An “overlooked” habitat for thermophilic bacteria: the phyllosphere

Jean Jacques Godon†, Amandine Galès§, Eric Latrille§, Pornpimol Ouichanpagdee†, Jean-Philippe Seyer§

† INRAE, Univ Montpellier, LBE, 102 avenue des Etangs, 11000 Narbonne, France
§ INRAE, Univ Montpellier, LBE, Narbonne, France
| Division of Biotechnology, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai, Thailand

Corresponding author: Jean Jacques Godon (jean-jacques.godon@inra.fr)
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Abstract

Thermophilic microbes are present everywhere around us and their only known natural biotope is far away and most usually associated with geothermal energy. To answer this paradox, we explore the hypothesis that the phyllosphere (surface of leaves), due to its exposition to the sun, could well be a thermophilic habitat for microbes and thus a source of thermophilic microbes growing around 50°C – 60°C. To support this hypothesis, we reviewed the heat sources on earth and associated microbial habitats, as well as the difficult identification of thermophilic microbes. We further present an experiment to show the presence and activity of thermophilic bacteria in the phyllosphere. Leaves were collected from eleven tree species from five locations on three continents belonging to three different biomes. On fresh leaves, 16S rDNA sequencing reveals the presence of 0.2 to 7% of clearly identified thermophilic bacteria. Moreover, after incubation at 55°C under aerobic and anaerobic conditions, 16S rDNA sequencing reveals the presence of 4 to 99% of clearly identified thermophilic bacteria. The accumulation of observations provides coherence to our hypothesis and allows the emergence of a new vision of leaves as a thermophilic biotope. We then propose a life cycle of microbes belonging to the thermophilic biotope associated with leaf surfaces.
Keywords

phyllosphere, thermophile, biotope, airborne, bacteria

Introduction

Thermophilic microorganisms are the Holy Grail in Biotechnology. Thermophilic microbes are extensively used in processes such as thermophilic anaerobic digestion, composting as well as other fermentation processes and the cellular components of such microorganisms (i.e. enzymes «thermozymes», proteins and nucleic acids) have considerable potential for many industrial applications (Bruins et al. 2001, Haki 2003). However, the real biotope of this unique microbial resource is still poorly known and in fact, the resource is under-exploited.

The average temperature of the earth is 15°C. The temperature, however, varies from 7000°C (centre of earth) to −89°C (Vostok Station in Antarctica). Over this huge range of temperatures, microbes can grow between -18°C and 113°C and can encounter four sources of heat: geothermal energy, self-heating (the organisms’ metabolism), human/animal activity and solar radiation.

The first one is considered as the true ‘thermophile biotope’, but geothermally-heated regions are rare, scattered, far removed from laboratories and to find them, the “hunter” of these microbes must travel to some of the most remote and inhospitable regions of our planet.

The heat from organisms' metabolism concerns mainly mesophilic microbes (through homeothermic animals), but it concerns also thermophilic ones through aerobic fermentation where microbial metabolic activity generates its own heat with temperatures up to 65°C (Diaz 1999). Known examples in nature have not been fully documented, except for their use by humans and certain animals which drive such thermophilic fermentations (Hechenleitner et al. 2015, see Table 1). All the self-heated fermentation observed has involved plant biomass.

| Habitat                  | Origin of the heat | Temperature Type | Ref                |
|--------------------------|--------------------|------------------|--------------------|
| hot springs              | geothermy          | 50 to 120°C      | Chaban et al. 2006 |
| deep sea hydrothermal vents | geothermy         | 50 to 120°C      | Chaban et al. 2006 |
| oil reservoir            | geothermy          | 50 to 120°C      | Chaban et al. 2006 |
| ocean crust              | geothermy          | 50 to 120°C      | Chaban et al. 2006 |
| deep marine sediment     | geothermy          | 50 to 120°C      | Chaban et al. 2006 |
| continental deep subsurface | geothermy         | 50 to 120°C      | Chaban et al. 2006 |
Mankind has developed various types of high-temperature biological processes where the heat is artificially produced. Such technological environments, inhabited by thermophilic microbes (optimal growing conditions between 50°C-65°C) are various or ancient, notably: (i) some cheese making processes (pressed cooked cheeses), (ii) anaerobic digestion reactors (van Lier et al. 2001, Table 1).

