

Ice nucleation active bacteria and their potential role in precipitation

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Abstract. Certain bacteria that are commonly found on plants have the capacity to catalyze the freezing of supercooled water at temperatures as warm as -1°C . This is conferred by a protein in the outer membrane of the bacterial cell. Because of the abundance of these bacteria and the warm temperature at which they function as ice nuclei, they are considered to be among the most active of the naturally-occurring ice nuclei. As plant pathogens, antagonists of plant pathogens and as causal agents of frost damage, these bacteria have well-studied interactions with plants. Here we propose that these bacteria also play a role in atmospheric processes leading to rain, given that they are readily disseminated into the atmosphere and have been found in clouds at altitudes of several kilometers. That they participate in a sort of biological cycle of precipitation - whereby they are transported into clouds from plant canopies and incite rain thereby causing favorable conditions for their growth on plant surfaces - was proposed about 20 years ago. Today, sufficient evidence and meteorological tools have emerged to re-ignite interest in bioprecipitation and in the ways in which plants play a role as cloud seeders.

1. INTRODUCTION

The growth and health of plants are closely linked to atmospheric conditions. Anyone with the slightest curiosity about agriculture or conservation of forests and prairies, for example, recognizes that plants are damaged by hail and air pollution and that they depend foremost on rain. But, as climatologists have demonstrated, the relationship between plants and the atmosphere is not simply a one-way affair. Plant canopies decrease the surface reflectivity (albedo) of the Earth, thereby fostering capture of solar energy by the Earth's surface that would otherwise be lost. This energy is essential for moving water molecules up into the atmosphere. Furthermore, plants are also a source of water that can become part of the atmospheric vapor content via evapotranspiration. Hence, one can understand why desertification seems to be a self-compounding process.

Are these the only ways in which plants influence climate? For over 20 years, a few meteorologists and plant pathologists have suspected that plants contribute another important player in the process of rain-making: ice nuclei. This idea is based on the fact that plants - whether healthy or diseased - are covered with bacteria and that some of these bacteria have the very unique capacity of catalyzing ice formation at very warm temperatures. As exciting and intriguing as this idea may be, it has been on the back burner for several decades. Numerous contemporary factors are bringing it into the forefront today. These include expanding and recurring droughts, the growing concern over possibly irremediable changes in our planet's climate and the availability of more and more refined analytical tools for understanding the microphysics of atmospheric processes.

2. THE BIOLOGICAL COMPONENT OF AEROSOLS

Outside of urban zones, the air over continents contains 3×10^9 to 5×10^{10} particles/m³. Mineral dusts are abundant in atmospheric aerosols. In polluted regions a major component of these aerosols can be soot. But, anyone afflicted with allergies to pollen knows that there is a biological component to atmospheric aerosols. In fact, up to 25 % of the insoluble part of aerosols can be of biological origin [1]. Pollen is only one of the biological components of aerosols and is only one of the types of particles that plants contribute to the atmosphere.

The seemingly abiotic atmosphere that surrounds our planet is a bouillon of biological bits including bacteria; protozoa; spores of fungi, ferns and mosses; virus particles; parts of insects and dust mites; algae and pollen grains. In some cases, these biotic particles are dead debris that are picked up into the air. But often, aerial dissemination is an integral part of the life cycle of organisms, a sort of boulevard to opportunities for species out-crossing and to supplemental food and water resources. At altitudes below about 15 m, there are hundreds to thousands of particles of biological origin per m³ of air. Over a suburban region the quantity of microorganisms, for example, in the air has been observed to be as high as 7000 culturable propagules of fungi and 1600 cultural bacteria/m³ [2]. The bulk of the popular interest in aerobiology concerns the presence – in the air we breath - of biological particles with direct negative impacts on human health such as allergens, causal agents of pneumonia and other lung diseases and of septicemias in hospital environments. The Andersen sampler, widely used in studies of aerobiology, was conceived to simulate uptake of particles by the human respiratory system [3].

This anthropocentric vision of the origin and impact of biological particles in the air is only one aspect of aerobiology. For bacteria, the protagonist of our story, airborne cells originate principally from plants and soil [4]. Thousands of bacteria/m³ have been detected in the air above forests and wheat canopies for example [4]. Agricultural activities such as combining and bailing can shoot this value up to 10⁶ to 10⁹ bacteria/m³ [5]. Flux of bacteria emitted from plant canopies has also been measured. For bean, alfalfa and wheat canopies, bacteria are emitted at a net upward rate of 50 to 500 culturable cells/m²/sec [6]. Combining and bailing can dramatically increase these values [5].

3. MICROORGANISMS ON AERIAL PLANT PARTS

The surfaces of aerial plant parts (leaves, shoots, flowers and fruits) harbor large numbers of microorganisms, mostly bacteria but also filamentous fungi, yeasts and protozoa. Here we focus on bacteria, unicellular organisms capable of a wide range of metabolic activities, many of them associated with decomposition but also important among the primary producers in Earth's food webs. They are typically rod-shaped, about 1-3 μm long and 0.3 – 0.5 μm wide, although sizes and shapes vary. When they are flagellated they are capable of autonomous movement when liquid is present. An image of a single cell of the plant pathogenic bacterium, *Pseudomonas syringae*, is presented in Fig. 1.

A typical healthy leaf of a mature plant may carry up to 10⁷ bacteria per cm² and a much smaller population of fungi and other microorganisms. If we think about the number of leaves on a tree or a bean or wheat plant and the number of plants on Earth, we can easily see that the plant kingdom is a significant habitat for microorganisms at a global scale. In fact, of the 10³⁰ bacterial inhabitants of earth [7], it has been estimated that about 10^{24–26} are found on leaf surfaces [8]. These microbes interact with each other and with their plant host in a number of ways. The most well-studied microorganisms of plant surfaces are plant pathogens because of their obvious economic impact on agriculture. They destroy or restrict growth and productivity of their plant hosts. But the majority of microorganisms reside on a leaf without being detrimental, and pathogens themselves often live benignly with plants.

