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Ice nucleation activity of bacteria isolated from cloud water

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HIGHLIGHTS

- Ice nucleation active (INA) bacteria were recovered from cloud water.
- 16% of the *Pseudomonas*-like bacteria isolated from cloud water are INA.
- Active strains identified as *Pseudomonas syringae*, *Xanthomonas* sp. and *Pseudoxanthomonas* sp.
- The *P. syringae* strains from clouds belong to rare clades in the environment.
- The number of bacterial IN in cloud water was estimated to fall between 0 and 500/mL.

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ABSTRACT

Some Gamma–Proteobacteria can catalyze ice formation thereby potentially contributing to the induction of precipitation in supercooled clouds and subsequently to bacterial deposition. Forty-four bacterial strains from cloud water were screened for their capacity to induce freezing. Seven strains (16%) were active at −8 °C or warmer and were identified as *Pseudomonas syringae*, *Xanthomonas* spp. and *Pseudoxanthomonas* sp. Phylogenetic analysis revealed that the *P. syringae* strains in clouds at the Puy de Dôme belonged to clades that are among the most infrequently detected in the environment, while widespread clades were absent suggesting some extent of selection or unusual biogeography of the bacteria at the sampling site. Three strains induced freezing at −3 °C while the others nucleated ice at −4 °C to −6 °C. The freezing profiles revealed that the peaks of activity were centered around −3.5 °C, −5 °C and/or −8.5 °C depending on the strain. The frequency of ice-nuclei (IN) per cell at −6 °C was generally below 0.5% and reached up to 4.2% in one strain. We estimated that clouds influenced by vegetated areas would carry between less than 1 and ~500 bacterial IN mL^{-1} of water active between −3 °C and −10 °C depending on the season. These data will contribute to modeling the impact of bacterial IN on precipitation at regional scales.

1. Introduction

Ice nucleation (IN) activity, *i.e.* the capacity to induce the crystallization of supercooled water (a metastable state whereby water is liquid at subzero temperatures), has been reported in a variety of microorganisms including diatoms (Knopf et al., 2011), pollen (Diehl et al., 2001), fungi (e.g. Iannone et al., 2011) and bacteria (e.g. Maki et al., 1974; Vali et al., 1976). In bacteria, this property is conferred by a single gene (for example, inaZ in *Pseudomonas syringae*; Green and Warren, 1985) coding for a membrane protein that acts as a template for the arrangement of water molecules in crystals. Gamma–Proteobacteria affiliated to the genera *Pseudomonas*, *Pantoea*, *Erwinia* and *Xanthomonas* are the most efficient IN active (INA) bacteria described so far: some of them can catalyze freezing of supercooled water at a temperature as warm as −2 °C (e.g. Cochet and Widehem, 2000). These bacteria are wide-spread on plants, except for coniferous trees; they are often plant pathogens and can cause frost injury thereby

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assuring entry into the leaf tissue (Lindow et al., 1978). Their presence and particularly that of the well-known epiphytic bacterium *P. syringae* has been reported in a variety of habitats associated with the water cycle, from vegetation to precipitation at concentrations ranging from $\sim 10^2$ to $\sim 10^5$ L^{-1} in freshwater environments (Constantinidou et al., 1990; Hirano et al., 1996; Morris et al., 2008; Šantl-Temkiv et al., 2009).

In the atmosphere as aerosols, such bacteria could play important roles by nucleating ice in supercooled clouds, which generally results in precipitation at mid-latitudes (Lohmann and Feitcher, 2005). Hence, it was proposed that INA bacteria in clouds could contribute to the induction of precipitation and cause at the same time their own redeposition (Sands et al., 1982; Morris et al., 2010). The capacity of INA bacteria to glaciate clouds was demonstrated in cloud chamber experiments (Maki and Willoughby, 1978; Möhler et al., 2008), but numerical simulations suggested that their presence in the atmosphere has probably no impact on the precipitation rate at the global scale, which does not exclude the existence of regional or local effects (Hoose et al., 2010; Sesartic et al., 2011). Within this context, there is still considerable debate about the extent of the importance of INA bacteria in the atmosphere and about the influence that the capacity to induce freezing could play on the distribution of bacteria between the different compartments of the water cycle. A substantial effort has been made over the last few years in order to detect, characterize and quantify INA particles and bacteria in the atmosphere. Observations indicated that about one third of the ice crystals sampled by aircraft at high altitude in a cloud event over Wyoming contained a solid residue with a biological signature (Pratt et al., 2009). In freshly-fallen snow collected worldwide, a large fraction (up to 100%) of the particles active as ice nuclei at temperatures of −7 °C or warmer were biological in origin, of which up to 85% were lysozyme-sensitive and assumed to be bacterial cells (Christner et al., 2008a). Moreover, rain invariably contained higher proportions of INA bacteria than air at the same locations (Stephanie and Waturangi, 2011), and contrarily to vegetation and water, all strains of *P. syringae* isolated from rain and fresh snow by Morris et al. (2008) were INA. Despite the fact that clouds are key components in the questions raised by the presence of INA bacteria in the atmosphere, to our knowledge only Ahern et al. (2007) targeted them in clouds collected in Scotland, but without success.

