g. Fröhlich-Nowoisky *et al.*, 2016 and references therein), and that SSA is associated with 1000 times fewer ice nucleating active sites per surface area compared to mineral dust (McCluskey et al., 2018b), it would be unexpected to find a marine IN isolate with an extremely warm freezing onset temperature. However, the presence of bacteria closely related to the *Brevibacterium* sp. in marine environments suggests that a marine origin is possible (Fig. S8, see also discussion in Sect. 3.2). Furthermore, the backtrajectory analysis for the sample from which *Brevibacterium* sp. was isolated indicates that North Pacific sources dominated the sampling period. Actinobacteria are common in marine environments (e.g. Bull et al., 2005) and have been identified in nascent SSA (Michaud et al., 2018).

To explore the role of the growth media on isolate IN properties, controls were run on nine washed isolates (Fig. S1 and Table S4, see Methods Sect. 2.4). Five of the selected isolates were found to not be significantly IN above sterile ZoBell background, while four were chosen from the subset of significantly IN isolates. Interestingly, the observed INP concentrations of washed isolates above that of the FASW were inconsistently related to activity when grown in ZoBell media, and were generally enhanced. Seven of the nine media-free isolates exhibited significant IN behavior, including 4 isolates that were not IN in ZoBell. Some of the observed differences in ice nucleation above background between isolates suspended in ZoBell and those suspended in FASW could be a result of the differences in the background INP concentrations present in the suspension media (i.e. concentrations of INPs in FASW are less than in ZoBell, thus increasing the temperature range in which IN activity could be detected). Another possibility is that the isolates' IN behavior varied depending on multiple factors, including their viability, environment, stress, and nutrient availability. As washing cells removes soluble molecules, the apparent IN activity of washed suspensions could indicate that the source of IN activity is membrane-associated, or alternatively, that expression of IN activity is sensitive to environmental factors. For example, limited nutrient availability has been shown to enhance IN behavior of both *Lysinibacillus* sp. and *P. syringae* sp. (Failor et al., 2017a; Nemecek-Marshall et al., 1993). The difference in IN activity between ZoBell and FASW suspensions indicates that *in situ* measurements of IN bacteria will be necessary to determine the abundance of active IN microbes in the atmosphere.

Another limitation of the cultivation approach is that the concentrations of the IN species in the precipitation samples from which we derived them are unknown. Considering that typically, only a fraction of an IN isolate's cells are actively ice nucleating, even for highly efficient IN microbes such as *P.syringae* (2-4%, Amato et al., 2015), concentrations of active IN microbes in the precipitation samples were likely below the limit of detection (0.675 mL^{-1}, see Sect. 2.1). For example, assuming a high active fraction of 2% and the maximum concentration of cultivable microbes in precipitation observed in (Failor et al., 2017b, ~22,000 L^{-1}), the concentration of actively ice nucleating microbes equal 0.44 mL^{-1}, which is below the limit of detection.

One study of note (Failor *et al.*, 2017) used similar cultivation and INP measurement techniques on precipitation samples. While Failor et al. (2017) did not report estimates of source regions or claim marine origin of the IN microbes that were cultivable in marine growth media, they report the presence and IN freezing temperatures of *Pseudomonas* sp. and *Pantoea* sp., both of which were also found here (see also Fall and Schnell, 1985). Additionally, whereas (Failor *et al.*, 2017) reports warm freezing temperatures between -4 and -12 °C for multiple *Pseudomonas* sp. samples, none of the *Pseudomonas* sp. isolated in our study exhibited detectable IN activity. Similarly, pseudomonads were common, but all lacked ice nucleation activity in rain and cloud water samples collected on the coast of Scotland (Ahern et al., 2007). IN observations for *Pantoea* sp. also differ. The *Pantoea* sp. isolate in our study exhibited a moderate IN freezing temperature of -17 °C, but (Failor *et al.*, 2017) reports warm freezing activity between -4 and -10 °C.