Microorganisms are capable of living in very diverse environments: the leaf surface, the soil, sea and fresh water, on the surface and inside of animals, insects, and fish. Some have evolved and adapted to life in extreme environments: hot springs or at the bottom of oceans with very little or no oxygen and at extreme salt concentrations. They have even been isolated from hydrothermal vents, one species recently

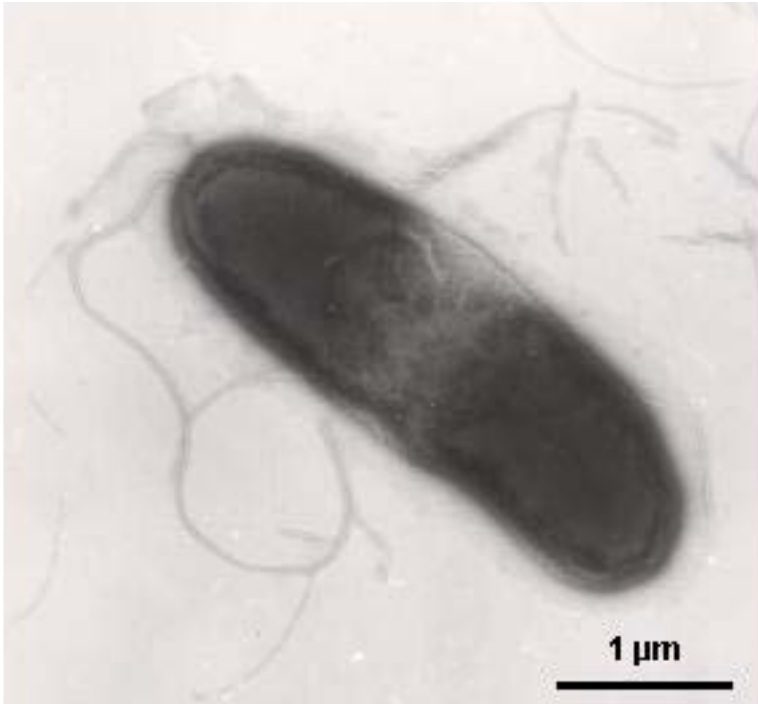


Figure 1. Transmission electron microscope image of a cell of the plant pathogenic and ice nucleation active bacterium *Pseudomonas syringae* pv. *aptata*. The cell is ca. 2.7 μm long. Several polar flagella and bits of broken flagella are visible.

found capable of withstanding a temperature of 121°C, the standard temperature of sterilization [9]. Although extreme, these environments might offer physical conditions that are more stable than those on the surface of a leaf where fluctuations in temperature, availability of water and nutrients and incident radiation are dramatic over both the short term (daily cycle) and the long term (seasons) [10].

The microbiology of aerial plant parts has been an active field of investigation since 1970 and comprehensive reviews and texts on the subject are published regularly [11-19]. In the next paragraphs we will describe some of the aspects of microbial life on aerial plant parts with particular reference to those that could influence the uptake into and fate in the atmosphere of ice nucleation active (INA) bacteria.

3.1. Origin and fate of leaf surface colonists: comings and goings

A newly emerging leaf from the growing top of a plant initially carries very few microorganisms. Yet, during the life cycle of a plant, this leaf will be colonized by large numbers of microorganisms, especially bacteria. The colonization of leaves by microorganisms has been likened to the colonization of islands where individuals can immigrate to a leaf, find a niche, survive and multiply. Individuals can also be lost from the population via death and emigration. These processes are affected by environmental conditions and plant species.

Where do these microorganisms come from? For several plant pathogens, a first source is the seed. When the seed germinates, microorganisms begin to grow on the exudates of the seed (rich in nutrients) and multiply on the seedling, colonizing the newly emerging leaves [20-23]. Some foliar pathogens have been reported to persist in the soil, thus being able to bridge the gap between an annual crop and the subsequent one [24]. In woody perennial plants, bacteria over-winter in dormant buds [25]. Plant

pathogenic bacteria can also over-winter in lake water used for irrigation [26]. Bacteria from plant surfaces can become part of atmospheric aerosols and then land on other plants via dry deposition. For example, Lindemann et al. [27] planted snap beans in fields situated either in a bean-growing area or in an area where no beans were cultivated. They found higher populations of bacteria on leaves from the commercial bean area and speculated that aerial dissemination of bacteria from neighboring crops was the reason for this difference. Likewise, bean cultivars that typically harbor low populations of epiphytic bacteria can in fact harbor significantly enhanced population sizes if grown among a heavily populated cultivar due to local aerial dissemination of bacteria [28]. During rain, these airborne bacteria are scrubbed from the air and their net flux is downward [29].

The atmospheric transport of bacteria implies that they are capable of escaping from the leaf surface. Bacteria are easily found in the atmosphere above fields at all times of the day. This happens especially on dry, sunny days. Detailed studies of airborne bacteria above bean or grass fields indicate that the highest numbers occur around noon, from about 1000 h to 1400 h, when the air is warmest and the atmosphere is the least stable [30, 31]. As mentioned above, upward movement of bacteria from plant canopies is on the order of 50 to 500 culturable cells/m²/sec [6]. Dry deposition rates are about 1 to 10 bacteria/m²/sec [6] suggesting that a much greater number of bacteria are moving up into the atmosphere than are falling back to the Earth's surface during the day. Some key aspects of bacterial immigration from aerial plant surfaces remain unknown: the mechanism by which bacteria leave the plant surface and enter the atmosphere and the necessary forces; the viability of airborne bacteria; and the nature of airborne particles containing bacteria (as single cells, carried on particles of plant origin, in aggregates or biofilms, etc.). We have suggested previously that one of the easiest means for leaf surface bacteria to be launched into the air would involve breaking-off of bacterial aggregates or biofilms from leaf hairs [32]. This corresponds to the way in which bacteria grow on leaf surfaces (described below) but remains to be explored.

