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Journal Article**Author(s):**

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Publication date:

2012-12

Permanent link:

<https://doi.org/10.3929/ethz-b-000059727>

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Originally published in:

Nature Reviews Microbiology 10(12), <https://doi.org/10.1038/nrmicro2910>

Microbial life in the phyllosphere

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Our knowledge of the microbiology of the phyllosphere, or the aerial parts of plants, has historically lagged behind our knowledge of the microbiology of the rhizosphere, or the below ground habitat of plants, particularly with respect to fundamental questions such as which microorganisms are present and what do they do there. In recent years however, this has begun to change. Cultivation-independent studies have revealed that a few bacterial phyla predominate in the phyllosphere of different plants and that plant factors are involved in shaping these phyllosphere communities, which feature specific adaptations and exhibit multipartite relationships both with host plants and among community members. Insights into the underlying structural principals of indigenous microbial phyllosphere populations will help to develop a deeper understanding of the phyllosphere microbiota and will have applications in plant growth promotion and protection.

Plants are populated by microorganisms both below and above ground. The phyllosphere (Fig. 1)¹ comprises the aerial parts of plants and is dominated by the leaves. The leaf surface habitat is vast: vegetation modelling² resulted in an estimated global leaf area of 508,630,100 km², which corresponds to 1,017,260,200 km² of upper and lower leaf surface and is an area approximately twice as great as the land surface. Assuming that there are on average 10⁶ to 10⁷ bacteria per cm² leaf surface³, the global bacterial population present in the phyllosphere could comprise up to 10²⁶ cells. The total size of the fungal population has not yet been estimated, but is expected to be lower[au: ok?]^{3,4}.

Annual plants complete their life cycle within a single growth season, perennial deciduous plants synchronously form and shed leaves every year, and evergreen plants do so sequentially. Consequently, the phyllosphere can be considered an ephemeral or short-lived environment as opposed to the rhizosphere, which comprises the area in the soil around plant roots [au: ok?].

Successful phyllosphere inhabitants can be expected to multiply and occupy newly formed niches while the leaves are expanding. Microbial epiphytes are exposed to the atmosphere and are subjected to the diurnal cycle; they are influenced directly by sunlight and indirectly by plant metabolism. Furthermore, the hydrophobic waxy cuticle that covers plant epidermal cells reduces evaporation of water as well as leaching of plant metabolites, thus resulting in an oligotrophic environment.

Despite these unifying aspects, there is substantial environmental heterogeneity at the macro- and microscale. At the macroscale, factors such as climatic conditions, the location of leaves within the vegetation and the chemical composition of the cuticle affect the environment. On the microscale, the presence of veins, stomata and surface appendages, including trichomes and hydathodes, alter nutrient availability^{5,6}. As well as nutrient limitation, microorganisms must also deal with UV radiation and low or fluctuating water availability and, as a consequence, are often unevenly distributed on the leaf surface and multiply unevenly⁷. In addition to both predictable and unpredictable environmental variability the microbial inhabitants of the phyllosphere also encounter antimicrobial compounds produced by plants or other microorganisms. Moreover, they can enter the apoplast (such microorganisms are then sometimes referred to as endophytes) where they trigger a range of plant defence responses. Phytopathogens can counteract such responses through the transfer of effectors⁸. An epiphytic lifestyle predominates in commensal phyllosphere bacteria [**au: just bacteria?**]; however, it also represents the initial phase of foliar colonization by many phytopathogens. In the case of bacterial pathogens, this phase precedes entry into the leaves via natural openings such as stomata or hydathodes and the onset of disease^{9,10}, or follows egress from the inner part of the leaf to the surface¹¹.

Although the phyllosphere has been less intensively studied than the rhizosphere, it has received considerable attention in recent years, and interest in the microbiology of leaf surfaces is now acknowledged to extend beyond pathogens. This review highlights recent cultivation-independent and -dependent studies that survey the microbial inhabitants of the phyllosphere and indicate conserved mechanisms by which predominating groups cope with the challenges of life on the leaf surfaces of terrestrial plants.

Microbial phyllosphere communities

Several recent cultivation-independent studies have revealed insights into the composition of microbial phyllosphere communities. It is apparent that these communities do not represent random assemblies of microorganisms, but instead undergo selection that results at least partially in predictable microbial communities with relatively few dominant (sub-)phyla¹²⁻¹⁸. This conclusion is derived from evaluations of the community composition with respect to plant host species as well as temporal and spatial factors. Most studies on the identity of organisms in the phyllosphere have focused on bacteria and, to a lesser extent, on fungi; archaea are apparently not abundant in the phyllosphere^{12,17,19}.

Bacterial aggregates. Most bacteria on leaf surfaces do not occur as solitary cells or small groups of cells as fungi tend to do²⁰, but in larger aggregates²¹; particularly at the depressions formed at the junctions of epidermal cells, along the veins and at the bases of trichomes^{3,4,22} (Fig. 1) where they are generally embedded within extracellular polymeric substances (EPS)^{3,4}. The EPS helps maintain a hydrated surface around the bacteria and eventually also to concentrate detoxifying enzymes (see below). Using experimental inoculations with a bacterial model strain and observations by epifluorescence microscopy suggests that most bacteria are located in aggregates of 1,000 cells or more and that the size of the aggregate positively correlates with water availability²². Aggregates usually contain a variety of bacterial and eventually also fungal species⁴. How these aggregates form, and the contributions of local replication and relocation, are key questions that remain to be addressed in further detail at the microscale^{23,24}. Notably, initial investigations using a bacterial dual-species model system have shown that different bacteria segregate in space to different extents²⁵.