Finally, the sun is the main source of heat on Earth, providing 3680 times more energy than the geothermal energy. Surfaces which directly receive this energy must provide a thermophilic environment but paradoxically, it does not define any known thermophilic biotopes.

We deal here with the ubiquity of thermophilic microbes but, as highlighted, there are only four origins for heat and three known biotopes, with two of them being artificial. Moreover, the only natural habitat (geothermal energy) is located in few very specific places. Thus, the biotope for the majority of thermophiles still remains unknown.

**Hypothesis**

To resolve this discrepancy between presence without the known biotope, we made the assumption that the leaf surface could well be the best candidate for natural biotopes of thermophilic microbes because they are widespread all over the planet.

Several observations allow us to make the hypothesis of the phyllosphere as a thermophilic habitat. First of all, we are looking for a biotope for thermophilic organisms...
that are present in abundance on mesophilic environments of the Earth. Secondly, we need to find a biotope for thermophilic organisms that is not linked to the Earth's geothermal energy. Finally, we need to find a biotope for thermophilic organisms, isolated from mesophilic environments which are directly or indirectly related to plant material and can utilise and grow on polysaccharides. This ability is incompatible with the scarcity of such polysaccharides in thermophilic biotopes, such as hot springs or deep sediments.

On the other hand, the phyllosphere ticks all the boxes as the main habitat for thermophilic microbes: firstly, leaf surfaces are everywhere: plants cover 90% of planet's land surface and the phyllosphere is estimated to exceed $10^8$ km$^2$ (Morris and Kinkel 2002). Moreover, plant leaves harbour a large number of microorganisms: the estimate is about $10^{24}$ to $10^{26}$ bacterial cells (Morris and Kinkel 2002) of the $10^{30}$ that live on Earth (Whitman et al. 1998). Up to $10^7$ bacteria per cm$^2$ are present on leaf surfaces (Morris and Kinkel 2002, Carotenuto 2017, Lindow and Brandl 2003, Bulgarelli 2013). Secondly, leaf surfaces are heated by the sun and plants have a very low albedo and absorb a large amount of the energy from solar radiation. Plant albedo values vary widely, ranging from 0.05 in coniferous forests to 0.25 in grasslands (Coakley 2003). The main feature of heat from the sun is its variability. The heat source stops each night and its intensity during the day depends on the latitude, seasons and climate. Finally, leaves are plant material and are rich in polysaccharides.

**Observations and supporting arguments**

The aim of this paper is to provide arguments in support of this hypothesis and to answer the following questions: (i) Can leaf surface provide the main biotope for thermophilic microbes? (ii) Are thermophilic microbes present on leaves? (iii) What are the ecology and the life cycle of microbes living on leaf surface hot biotope?

The thermophilic identification, based on the ability to grow at temperatures higher than 45°C, was checked in the bibliography. By using this method, 44 out of 582 OTUs identified on fresh leaf samples were close to known thermophilic bacterial species, 40/368 OTUs after aerobic incubation at 55°C and 20/168 OTUs after anaerobic incubation at 55°C (Fig. 1). Comparison of the abundances showed a complete shift of the microbial communities, with a large increase in the abundance of the identified thermophilic phylotypes from 3% on fresh leaves to 45% and 10% on aerobic incubation at 55°C and on anaerobic incubation at 55°C, respectively (Fig. 1). In fact, simply, thermophiles present, but rare (not detected), on fresh leaves grow at 55°C. Anyway, these values are only the default minimum values. The trend is stronger if we consider species described as non-thermophilic, but belonging to genera containing certain thermophilic species, from 10 to 74 and 72%, respectively (Fig. 1).

These results confirm data in literature. Indeed, 16S rDNA corresponding to bacteria described as thermophilic are often found on leaf surface communities (Gandolfi 2017). Thermophilic fermentation of plant products leads to an increase in thermophiles on fermented products such as tea (Lv et al. 2013) or compost. A comparison between leaf
bacteria after thermophilic incubation with bacteria found in compost (Silva et al. 2016) reveals a great similarity between the microbial compositions of compost plant and the leaf surface. Thus, leaf surface is a biotope for many thermophiles, but all microbes that inhabit leaf surfaces are not thermophiles or cannot be identified as thermophiles.