We reported in the past the presence of *Pseudomonads* species in cloud water samples collected at the Puy de Dôme summit, France (Amato et al., 2005, 2007; Vaïtilingom et al., 2012), but their ice nucleation activity was not investigated. Here, we tested 44 of these Gamma–Proteobacteria isolated from 14 individual cloud water samples for their capacity to catalyze the formation of ice. The ice nucleation frequency profiles of the 7 strains detected positive were established, thereby allowing us to estimate the concentration of INA cells in an average cloud over rural landscapes such as the Puy de Dôme.

2. Material and methods

2.1. Cloud water samples and microbial strains

A total of 257 bacteria isolated from 25 distinct cloud water samples collected at the Puy de Dôme site (1465 m a.s.l., Central France) were investigated: 71 strains reported in Amato et al. (2007), 185 strains reported in Vaïtilingom et al. (2012) and 1 strain not yet reported. Among them, we identified 44 *Pseudomonas*-like bacterial species potentially INA: 8 strains were isolated in 2003–2004 (reported in Amato et al., 2007), 35 strains were isolated in 2008–2010 and 1 additional strain was isolated later in 2011. These are listed in Table 1, along with the accession numbers of their 16S rRNA gene sequences when available; they were identified as *Pseudomonas* sp. (10 strains), *P. syringae* (9 strains), *Pseudomonas graminis* (5 strains), *Pseudomonas fluorescens* (3 strains), *Pseudomonas trivialis* (2 strains), *Pseudomonas poae* (1 strain), *Pseudomonas reactans* (1 strain), *Pseudomonas veronii* (1 strain), *Pseudomonas viridiflava* (1 strain), *Erwinia* spp. (5 strains), *Xanthomonas* spp. (5 strains) and *Pseudoxanthomonas* sp. (1 strain). Details concerning the procedures of sampling, the abiotic characteristics of the samples including chemical composition, and the isolation and identification of the strains are given in the corresponding references. Briefly, sterile cloud droplet impactors installed at the summit of the Puy de Dôme were used for sampling. Viable microorganisms were recovered by platting of 0.1 mL of the samples on R2A (Reasoner and Geldreich, 1985; DIFCO), R2A + NaCl 0.4%, King's B (King et al., 1954), Sabouraud (DIFCO) or TSA (DIFCO) medium and incubating at 17 °C or 5 °C until appearance of colonies (typically 5 days at 17 °C or 8 days at 5 °C). *Pseudomonas*-like colonies (yellowish and smooth colonies, eventually fluorescent under UV light), *i.e.* the bacteria that were presumably INA, were then isolated on R2A medium at 17 °C. Stock cell suspensions were made from pure cultures in their stationary phase of growth in R2 medium (17 °C, 200 r.p.m.), supplemented with 10% glycerol (final concentration) and kept at −80 °C. Cultures for further investigations were then grown from these stocks. The taxonomic affiliations of the isolates were determined according to their 16S rRNA gene sequences, the GenBank accession numbers of which are given in Table 1.

2.2. Phylogenetic analyses

The phylogenetic context of fluorescent pseudomonads isolated from clouds was determined according to the similarity of DNA sequences of three housekeeping genes: *gyrB*, *cts* (*glt*) and *gapA* (*gap1*). PCR amplifications of gene fragments and subsequent sequencing were conducted as previously described by Morris et al. (2010). Phylogenetic relationships of strains from clouds were determined relative to the most detailed phylogeny of *P. syringae* published to date (Morris et al., 2010). DNA sequences of each of the gene fragments from 16 cloud strains were aligned with the corresponding sequences of 48 strains of *P. syringae* from crops and non-agricultural habitats and 3 strains of *P. fluorescens* (Morris et al., 2010). Alignment was achieved with the ClustalW routine and sequences were cut to equal lengths with DAMBE version 5.1.1 (Xia and Xie, 2001). The phylogenetic tree was constructed from the concatenated sequences of the three genes (1493 bases) according to the neighbor-joining method in MEGA, version 5 (Kumar et al., 1994) based on the Tamura-Nei model with gamma correction and 3000 bootstrap replicates. Strains were grouped into clades according to the previously described criteria (Morris et al., 2010).

2.3. Ice nucleation assays

The *Pseudomonas*-like bacteria isolated were first screened for ice nucleation activity at −8 °C using the drop-freezing method described by Vali (1971). Cells were suspended in sterile distilled water ($OD_{575\ nm}$ of 0.3–1.9) from R2A cultures at 17 °C and incubated in ice for 1 h to allow the expression of the IN protein. Five droplets of 20 μL of these cell suspensions were deposited on the surface of an aluminum plate floating on a cooling bath (model F34-ED, Julabo Colmar France) set at −8 °C. Controls consisting of sterile distilled water droplets were tested in parallel. Strains for which at least one droplet was visually frozen after 8 min were considered INA positive, whereas those for which none of the