From Grains to Rain:

the link between landscape, airborne microorganisms and climate processes

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Preface and Acknowledgements

We have conducted research for several decades on the biology and ecology of the bacterium *Pseudomonas syringae*. This bacterium, well-known for its ability to grow on plants and to sometimes cause plant disease, also has the unique ability to catalyze the formation of ice – in other words to be ice nucleation-active. The ice nucleation activity of *P. syringae* seems to leave little room for indifference. We have seen classrooms of a hundred undergraduates rise to their feet to applaud the professor of Introduction to Plant Pathology who conducted a demonstration of ice catalysis by this bacterium. In the early 1980's researchers snipped out the gene for the ice nucleation protein in a strain of *P. syringae* and then made a request to the US government to release this strain into the environment to test it efficiency in controlling frost damage to crops caused by this bacterial species. This constituted the first official request for deliberate release into the environment of an organism whose genome was expressly modified with the techniques of modern molecular biology. This single event snowballed into the very vocal and active movement against the release of "genetically modified organisms" into the environment that has waxed and waned across North America, Europe and Asia. *P. syringae* has also been turned into a commercialized product for facilitating the productivity of snow cannons at ski resorts. Initially used at ski resorts across the world, it now is the object of self-imposed moratoria or of interdiction by national legislation in some countries because of concern about potential environmental impacts. Research with this bacterium is now also opening questions about its role, and that of other ice nucleation active microorganisms, in processes leading to snow and rainfall. Through our work with *P. syringae* we have traversed the vagaries of all of these sagas. The question of the impact of ice nucleation active organisms on atmospheric processes is the most compelling. And its treatment requires the great challenge of melding disparate disciplines from the life sciences and the physical sciences in the heated surge to understand the implications of the changing climate of our planet. Because of our experiences, we have been asked numerous times to explain to physicists the biology of microorganisms that can nucleate ice. And as biologists we have posed many questions about basic atmospheric physics and chemistry to our colleagues who master these disciplines. Via these exchanges we have honed a certain ability to simplify the vocabulary associated with these phenomena. We want to share this accumulated knowledge to broaden the interest in this subject. Therefore we decided to present the whole of the scientific background we have garnered in a way that should be comprehensible to a wide range of readers with some training in the natural sciences. We have also presented our perspective of how these questions evolved and have deployed a writing style that we hope will engage the reader's interest. This essay is also an expression of our passion for this subject. Therefore, at times we have allowed ourselves a bit of literary license in imaging that we were writing a great adventure novel.

In preparing this text we have consulted various colleagues to validate the accuracy of the information presented, and in particular that concerning physical processes in the atmosphere. We thank these colleagues for their input. Nevertheless, we assume full responsibility for the content.

If you find the information in this essay useful, it is thanks to the many people who contributed to its existence:

- to those who have encouraged and supported us to pursue our dreams about this subject: Gabor Vali, Zev Levin, Ulrich Pöschl, Bernard Sequin, Nicolas Métro and Gerry Ohrstrom.
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I. The Seeds of Aerobiology

The cultivation of grains has had enormous impacts on the growth and health of the human population, on urbanization, and consequently on the environment of our planet and on how we perceive nature (50). Human endeavors to expand the regions where grains are cultivated, in particular wheat, rice, maize and barley, and to increase their yields have had consequences on demographics, politics and the state of Earth as a habitat for living organisms. The ensuing changes in the vegetated landscape of the Earth, and most notably the homogenization of the types of plants grown across extensive swaths of land, has also created ripe conditions for the proliferation of certain microorganisms. Investigation into the life cycles of some of the plant pathogens that have become particularly important in this new context of the Earth's landscapes has inadvertently been the starting point for even further changes in how we perceive nature. We have begun to wonder if microorganisms might be involved in processes in the troposphere that subsequently influence Earth's climate.

In 1900, cultivation of winter wheat spread widely across the Great Plains of North America. This was due to the successful release, by scientists from the US Department of Agriculture, of new hardier varieties of hard red winter wheat derived from Crimean wheat varieties introduced into the US in the late 1800's. The vast belt of wheat created by the cultivation of these new varieties, from northern Mexico to Canada, was subject to severe epidemics of stem rust (caused by the fungus *Puccinia graminis* f. sp. *tritici*). The characteristics of the disease epidemics caused by this prolifically sporulating fungus led plant pathologists to investigate the extent to which aerial dissemination was involved in the spread of this disease. In 1921, a team of scientists from the University of Minnesota, led by Elvin C. Stakman, hung a variety of spore-sampling devices out of US Army fixed-wing planes as they flew over the front of an epidemic as it moved in one growing season from southern Texas to southern Wyoming. More than 50 sampling flights were conducted from April to July at altitudes up to 3300 m (46). Spores of rusts and other plant pathogenic fungi were regularly detected during all flights and at all altitudes. These experiments demonstrated for the first time that microorganisms are present in the atmosphere at the altitudes of clouds and beyond, and opened the way for a series of subsequent high altitude sampling efforts, some involving eminent pilots including Charles Lindberg.

The initial impact of these observations was on the science of Plant Pathology itself and on the foundation of Aerobiology. It led to studies of long distance spore dissemination. Its importance for the epidemiology of plant diseases is illustrated in the classical works of Gregory (22) and Stakman and Christensen (45). The critical importance of long distance aerial dissemination of plant pathogens is still pertinent for plant health. The spread of a new race of stem rust of wheat, strain UG99, is being closely monitored in an attempt to avoid a major pandemic that could significantly reduce wheat yields.

Since the inception of Aerobiology, the principle questions asked about microorganisms have focused on how the atmosphere affects the microorganisms it transports – mostly in terms of the geographical range of transport and viability during transport. This information is particularly critical for estimating the potential spread of human, animal and plant diseases. But in the last decade, research into the questions of reciprocal impacts has been launched. Microorganisms have some of the same properties that enable inert aerosol particles to influence physical and chemical processes in the atmosphere. Furthermore, they are lofted to sufficiently high altitudes and remain aloft long enough to permit them to participate in these processes. Might the microorganisms in the atmosphere influence the atmospheric processes around them, and in particular those involved in their transport?

