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Characterization of airborne ice-nucleation-active bacteria and bacterial fragments

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HIGHLIGHTS

- Wet deposition of ice nucleation active (INA) *Pseudomonas* strains was observed.
- Precipitation contained high density (199–482 L^{-1}) of submicron INA cell fragments.
- INA cells either had a long-distance continental or a local origin.
- 12% of all cultivable bacteria carried *ina* genes and were INA at ≤ -7 °C.
- Isolated INA strains excreted INA outer membrane vesicles at low concentrations.

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ABSTRACT

Some bacteria have the unique capacity of synthesising ice-nucleation-active (INA) proteins and exposing them at their outer membrane surface. As INA bacteria enter the atmosphere, they may impact the formation of clouds and precipitation. We studied members of airborne bacterial communities for their capacity to catalyse ice formation and we report on the excretion of INA proteins by airborne *Pseudomonas* sp. We also observed for the first time that INA biological fragments <220 nm were present in precipitation samples (199 and 482 INA fragments per L of precipitation), which confirms the presence of submicron INA biological fragments in the atmosphere. During 14 precipitation events, strains affiliated with the genus *Pseudomonas*, which are known to carry *ina* genes, were dominant. A screening for INA properties revealed that ~12% of the cultivable bacteria caused ice formation at ≤ -7 °C. They had likely been emitted to the atmosphere from terrestrial surfaces, e.g. by convective transport. We tested the ability of isolated INA strains to produce outer membrane vesicles and found that two isolates could do so. However, only very few INA vesicles were released per INA cell. Thus, the source of the submicron INA proteinaceous particles that we detected in the atmosphere remains to be elucidated.

1. Introduction

Cloud development is among the major processes shaping the meteorological and climatic conditions on Earth. Precipitating clouds cannot develop purely through the process of condensation, but also rely on cloud droplets coalescing and on ice crystals getting formed and growing. In fact, a major part of precipitation in

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temperate regions depends on the process of freezing. But as pure water freezes at about −38 °C, the ice formation at temperatures between −38 °C and 0 °C depends on the presence of particles that lower the energy barrier for the freezing of water and thus act as ice nuclei (IN). Mineral dust is often considered the most prominent class of IN in the atmosphere, but the role of biological aerosols recently drew attention (DeMott and Prenni, 2010; DeMott et al., 2010; Despres et al., 2012) as bioaerosols promote ice formation at much higher temperatures than mineral aerosols. While atmospheric ice nucleation below −15 °C is dominated by mineral dust, biological IN are the only reported particles that promote ice formation above this temperature (Murray et al., 2012; Hoose and Möhler, 2012). Especially, some bacterial plant pathogens, belonging to the genera *Pseudomonas*, *Xanthomonas* and *Erwinia*, are efficient IN and could therefore be involved in precipitation processes of mixed phase clouds (Möhler et al., 2007).

Freezing induced by bacteria is associated with ice-nucleation-active (INA) proteins, which are synthesised at low temperatures and under starving conditions (Nemecek-Marshall et al., 1993). After synthesis, INA proteins, which are encoded by highly conserved *ina* genes (Wolber and Warren, 1991), get anchored in the outer membrane of the cell wall, where they form oligomers (Govindarajan and Lindow, 1988; Southworth et al., 1988; Mueller et al., 1990; Schmid et al., 1997; Garnham et al., 2011). It is proposed that these INA protein oligomers provide a template for the formation of ice crystals as it has been shown that the probability of ice formation at higher temperatures increases with increasing number of INA proteins in oligomers (Schmid et al., 1997). Although the presence of the outer cell membrane is crucial for INA protein function (Govindarajan and Lindow, 1988), viability of cells is unnecessary and cell fragments carrying INA proteins are sufficient to induce freezing (Maki and Willoughby, 1978; Lindow et al., 1989; Hartmann et al., 2013). The cell benefits from INA proteins, as these can promote frost damage on plant leaves and thus provide access to nutrients leaking from disrupted plant tissues (Lindow et al., 1982; Buttner and Amy, 1989). It has also been proposed that INA positive cells have an additional advantage due to the increased wet deposition probability out of the atmosphere facilitated by INA proteins that thus help the INA bacteria to colonise novel environments on ground (Morris et al., 2004). As viable INA *Pseudomonas syringae* cells were found in all parts of the water cycle around the globe, Morris et al. (2008) suggested that this species is evolutionary adapted to dissemination via aerosolization and wet deposition.