Community composition. For the past 15 years, 16S/18S-ribosomal RNA gene PCR amplification followed by sequencing of clone libraries or, more recently, amplicon pyrosequencing, has been the most common approach by which the composition of the microbial phyllosphere communities has been analysed (see e.g.^{16-18,26}). Overall, species richness in phyllosphere communities is high. It appears fungal communities are extremely variable in the phyllosphere in temperate regions and

exhibit even greater diversity in the phyllosphere of tropical trees^{16,27}. The bacterial species richness is substantial in both temperate and in particular in the subtropical and tropical regions^{12,15,18,19,28}; however, the bacterial communities are lower **[au: do you mean community size is smaller (total number is less) or community diversity is reduced (fewer species)?]** compared to the rhizosphere or bulk soil, as evidenced by rarefaction analysis^{12,19}. This is not surprising, given that leaves usually have a relatively short lifespan and are discrete entities, in contrast to the roots which are surrounded by soil where microorganisms can survive in a dormant state for many years or even decades. Some bacterial phyla such as the Proteobacteria (and among them in particular Alphaproteobacteria and Gammaproteobacteria) have been shown to be predominant in the phyllosphere of distinct plant species using various PCR methods^{13,26}. Metagenomic shotgun sequencing of total genomic DNA extracted from environmental samples is an alternative approach that generates taxonomic information based on single copy protein-coding marker genes. Although extraction and sequencing protocols are also prone to biases, metagenome shotgun sequencing avoids the PCR amplification biases that result from the use of target-specific primers²⁹. **Figure. 2** shows the results of an MLTreeMap analysis³⁰ from four different phyllosphere samples: the dicots *Glycine max* (soybean), *Trifolium repens* (clover) and *Arabidopsis thaliana* and the monocot *Oryza sativa* (rice)^{12,19}. Proteobacteria was the most represented phylum, with the Alphaproteobacteria representing up to 70% of the community in each of the four phyllosphere samples. Of these, Methylobacteriaceae were found in all four samples and Sphingomonadaceae was the most abundant bacterial family detected on the three dicot plants. In addition to the Proteobacteria, the Bacteroidetes phylum was abundant in the soybean, clover and *A. thaliana* communities, but constituted only a minor fraction of the rice sample. By contrast, Actinobacteria accounted for ~40% of the rice community but constituted a lower fraction of the other samples. The most frequently detected genera are listed in **Table 1**; some bacterial genera are recurrently found on different plants although their relative contribution to the overall community might differ. Some of these genera such as *Agrobacterium*, *Burkholderia*, *Clavibacter*, *Leifsonia*, *Pantoea*, *Pseudomonas* and *Xanthomonas* include phytopathogenic bacteria. Comprehensive cultivation-independent studies paired with strain isolation has also allowed the identification of representative bacterial strains of various genera including *Pseudomonas*, *Xanthomonas*, *Pantoea*,

Dadantii, *Methylobacterium* and *Sphingomonas*³¹⁻³⁶, as well as fungal model strains such as the yeast-like fungus *Aureobasidium pullulans*³⁷, for functional studies (see further below).

Another interesting aspect is that at the genus or order rank, there is a relatively high consistency in the bacterial phyllosphere communities (Fig. 2); however, at a greater phylogenetic resolution, remarkable differences can be observed; for example, at the strain level, community profiling shows striking differences between plants of different species growing adjacent to each other (see for example^{12,38}).

Drivers of community structure. What drivers shape microbial communities is a fundamental question in microbial ecology. For the phyllosphere, it is well established that environmental factors, including radiation, pollution and nitrogen fertilization, as well as biotic factors, such as leaf age and the presence of other microorganisms, affect bacterial community structure³⁹⁻⁴² and eukaryotic microbial diversity is probably influenced by the same factors^{43,44}. Although the identification of assembly rules for bacterial and fungal communities is very difficult due to the complexity of the abiotic and biotic factors and stochastic events involved, several recent studies have reported that the plant genotype is an important factor influencing the bacterial composition of the phyllosphere. Redford *et al.* analyzed the bacterial community composition in the phyllosphere of pine trees and found that intra-plant species variability was lower than inter-plant variability, even over large geographical distances⁴⁵. Similarly, a significant but weak association between tree species and phyllosphere communities have been described for tropical trees¹⁸. Another study focused on members of the ubiquitous plant colonizing genus *Methylobacterium* in the phyllosphere of *A. thaliana* plants under natural conditions and also found that intra-plant variability was lower than inter-plant variability, although the site was also identified as an important factor that influenced this variability³⁸. Pronounced site effects were also found for bacterial and fungal communities of the salt-excreting *Tamarix* tree¹⁷, as well as bacterial communities on field-grown lettuce²⁶ as shown by comprehensive amplicon sequencing. Other studies have focused on the effect of plant genotypes and involved field trials with a collection of *A. thaliana* and maize plants (accession and selected plant mutants) and indicate that plant factors shape the bacterial phyllosphere community structure; possible mechanisms for such genotype effects

might originate from leaf surface properties⁴⁶, hormone jasmonic acid signaling⁴⁷ or signaling via the gamma-aminobutyric acid (GABA) pathway⁴⁸. The identification of plant genes crucial to the microbial composition of the phyllosphere will be of greatest interest in future studies and help elucidate whether plants have evolved to actively recruit these communities. This has been suggested for the rhizosphere^{49,50}, and was further substantiated by recent in-depth analyses of the root associated microbiota, demonstrating that a small fraction of root endophytes varies between plants of different genotype^{51,52}. Plants may also recruit founder or keystone species that play a fundamental role in structuring the overall community through microbe-microbe interactions. The finding that numerous bacterial genera on one plant species were represented on neighbouring plants by different strains^{12,38} indicates that plants offer distinct niches to different strains of the same bacterial genus and may thus contribute to intra-genus variation.