3.2. Life on a leaf

Aside from immigrating and emigrating, plant surface bacteria eat, reproduce, probably spend large periods of time waiting (surviving) when conditions for growth are hostile, and die. The leaf environment can change dramatically during the day in terms of temperature, moisture and solar radiation [10]. Plant surface bacteria have adapted to these conditions by developing certain traits. Many species of these bacteria produce pigments, which protect them from the detrimental effects of UV radiation, which causes mutations and eventual death, depending on intensity and time of exposure [33]. Other species produce a thick coat of extracellular polysaccharides for protection against desiccation. This extracellular material leads to the formation of bacterial aggregates called biofilms (Fig. 2) which might offer a wide range of survival advantages for bacteria on plant surfaces [32].

The variability of the plant surface environment leads to marked variability in bacterial population sizes at various scales of time and space. In general, population sizes on leaves are initially very low on young plants but as the season progresses, these sizes increase. Bacterial growth on plants depends on plant species and variety, age, and the micro-architecture of plant surfaces. All of these contribute to the micro-environmental conditions that bacteria witness. Large differences in bacterial population sizes between plant species [34], among cultivars of the same species [35] and among leaves in a stand of a single cultivar [36] have been observed. Bacteria use nutrients on the plant surface for growth and the variability in the available quantities among plants and at different sites on plant surfaces may be a factor contributing to variability in population sizes. These nutrients (sugars, organic acids, amino acids, etc.) diffuse either from natural openings (stomata, involved in respiration and evapotranspiration) or wounds of the leaf epidermis [37, 38]. Part of the nutrients may also originate from dead, lysed microbial cells. These nutrients concentrate in cavities of the leaf surface after rounds of hydration (rain, dew), water flow and dehydration. Nutrients also leak from leaf hairs and glandular trichomes which are often densely populated with bacteria [39]. Plant architecture and age also play roles: bacterial populations frequently

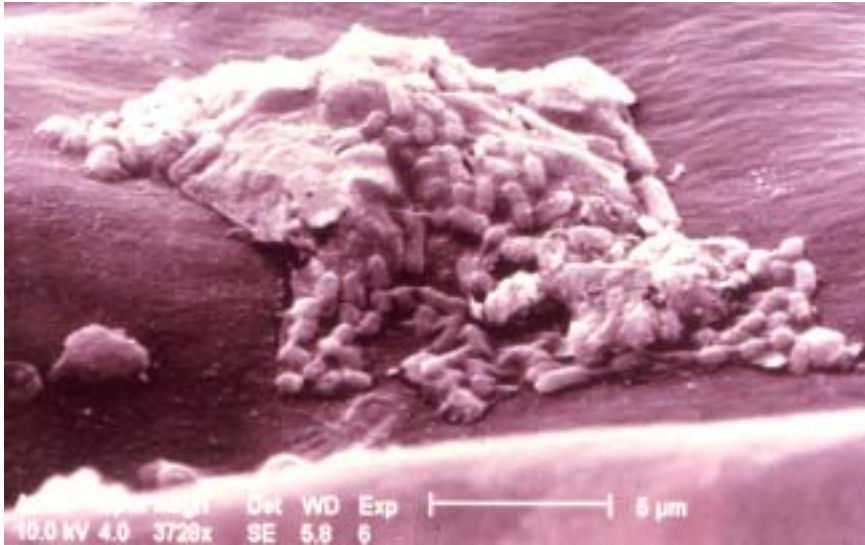


Figure 2. Scanning electron microscope image of a bacterial biofilm naturally occurring on the leaf of broad-leaved endive. The biofilm is sitting on the amorphous cuticular wax characteristic of the leaf surface of this plant. The exopolymeric matrix enrobing this biofilm, which is normally well hydrated, has been partially destroyed during the desiccation of the sample necessary to prepare it for microscopic observation.

are higher on older leaves at lower parts of the plant [40, 41] which are shielded from direct sunlight, may experience higher relative humidity or may exude more nutrients. There is also considerable fluctuation of bacterial populations over short time periods and over an entire growing season. Monitoring populations of INA bacteria on bean leaves at regular time intervals during 24 hour time periods showed that this fluctuation can exceed one order of magnitude (over 10-fold differences between high and low population sizes per leaf). The lowest populations of bacteria were observed during the warmest time of the day [42]. Interestingly, the decrease in population sizes coincided with the period of maximum immigration of bacteria from the plant surface. Flushes of bacterial populations on leaves also seems to coincide with rain, but in particular with intense rain falls. The momentum of the rain, rather than simply the quantity of rain water falling, is an essential factor for these flushes [43].

As mentioned previously, there is an important diversity of microorganisms on aerial plant surfaces. Here, we have made particular reference to bacteria that are plant pathogens or that are active as ice nuclei, the latter group occurring commonly on a wide variety of plants particularly in temperate regions [44]. But it must be kept in mind that these bacteria coincide with and are likely to interact with hundreds of other species of bacteria and other types of microorganisms and insects. The processes involved in colonization of plants and in aerial dissemination evoked for INA and plant pathogenic bacteria are also pertinent to these other organisms.

4. DISCOVERY OF THE ICE NUCLEATION ACTIVITY OF BACTERIA

Historical themes in the various branches of science have an amazing similarity. Discovery marches through several layers of complexity, starting with some initial and often startling observation, followed by a description phase, then by explanation and even application and an over-all manipulation [45]. The first observation is often made by someone in a peripheral field. This certainly appears to be the case for INA bacteria. Although all of the groups of bacteria capable of ice nucleation are usually associated with plants, they were first recognized as catalysts of ice formation by meteorologists [46-48]. The French meteorologist Soulage was the first to identify bacterial cells in an ice crystal [49] although he did not

know at the time about the potential ice nucleation activity of bacteria. Vali in Wyoming observed some association of ice nucleation with plant debris and reported the presence of bacteria in the center of atmospheric ice crystals [48]. In short, the field of biogenic ice nucleation was born.