A few studies using climatic models to investigate the relevance of INA bacteria question their importance for atmospheric processes (e.g. Phillips et al., 2009; Hoose et al., 2010; Sesartic et al., 2011), citing the low densities of INA bacteria in the atmosphere, which were proposed to be up to 3 orders of magnitude less abundant than inorganic IN (DeMott et al., 2010). To date, however, the densities of bacterial INA proteins in the atmosphere have not been well constrained, which is in large part due to methodological difficulties. INA proteins can be found in the atmosphere in different forms, including (i) viable INA cells, (ii) non-viable INA cells, (iii) single INA cell fragments, and (iv) INA cell fragments attached to mineral particles. Several studies that either analysed viable INA cells, *ina* genes or total proteinaceous IN have been conducted in order to infer the atmospheric densities of INA proteins. Christner et al. (2008a) showed that total proteinaceous IN can be found in precipitation at high densities of up to 290 bacterial proteinaceous IN per L. Screening viable cells from cloud water belonging to γ-Proteobacteria, Joly et al. (2013) found that 2.7% of cultivable strains were ice-active at ≤ −8 °C. A study using molecular methods to investigate the presence of *ina* genes in bacterial isolates (Ahern et al., 2007) failed to detect INA bacteria in cloud water. Trying to quantify *ina* genes in air over agricultural fields, Garcia et al. (2012) found that they were below the detection limit in all but one sample collected during corn harvesting, during which they found 19 100 *ina* genes per m^3 of air. Recently, Hill et al. (2014) successfully quantified *ina* genes in a series of precipitation samples and found that there were between < 10 and 1200 bacterial *ina* genes per L of melted snow or hail. It is unlikely that the atmospheric density of *ina* genes would be reflected in the density of INA proteins, as often only a low proportion of cells that possess an *ina* gene also produce INA proteins. When Garcia et al. (2012) and Hill et al. (2014) compared the numbers of *ina* genes to the density of total proteinaceous IN they found a 0%–30% concomitant presence of INA proteins. Only in one case the density of proteinaceous IN corresponded to the *ina* gene density (Garcia et al., 2012). There is a general discrepancy in the reported densities assessed by different methodologies, such that the densities of total proteinaceous IN are usually much higher than the densities of viable INA bacteria or *ina* genes. This may be a result of biological fragments that get airborne either as individual particles or in association with mineral soil particles. They may be much more abundant than the intact cells, but are difficult to detect and characterise *in situ*. Lately, it was demonstrated that proteinaceous and biogenic organic matter of unknown origin, associated with mineral soil particles, may be important for atmospheric ice nucleation at low supercooling, i.e. between 0 and −12 ° C (Conen et al., 2011; O'Sullivan et al., 2014). Thus, INA bacterial fragments may be more important for formation of precipitation than intact cells.

A common mechanism that enables bacterial cells to actively secrete insoluble molecules, such as INA proteins, is the formation of outer membrane vesicles (OMV). These OMV are small circular structures that bulge off the bacterial outer membrane and are between 20 and 250 nm in diameter (Kulp and Kuehn, 2010). Phelps et al. (1986) were the first to show that INA bacteria could secrete INA proteins associated to their OMV into the environment. Later, the transport of INA proteins into OMV was demonstrated by immunohistochemical methods (Michigami et al., 1995). Hitherto, the importance of this excretion mechanism for INA proteins in terms of its atmospheric effects has not been well understood. Although OMV that carry INA proteins were shown on several occasion to have a similar ice nucleation activity as intact INA bacterial cells (e.g. Phelps et al., 1986; Obata et al., 1990), the fraction of OMV that are ice-active is not known. In addition, particles that are in the size range of OMV and carry INA proteins have not yet been detected or quantified in the atmosphere.

We studied members of airborne bacterial communities in 14 precipitation samples for their capacity to catalyse ice formation and we report for the first time on the presence of submicron proteinaceous INA fragments (<0.2 μm) in samples from two precipitation events, which confirmed that INA biological fragments are present in the atmosphere. INA strains of *Pseudomonas* sp. isolated from rain samples were able to excrete outer membrane vesicles (OMV), which we characterised for their size and ice nucleation activity.

2. Material and methods

2.1. Sample collection and concentration

Between February and October of 2009, 9 rain and 5 snow samples were collected using a sterile stainless steel funnel (Table 1) at ~30 m above ground level (N55°41′29.51"/