Origin of community members. Another basic question relates to the source of phyllosphere microorganisms. The finding that bacterial populations re-establish on annual plants in patterns similar to those seen in previous years³⁸ argues for local reservoirs of bacteria. Although a fraction of microorganisms in the phyllosphere can be expected to be ad-hoc colonizers from the air, for bacteria at least, 16S-rRNA gene based profiling suggest that early recruitment of a ‘core’ community from seeds, the soil or other plants could explain the inter-annual consistency in the dominant bacterial community members on individual plants³⁸. While seed-born transmission has been documented for *Xanthomonas* spp.⁵³, a lack of knowledge of the relative contribution of microorganisms from different sources to the developing phyllosphere community makes it difficult to draw general conclusions. Dedicated analyses will be required to elucidate the relative contribution of vertical and horizontal transmission [**au: examples of each?**].

Microbial adaptation to the phyllosphere

Successful reproduction in the phyllosphere requires adaptation to this habitat, including the ability to withstand abiotic and biotic stresses such as scarce nutrient supply and the presence of anti-microbial secondary metabolites of plant or microbial origin. Phyllosphere-inhabiting bacteria can be expected to

share common strategies in addition to genotype[**au: species?**]-specific strategies to survive these stresses. The identification of traits that are important for survival and the elucidation of their molecular basis is not an easy task, and it has been suggested that many different traits contribute incrementally to epiphytic fitness⁵⁴. Gene-expression studies including transcriptomic and proteomic analyses have helped to identify genes and protein families that are upregulated upon plant colonization using various biological model systems (Table 2). These data serve as a proxy by which to identify the bacterial protein functions that are required for survival in the environment. Additionally, meta (or community) proteogenomic approaches allow the identification of abundant proteins in microbial community members which can help infer their *in planta* physiology^{12,19} and stimulate targeted analysis of individual proteins or protein families to elucidate their importance.

The following section focuses on general traits of non-pathogenic bacteria and pathogenic bacteria in their epiphytic phase (Fig. 3). However, it should be kept in mind that the type-III secretion system (T3SS) of phytopathogenic bacteria is also crucial to attain a maximal epiphytic population size^{9,55}. In this context it is interesting to note that the importance of specific effectors for the development of aggregates on leaf surfaces was recently demonstrated for *Pseudomonas syringae* B728a and was shown to be mediated by a subpopulation of the epiphytic population and be host-dependent⁵⁶[**au: ok?**]. Moreover, bioactive molecules produced by *Pseudomonas* strains such as coronatine⁵⁷ or syringolinA⁵⁸ counteract pathogen-triggered stomata closure and thus affect entry into the apoplast.

Resistance to abiotic stresses. The phyllosphere is exposed to oxygen from plant photosynthesis and also to light during the day. Consequently, microbial colonizers are particularly prone to reactive oxygen species (ROS)-mediated direct damage of DNA and indirect damage to proteins. Protection mechanisms that have been shown experimentally to be important in bacterial epiphytic fitness involve the production of pigments⁵⁹ and the activation of DNA-repair mechanisms, which include but are not limited to, photolyases⁶⁰. Additionally, the DNA-protection protein Dps is important in stress protection and is present in high amounts during phyllosphere colonization^{12,61}. Catalases and superoxide dismutases can also detoxify ROS.

Desiccation is another environmental factor that is of relevance in the phyllosphere⁶². Two principal traits are known to help bacteria cope with this challenge, aggregate formation and the excretion of bioactive compounds. The importance of aggregate formation was demonstrated by analyzing the survival of solitary versus aggregated *P. syringae* cells on bean-leaf surfaces that were periodically exposed to desiccation stress and humid conditions⁶³. Aggregation involves the formation of EPS, which helps in maintaining a highly hydrated layer surrounding the bacteria and contributes to epiphytic fitness⁶⁴⁻⁶⁶. In addition, biosurfactants that increase wettability are commonly found to be secreted by phyllosphere bacteria^{67,68}. Not only is sufficient water availability a crucial parameter, but fluctuations in water availability result in osmotic stress, as has been illustrated on the microscale through the use of bacterial bioreporters⁶⁹. *Pseudomonas* spp. and other epiphytes can protect themselves against osmotic stress by accumulating compatible solutes such as choline or trehalose, either by *de novo* synthesis or by importing plant-derived osmoprotectants^{70,71}.

Resistance to (and production of) antimicrobial compounds. Plants produce a wide range of structurally diverse secondary metabolites with antimicrobial activity⁷². Additionally, antimicrobial compounds might be produced by microorganisms and thus be involved in structuring the community *in planta* at the local scale. Numerous bacteria produce antibiotics under laboratory conditions; however, the significance of their production *in planta* is not well established, with very few exceptions (e.g.,⁷³). The relevance of mechanisms to counteract toxic compounds *in situ* is shown by the finding that resistance-nodulation-cell division (RND) efflux pumps of plant-pathogenic *P. syringae* pathovars are crucial for *in planta* reproduction and evading the native immune response^{74,75}.

Motility versus adhesion. Motility allows active movement to favourable sites on leaf surfaces and is likely to be assisted by chemotaxis towards nutrients or plant signalling molecules. *Pseudomonas* model strains have been shown to dynamically regulate motility in response to various endogenous and exogenous signals, including quorum-sensing molecules (Box 1). Motility might thus help *Pseudomonas* strains to search for more favourable sites, to facilitate spreading and also to locate their

ultimately preferred site, the leaf apoplast, where high growth rates of phytopathogens have been observed^{10,76}. The degree to which the motility of commensal bacteria is of importance for epiphytic fitness is currently not clear. Notably, no evidence for flagellin production has been found so far in commensal phyllosphere members *in planta*¹², provoking the question whether commensals suppress the expression of the genes involved in flagellum formation. Downregulating flagellin synthesis favours local aggregate formation and might prevent plant recognition of Flg22, a conserved peptide in flagella and a well-described microbial associated molecular pattern (MAMP) that is recognized by the plant immune receptors⁸. Adhesion and local replication, on the other hand, might represent the dominant lifestyle of commensal bacteria that are generally found as epiphytes on leaf surfaces and to a lesser extent in the apoplast^{10,34}. Adhesion is likely to favour resistance to removal by rain fall and splash from rain drops and is a prerequisite for the formation of EPS-containing mucus that provides protection from desiccation. Consequently, adhesion may favour successful epiphytic colonization and allow colonization following leaf expansion.