Irrigated monoculture of barley at the foot of the Grand Teton Mountains in Wyoming. Less than 200 years ago these fields were wild prairies of diverse plant species, of both monocotyledonous and dicotyledonous plants. (photo C.E. Morris)

II. Abundance, Abodes and Flight of Microorganisms in the Atmosphere

Turbulent wind movements are very efficient at lofting all sorts of matter into the atmosphere. The remarkable and rare event of fish (23) or frogs being swept up into the sky and later falling out as rain can occur at stormy wind speeds of 160 km/hr. Hence, it is easy to comprehend that common gentle breezes of 20 km/hr or light air of 5 km/hr would regularly uplift bacterial cells whose weight is measured in picograms (1 picogram = 10^{-12} g) or fungal spores that weigh under a microgram (10^{-6} g). Microorganisms are particularly abundant during periods favorable for disease of crop plants caused by fungi with aerially disseminated spores and during periods of human activities that are effective in releasing microbial particles into the atmosphere such as combining and other practices associated with crop harvest.

The list of microbial species found in the atmosphere is too long to be described in detail (see the work of Després and colleagues for details (15)). Among the bacteria detected in the atmosphere, many have a Gram-positive cell wall and include spore-formers such as *Bacillus* and *Microbacterium* species (spp.). But Gram-negative bacteria, having a cell wall that is considerably more fragile than that of Gram-positive bacteria, have also been found. Among the fungi, spores of *Cladosporium*, *Aspergillaceae*, *Alternaria*, *Botrytis*, and various basidomycetes (*Coprinus*, *Ustilago*) are often observed in the atmosphere, but spores of *Cladosporium* spp. seem to be numerically the most dominant. Algae such as *Chlorella* spp. are regularly found in the air. Viruses have also been observed, in particular in aerosols over the sea surface and in clouds. Special mention should be made of *Pseudomonas syringae*. This Gram-negative plant pathogenic bacterium is found in the atmosphere, in clouds and in a range of habitats associated with the water cycle (36). *P. syringae* will very likely become one of the most highly studied organisms with regard to potential impact on atmospheric processes as we describe in the following chapters.

Overall, the abundance of microorganisms in the atmosphere is in the range of several to hundreds or thousands of particles per m^3 (except for the occasions of important flushes of microbial particles released during harvesting of crops (27)), while atmospheric particles in general are numbered in the tens of thousands per cm^3 (34). Abundance is related mostly to the presence of sources of microorganisms that can be incorporated into aerosols and to the influence of meteorological conditions and human activities favorable for creating aerosols. Plants and erodible soils are currently seen as the most important sources of microorganisms in outdoors air in non urban environments. The most prevailing and well-studied meteorological parameters that influence microbial abundance in the atmosphere are wind speed and direction, relative humidity, rainfall and solar radiation. Solar radiation has a particular influence on the diurnal cycles of the atmospheric concentrations of microorganisms. It contributes to the concomitant diurnal cycle of vertical turbulent fluxes of moisture and heat that are directly involved in driving aerosol materials up from the surface of the earth and into the atmosphere. Above water surfaces, creation of aerosols containing microorganisms can occur via a process called bubble bursting and can explain the presence of microorganism-like particles in the atmosphere in remote regions such as above the central Arctic Ocean.

The size, buoyant density and surface properties of microbial spores and other types of cells determine their capacity to remain aloft in the air. These traits give the microbial particle its aerodynamic properties. As for airplanes, the ease of flight of microorganisms is not necessarily determined by their physical dimension (43). In some cases, the aerodynamic properties are such that the effect of gravity will override turbulent and convective air movements bringing the microbial particle back to the ground. But very often, because of the aerodynamically ultra-light nature of microbial particles, air movements are sufficient to keep the microbe aloft for a rather long duration. Residence times of various microorganisms have been estimated to be on the order of weeks (6, 51). Masses of air that bring rain and storms can cross continents and oceans in the matter of days. So it is easy to understand that microorganisms have a very great potential for long distance flight. Direct measurement of the distance that microorganisms travel in real-life situations is difficult because their concentrations in the atmosphere become rarefied as they move away from the source. Mapping the spread of certain plant diseases, such as those caused by obligate parasites such as the rusts mentioned in the first chapter, provides some of the most concrete measures of the distance travelled. Evidently, the wheat rust studied by Stakman and colleagues spread from the northern boarder of Mexico to the southern boarder of Canada - nearly 2000 km - but the trajectory might have been in multiple leaps. However, the movement of coffee rust from the Republic of Angola to Brazil and of sugarcane rust from Cameroon to the Dominican Republic (39) are clearly cases of uninterrupted transatlantic flight.

The presence of microorganisms in the air has been assessed mostly near ground level: just above plant canopies (5, 7) and up to 7.5 m in attempts to measure microbial flux from ground covers (28, 29). Aircraft has been used to observe the presence of microorganisms throughout the rest of the troposphere, which at middle latitudes is about 10 km above sea level. Rockets and balloons have been deployed in addition to aircraft to ferret out microorganisms in the stratosphere. In the late 1970's scientists in the Soviet Union, by using meteorological rockets, observed microorganisms at far reaches of Earth's atmosphere, at altitudes of 48 to 77 km. The organisms detected at these outposts of the atmosphere have, so far, shown particular types of resistance – such as to ultraviolet light – as expressed by the new species of *Deinococcus* recently isolated from the lower stratosphere at 10 – 12 km altitude (52).

Although microorganisms have been found throughout the troposphere and up into the stratosphere, the troposphere is the main target of study because it contains 99% of the Earth's water vapor and it is where all weather processes happen. The presence of microorganisms in or near the stratosphere, although less important for understanding the interaction of microorganisms with atmospheric processes, explains how microorganisms could be transported long distances and between the Northern and Southern hemispheres. Their presence in the upper stratosphere also evokes controversial but exciting questions about their origin, if they are in fact from Earth or rather from other planets and thereby are proof of the notion of panspermia. Nevertheless, because of the potential importance of microorganisms in atmospheric processes occurring in the troposphere, and in particular those occurring in clouds that could yield rain, concerted effort has been made to verify that microorganisms are present in cumulus clouds. The microbiology of cloud droplets has been investigated mostly by taking advantage of geographic sites where one can simply walk into clouds that cloak mountains, such as clouds in the lower troposphere (1400 – 1600 m) over the Alps in Austria and over the *Massif Central* in France and for particularly low-lying clouds (340 m) in the planetary boundary layer over the Hebrides (1, 2, 4). But samples of clouds at higher altitudes (up to 2500 m) have also been collected from airplanes for microbiological studies (44). The presence of microorganisms in freshly fallen rain and snow (12, 36), similar to those in the atmosphere at large and in clouds, offers further support to the general notion that microorganisms in the atmosphere are in the right place at the right time to have the opportunity to get involved in the atmospheric processes occurring around them.