It was not immediately clear what bacteria were responsible. By using large arrays of tests of physiological properties of bacteria, plant pathologists at that time (the early 1970's) were just learning how to differentiate plant pathogenic bacteria from each other and from the numerous plant-associated bacteria that did not cause disease. A numerical taxonomy using computer analysis that made such large scale physiological studies (200 tests \times 200 strains of bacteria) possible was first developed in biology - by entomologists - to differentiate groups of insects [50]. Via such analysis, the *Pseudomonas syringae* group had just been found to be a rather homogenous physiological entity [51] that for practical reasons was subdivided into a multitude of pathotypes or pathovars based on the principle plants to which they caused disease [52, 53]. For example, *P. syringae* pathovar *syringae* denotes strains originally isolated from lilac (a plant in the genus *Syringa*) and *P. syringae* pathovar *pisi* denotes strains from pea (genus *Pisum*). Similarly, other plant-associated bacteria such as *Erwinia herbicola* and *Xanthomonas campestris* were being differentiated in terms of their physiology and host range. It became clear that only a few physiologically distinct groups of bacteria associated with plants were capable of ice nucleation "in vitro" and that they could "cause" frost injury of plants [54].

Most bacteria do not have a surface protein that interacts with the structure of liquid water and serves as a template for ice crystallization as described in section 6, below. Hence, answers about the nature of this unique property came quickly with the advent of molecular genetics. Discovery of the gene controlling the ice nucleation capacity of bacteria was an early advance in the study of how bacteria cause disease [55]. The gene encoding the INA protein was cloned, sequenced and a protein sequence region of eight amino acids in tandem repeats was clearly demonstrated to be the actual protein sequence associated with lining up the water crystal, in essence serving as a rigid template on which ice could reach critical mass [56]. The role of INA plant pathogens in causing frost injury to crop plants was firmly established [57]. The focus quickly moved from a science based on field observation coupled with diagnostic bacteriology to a subset of molecular biology where genes and their structure and regulation, and the corresponding proteins and their structure, were given great importance. Yet despite numerous publications reporting molecular aspects of INA genes and proteins and their regulation over the ensuing 30 years, precious little has been done to protect plants from frost injury as a result of these studies. The few practical applications of this intense research are described in section 7, below. The combination of bacterial physiology, computer science in the form of cluster analysis, and genetic reductionism that marked this intense period of study in the 1980's could have led the field of plant pathology into an area with global implications. Some key incentives and tools were missing for the serious investigation of questions about a more global impact of these bacteria. These questions might find their awakening in the 21st century.

5. ROLE OF HETEROGENEOUS ICE NUCLEATION IN FREEZING

In the absence of catalysts for freezing, water can remain in a metastable liquid state at temperatures well below 0°C. Water in the liquid state at temperatures below 0°C is referred to as supercooled water. Spontaneous freezing of supercooled water occurs below -39°C. Hence, freezing catalysts are clearly essential for natural freezing processes outside of polar regions and extreme winter seasons on Earth and in much of the corresponding troposphere.

The phase change from supercooled liquid water to solid ice involves the clustering of a few molecules of water into a condensed phase. When this cluster reaches a critical size it can grow into an ice nucleus. This nucleus then initiates a rapid cascade of binding of water molecules leading to the formation of solid ice. The growth of initial clusters, or embryos, into nuclei is ruled by the quantity of energy necessary for attachment and detachment of water molecules from embryos relative to the overall energy budget of the bulk system. For ice formation in pure water at -40°C, an embryo of critical size to permit growth requires

the clustering of roughly 70 water molecules. For ice to form in pure water at -5°C , 45000 molecules would be needed to overcome energy barriers to growth of the embryo and subsequent ice formation [58]. A schematic representation of the process has been created by Matsumoto and colleagues [59] via a virtual molecular simulation of nucleation. Further details of the physics involved in ice nucleation are described in detail in Vali [58].

When the ice embryo forms on certain foreign (non water) surfaces, the energy barriers leading to nucleation can be overcome more readily than when the ice embryo forms in an environment of pure water. In this case, the energy of interaction between the foreign surface and the ice embryo is defined by the contact angle of the cluster of water molecules with the surface. The energy barrier to nucleation decreases as the contact angle decreases. From this simple geometric model of the physics of ice nucleation on foreign surfaces (called heterogeneous ice nucleation), it has been estimated that only 600 molecules of water would be needed to form an embryo of critical size to permit growth at -5°C if the cluster had a 30° contact angle with the surface [58].

A wide range of substances are able to catalyse heterogeneous ice nucleation. Various inorganic crystalline solids (the most well known of which is silver iodide), amino acid crystals, monolayers of long chain alcohols and organic compounds such as phloroglucinol and metaldehyde can catalyse the freezing of supercooled water [60]. Substances that are abundant in atmospheric aerosols, such as soot [61], mineral dusts and metallic particles [62] are also ice nucleation active. Some of these, such as aluminium oxide, originate from the combustion of rocket fuels [63]. Under laboratory conditions, combustion of one gram of rocket propellant produces up to 10^{11} ice nuclei active at -20°C (rockets use about 10^9 g of propellant per launch), but these nuclei seem to be short lived [63]. In the atmosphere near forests, combinations of terpenes and of other tree oils with iodine have been detected [64]. Some of these substances are active as ice nuclei at -4°C . The burning of plant material can also generate INA particles in the atmosphere, and may be the basis for certain ancient rituals to secure rain. It has been proposed that the ice nucleation activity of these particles is due to iodine contained in the plant [63].

Diverse organisms produce INA materials. Almost all of the INA materials of biological origin are proteins or proteinaceous compounds that may be associated with lipids, phospholipids and/or carbohydrates [65]. INA organisms include plants, fungi, bacteria, vertebrates and invertebrates. When ice nucleators occur in freeze-tolerant organisms, it is believed that they generally play a role in controlling and stabilizing the growth and morphology of ice crystals, thereby allowing the organism to avoid damage to cell membranes. However, in some cases the occurrence of ice nucleators in organisms might simply be incidental [65].

The flowers of *Lobelia telekii* [66] contain substances active as ice nuclei at -4.5°C . Proteinaceous ice nuclei have been detected in plants such as *Prunus* spp [67] and winter rye [68]. Pollen of certain plants can also be active as ice nuclei. Pollen from grasses and from pine and oak trees is active between about -8 and -11°C whereas that of birch is active at temperatures as warm as -5°C depending on how the freezing test is conducted [69]. The free-living fungi known to produce INA materials are all species of the genus *Fusarium* [70-72]. These fungi and their culture filtrates can be active as ice nuclei at temperatures as warm as -5°C [73]. The fungal component of several species of lichens (symbiotic associations of fungi and algae) are also ice nucleation active at relatively warm temperatures (warmer than -5°C) [74]. The body fluids of a wide range of insects (both larvae and adults) contain ice nucleators [75]. The haemolymph (circulatory fluid) of other invertebrates such as mussels also contains ice nucleators whose presence is influenced by day length and temperature [76]. Among vertebrates, ice nucleators have been found in the blood of certain fish, amphibians and reptiles [77].