So far, few studies have addressed the presence of surface structures and specific proteins that allow adhesion to the hydrophobic cuticle. Notable exceptions include studies on adhesion by the hemagglutinin family proteins in *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) and *Xanthomonas* strains, which have been shown to be important for epiphytic attachment and aggregation in these species^{77,78}.

Metabolic adaptation. In contrast to the rhizosphere, where large quantities of plant assimilates are accessible to microorganisms, nutrients on leaf surfaces are scarce³. Carbon sources that have been identified on leaf surfaces include carbohydrates, amino acids, organic acids, and sugar alcohols⁷⁹⁻⁸¹. The heterogeneous nature of the nutrient availability on leaf surfaces has been well documented by studies using bioreporters^{5,6,82}. It is currently not clear whether leaf-surface waxes support microbial growth. Bacteria themselves may influence substrate availability by the production of biosurfactants that result in increased wettability and thus increased substrate leaching⁶⁸. Additionally, bacteria (and fungi) may also produce plant hormones, including indole-3-acetic acid (IAA; also known as auxin), (see e.g.^{36,83,84}). There is evidence, that auxin increases nutrient availability by stimulating cell wall

loosening and release of saccharides from the plant cell wall^{3,85}. The higher substrate availability as a result of bacterial auxin production is substantiated by epiphytic fitness tests⁸³.

In addition to the above-mentioned carbon sources, there are other, volatile plant-derived substrates including isoprenes and one carbon compounds⁸⁶. A prominent one carbon source is methanol, which is primarily formed as a by-product of cell wall metabolism by pectin methyl esterases and is released in diurnal cycles⁸⁶. The global emission of plant-produced methanol has been estimated to be 10¹⁴ g per year⁸⁷. Methanol has been documented as a substrate for epiphytic bacteria and methylotrophic yeasts, conferring a growth advantage *in situ*⁸⁸⁻⁹⁰. Notably, essentially all methylotrophic bacteria in the phyllosphere belong to the genus *Methylobacterium* and are facultative methylotrophs with a restricted substrate range^{32,91}. Besides methanol, it has been documented that plants produce halogenated methane derivatives⁹². Methyl chloride-degrading Alphaproteobacteria of the genus *Hyphomicrobium* have been isolated from the phyllosphere⁹³ although the significance of this one-carbon substrate for epiphytic fitness requires further investigation. Yet another one carbon compound that has been postulated to be produced at trace amounts is photochemically formed methane⁹⁴. However, there is so far no evidence that methane formation by plants can sustain a population of methanotrophs and they do not appear as abundant players in the microbial community in the phyllosphere of rice plants¹⁹.

Carbon-source phenotyping of bacteria under laboratory conditions is a standard method applied to phyllosphere bacteria and is frequently used to determine the overlap in substrate utilization among strains as a measure of their potential to co-exist^{95,96}. An extension of such carbon profiling was recently performed⁹⁷; apoplastic fluid extracted from healthy tomato leaves was used to preincubate *P. syringae* before recording the repertoire of nutrients that could be consumed without further preadaptation of the cells. Such substrate profiles revealed that *P. syringae* uses a restricted number of sugars, organic acids and amino acids.

Gene-expression profiling *in situ* is an alternative approach to investigate the metabolic adaptations of particular microorganisms^{12,61}. The high abundance of enzymes involved in methylotrophy of *Methylobacterium* spp. is in-line with the importance of these proteins in the physiological adaptation to, and methanol consumption in, the phyllosphere^{88,90}. The presence of

numerous ABC transporters from *Pseudomonas* spp., including those involved in the uptake of mono- and disaccharides and amino acids suggests the uptake of these substrates by these species. The production of a large number of TonB-dependent transport systems by *Sphingomonas* strains may indicate scavenging of various nutrients present at low concentrations. This finding corroborates an earlier report on the importance of the TonB-dependent transporter for sucrose uptake and metabolism in *Xanthomonas* spp.⁹⁸. To gain further insight into metabolic activities of epiphytic communities it will be necessary to develop *in situ* metabolomic approaches for epiphytic bacteria; although this will be challenging it is likely to offer new insights into the physiology of these bacteria.

There is evidence that bacterial communities on well-fertilized plants are limited primarily by carbon availability and only secondarily by nitrogen availability⁹⁶. Bacteria can use several nitrogen sources including organic nitrogenous compounds such as amino acids which may represent a valuable source of nitrogen for phyllosphere bacteria. In addition, ammonia is probably used as a nitrogen source in the phyllosphere^{12,99}, and nitrogen fixation has been reported by phyllosphere bacteria¹⁰⁰.

Aside from carbon and nitrogen, phyllosphere bacteria require further macro- and also microelements for growth. The expression of several transport systems for phosphate, sulfate and iron has been observed *in situ*^{12,61,101}. Although several studies found that siderophore production was involved in epiphytic growth and competition with other microorganisms during colonization of leaf surfaces, others found only moderate or no iron limitation^{102,103}. Such controversial results were recently suggested to be a consequence of plant secondary metabolism, as different plants produce various polyphenolic compounds (including tannins) with different iron-sequestering abilities¹⁰⁴. Thus, the importance of siderophore production for iron sequestration may depend on the host plant.

Recent studies have reported that phyllosphere bacteria that have the potential to benefit from light by expressing bacteriorhodopsins and the presence of aerobic anoxygenic phototrophs that may be able to capture light in photosynthetic reaction centers^{105,106}. Light-harvesting in the phyllosphere would allow epiphytic bacteria to use light as an additional energy source and might supply maintenance energy in case of nutrient deficits.