III. Microorganisms and the Dynamics of the Earth's Atmosphere: Confirmed and Hypothetical Roles.

While they are bound to their terrestrial and aquatic habitats, microorganisms have well-recognized effects on the dynamics of the atmosphere. The most well-known and critically significant for life on Earth was the rise in the atmospheric concentration of O_2 (the oxygen that we breath) due to photosynthesis by marine cyanobacteria (25). This rise began about 2.3 billion years ago and led to the current abundance of oxygen as 20% of the total gas content of our atmosphere from a starting point of only 2%. As examples of modern phenomena, microorganisms in soil, in landfills, in the guts of ruminants and termites, and in sewage treatment plants produce about 70% of the greenhouse gas methane that is released into the atmosphere (11). While reducing nitrates in soils, denitrifying microorganisms produce nitrogen oxides such as nitrous oxide (N_2O) and nitric oxide (NO). These gases, for which considerable effort has been made to eliminate them from automobile emissions, can lead to the destruction of ozone in the troposphere and the stratosphere. Marine phytoplankton also produce the precursor of dimethlysulfide (DMS) which is the major natural source of sulfur in the atmosphere (3). The precursor molecule, dimethylsulfoniopropionate (DMSP), is a cellular component of many marine phytoplankton thought to protect these organisms from osmotic, cold and oxidative stress. When cells of phytoplankton die due to attack by viruses or other causes, DMSP is released into the ocean waters and readily metabolized into volatile DMS. This latter product seems to repel herbivores that would otherwise compete with the surrounding bacteria for nutrients during phytoplankton blooms. DMS also diffuses into the atmosphere where it is oxidized into sulfur aerosols that are hygroscopic and hence are very efficient in forming water droplets to make clouds. Initial observations that cloud coverage of oceans was correlated with phytoplankton blooms led to the establishment of the CLAW hypothesis (named after the founders). According to this hypothesis, DMS is linked to the global climate because enhanced cloud coverage fosters cooling of the planet thereby leading to a negative biogeochemical feedback cycle stabilizing the Earth against global warming.

Although recent work suggests that the biogeochemical feedback proposed in the CLAW hypothesis is only weak or very localized at best, the intense investigations of the processes involved in this theory brought Earth systems science to the forefront in this century (see the Planet Earth Summer 2010 document). It is in this context that new questions about the impact of microorganisms on the atmosphere are being posed.

As part of this trend, aerobiology is now moving toward a vision of the atmosphere as a global biome. In the model emerging from this new vision, the role of microorganisms is suspected to be analogous to the role that microorganisms play in what is known as "superorganisms". Humans and plants are very visible examples of superorganisms whereby some of their seemingly intrinsic behaviors, such as production of hormones or metabolism of nutrients, are in fact achieved by their resident microbial flora. Hence, in this emerging perspective of the atmosphere as a biome, microorganisms could contribute to some of the physicochemical properties of the atmosphere that govern the processes that define climate. Apart from their ability to respire, metabolize and multiply, microbes are essentially just another component of the particulate matter suspended in the atmosphere – particulate matter that is called aerosols or sometimes referred to as dust. Aerosols in general play four roles in the atmosphere that are of major importance to the climate and air quality. They can reflect light, they can participate in chemical reactions, they can be cloud condensation nuclei (CCN), and they can be ice nuclei (IN). But what is special about microorganisms as aerosols? What can they do that common dust cannot? The current hypotheses around these questions suggest that, for the latter three roles (chemical catalyzers, CCN and IN), microbial aerosols function under situations where other aerosols are less efficient. To express it in biological jargon, microbial aerosols occupy niches that are poorly occupied by other aerosols. Furthermore, as would be expected for living organisms, some of their atmospheric functions might incite feedback processes that are beneficial to their proliferation. This sounds reminiscent of the proposed CLAW hypothesis and hence it is not surprising that this subject generates so much interest.

As indicated above, aerosols in general have a wide range of potential impacts on the atmosphere (31) (see also: NASA Earth Observatory). The roles that could be played by microorganisms in the atmosphere are listed briefly below.

Catalyzing chemical reactions leading to the degradation of organic compounds.
Oxidative reactions and other transformations of organic compounds in the atmosphere are particularly important in the acidification of the atmosphere, in the subsequent formation of secondary aerosols and in their ensuing impacts on climate, air quality and geochemical cycles. Most chemical reactions in the atmosphere are driven by light (photochemistry). This is a powerful process because it can occur for materials in dry suspensions. However, the metabolic activity of microorganisms suspended in wet aerosol particles has a catalytic potential similar to that of photochemistry when the sun goes down (13).

Scattering or absorption of light.
The interaction of atmospheric particles with incoming radiation can contribute to warming or cooling of the atmosphere. Due to the relatively low abundance of microbial aerosols compared to mineral or non biological aerosols in the atmosphere, this role is likely to be insignificant.

Catalyzing the condensation of water vapor.
Particles in the atmosphere can serve as surfaces on which water vapor condenses to liquid under the appropriate conditions of vapor pressure and temperature. Particles that participate in the condensation of water are called cloud condensation nuclei (CCN), and this name indicates the key contribution of this

phenomenon to atmospheric processes. Although the chemical surface properties of particles can influence their capacity as CCN, size of particles has an overriding influence on CCN efficiency above certain dimensions. Particles of 1 μm or more in diameter are well into the range of sizes that are highly efficient at causing condensation, with size being more important in this regard than their surface chemistry (17).

Catalyzing the freezing of atmospheric water droplets.

The freezing of water is not a spontaneous process at temperatures below zero. In fact, it is not spontaneous above about -37° C, and most importantly not under the environmental conditions and life-time typical of cloud droplets. At sub-zero temperatures warmer than the spontaneous freezing temperature, the freezing of water in the environment necessarily involves catalysts, termed 'ice nuclei', that foster the aggregation of water molecules into the configuration needed for the formation of the crystalline structure of ice. Hence, cloud droplets are able to super cool to temperatures approaching -37°C without freezing. If they do not freeze, then they can remain suspended in the air because at mid-latitudes freezing is often necessary for them to aggregate into larger, heavier precipitation-sized ice particles that can fall toward the ground as rain or snow. It should be noted that under some atmospheric conditions and especially in tropical regions, cloud droplets that are large enough can collide efficiently and coalesce to form raindrops directly in the liquid phase. In these cases freezing is not a critical step for rain to form.