The most widely studied ice nuclei of biological origin are associated with bacteria. The outer membrane of the cells of some bacteria contains a protein that can function as an effective ice nucleator at temperatures as warm as -1°C . Only a few species of bacteria are known to produce INA proteins. INA bacteria are generally species that are frequently found in association with plants (as saprophytic epiphytes and/or as plant pathogens) and include *Pseudomonas syringae*, *P. viridiflava*, *P. fluorescens*, *Pantoea agglomerans* (formerly called *Erwinia herbicola*), and *Xanthomonas campestris* [56, 78].

6. MOLECULAR BASIS OF ICE NUCLEATION ACTIVITY IN BACTERIA

6.1. The ice nucleation protein and gene

The ice nucleation activity of bacteria is due to a protein anchored on the outer part of their cell membrane and exposed to the external environment. The bacterial cell membrane (Fig. 3), like all biological membranes, is a phospholipid bilayer in which numerous proteins are embedded. Many of these proteins play important roles in sensing the conditions of the external environment where the bacterial cells reside or in transporting molecules between the external environment and the cytoplasm. The precise role of the ice nucleation protein with regard to bacterial physiology is not known. Nevertheless, it can initiate the formation of ice nuclei by orienting water molecules into an ice-like structure thereby catalyzing the formation of ice at temperatures slightly below 0°C. Among different species of bacteria, the different forms of the INA protein and of the gene responsible for their biosynthesis share some common features. This suggests that they evolved from a single original protein and bacterial ancestor. Moreover, this unique protein has no resemblance to other bacterial proteins, either in function or amino acid sequence.

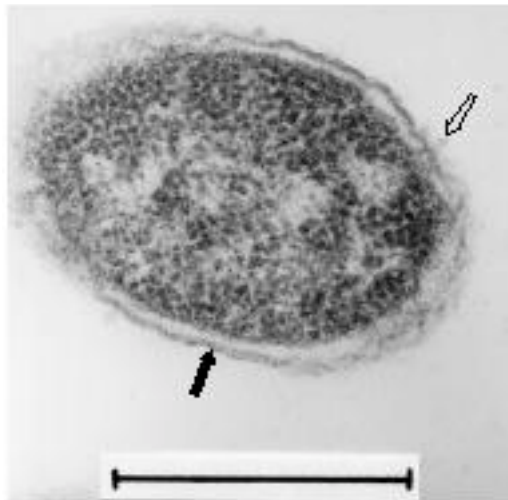


Figure 3. Transmission electron micrograph of a cross section of a cell of ice nucleation active strain CC94 of *Pseudomonas syringae* grown on the surface of cantaloupe leaves. The solid arrow points to a section of the cell membrane. The hollow arrow points to protuberances that are probably ejected parts of the membrane. Bar represents 0.1 μm .

The gene controlling the ice nucleation activity of bacteria has now been cloned from seven different bacterial strains and the sequences of their nucleotides (building blocks of DNA) have been determined. The nucleotide sequences of these different forms (alleles) of the *ina* gene have been compared. Furthermore, from the nucleotide sequence of a gene it is possible to determine the putative amino acid sequence of the protein, the product of expression of the gene by the bacterium. Subsequently, the amino acid sequence of the protein can be used to construct theoretical models of secondary and tertiary protein structure, determine basic biochemical properties such as hydrophobicity or hydrophilicity, as well as its potential location and activity in the bacterial cell. All the above analyses were done for *ina* genes and INA proteins and their degree of similarity was determined [79].

The different alleles of the *ina* gene in the chromosome of the seven different bacterial strains studied are not the same length, but they share some common features. The part of the gene responsible for the N-terminal of the protein, *i.e.* the end with the free amine (NH_2) group, codes for a hydrophobic part of the protein that has properties expected of protein domains that would be inserted in a membrane.

The part of the gene responsible for the C-terminal of the protein codes for hydrophilic domains that are highly variable among the different alleles. The largest portion of the gene codes for the central core of the protein. In all alleles studied, this core has a common motif – a sequence that is repeated - of 24, 48 and 144 nucleotides. This translates into repeats of 8, 16 and 48 amino acids. The details of these repeated sequences are highly similar ("conserved") among the predicted corresponding INA proteins, particularly within the 48-amino acid repeat. The shorter repeats are less similar, with stretches of higher and lower repetition. This periodicity is in accordance with ice nucleation activity in orienting molecules of identical structure (water) and acting as a template for ice formation. A model of the protein encoded by the *inaZ* gene from the bacterium *P. syringae* (proposed by Kasava and Lindow [80, 81]) depicts the structure as a chain of repeated octapeptides (segments of 8 amino acids) forming a series of loops, a secondary protein structure called β -strands. As part of the mechanism by which this protein initiates ice formation, it has been proposed that there is a conformational change on this protein that is driven by bacterial metabolism and that involves redistribution of ionic charge leading to an exceptionally good ice nucleating site – especially at warm temperatures [82].

6.2. Expression of the ice nucleation activity

The physics underlying the mechanisms by which INA proteins catalyze ice formation require further exploration. But even if we understood the complexities of the physics, they would likely be outshone by the biological variability in the production of effective ice nuclei by bacteria. Firstly, bacteria produce and maintain active INA proteins in their cell membrane as a function of environmental conditions. Environment is the overriding cause of variability in bacterial ice nucleation activity; differences in the structure of the gene among bacteria have not been shown to be related to expression of effective INA proteins. The main triggers for expression of ice nucleation activity by bacteria are cool temperatures ($<15^{\circ}\text{C}$) and low availability of nutrients [83]. Secondly, there is marked variability among individual cells in the production and assembly of the INA protein. Although all cells in a population of INA bacteria have the gene and are subjected to the same environmental conditions, they do not produce the protein to the same degree, even if the cells are essentially clones (originating from the same parental cell via only one or a few cycles of cell division).