Regulation of fitness traits. Uncovering global and specific regulatory systems as well as the perceived environmental cues, will be crucial for understanding microbial adaptation to the phyllosphere. Following the identification of genes that are up-regulated during phyllosphere colonization, the response regulator PhyR (phyllosphere-induced regulator) was discovered to be crucial for colonization by *Methylobacterium* spp.⁶¹ and later also shown to be important for the plant epiphyte *Sphingomonas* sp. Fr1¹⁰⁷. PhyR regulates a number of genes typically associated with stress responses, such as heat-shock proteins, the osmo-protectant trehalose, and proteins involved in carotenoid biosynthesis. The response regulator is part of the PhyR-Nep- σ^{EcfG} signaling cascade, which governs the general stress response in Alphaproteobacteria¹⁰⁸. As stressful conditions in environmental settings are the rule rather than the exception, it will be interesting to determine the degree to which such global systems constitute “alternative” regulatory systems, as is commonly assumed. In contrast to the Alphaproteobacteria, analysis of two gammaproteobacterial *Pseudomonas fluorescens* strains showed that sigma factor σ^{S} , which governs general stress response in these bacteria, seems to play only a minor role in epiphytic fitness¹⁰⁹. This finding argues against an oversimplification of common stress-response models, as highly environmentally resistant strains may use multiple, redundant regulatory mechanisms to ensure survival.

In *P. syringae*, another sigma factor belonging to the ECF family, AlgT (AlgU, RpoE, or σ^{22}), is important for *in planta* fitness^{64,110}. However, the regulatory phenotypes have not yet been causally connected to fitness in terms of surviving desiccation and osmotic stress. Obviously, the analysis of the regulons controlled by these factors will help to further dissect the significance of individual traits. Beyond sigma-factor regulation, other global regulatory systems, including the GasSR two-component system in pseudomonads, have been uncovered.

Another layer of complexity in these regulatory networks lies in second-messenger molecules such as cyclic-di-GMP, which is often involved in controlling different traits including adhesion¹¹¹. The production of these molecules is regulated by various inputs as has been demonstrated for *X. campestris*¹¹². Although insights into the importance of the secondary metabolites of *Pseudomonas* isolates in the rhizosphere are emerging¹¹³, systematic analyses of the importance of cyclic-di-GMP signaling in most epiphytic bacteria are currently lacking.

Last but not least, quorum sensing has been established as an important regulatory system in plant-pathogenic bacteria¹¹⁴, for example during the epiphytic phase, which precedes the endophytic pathogenic lifestyle, in *Pseudomonas* spp.^{115,116}. Quorum sensing represents a means by which bacteria process information on cell density and/or diffusion^{114,117}. This system depends on acyl homoserine lactones as signals in *Pseudomonas*¹¹⁴ and *Methylobacterium* spp.¹¹⁸ as well as on the diffusible signal factor DSF in *Xanthomonas* spp.¹¹⁹. Further details on quorum sensing in *P. syringae* B728, which is the best-studied strain with respect to the importance of this regulatory system for epiphytic growth are given in **Box 1**.

Interactions with the host and among microbial community members

Complex interactions are expected to exist in the phyllosphere between various microorganisms, including bacteria and fungi, as well as between microorganisms and host plants (**Fig. 4**), and even among microorganisms and higher eukaryotes¹²⁰. The relationships between microorganisms and their hosts include parasitic, commensal, and mutualistic interactions. A clear classification can be difficult, particularly discriminating between commensals and mutualistic symbionts, and it has been suggested that these interactions represent a continuum^{121,122}. In the human gut, many community members that were once considered commensals are now regarded as beneficial symbionts because of their contributions to host metabolism and immunity¹²³. The thought-provoking statement by Janzen, “Plants wear their guts on the outside”¹²⁴ suggests that similar questions on host benefit and microbe-microbe interactions should be applied to phyllosphere communities. For phyllosphere microorganisms, the benefits provided by the plant are obvious and include nutrient supply but the advantages provided by phyllosphere inhabitants to their host plants are not necessarily as apparent. It has been suggested that plants benefit from microbial production of plant hormones, such as cytokinins and IAA; however, studies carefully analysing the effects of defects in these traits are not yet available. While the reactions of the plant to leaf pathogens have been studied on the transcriptional level, the responses to “commensal” phyllosphere bacteria remain poorly characterized; however preliminary analysis indicates that plants respond to the presence of a non-pathogenic *Pseudomonas* strain¹²⁵. Research on induced systemic resistance (ISR), a mechanism that provides

resistance to a wide range of pathogens and is induced by beneficial microorganisms in the rhizosphere, is not yet well established for phyllosphere bacteria. Likewise, functional studies that might uncover the potential of microorganisms to assist in plant defense, e.g. through the production of secondary toxic metabolites or scavenging of essential macroelements as observed in the rhizosphere^{49,126}, are yet to be done in the phyllosphere.

The term “barrier effect” was coined¹²⁷ to describe the resistance to infection by pathogens that is conferred by the human gut microbiota. Indigenous microbial populations may also affect the outcome of plant-pathogen interactions in the phyllosphere^{11,128}. As a consequence, it is not surprising that axenic plants are more susceptible to infection, by analogy to animal model systems³⁴. In general, the interactions that prevail *in planta* are far from being understood, and it will be important to attain more system-level insights into the complex interactions that govern outcomes among community members in the context of the plant host. The use of gnotobiotic conditions will be crucial to address the importance of indigenous bacteria and to study microbial interactions in the phyllosphere. Recently, the potential of *Sphingomonas* isolates in plant protection against bacterial pathogens in a three species *Arabidopsis* model system has been demonstrated³⁴. Although the molecular basis for reduced pathogen growth in the presence of *Sphingomonas* is not yet understood, evidence is available to suggest that several traits might contribute to the outcome of plant protection¹²⁹.