Aerosol particles that are ice nuclei are generally insoluble materials whose surface properties foster the binding of water molecules into a crystal embryo of a size that can grow, permitting the surrounding water molecules to overcome the thermodynamic constraints to binding into the spatially organized crystalline structure of ice. Without a catalyst, at -5°C for example, a total of 45000 molecules of water would be needed to overcome energy barriers to growth of the embryo and subsequent ice formation. It is highly improbable that random movement of molecules would lead to such an organization. Hence, materials that are active as ice nuclei reduce the energetic constraints of crystal formation. Numerous materials in the atmosphere can act as ice nuclei including dust and soil particles (10, 38). Most mineral and non-biological aerosol particles in the atmosphere are capable of catalyzing the freezing of water only when the temperature is below -10°C or even -15°C. However, several types of microorganisms are active as ice nuclei at considerably warmer temperatures. Among these "ice nucleation active" microorganisms are several species of bacteria and fungi that are commonly associated with plants as will be described in the following sections.

The reader who is curious for more details on ice nucleation can consult the numerous reviews on ice nucleation in clouds (8, 14, 26, 32, 47-49). The review of Cantrell and Heysfield also reveals an interesting story from popular culture that can provide a tool to facilitate understanding of the mechanism by which ice nucleators orient water into a crystal. The concept of ice nucleation was popularized by the brother of Bernard Vonnegut, the atmospheric physicist who discovered the effectiveness of silver iodide as an ice nucleator in 1946. This compound has been used ever since in attempts to seed clouds to incite rain or to reduce the risk of damaging hail. In the novel *The Cat's Cradle*, by the science fiction writer Kurt Vonnegut (brother of B. Vonnegut), one of the characters uses the analogy of a stack of canon balls to describe the process of ice nucleation. The balls in each layer teach those in the layer above them where to position themselves in order to stabilize the structure.

IV. Ice Nucleation Activity in the Biological World

A wide range of organisms produce molecules that are ice nucleation-active (INA). Although INA materials can 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 freeze-sensitive organisms to avoid damage to cell membranes. The ice nucleators produced by organisms described at present are almost universally proteins or proteinaceous compounds that may be associated with lipids, phospholipids and/or carbohydrates. An in-depth investigation of the ice nucleation activity of certain pollen suggests that carbohydrates are principally responsible for their activity (42).

Some plants produce ice nucleation-active materials such as those in the flowers of *Lobelia telekii*, a plant in the bellflower family found only on in alpine zones of mountains in eastern Africa, and in various internal tissues of trees in the plum family (*Prunus* spp.) and in winter rye. 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. The absolute temperature of their activity depends on how this activity is measured (if the particles were immersed in water before they were cooled to freezing or if they were allowed to collide with supercooled water droplets, for example) (16). Therefore, the values for temperature of activity presented here are most informative about relative differences in activity.

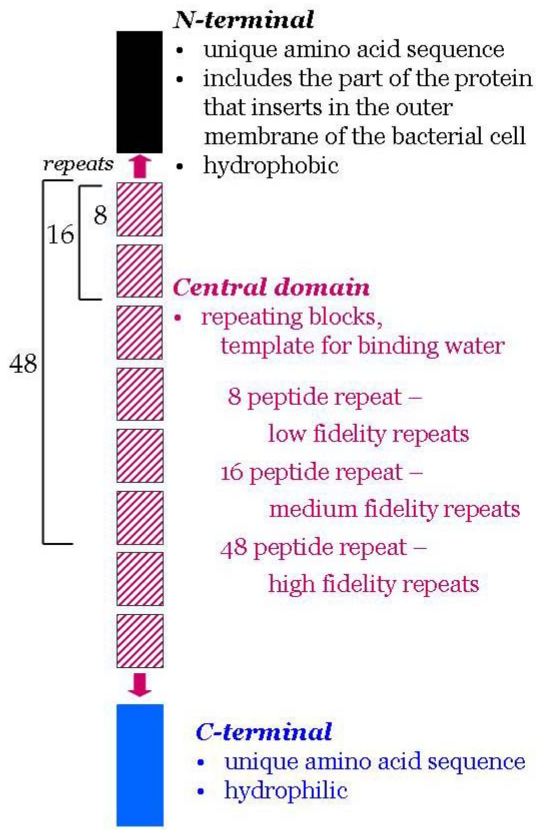
Insects and other invertebrates are well-known for their capacity to deploy autocatalysis of ice as a means of cold tolerance. The indigenous INA materials involved are usually part of body fluids such as haemolymph. Insects can also ingest or become covered with some of the INA microorganisms described below. Various vertebrates such as certain fish, amphibians and reptiles can have ice nucleators in their blood. But little of this material of animal origin is likely to be in the atmosphere in a form that could interact with cloud water droplets.

Several types of microorganisms produce INA materials including algae, fungi and bacteria. Some algal species in the genus *Chlorella*, ubiquitous in soils and waters, can catalyze ice formation at relatively warm temperatures such as -6°C. *Chlorella* spp. in particular are among the microalgae that are readily transported by air. But their contribution to the pool of biological ice nucleators in the atmosphere is unknown. The free-living fungi currently known to produce INA materials are all species of the genus *Fusarium*. Bulk hyphal growth and culture filtrates of these fungi can cause water to freeze at temperatures as warm as -5°C. But it is their spores that are most likely to be disseminated in the atmosphere. Strains of *Fusarium* spp. have been found in clouds and were INA when tested in the laboratory, but it is unknown if the spores disseminated into the clouds also carry the INA materials. The fungal component of several species of lichens (symbiotic associations of fungi and algae) are also ice nucleation active and are likely to be air-borne during part of their life cycle.