Based on this variation, three types of bacterial ice nuclei have been identified: Type I, able to catalyze freezing of water supercooled between -2°C and -5°C ; Type II, active from -5°C to -7°C ; and Type III, active below -7°C [84]. Irradiation studies [85] and biochemical data [86] have shown that this variation in nucleation frequency and temperature is due to the size of the nucleation site on the bacterial cell membrane. A proposed model suggests that the nucleation site is made of assembled monomers of the INA protein and that the ability for the protein to catalyze ice formation is more efficient with increased aggregate size [56]. In other words, the larger the assembled protein, the warmer the temperature at which freezing can be catalysed. The bacterial cell membrane plays an important role in the stability of the protein aggregate. Not surprisingly, purified protein preparations exhibit lower activity than whole, intact cells [87].

The Type III proteins seem to be relatively stable. On the other hand, Type I and II forms of the INA protein have fleeting life spans, most probably as a function of the ability of the membrane to hold together these large complexes. For example, Rogers and co-corkers [88] found that shifting a bacterial culture from 30°C (the optimal temperature for growth) to 5°C increases the appearance of Type I ice nuclei. Furthermore, shifting a bacterial culture to higher temperatures causes the disappearance of mainly Type I proteins [83, 89] such that cycles as short as 4 hours between temperature shifts can rock the abundance of protein types back and forth. Appearance of Type I protein is also favored by nutrient starvation of bacteria, especially for phosphates [90]. It is important to note that the leaf surface is not a nutrient-rich environment for bacteria and that temperature fluctuation during the day-night cycle may well influence the ice nucleation activity of bacterial populations growing on leaves. However, the effect of *in situ* conditions of the leaf on ice nucleation activity of bacteria has not been investigated.

A standard method for testing the ice nucleation activity of a bacterial culture is derived from the drop-freezing method described by Vali [91] and modified by Lindow and co-workers [92]. This can be accomplished by placing droplets of a series of concentrations of a bacterial suspension on a hydrophobic surface, usually an aluminum foil boat coated with paraffin and floated in a cooling bath or on a cooling plate. The frequency of frozen droplets is then determined at a range of temperatures, usually between -1° and -10°C . Such tests have revealed that nearly all cells of certain strains of *P. syringae* can produce Type I ice nuclei when exposed to an optimal growth and pre-conditioning environment [89], whereas for most strains only 1% or as few as 10^{-8} cells produce such ice nuclei [93]. Such tests have revealed the INA inhibitory and enhancing effects on of nutrients, metabolic inhibitors, pH and other parameters that can be modified in a laboratory culture [89, 94]. Unfortunately, they do not give insight into the activity of bacterial ice nuclei in natural environments. Determining the freezing temperature of individual leaves and relating it to the size of bacterial population on leaves (the so-called tube-freezing test proposed by Hirano et al. [95]) gives a rough estimation of the frequency of INA bacterial cells on leaves in the field. However, to date there are no measurements of the frequency of INA bacterial cells (or bits of cells) in the atmosphere – in the state in which they exist *in situ* - nor of the extent to which pollution, UV light, cell desiccation, and contact with cloud water and its associated chemicals can effect bacterial ice nucleation activity.

7. PRACTICAL APPLICATIONS OF BACTERIAL ICE NUCLEATION: AGRICULTURE, FOOD PROCESSING, BIOTECHNOLOGY AND RECREATION

Ice nucleation in and on plants has been a widely studied phenomenon, clearly complicated by a diversity of interacting biological and physical factors. There are several modes of a metastable state where ice crystallization is prevented in plants, including *supercooling* where an ice structure template is not present, *supersaturation* – where sugars or other solutes due to colligative properties depress freezing point and prevent nucleation, *tension stressing* – where water is under negative stress due to suction of water up to the top of a tree, and *glassed cell solutions* - where highly viscous solutions do not crystallize [96]. With the possible exception of glassed cell solutions, these metastable states are not optimal for plant growth, and they usually are expressed during a shut-down of metabolism such as during seed formation or during physiological changes at or before the advent of winter, and in xylem vesicles that serve as water conduits relatively devoid of metabolic activity. In short, there seems to be a trade-off between active metabolism and a survival state, and plants demonstrate different adaptations to handle this dilemma. Many frost sensitive annual plants such as tomato and cucumber may not have the ability to avoid ice crystallization by lowering their freezing point either by supercooling or by increasing solute concentration. In perennial winter hardened plants that have evolved to withstand freezing temperatures, extensive supercooling actually leads to more destruction than if freezing occurs close to 0°C because nonequilibrium freezing unevenly tears tissue [97]. Hence, the small group of aforementioned bacteria can clearly disrupt the apperant of orderly adaptation to sub zero temperatures. Their presence on the surface of leaves, buds and fruits of vegetable, grain and fruit crops; wine grapes; olives; tea; forest, hedge and ornamental trees, and ornamental herbaceous plants can enhance the degree of frost damage sustained by these plants by causing water on or in these plants to freeze where it might have otherwise simply supercooled [54, 78].

Traditional methods of limiting frost damage to crops involve inputs of energy to physically isolate plants from cold temperatures. Heating, circulating air or watering (to garner the heat of fusion) in orchards, vineyards, greenhouses or fields can be costly means of frost control. The discovery of the role of INA bacteria in frost damage to plants opened the possibility of managing frost as one would manage disease by limiting or inhibiting the causal organism. In the 1980's considerable effort was invested in research on inhibiting INA bacteria by biological control via antagonistic epiphytic bacteria that were not ice nucleation active and by use of bactericides. The latter has shown important efficiency [98] and corresponds to treatments readily used by growers for disease control. The former has also

proven to be effective under experimental conditions. However, much of this work was oriented around strains of bacteria that scientists rendered inactive as ice nuclei by excising out the gene coding for the ice nucleation protein. This opened the heated debate, that still festers today, about the deliberate release of genetically modified organisms into the environment, and frost control products based on such bacteria never saw the light. Furthermore, the cost of this technology would have likely been prohibitive for farmers [99]. A formulation of a naturally-occurring non INA bacterium that is effective in controlling frost damage was registered in the USA and marketed for a few years in the early 1990's [47]. Nevertheless, the image of biological control of frost via antagonistic bacteria sprayed on crops was severely tainted. This naturally occurring antagonistic strain is still marketed, but under the name Blightban A506[®] to highlight its effectiveness in control of a family of bacterial diseases known as blight [47].