The traits important for successful epiphytic growth, which were discussed in the previous section, link directly to interactions with other community members; these traits will ultimately allow coexistence or exclusion of other phyllosphere bacteria (see also¹³⁰). In microbial communities, competition for space and nutrient resources as well as the production of antibiotics and interference with cell signaling systems are considered to be the principal mechanisms by which indigenous bacteria and fungi antagonize each other^{3,96,131}. These traits all appear important and have been shown to be significant in correlational studies; however, experimental proof on the molecular level is lacking or has failed to support previously hypothesized traits. Although antibiosis is the most commonly studied mechanism, it is well known that *in vitro* antibiosis is not necessarily predictive of *in planta* antibiosis^{95,128}. At present, the factors that determine interactions between epiphytic microorganisms and that ultimately shape microbial communities are poorly elucidated.

Applications for phyllosphere microbiology

A prominent area of applied phyllosphere microbiology is the improvement of plant health to increase plant biomass production and prevent biomass losses. As mentioned above, many foliar bacterial pathogens colonize plant surfaces prior to infection, and the size of these populations is correlated with disease severity^{34,132,133}. This suggests that a reduction in pathogen numbers could lead to plant protection. Possible mechanisms to suppress pathogen proliferation include exploiting competition for nutrients and space, antibiosis, and stimulating systemic host responses^{128,134}. Alternative biocontrol strategies that have been proposed use individual protective strains or strain combinations, see e.g.^{135,136}. For a recent review on plant protection in the rhizosphere and phyllosphere see Ref. ¹³⁷. A better understanding of the principal drivers of community structure and multitrophic interactions in the phyllosphere will be the key to developing new strategies for plant protection. Biocontrol applications will benefit from fundamental research; in other words, the better we understand the roles and importance of indigenous bacteria, the better we will be able to predict and protect against pathogen infection. Other aspects of applied phyllosphere microbiology relate to the occurrence of human pathogenic bacteria in food including fruits, vegetables and salads. This area of research has been stimulated in the past years by numerous outbreaks of infection associated with foodborne pathogens such as *Salmonella* and *Escherichia coli* and advances in the research area have been reviewed recently¹³⁸. Another intriguing area of interest is phytoremediation, using the phyllosphere microorganisms to remove gaseous pollutants such as phenol or benzene from the air (“phylloremediation”)^{139,140}.

Conclusions

The phyllosphere was once termed a neglected milieu¹. This situation has changed, and there is now an increased interest in phyllosphere microbiology. The phyllosphere is an well-suited model system in which to study basic principles of ecology in particular due its amenability to experimentation and visual inspection,¹⁴¹ and this area of research field has the potential to have an impacts on diverse areas such as plant health and atmospheric chemistry. There is still a major focus on understanding

pathogen/plant interactions; however, there is growing awareness of the importance of the indigenous microbiota and their role in microbe-microbe and host-microbe interactions. There have been substantial advancements in identifying community members and epiphytic fitness traits. New approaches that would not have been possible a few years ago, such as community profiling based on next generation sequencing technologies as well as proteogenomics or metaproteogenomics, help to understand how microbial communities are shaped and to identify new targets for hypothesis-driven research with the aim of uncovering novel protein functions of importance for survival and growth in the phyllosphere. There is a great need to study phyllosphere microorganisms and their interactions *in situ* and at the microscale using complementary approaches.

Many important questions are still unanswered. What are the drivers of microbial community structure in the phyllosphere? Are there epistatic effects? How stable or easily disturbed are microbial communities? How do closely related microbial genotypes coexist in the phyllosphere? What are the impacts of microorganisms on the host plant? Future research directed at answering these questions will improve our understanding of microbial inhabitants of the phyllosphere and the potential for biotechnological applications. These research efforts should ideally lead to an increase of the availability of genome sequences for representative model strains throughout all phylogenetic taxa detected in the phyllosphere. Such strains will allow the design of synthetic communities and enable a union of genetics studies to obtain direct experimental proof for interactions and to link observations that have been and will be made under environmental conditions with those that can be traced to the molecular level.

Glossary

Perennial plants

Plants that lives for more than two years

Deciduous plants

Plants that lose their leaves seasonally

Evergreen plants

[au; please define]

Epiphytes

AU: please define

Oligotrophic

AU: please define

Stomata

Openings of leaves that control gas exchange (in particular oxygen and carbon dioxide) and water transpiration between the plant interior and the environment.

Trichomes

Epidermal outgrowth of plant surfaces including the leaves. A common type is a hair which might be branched or unbranched. Glandular trichomes excrete various exudates.

Hydathodes

AU: please define

Apoplast

Au: please define

Coronatine

Phytotoxin produced by several pathovars of *Pseudomonas syringae*. It consists of coronafacic acid (CFA), an analog of methyl jasmonic acid (MeJA), and coronamic acid (CMA), which resembles 1-

aminocyclopropane-1-carboxylic acid (ACC), a precursor to ethylene. Has been shown to open stomata.

Syringolin A

Major variant of a family of structurally related small cyclic peptides that are secreted by many phytopathogenic bacterial strains. Syringolin A counteracts stomatal closure by proteasome inhibition.

Photolyase

Enzyme involved in DNA repair caused by UV light. This flavoprotein reversibly binds to pyrimidine dimers and converts them back to the original base, a reaction for which visible light is required.

Trehalose

Disaccharide of two glucose units, formed by an α,α -1,1-glucoside bond. Important for desiccation resistance.

Microbial associated molecular pattern (MAMP)

Molecular component characteristic for microorganisms; their recognition plays a key role in innate immunity.