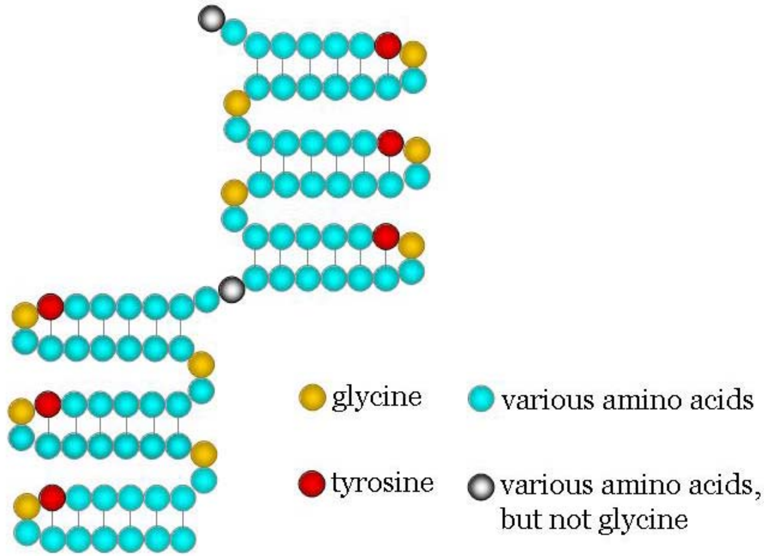
The most well-described microbial ice nucleators are bacteria. All of the known species of INA bacteria are in a single branch of the phylogenetic tree of prokaryotes called the γ-Proteobacteria, and many of them are commonly associated with plants, either as plant pathogens or commensals. Bacteria with distinctive ice nucleation activity include strains of *Pseudomonas syringae*, *P. viridiflava*, *P. fluorescens*, *P. borealis*, *Pantoea agglomerans*, *Pantoea ananatis* and *Xanthomonas campestris* pv. *translucens*. The material that engenders the INA of these bacteria has been particularly well described. These bacteria produce a protein anchored to the outer cell membrane that can constitute up to 1% of the total dry weight of the cell under optimal conditions for its production. In its ice nucleation-active form, the protein is a complex of multiple copies of the same β-helix sub-unit. The sub-units align in a manner that permits water molecules to bind in a pattern that favors the formation of ice embryos for subsequent crystal growth (This is illustrated in the Feb. 2012 issue of Microbe magazine). The complex is held in place by the lipids of the outer membrane and in particular by the phosphatidylinositol component. This protein complex is particularly responsive to environmental conditions. Its degree of aggregation changes rapidly in the face of temperature, availability of various nutrients and age of the bacterial cell. In its optimal configuration, the protein can catalyze the freezing of water at -1°C. But this is a very rare event that seems to occur for only a few cells of a few strains of these bacteria. The protein is the product of a single gene of about 4000 pairs of nucleotide bases in length. The gene is highly conserved among INA bacteria and likely is from a common γ-Proteobacteria ancestor. Descendants of this common ancestor that are not INA probably lost the gene completely or partially.

A cell of *Pseudomonas syringae* as seen under Transmission Electron Microscopy (photo: B. Delecolle & C.E. Morris, INRA)

The intensity and efficiency of the bacterial ice nucleation protein in catalyzing ice formation is the result of a dynamic processes dependant on the physiological activity of the bacterial cell. The initial step of production of the protein is its biosynthesis from the DNA sequence of the INA gene ①. The protein modules that are produced are then transported across the cells wall, they insert in the outer membrane and begin to aggregate ②. Additional compounds are needed to enhance the INA that is conferred simply by the protein. Mannonse and glucosamine transported to the outer membrane form a glycoprotein complex that enhances the INA ③. In addition, phosphatidylinosition transported to the outer membrane covalently anchors to the glycoprotein complex thereby re-enforcing the association of the protein modules and enhancing even more the INA ④. De novo synthesis of the protein modules can occur in response to environmental stimuli such as cold and nutrient limitation. The protein complex can also be stabilized and destabilized repeatedly in response to environmental stimuli.



The overall structure of the bacterial ice nucleation protein consists of a central domain of repeated peptides flanked by the N and C terminals. The N terminal of the protein is the part that is anchored in the outer cell membrane of the bacterial cell. The repeats in the central domain of the protein lead to the capacity of the protein to fold back on itself into what is referred to as a ß-pleated sheet, described in the next figure.



The particularities of the amino acid sequence of the bacterial ice nucleation protein lead to the formation of a folded configuration called a ß-pleated sheet. Tandem peptide repeats play an important role in self-assembly and aggregation of proteins. The necessary conformation for the ß-pleated sheet is facilitated by the presence of the amino acid glycine (the "smallest" of all the amino acids in terms of the space it occupies) before or after an aromatic amino acid in the repeated octapeptide region of the protein. Tyrosine is an aromatic amino acid. The presence of glycine, in particular, next to tyrosine allows for a high degree of conformational freedom of the peptide chain and facilitates folding into a ß-pleated sheet structure that is essential for protein aggregation (19). The folding is stabilized by internal serine and glutamine ladders. A recent model predicts that each chain of dimers contains the water-binding sites responsible for creating ice nuclei. They are located opposite to each other and consist of two different series of four amino acids: TQTA (threonine-glutamine-threonine-alanine) on one side and SLTA (serine-leucine-threonine-alanine) on the other (18). According to a model of the ice nucleation protein developed from data from the INA bacterium *Pseudomonas borealis*, a dimer of 2 protein strands, each consisting of 64 hair-pin folds, would form a protein 320 Å in length and 40 Å in width with two active surfaces for binding water. This dimer complex would be able to catalyze freezing at -12° C. An oligomer of at least 50 of these dimers would be required to catalyze freezing at -2 to -3° C according to this model (18).

V. The Case for Microbial Orchestration of Vapor-Liquid-Ice Dynamics in Clouds

It is the potential interaction of ice nucleation-active microorganisms with processes leading to rainfall that we consider the most tantalizing. It has also captivated the interest of many other scientists who are now striving to overcome the obstacles of bringing together a wide range of disparate scientific disciplines to explore this question. There has also been controversy around the idea that microorganisms could have a marked role in atmospheric processes leading to rainfall. A main point of contention concerns the abundance of biological ice nucleators in the atmosphere – are they sufficiently abundant to set off rainfall and if so, where and when could this happen? Part of the controversy arises from the fact that there has been much more speculation than there have been measurements of the activity of biological ice nucleators in the atmosphere. These measurements are only just beginning, and they are critically needed to set the limits in which atmospheric models will be run (in other words, to constrain or parameterize the models). To understand the arguments, we need to understand some of the basic processes involved in the transformation of cloud drops into rain drops.