The biotope

The temperature of leaf surfaces varies with the local meteorological parameters and regularly fluctuates in accordance with the day/night alternation. In addition, even for heliophytes, only one side of the leaf is generally exposed to the sun and thanks to the sun's location, all the leaves are not exposed at once. For living leaves, the highest temperature reported on the surface was 53.1°C for a succulent plant (agave) and 50.4°C for a non-succulent (liriodendron) (Kuraishi and Nito 1980). These temperatures are highly

Figure 1.
Phylogenetic, geographical, environmental parameters of leaf samples and thermophilic state of OTU identification. 1, average annual sum of solar potential; 2, average temperature corresponding to the month of sampling. 3, abundance of identified OTUs are shown for each leaf sample: T+: correspond to closest species described as thermophilic or thermo-resistant; T?: correspond to closest species described as non-thermophilic but belonging to genus containing some thermophilic or thermo-resistant species; T-: correspond to closest species described as non-thermophilic or thermo-resistant or thermophilic status unknown due to a distant phylogeny or low abundance of the OTU.
dependent on the plant type (conifer, evergreen, herbaceous etc.) (Kuraishi and Nito 1980), as well as on environmental conditions (i.e. solar radiation, wind speed, air temperature and humidity) (Pincebourde and Casas 2015). Evapotranspiration enables plants to keep leaves below 50°C. Leaves have complex structures covered by a cuticle. The cuticle, composed of wax, reflects radiation and regulates the temperature. Furthermore, the leaves 3D structure is complex, with trichomes and stomatal cavities which, by diffusion, increase the protection against solar radiation. At the microbial scale, the actual temperature remains unknown. To sum up, temperatures on the phyllosphere are basically very variable, ranging from cold to hot and often even reaching 50°C, but in the absence of evapotranspiration on dead leaves or partially-dead leaves, the temperature may increase.

Which foods can microbes find on the leaf surface? Living plant cells are well protected and only a few specialised pathogens can penetrate to feed inside the cells. At the microbial level, however, leaf surfaces can provide water, exudates or volatile compounds (terpenoids). The vast surface area of foliage facilitates access to compounds in the air such as O₂, CO₂, CO, CH₄, volatile organic compounds or alkanes in urban pollution (Gandolfi 2017). The life cycle of leaves is, however, from living cells to dead cells. Microbes can feed from dead cells on plants (e.g. yellow dots) or dead leaves on the soil (see section: Transportation and cycle of life and Fig. 2). As examples, the production of phytase from thermophilic fungi (Singh and Satyanarayana 2009) or of cellulose from thermophilic bacteria (Acharya and Chaudhary 2012) indicates, unambiguously, the substrate of these organisms.

Figure 2.
Life cycle of thermophilic microbes associated with leaf surfaces.
The inhabitants

Based on the phylogeny within bacteria, only two phyla contain hyperthermophiles (Aquificae and Thermotogae), whereas thermophiles are present within several phyla, including Cyanobacteria, Firmicute and Actinomycyes. Within Archaea, 7 out of 10 phyla contain hyperthermophiles and thermophiles. Within Eukarya, three groups contain thermophiles, two belonging to Ascomycetes (Eurotiomycetes and Sordariomycetes), the other to the Mucorales group (Morgenstern et al. 2012). These three groups contain organisms associated with living plants (pathogens or endophytes). Thus, for bacteria and archaea, thermophiles cannot be determined by phylogeny, whereas for eukarya, phylogeny allows identification of thermophile groups.

One may think that thermophilia, as a trait, is encoded in the genome of thermophiles. However, there is no signature of thermophilic adaptation on the 16S rDNA sequence (Galtier and Lobry 1997) and the existence of other signatures, based on DNA composition, remains controversial (Musto et al. 2006). Amino acid composition signature can define some thermophiles, but not all (Puigbò et al. 2008).

Thermophilic microbes can be identified either by physiological data from isolated microbes or by 16S rDNA from microbial communities growing in thermophilic conditions. The paradox is that, for isolated microbes, the possibility of identifying them as thermophilic is based on the growth at high temperature but, unfortunately, the growth at high temperatures is rarely tested for environmental microbes. Growth in thermophilic conditions is, indeed, only tested in two circumstances: when microbes are isolated from either known thermophilic or hyperthermophilic environments, such as hot springs or, paradoxically, from psychrophilic environments (McBee and McBee 1956). Otherwise, information is lacking. Thus, the link assumed between the growth temperature limits and the environment has given rise to curious and inexplicable examples in literature: (i) a thermophilic enzyme extracted from Acinetobacter baylyi which was assumed as a mesophilic species (Uttatree et al. 2010); (ii) the mesophilic Staphylococcus aureus became thermophilic (grew at 48°C) after the addition of 5.8% sodium choride (NaCl) in slurry (Hurst and Hughes 1983); (iii) a single mutation transformed Pseudomonas fluorescens from a mesophile to a thermophile (DeCicco and Noon 1973). To sum up, using physiological data from literature enables us to affirm only that a microbe is thermophilic, but almost never to affirm that it is not.