Breeding of plants to obtain disease resistance is also prominent in the arsenal of disease control strategies and is a possible means of frost control. Plant breeders selecting for frost resistance may select plant lines with altered physiology or they may in fact be selecting for plants that inhibit INA bacteria from growing in or on the plant [100]. In this case a consistent inoculation scheme employing appropriate INA bacteria should by rights differentiate between these different bases for frost tolerance or resistance. However there are many complicating factors including the fact that such bacteria can produce antimicrobial compounds inhibiting other disease causing bacteria and fungi, as illustrated above for the case of the Blightban A506[®] bacterial strain. If only we could enjoy the pure and serene science of the subatomic world, or even in a test tube, rather than the multidimensional chess game that we see in field biology. Perhaps we should simply note that INA bacteria left to their own devices, can cause frost injury where it might otherwise not happen, and that few if any advances have been made in control of these bacteria. Perhaps we will have more success using specific bacteria to cause frost injury on undesirable weeds, as an alternative to chemical control. One caution to breeders might be in order: the strong selection against phytopathogenic bacteria in plant variety selection may have an unanticipated consequence in reduced production of INA bacteria that naturally seed clouds as we will see in the next section. In short, one must be careful for what one desires.

There are a few notable and practical applications of the research involving INA bacteria and the nucleation protein. The product Snomax[®], cells of *P. syringae* that have been freeze-dried and then killed by gamma radiation, is widely used to seed snowmaking canons at ski resorts [47]. This has led to significant reductions in the energy consumption of snow-making. But because of its efficiency, use of this product has probably also led ski resorts to greatly increase water consumption thereby putting a significant drain on local lakes and rivers [47]. Use of the ice nucleation protein in freeze-concentration of foods and in texturing proteins is under intense study [94]. These applications are of interest particularly because the rate of the freezing process and the size of the crystals formed, essential to the quality of the products, can be finely controlled in the presence of high temperature freezing catalysts such as bacterial ice nuclei. This work also benefits from bacterial strains that produce cell-free ice nuclei, *i.e.* those that are liberated from cells in the form of vesicles and from the apparent innocuousness of the nucleation protein for humans [94]. One of the well-characterized properties of the ice nucleation protein is its N-terminal. This end of the ice nucleation protein anchors the protein into the outer membrane of the bacterial cell as described above. The sequence of DNA coding for this part of the protein has been combined with DNA sequences for a wide range of proteins of practical or fundamental research interest and introduced into the genome of bacteria destined to translate this DNA into protein. This technique, called protein display, allows microbiologists to ornate the cells of these bacteria with the jewellery (*i.e.*, proteins) of their choice [94]. This can facilitate the study of proteins that are difficult to produce under natural conditions such as those associated with viruses pathogenic to humans [101] or can also enhance the efficiency of enzymes involved in bioremediation of polluted sites [102]. The ice nucleation protein and its associated gene have also been deployed in detection of bacteria or in reporter systems to verify expression of genes of interest as described by Cochet and Widehem [94].

8. BACTERIAL ICE NUCLEI AND THE CONCEPT OF BIOPRECIPITATION

The microorganisms that inhabit the aerial surfaces of plants have an incredible propensity to be disseminated long distances via wind movement. The pioneering work of Stakman and colleagues in 1923 revealed that the spores of important fungal pathogens of cereal crops could be carried up to altitudes of over 5000 m [103]. Numerous other sampling campaigns from airplanes, including the flights of Charles Lindberg, confirmed the presence in the upper atmosphere of fungi that live on plants either saprophytically or as pathogens [104]. Although bacteria were observed in many of these sampling campaigns, it wasn't until the early 1980's that cells of INA bacteria were clearly identified from air samples taken at altitudes of several thousand meters [105, 106]. Because of the important implication of these observations for long distance dissemination of plant diseases, there has been considerable interest in the impact of atmospheric conditions (relative humidity, UV radiation, etc.) on microbial survival. Conversely, because of the capacity of some of the airborne bacteria to catalyze freezing of supercooled water, it has been proposed that airborne microorganisms can have an impact on the atmosphere by playing a role in processes leading to rain [106, 107].

There are two lines of reasoning that lead to the idea that INA bacteria can have an impact on rain. Since the 1930's, an important tenet of cloud microphysics has been that ice is required for most heavy rainfall from supercooled clouds [108]. In lower troposphere clouds where temperatures are below freezing but warmer than -40°C , heterogeneous ice nuclei have important roles in this ice formation [109]. This is particularly true in stratocumulus and small cumulus clouds where summit temperatures are often warmer than -5°C and where there are no processes for multiplication of ice nuclei (such as fractioning of ice crystals). Hence, some of the principal strategies for weather modification, such as seeding of clouds with ice nuclei, are based on the assumption that the formation of new ice particles around the 'seeds' can lead to enhancement of precipitation under certain conditions [108]. The second line of reasoning is related to the observation that land surface properties seem to be a driving force of atmospheric variability [110]. In particular, there is evidence that vegetation patterns and irrigation intensity can have a significant influence on rainfall. It has been proposed that the denuding of western Africa and the deforestation of certain of the Canary Islands have compounded processes leading to decreased precipitation [110], whereas irrigation and the expansion of vegetated (cropped) areas can lead to enhanced rainfall [110-113].