Interestingly, Failor *et al.'s* (2017) results show discrepancies between IN behavior of isolates directly plated from precipitation samples and those from suspensions of purified strains, supporting our findings that IN behavior can vary between different isolate suspensions. (Failor et al., 2017) suggests that changes in an isolate's IN activity may be explained in part by growth conditions not conducive for the expression of INA, and that INA molecules might generally be produced in higher amounts in oligotrophic conditions, like those found in the atmosphere. In addition to environment-dependent changes in isolate IN activity, the differences between the two

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studies could also be the result of inherent differences in IN activity between different strains of the same species (Morris et al., 2008). Finally, whereas (Failor *et al.*, 2017) report only IN Gammaproteobateria that were cultivable in marine growth media, we find greater diversity among the IN isolate taxonomies, including Actinobacteria, Bacilli, Saccharomycetes, and Tremellomycetes.

4 Conclusions

Through isolation and identification of multiple IN microbes in precipitation and aerosol, this study reveals two specific marine INP identities, *Idiomarina* sp. and *Psychrobacter* sp. 1c2, confirming previous mesocosm studies' implication of marine microbes as INP candidates (McCluskey et al., 2017, 2018a). Furthermore, we isolated six new IN gram-positive bacteria capable of ice-nucleation, as prior to this study, *Lysinibacillus* sp. was the only gram-negative species capable of ice nucleation (Failor et al., 2017). Additionally, through cell washing experiments in which soluble molecules and growth media are eliminated from isolate suspensions, we find that most isolates' IN activities are dependent on growth conditions.

Due to the challenge of distinguishing marine from terrestrial INPs in environmental samples, it is impossible to definitively claim marine or terrestrial origins for 10 of the 14 IN isolates featured in this study. In order to survive atmospheric transport and deposition through rainwater, cultivable isolates derived from precipitation must be tolerant of near-freshwater conditions. However, marine origins are possible for the following reasons: aerosol back-trajectories and INP observations during sampling events indicate that marine sources were dominant (Figs. 1-2), multiple isolate sequences show similarity to marine isolation sources in reference sequences (Figs. 3, S8), and isolate freezing temperatures are generally in agreement with previously documented nascent SSA IN freezing temperatures (DeMott et al., 2016; McCluskey et al., 2017, 2018a).

While cultivation methods preclude quantification of atmospheric abundance and exclude a large fraction of uncultivable microorganisms, we captured several possible contributors to precipitation IN populations and through isolation maintained the ability to assess their IN activity and other characteristics. Considering the general rarity of atmospheric INPs (1 in 10^5 at -20 °C) (Rogers et al., 1998), the relatively lower concentrations of INPs in marine air masses (DeMott et al., 2016; McCluskey et al., 2018c), and the rarity of cultivable microbes, it is quite surprising that a substantial fraction of the cultivable microbial isolates from precipitation samples were found to be IN at temperatures above -17 °C (11 out of 34 total, or 32%), and suggests that there are more – i.e., a significant fraction – of IN species in aerosols among the substantially larger uncultivable community.

Finally, as cultivable populations represent a small fraction of the total microbial community, future studies should combine INP measurements with state-of-the-art sequencing approaches to better identify the putative IN microbes that may not be recovered by cultivation techniques. Further study is also needed to understand the factors, such as atmospheric processing or nutrient limitation, that inhibit or enhance microbe IN behavior, as well as the factors that modulate the emissions of IN bacteria emission from the ocean surface.

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Data Availability: The data set supporting this manuscript is hosted by the UCSD Library Digital Collections (https://doi.org/10.6075/J0GQ6W2Z).

Supplement Link: The supplement related to this article is available here:

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Author Contributions: CMB and JMM wrote the manuscript, prepared figures, and conducted the field campaign and laboratory measurements. MAF contributed HRRR analysis and characterized meteorology during sampling periods. JD provided phylogenetic analyses and prepared figures. GCC supported FLEXPART simulations. MDS, MDB, TCH, PJD and KAP provided feedback on the study design, analyses and manuscript. KAP and PJD are principal investigators on awards CHE-1801971 and AGS-1451347, and MDB is the principal investigator on award IOS-1516156.

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