In the same way, the majority of microbes, molecularly identified by their 16S or 18S rRNA genes growing in thermophilic conditions, are not recognised as thermophilic through the physiology of their close relatives. Their 16S rDNA sequences are similar to microbes considered as mesophilic in both anaerobic (Town 2014 and aerobic conditions (Silva et al. 2016).

Candidate which can belong to the thermophilic phyllosphere?

We here propose, by the way of an example, the ecology of three bacteria without a clearly identified biotope, although a thermophilic leaf surface biotope would correspond to their physiology: (i) Geobacillus is a thermophile, endospore-forming, present in the air,
compost and on leaves, resistant to radiation and able to decompose complex polysaccharides found in plant biomass. The article entitled ‘the Geobacillus paradox: why is a thermophilic bacterial genus so prevalent on a mesophilic planet’ (Zeigler 2013) provides a resume of the paradox of microbes orphan of habitat. (ii) *Saccharopolyspora rectivirgula* is a good example of a bacterial species living in the phyllosphere. It has already been found in air and on leaves (Le Goff 2010, Gales 2014) and in several anthropic biotopes involved in plant material degradation such as dairy barns, compost facilities and mushroom cultivation rooms (Blais Lecours et al. 2012, Schäfer et al. 2013). (iii) *Sphingomonas* is present in air, in compost and on leaves (Vogel et al. 2016), but is considered as psychrophilic and mesophilic (Bowman 1997). It has never been tested for its thermostolerance. Nonetheless, *Sphingomonas* spp. are able to produce extracellular thermoresistant polymers, such as sphingans (Lin and Casida 1984).

**Identification of thermophiles by experiment**

Despite these limitations in identifying thermophiles, we carried out experiments with the objective of identifying the leaf surface as a thermophilic biotope: 11 sets of fresh leaves were collected from different tree species (Fig. 1) belonging to three different biomes (deciduous forest, chaparral, tropical rain forest) and exposed to different temperatures and solar radiation intensities (Fig. 1). Subsequently, these leaves were incubated for two months under aerobic and anaerobic thermophilic conditions at 55°C. From these three contexts and in 19 samples (7 fresh leaf samples, 9 leaf samples after aerobic incubation and 3 leaf samples after anaerobic incubation), the presence of thermophilic bacteria within bacterial communities was tracked using 16S rDNA gene sequences (Material and Methods on Suppl. material 1).

**Evaluation of the hypothesis**

Our observations show the presence of live thermophilic bacteria on the surfaces of many different leaves. However, this does not prove the hypothesis of the phyllosphere as a thermophilic biotope.

Consequently, rather than characterising a physiology (thermophilic) or a biotope (leaf surfaces), it is necessary to consider the cycle life of these organisms. Their cycle is the same as the leaf biomass, with three phases: (i) the living leaf, (ii) the decomposing leaf and (iii) the air (Fig. 2).

First, leaves are non-perennial and non-contiguous biotopes; each new leaf must be colonised by new microbes. This colonisation from old leaves or from other environments is done mainly by airborne transportation. Atmospheric depositing must be considered as essential in the ecology of these thermophilic microbes. To survive the stress of transportation (UV radiation, desiccation, cold), many thermophiles are spore-forming organisms (firmicutes, actinomycetes, dycarya) (Setlow 2014).
The presence of thermophilic micro-organisms in the air can be explained by the carrying off of their putative biotope from their leaf surfaces. Thus, thermophilic microbes are present in air (Marchant et al. 2008) and transportation by air can explain the presence of thermophiles in many unexpected environments, for example, in the Arctic or Antarctic (Hubert 2009, Marchant et al. 2008). For microbes, air is an extreme environment (UV, dry, cold), but not a thermophilic environment since, due to the size of microbes, radiation from sun diffuses rapidly. Moreover, air and the phyllosphere have microbial communities with different structures (Vokou et al. 2012) and leaves cannot be just a convenient landing surface for airborne microbes. Indeed, airborne bacteria can land on leaf surfaces, but they can also land on other surfaces everywhere (Ottesen et al. 2016). Thus thermophiles can be found everywhere.