What happens in clouds that leads to rainfall?

A cloud is composed of ultra-light droplets of liquid water that remain suspended in the air. Cloud droplets form when water vapor condenses onto aerosol particles that are competent as cloud condensation nuclei. In clouds that are not yet yielding rain, these droplets have diameters between about 1 and 100 µm, with most of them being smaller than about 20-30 µm in diameter. The resistance of the air is such that droplets of this size can not fall to the ground. To reach a size sufficient to overcome the resistance of the air, cloud droplets must grow. In most clouds, with the exception of deep convective clouds, the growth of cloud droplets to form rain drops is mediated by ice. Ice plays a role in a series of physical processes that are favorable for growth of the droplets. The concentration of droplets in a cloud is variable along the altitude of the cloud, with 10^8 droplets /m^3 being among the higher concentrations observed. As air currents move droplets around, in many clouds these droplets are not large enough to form rain drops simply by coalescing with each other because their efficiency at colliding during any given droplet-droplet interaction is too low. Ice enhances this process.

As the first step in the process of droplet growth, droplets must attain a temperature below 0°C. As explained in the chapter III, this will not necessarily lead to freezing. The droplets might remain supercooled, in other words they remain in the liquid phase below 0°C. But at temperatures above 0°C, freezing is impossible. So, the first key event is the freezing of some supercooled cloud droplets. Freezing of a droplet will depend on the droplet harboring a particle of foreign material, an impurity that can initiate freezing (material that is ice nucleation active as described in chapter IV) at the temperature of the surrounding cloud. Once a droplet freezes, it can attract more water and grow by three principle processes. The newly formed ice crystal can grow by diffusion of vapor onto it. This is rapid in any mixed-phase (liquid + vapor) cloud. As the growing ice crystal starts to fall it can also collide with suspended supercooled droplets thereby causing them to freeze onto the growing crystal. This process of accretion known as riming can, in extreme cases, lead to the formation of graupel or hail. And finally, if there are other ice crystals, they can aggregate simply by colliding and sticking to each other. This latter process of ice-ice aggregation is facilitated at certain temperatures such as near -15 °C where branches of dendritic ice crystals can inter-lock and near 0 °C where the ice surface can be liquid (e.g. due to melting or wet growth of riming). As the crystal falls toward the ground it can experience cycles of melting and then evaporation or re-freezing depending on ambient conditions outside of the cloud. A raindrop that successfully reaches the ground might be as large as 2000 µm in diameter, could weigh 30 mg and contain 0.03 ml of water.

The droplet-ice crystal ratio

As we can understand from the description above, rainfall depends on an appropriate ratio between ice crystals (i.e. frozen cloud droplets) and unfrozen supercooled droplets in a cloud. If there are too many ice crystals relative to supercooled droplets, the liquid from the supercooled droplets will evaporate away and there will be considerable competition among crystals to recover this vapor. None might attain the mass necessary to start growth by aggregation or riming and will not fall as precipitation. If there are too few crystals, there will be an insufficient number of raindrops to lead to measurable rainfall. The optimal ratio is 1 ice crystal for 10^5 or 10^6 supercooled droplets. This ratio is at the heart of the debate about the significance of biological ice nucleators in the formation of rainfall. The available data about microorganisms in the atmosphere only allow us to make approximations about the concentrations of biological ice nucleators that could be in clouds; direct measurements of the activity of biological ice nucleators in clouds are only just beginning. Bacteria are the most abundant of the different microorganisms in the atmosphere. In cloud water there are from 10^3 to 10^5 bacterial cells/ml (data are summarized by Delort and colleagues (13)). Only a small fraction of these are known to be ice nucleation active. Among the bacteria isolated from clouds, the only ones with known ice nucleation activity are *Pseudomonas syringae*, but there are no quantitative data about their concentrations in clouds. In freshly collected rain water in southeastern France, we have found that *P. syringae* – when it is present – constitutes from 0.1% to 10% of the total bacterial population in the rain water (36). This lets us make a first estimate that there could be from 10 to 10^4 cells of a potentially ice nucleation active bacterium per ml of cloud water. On average, clouds contain between about 0.01 g (stratiform clouds) to 1 g (convective clouds) of water per m^3 of cloud. If we take the case of convective clouds, this would mean that there could be from 10 to 10^4 ice nuclei – one for every cell of *P. syringae* depending on the temperature – among the 10^8 cloud droplets per m^3. But we know that not all cells of *P. syringae* are ice nucleation-active. The capacity of this bacterium to act as an ice nucleus varies even among cells that are otherwise genetically identical. Laboratory studies of the optimal conditions of ice nucleation activity by *P.*

syringae indicate that for most strains less than 0.1% of cells are active at -4° or -5°C but that at -8°C about 10% of cells can be active. Furthermore, in the only direct measurements of the activity of biological ice nucleators in environmental samples, the greatest values detected in rain were on the order of 0.1 ice nuclei per ml active at -7°C (9). The whole of the available data suggests that it is not unlikely that there are conditions under which there are sufficient numbers of ice nucleation-active bacteria to incite the processes that lead to rainfall.