Bacteriorhodopsins

Retinal-containing transmembrane proteins that act as light-driven proton pumps.

hrp genes

Encode for proteins that constitute the type III secretion system of phytopathogenic bacteria, which is used to transport effectors into plant cells and elicit a hypersensitive response when recognized by resistance proteins.

Axenic

Culture of an organism, here a plant, that is free of contaminating organisms.

Antibiosis

Biological interaction between two species whereby one acts antagonistic to the other one by producing a substance that inhibits growth of the latter or kills it.

Acknowledgements.

Work in the author's laboratory is supported by research grants from the Swiss National Foundation (SNSF), ETH Zurich, the Vontobel Foundation, the Swiss Agency of Innovation in Technology (KTI/CTI), the Swiss Initiative for Systems Biology SystemsX, the European Science Foundation (ESF), and the European Union (FP7). Claudia Knief, Mitja Remus-Emsermann and Natacha Bodenhausen are greatly acknowledged for comments on the manuscript. I like to thank the anonymous reviewers for helpful suggestions that improved this review. I am grateful to G. Innerebner and M. Remus-Emsermann for providing pictures for Figure 1 and to Manuel Stark and Christian von Mering for preparing Figure 2.

Table 1. Major bacterial genera detected in phyllosphere samples.

Asking au to rethink presentation

Class	Order	Genus	<i>A. thaliana</i>	Soybean	Clover	Rice
Alphaproteobacteria	Rhizobiales	<i>Agrobacterium</i>	■	■	■	■
	Rhizobiales	<i>Bradyrhizobium</i>	■	■	■	■
	Rhizobiales	<i>Brucella</i>	■	■	■	■
	Caulobacterales	<i>Caulobacter</i>	■	■	■	■
	Sphingomonadales	<i>Erythrobacter</i>	■	■	■	■
	Rhodospirillales	<i>Magnetospirillum</i>	■	■	■	■
	Rhizobiales	<i>Mesorhizobium</i>	■	■	■	■
	Rhizobiales	<i>Methylobacterium</i>	■	■	■	■
	Sphingomonadales	<i>Novosphingobium</i>	■	■	■	■
	Rhizobiales	<i>Ochrobactrum</i>	■	■	■	■
	Rhizobiales	<i>Rhizobium</i>	■	■	■	■
	Rhizobiales	<i>Rhodopseudomonas</i>	■	■	■	■
	Rhizobiales	<i>Sinorhizobium</i>	■	■	■	■
	Sphingomonadales	<i>Sphingobium</i>	■	■	■	■
	Sphingomonadales	<i>Sphingomonas</i>	■	■	■	■
	Sphingomonadales	<i>Sphingopyxis</i>	■	■	■	■
	Sphingomonadales	<i>Zymomonas</i>	■	■	■	■
Betaproteobacteria	Burkholderiales	<i>Acidovorax</i>	■	■	■	■
	Burkholderiales	<i>Burkholderia</i>	■	■	■	■
	Burkholderiales	<i>Variovorax</i>	■	■	■	■
Gammaproteobacteria	Pseudomonadales	<i>Acinetobacter</i>	■	■	■	■
	Enterobacterales	<i>Enterobacter</i>	■	■	■	■
	Enterobacterales	<i>Pantoea</i>	■	■	■	■
	Pseudomonadales	<i>Pseudomonas</i>	■	■	■	■
	Xanthomonadales	<i>Stenotrophomonas</i>	■	■	■	■
	Xanthomonadales	<i>Xanthomonas</i>	■	■	■	■
Actinobacteria	Actinomycetales	<i>Aeromicrobium</i>	■	■	■	■
	Actinomycetales	<i>Arthrobacter</i>	■	■	■	■
	Actinomycetales	<i>Clavibacter</i>	■	■	■	■
	Actinomycetales	<i>Leifsonia</i>	■	■	■	■
	Actinomycetales	<i>Mycobacterium</i>	■	■	■	■
	Actinomycetales	<i>Nakamurella</i>	■	■	■	■
	Actinomycetales	<i>Nocardioides</i>	■	■	■	■
	Actinomycetales	<i>Streptomyces</i>	■	■	■	■
	Actinomycetales	<i>Rhodococcus</i>	■	■	■	■
Bacteroidetes	Bacteroidales	<i>Bacteroides</i>	■	■	■	■
	Sphingobacteriales	<i>Chitinophaga</i>	■	■	■	■
	Cytophagales	<i>Cytophaga</i>	■	■	■	■
	Cytophagales	<i>Dyadobacter</i>	■	■	■	■
	Flavobacteriales	<i>Flavobacterium</i>	■	■	■	■
	Sphingobacteriales	<i>Pedobacter</i>	■	■	■	■
	Cytophagales	<i>Spirosoma</i>	■	■	■	■

The length of the bars represents the relative frequency of reads with predicted proteins and ribosomal RNA genes annotated to the genus level within the metagenomes of microbial communities^{12,19} using the MG-Rast (metagenomics.anl.gov/). A threshold of greater than 1% of the overall community was set.

Table 2. Global approaches applied to phyllosphere bacteria to uncover epiphytic fitness traits under *in situ* conditions.