The thermophilic inhabitants of the phyllosphere must be able to cope with high temperatures coming from two origins: the sun on the surface of the leaves, but also in the soil during self-heating compost-type fermentation. These microorganisms must also be able to withstand low temperatures: (i) on the surface of the leaves during daily and seasonal variations; and (ii) in the air during their dissemination. This may explain the resistance to low and high temperatures observed in certain bacteria whose biotope is still poorly defined (Hubert 2009).

Responses to stresses such as UV radiation, reactive oxygen species (ROS) and desiccation have been identified as important functional traits of the leaf-colonising bacteria (Vorholt 2012) and metaproteomic analysis of these phyllosphere bacteria confirms this assertion (Delmotte 2009). Such stresses are similar to stresses undergone during an air journey (Potts 2005). Known thermophiles present physiological responses to various stress conditions which include the stresses within the phyllosphere (Ranawat and Rawat 2017).

Relevance of the hypothesis

The optimal growth temperature for microbes covers a broad range, from -18°C to 113°C, but not as a continuum. Two temperatures seem to be most favoured: one around 30°C-40°C (mesophilic range), a second around 50°C-60°C (thermophilic range). This observation may be biased by the sampling. However, unbiased measurements, based on cold Arctic seabed populations and on sulphate-reduction activity, also reveals mesophilic and thermophilic optima (Hubert 2009). The mesophilic range can be easily explained by the temperature prevailing in the bodies of homeothermic animals (birds and mammals). This present study provides an explanation for the thermophilic range which may correspond to the maximum temperature which occurs on leaf surfaces exposed to the sun.

The central interest in our hypothesis is that it reconsiders thermophilic microbes through their biotope and their life cycle. The phyllosphere is a carbon-rich habitat (heterotroph) which must cope with day/night variations, strong radiation and water-related stresses; this habitat is completely different from the widely-known thermophilic habitat which is stable in...
temperature, poor in carbon and undergoes chemical stresses (chemolithotroph), but exhibits no stresses due to water deficit or radiation. Moreover, the phyllosphere is a discontinuous habitat and the life cycle of leaves goes from living plant (from less than 6 months for annual plants or deciduous trees to several years for evergreen plants) to decayed plant on the ground. This involves for associated microbes: self-seeding by air, colonisation and resistance to the associated stresses (UV radiation and desiccation). Thus, thermophilic organisms or thermophilic enzymes from the phyllosphere will, in all probability, be more suitable for use in biotechnology (Blumer-Schuette 2012) than those counterparts from the geothermal energy habitat.

Conclusions

The living phyllosphere and the dead phyllosphere on the ground cover the majority of the Earth's land surface and this surface is warmed up by the sun. Physiology of many thermophilic microbes corresponds to a life associated with plant material. Literature and our experiments show the presence of thermophiles within the phyllosphere microbiota. Despite these converging facts, final proof that the phyllosphere is a major thermophilic habitat cannot be proved because: (i) the temperature conditions at leaf surfaces are not really known; (ii) the thermophilic status of the majority of environmental microbes remains unknown; (iii) thermophiles are present in the air and they are spread everywhere by the air. Nevertheless, our hypothesis does not simply offer a newly-discovered habitat for homeless thermophiles, but it also allows a different interpretation of data related to the physiology of our neighbour's thermophilic microbes and, in this way, can improve their use for biotechnology purposes.

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Author contributions

JHG: concept, acquisition, interpretation, drafting; AG: acquisition, interpretation, drafting; EL: concept, interpretation; PO: acquisition, interpretation; JPS: interpretation, drafting.

Conflicts of interest

No conflicts of interest
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An “overlooked” habitat for thermophilic bacteria: the phyllosphere

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Supplementary material

Suppl. material 1: An “overlooked” habitat for thermophilic bacteria: the phyllosphere

doi

Authors: Jean-Jacques Godon, Amandine Galès, Eric Latrille, Pornpimol Ouichanpagdee and Jean-Philippe Steyer
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