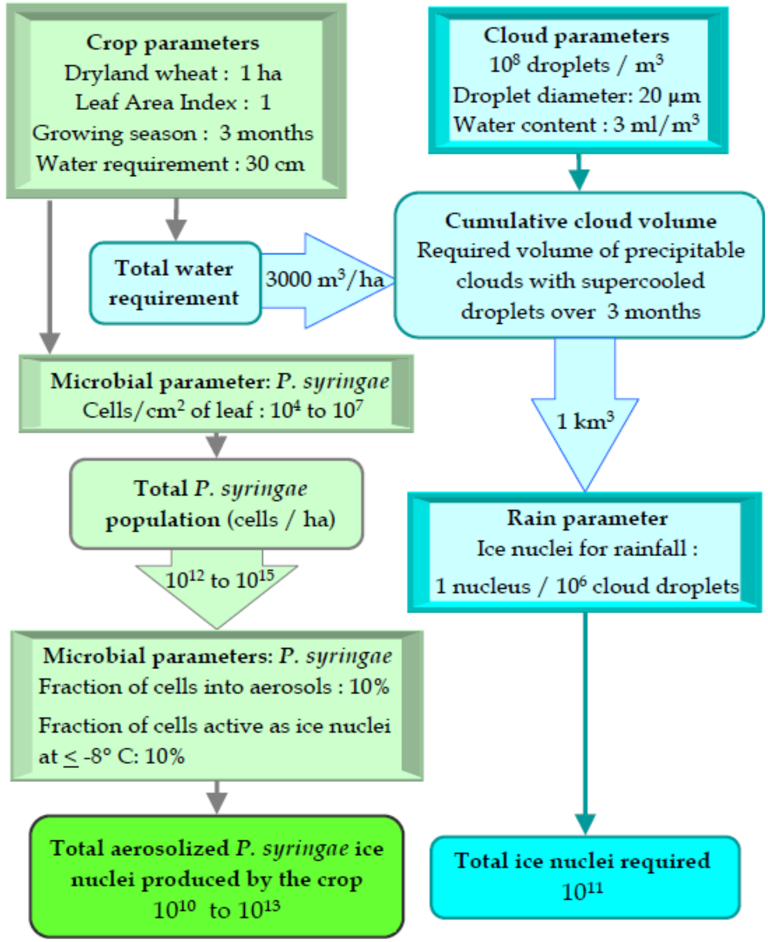
The greatest window of opportunity for biological ice nucleators to incite rainfall is in clouds that have relatively warm minimum temperatures, above -10°C. At such warm temperatures there are no other naturally-occurring materials that are ice nucleation active under the conditions that govern the microphysics of freezing in clouds. Furthermore, temperatures between -3° and -8°C are propitious for ice multiplication, known as the Hallett-Mossop process (24, 37). As crystals grow via accretion they can become draped in a filigree of rime that can eject ice splinters or shards. These shards can then grow via the same processes as other crystals. Hence, the apparent lack of sufficient abundance of biological ice nucleators in the atmosphere might be compensated by proliferation of ice crystals by fracturing, thereby leading to the formation of enough raindrops to fall to the ground as palpable rain.

The processes we have presented above represent a simplified synthesis of a large body of scientific literature on the physics of clouds. The details can be found in Pruppacher's seminal work on cloud processes (41) and in numerous reviews on ice nucleation in clouds as listed above (8, 14, 26, 32, 47-49).

VI. Landscapes, Plant Microflora and Rain

The story of ice nucleation-active bacteria and rainfall is deeply rooted in agriculture. Most of the known ice nucleation-active microorganisms colonize plants and many can also cause diseases of plants. Plants are the principle sources of these microorganisms in the environment. Rain makes plants grow. But dare we ask if the corollary is true – that growing plants contribute to making rainfall? Clearly, evapotranspiration of water from plants is a major source of water vapor needed for cloud formation over continents. The types of plant cover and the associated management of soils also influence heat exchanges and other processes that underlie the ascent of water vapor and aerosols into the atmosphere and the formation of clouds. But the more subtle aspect of this question concerns the ice nuclei that could be generated by plants, and in particular the ice nucleating microorganisms that they harbor. To pose the question this directly makes the idea seem like an oversimplification. However, one of the driving motivations for the recent interest in the impact of biological ice nucleators on rainfall comes from this simple question. Above all, there is significant documentation on the effect of regional landscape changes on rainfall (40). In light of the importance of plants, and in particular cultivated plants, on the abundance and variety of microorganisms in the atmosphere, it is reasonable to wonder if the amount of air-borne biological ice nucleators liberated from plants could be sufficient, under some conditions, to have a tangible effect on the atmospheric processes leading to rainfall. A rigorous evaluation of this question will require concerted efforts to obtain additional data on the abundance and activities of biological ice nucleators in the atmosphere, to improve regional atmospheric models and to develop approaches to validate model predictions under field settings. But we are a bit impatient, and we are striving to construct arguments to whet the appetites of the corps of scientists and research institutes who could contribute to achieving this evaluation. So we have made some simple calculations, presented in the figure below in this chapter.

If a landscape contributes to producing sufficient ice nucleators to influence rainfall, we must evidently wonder about the consequences of changes in landscapes. What are the effects of overgrazing, desertification and urbanization on the abundance of microbial ice nucleators in the atmosphere? What is the importance of the cultivars of crops that we choose to plant for their specific capacity to resist certain diseases or to produce high yields? Different crop cultivars and plant species in general are very different in terms of the abundance of microbial ice nucleators that they harbor (20, 30), but there has been no work to examine how this is related to other agronomically important traits such as yield or disease resistance. The overall layout of agricultural and peri-urban landscapes also creates topographies that in turn favor the turbulent and convective movements of air that will lift them into clouds. This has led us to wonder if we can identify the traits of vegetated landscapes that would be most favorable for them to have an impact on precipitation. If these traits can be identified, then would it be possible to alter vegetated landscapes in such a way that the plants we cultivate effectively seed clouds with sufficient numbers of ice nucleation active microorganisms to enhance rain on a regional scale? Although there are those who might consider these questions outlandish, we believe that they are questions that cannot be simply disregarded. In the private sector there are mounting efforts to restore the disappearing vegetation of our planet and to restore forests in particular. Monumental efforts, led by foundations such as Forest&Life and WeForest and regional efforts such as the Rain Drop Project are motivated by the key role that plants play in a wide range of ecosystem services and their contributions to the water cycle in particular. The massive re-forestations planned by these movements could be an opportunity for scientists to explore the multiple ways in which landscape is linked to rainfall and to contribute to addressing the issues of landscape-precipitation interaction on a regional scale already raised in the scientific community (40).



A simple simulation of the potential of crops to seed the rain that they need. In a real-life setting the parameters indicated here are dynamic in space and time. Here they have been simplified to make a rough estimate.

If plants generate ice nucleators for rain, it is most likely that the rain will fall somewhere else than above the plants that generated the ice nuclei. So perhaps the real beneficiaries of a rainfall cycle are the microorganisms themselves. Curiously, ice nucleation activity is a rare trait among microorganisms, and bacteria such as *P. syringae* are usually a minor component of most of the microbial communities in which it is found. Yet this bacterium is very widespread in numerous habitats and across the planet. In its relative scarcity it assures that the ice crystals that it induces in a cloud will not have many competing crystals and will be able to grow to form a raindrop or a snowflake that brings it down to a new terrestrial habitat.