Approach	Biological system	Major findings	Ref.
Transposon mutagenesis	<i>P. syringae</i> pv. <i>syringae</i> B728a - bean phyllosphere	Various traits contribute incrementally to epiphytic fitness; identified traits concern motility and stress tolerance	54
Promoter trapping	<i>P. syringae</i> pv. <i>tomato</i> DC3000 - <i>A. thaliana</i>	Identification of virulence genes, genes for proteins involved in stress tolerance, polysaccharide synthesis, transport and carbon metabolism, unknown loci	142
	<i>P. syringae</i> pv. <i>syringae</i> B728a - bean phyllosphere	Identification of virulence genes, genes for proteins involved in regulation, stress tolerance, polysaccharide synthesis, transport and carbon metabolism, unknown loci	101
Transcriptomics	<i>X. campestris</i> pv. <i>vesicatoria</i> - tomato	Identification of a citrate transporter, which was shown to be required for virulence	143
	<i>D. dadantii</i> (<i>E. chrysanthemi</i> 3937)	Identification of genes for proteins involved in metabolism, regulation, transport, unknown loci	144
	<i>D. dadantii</i> (<i>E. chrysanthemi</i> 3937)	Identification of virulence genes, genes for proteins involved in iron uptake, transport, xenobiotic resistance, chemotaxis, stress responses	145
Proteomics	<i>M. extorquens</i> AM1	Up-regulation of stress proteins and enzymes involved in methylotrophy; discovery of PhyR (general stress response regulator of Alphaproteobacteria)	61
Metaproteogenomics	Microbial community - Soybean, clover, <i>A. thaliana</i>	Common repertoire of proteins expressed by bacterial communities on different plants	12
Metaproteogenomics	Microbial community - rice	Large set of proteins related to one carbon metabolism	19

[fig legs may (will) change]

Figure 1. The phyllosphere environment.

Electron micrograph pictures showing the edge of a leaf (a), and epiphytic bacteria that become visible after higher magnification (b). Schematic view of a leaf surface and the apoplastic space (c). Epifluorescence pictures of tagged phyllosphere bacteria (*P. agglomerans* strain 299R in cyan and *P. syringae* DC3000 in red) (d).

Figure 2. Microbial phyllosphere diversity.

An ML-TreeMap analysis³⁰ was performed to assess the microbial community composition in the phyllosphere of *A. thaliana* (yellow), soybean (green), clover (blue), and rice (red) from samples obtained after shotgun metagenome sequencing of assembled and unassembled reads as described in Delmotte et al. 2009¹² and Knief et al. 2012¹⁹ (publically available through the MG-RAST server). The backbone tree was calculated based on aligned sequences of 40 phylogenetic marker genes from fully sequenced organisms. Dots indicate the placement of metagenomic sequence reads containing these marker genes, with the size of the dot corresponding to the frequency of recovery. Archaea (green part of inner cycle) contributed less than 1% to the microbial community of the samples. Note, the low contribution of eukaryotes (red part of inner cycle) is in accordance with the design of the microbial harvesting procedure, which included a physical depletion step for eukaryotic cells.

Figure 3. Proposed bacterial traits involved in adaptation to the phyllosphere acting either at the community level, with the plant or the level of the individual cell.

Figure 4. Multipartite interactions occurring in the phyllosphere among commensal and pathogenic microorganisms as well as the plant.

Box 1. Quorum sensing in *Pseudomonas syringae*.

Many Gram-negative plant associated bacteria including *Pseudomonas syringae* produce acyl homoserine lactones (acyl-HSL) as quorum sensing signal molecules. One of the best studied phyllosphere bacteria regarding the importance of HSLs is *Pseudomonas syringae* pv. *syringae* B728a. It has been shown that AhlI, which is the synthase responsible for 3-oxo-hexanoyl HSL production, is under the control of AhlR and other regulators. Quorum sensing in *P. syringae* B728a is important for epiphytic fitness by inducing EPS production and indirectly desiccation and resistance to hydrogen peroxide, as well as suppressing motility^{115,116}. In addition, it has been shown that *P. syringae* acyl-HSL-dependent signalling in the phyllosphere was strongly influenced both by the size of cell aggregates and by the availability of water on leaves¹⁴⁶. EPS production by acyl-HSL was thus postulated to increase the survival of epiphytes under desiccating conditions where acyl-HSL accumulates faster. On the other hand, motility, which is negatively regulated acyl-HSL-signaling, might reflect an adaptation to dry conditions that would allow the bacterium to conserve the resources invested in flagella when movement is impossible or disadvantageous.

Quorum sensing signaling mechanisms have in common that they principally allow cross talk and are prone to signal quenching. Both phenomena have recently begun to be studied in the phyllosphere, and may be relevant and contribute to the complex interaction of microorganisms in this niche [au: ok?] ¹⁴⁷⁻¹⁵⁰.

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About the author

Julia A. Vorholt performed her PhD work at the Max-Planck-Institute of Microbiology in Marburg, Germany. She did a postdoc at the University of Washington, Seattle, USA and worked as a group leader at the CNRS in Toulouse, France. She is now Full Professor at the Institute of Microbiology, ETH Zurich, Switzerland. The research in her group is driven by her interest to learn how the environment shapes bacterial physiology, with an emphasis on metabolism, novel protein function and gene regulation. The habitat of particular interest to her is the phyllosphere.

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At a Glance Summary

- ❖ The surface area of the phyllosphere is approximately twice as great as the land surface area and provides a habitat for numerous microorganisms, which colonize the surfaces (where they mostly form aggregates) and the inside of leaves.
- ❖ Most phyllosphere microorganisms are bacteria, are non-pathogenic and belong to a few predominant phylogenetic groups including Alpha- and Gammaproteobacteria, Bacteroidetes and Actinobacteria. The fungi that are also detected in the phyllosphere appear hyperdiverse.
- ❖ Numerous abiotic and biotic factors drive the microbial community structure in the phyllosphere, including the plant itself.
- ❖ Targeted and large scale metaproteogenomic studies have helped to identify important mechanisms by which bacteria adapt to the phyllosphere; among these are aggregate formation, surface alterations by production of biosurfactants, induction of stress responses and metabolic adaptations ranging from utilization of the one carbon compound methanol to amino acids and sugars.
- ❖ The phyllosphere is a discrete habitat (a sum of discrete habitats) and is a tractable model system for understanding the relationships between microorganisms and hosts. An improved understanding of phyllosphere microbiology is also of practical importance for biocontrol of the phyllosphere as the primary carbon fixing unit in terrestrial systems.