Vegetation alters numerous land surface properties linked to the formation of rain. The properties described by meteorologists generally involve the availability of 1) water that can be transferred as vapor into the air and 2) the energy necessary for this vaporization. These include surface albedo, emission of infrared radiation, turbulence, moisture retention, etc. [110]. But, as indicated above, plants harbor large numbers of microorganisms on their surfaces. Cultivated plants, in particular, are considered to be a major source of the microorganisms – and especially the bacteria – in the air [4, 6]. Lighthart and colleagues [114] observed a total upward flux of 76000 bacteria/cm² per day during the daylight period in June over grass and sagebrush in a nuclear reservation in the state of Washington. Canopies of snap bean, a host for disease caused by *P. syringae*, emitted about 30 bacteria/m²/sec of INA strains of this bacterium [6]. Furthermore, a large downward flux of INA bacteria has been observed during rain [29]. Such observations led Sands and colleagues [106, 107] to propose the existence of a biological cycle whereby colonization of plants by INA bacteria contributes to enhanced precipitation which in turn enhances plant and microbial growth and contributes to the dissemination of bacteria to new plants. This general cycle has been christened "bioprecipitation".

In light of the numbers of INA bacteria emitted from plants, plants could be considered to be cloud seeders. To extrapolate on this idea, enhancing the cultivation of crops favorable to colonization by INA bacteria and halting the eradication of wild plant stands favorable to these bacteria could be considered to be strategies for weather modification [107]. To date, the concept of bioprecipitation and its possible exploitation for enhancing rain are exciting hypotheses waiting to be tested. Up until recently, they have suffered the same fate as many of the other ideas for weather modification that have emerged since

the late 1940's. Their experimental validation is extremely difficult to achieve due to the experimental designs required [115]. Furthermore, the temporal and spatial scales of this cycle have not been defined. Do we expect to see effects within a season, over several years, within a predictable geographic region, etc.? The mathematical and simulation models that could reveal the impact of bacterial ice nuclei on a precipitation cycle are just now seeing the light.

9. ENHANCING BIOPRECIPITATION : WEATHER MODIFICATION VIA AGRICULTURE AND LAND MANAGEMENT

Up to this point we have kept within the confines of scientific discourse based on our best assessment of data. We have not directly asked the compelling question: might there be an effect of man's activity on rainfall, in the past, the present, and in the future? If there is a bioprecipitation cycle, then to what extent do human activities have consequences on this cycle? For example, what have the cultivation, eradication and breeding of plants done to influence rain? How much desertification is caused by man's use of grazing animals beyond a sustainable level? Might our preference of crops and the selection and breeding of their varieties have been different if we had taken into account the number of ice nucleation bacteria on plant varieties? It seems that these somewhat overwhelming questions cannot be answered without at least some database gathered in a coordinated way among laboratories representing the diverse scientific disciplines needed to address this question. Given such a data set, we might most likely end up finding an additional *mea culpa* placed at our doorstep as we are now facing in the case of global warming.

Today, we seem to be stepping into that brave new world where we recognize that human activities in fact have affected climate, and that we had best set a series of correction courses, perhaps with some extreme urgency. In the case of global warming, we tend to focus on stopping or reducing activity that has a nefarious impact on climate. Perhaps in the case of bioprecipitation it would be more useful to think about initiating or intensifying certain modern human activities having positive (and not simply neutral) effects. If we wish to enhance the bioprecipitation cycle in some drought-prone areas, with the objective of increasing rainfall, we might have to make decisions about cropping, grazing and other land management systems to enhance numbers of airborne INA bacteria. Certain species and strains of bacteria could be more effective than others, and large scale production and inoculation of plants with these bacteria might increase the efficiency of our intervention. INA bacteria can play multiple roles in the plant environment (as plant pathogens and as antagonists of other plant pathogens, for example) and their overall impact needs to optimize the benefits for all concurrent interests. Crops can be selected that support high numbers of INA bacteria, and if these bacteria also mitigate foliar fungal disease, the overall effect will likely be positive. Reduction in grazing also presents a similar conflict of interest, unless plants selected to produce high numbers of INA bacteria are not preferred by livestock. Some INA bacteria release cell-free membrane vesicles and some maintain ice nucleation activity when they are dead. Plant breeding and cultivation strategies that enhance these forms could be investigated to reduce the spread of plant pathogenic INA bacteria. Inoculation of plants with INA bacteria could be achieved via inoculation of seeds which could assure perhaps a more efficient colonization and would reduce technical inputs for growers. A considerable knowledge base integrating research in agronomy, microbiology, meteorology, physics, sociology and economics will be needed to bring us to the point of such choices.

10. CONCLUSION

Ice nucleation active bacteria are catalysts. We have explained how they catalyze the freezing of supercooled water and have illustrated some of the environmental and industrial consequences. The consequence of this phenomenon for the bacteria themselves is unknown. A protein capable of orienting water molecules and inducing a phase change might also act as a condensation nucleus [116], thereby providing a powerful tool for INA bacteria to sequester liquid water. INA bacteria have also been

catalysts of social phenomena and changes in scientific paradigms. In the mid-1980's, as a means to control frost damage, a group of scientists in California made the first request to release a genetically engineered organism into agricultural fields – an INA strain of *P. syringae* whose *ina* gene had been snipped out rendering it non active as an ice catalyst. Metaphorically, the latent heat of fusion from bacterial ice nucleation heated an intense public debate and sparked the first militant acts against the scientific community over genetically engineered organisms. Today we witness the world-wide social and economic consequences of this debate and its impact on research infrastructure. Understanding the role of INA bacteria in frost damage to crops has also led to recognizing frost damage as a disease, with a biological causal agent. Hence, it is not simply the result of sub-zero temperatures, conditions which are now perceived as necessary but not always sufficient for frost to occur. Methods for limiting frost damage can now be extended to means directed at limiting bacterial populations, as would be done for disease control, and the notion of frost tolerance of crops now encompasses both plant physiology and the bacterial partners of these plants.

INA bacteria might be the catalysts of another possible change in scientific paradigm, toward a vision of drought as a disease – not a disease of plants but of the biosphere. Perhaps drought could be viewed as a disease of the biosphere with biological causal agents as well as pre-disposing environmental factors. As we discussed above, this notion is hypothetical but it may offer a new mind frame for investigating certain atmospheric processes. In this context, studies of the etiology and epidemiology of planetary or regional drought would bring into play a unique interaction of scientific disciplines and shed new light on the causes and potential remedies for drought.

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