As a graduate student, C. Monteil at INRA in Avignon, France sampled snowpack throughout the winter in the southern French Alps and demonstrated that the snowpack, and in particular the litter-snow interface, is a habitat for *Pseudomonas syringae* (33). In the spring, the melting snow carries the bacterium into the rivers of the local water sheds. (photo F. Lafolie, INRA, Avignon)

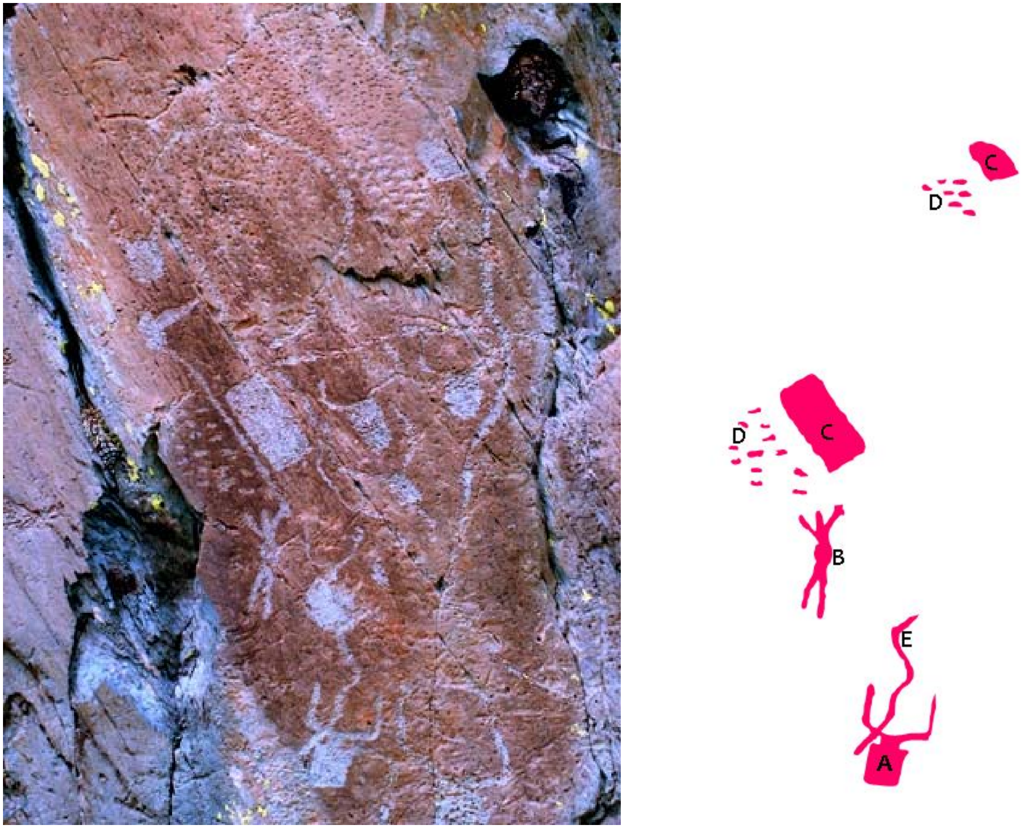
VII. Epilogue: A Midsummer Night's Dream of Ice

Near the end of Shakespeare's famous play *A Midsummer Night's Dream*, Hippolyta cries "The skies, the fountains, every region near seem'd all one mutual cry: I never heard so musical a discord, such sweet thunder". When this phrase was pronounced by an outdoor theater troop in the plains of Wisconsin in the mid 1980's, coincidently at the same moment that a summer storm burst onto the sky's stage with a great bang of thunder, the play came to a halt. The audience and actors turned in awe to watch nature's spectacle. Lightning is primitively alluring. This is particularly so in the Southern Alps where the lightning that strikes near the Bégo Mountain has allured pilgrims for thousands of years. Bégo Mountain, in the current day Mercantour National Park, is the first in the region to trap air masses from the Mediterranean Sea where atmospheric processes can be set off into storms. The unusually high iron content of this mountain is propitious for lighting. The majestic bolts can be seen from hundreds of kilometers away. Rocks in the valleys at the base of the Bégo are covered with engravings made by pilgrims attracted by the allure of this powerful site. Over 40000 engravings were made during the Neolithic period (ca. 5000 years ago), and many testify to human's deep veneration of the water cycle and recognition of its importance for agriculture even at the very advent of agriculture in this part of Europe. Walking through the Fontanalba Valley on the eastern slopes of Bégo Mountain with a historical interpreter, we had our own mid-summer's dream about ice nucleation active bacteria.

The bulk of the symbolism of the engravings in the Fontanalba Valley concern lightning, water that flows through irrigation channels into interconnected Cartesian agricultural fields, and the life-giving oxen whose force is used to plow fields and whose upright horns maintain a spiritual link with the powers of the sky. Underlying the atmospheric processes, as they were perceived from the perspective of Neolithic humans, are the physical and biological mechanisms that we understand today. Ice crystals are a key factor needed for the formation of lightning; ice-ice collisions produce the charge separation needed for the flow of electricity in lightning discharges within or from a cloud (21). In summer, minimum cloud temperatures could be warmer than -10°C. Under such conditions, and in our mid-summer's dreaming, ice nuclei just might become a limiting factor for lightning formation if biological ice nucleators such as

INA bacteria weren't present in clouds to initiate ice formation. And likewise for rainfall, ice nuclei could also be limiting under warm conditions in the absence of biological ice nucleators. From our own research we know that the region of the Southern Alps is particularly rich in ice nucleation active bacteria such as *P. syringae*: it is in wild plants, in leaf litter, in freshly fallen rain and snow, and in the mountain lakes and rivers (33, 35, 36). These reservoirs are indicators that the bacterium precipitates with rainfall, but they are also indicators of potential sources for aerosols that could transport bacteria into the clouds. What types of engravings might have been made in the Fontanalba Valley if the association of bacteria with the water cycle were not invisible?

A plateau in the Fontanalba valley with a view on Bégo Mountain (upper left hand corner). (photo: CE Morris)



Engravings in the Fontanalba Valley. This example depicts what has been interpreted as oxen heads (A), a human figure (B), fields of crops (C), seeds (D), and either a bolt of lightning or a part of an irrigation channel (E). The long lines between fields that traverse many of the engravings are interpreted as irrigation channels. Nuggets of iron oxide can be seen in the upper right and lower left hand corners of the photo (photo CE Morris).

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