New insights into the structure and function of phyllosphere microbiota through high-throughput molecular approaches

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Abstract

The phyllosphere is an ecologically and economically important ecosystem that hosts a large and diverse microbial community. Phyllosphere microbiota play a critical role in protecting plants from diseases as well as promoting their growth by various mechanisms. There are serious gaps in our understanding of how and why microbiota composition varies across spatial and temporal scales, the ecology of leaf surface colonizers and their interactions with their host, and the genetic adaptations that enable phyllosphere survival of microorganisms. These gaps are due in large part to past technical limitations, as earlier studies were restricted to the study of culturable bacteria only and used low-throughput molecular techniques to describe community structure and function. The availability of high-throughput and cost-effective molecular technologies is changing the field of phyllosphere microbiology, enabling researchers to begin to address the dynamics and composition of the phyllosphere microbiota across a large number of samples with high, in-depth coverage. Here, we discuss and connect the most recent studies that have used next-generation molecular techniques such as metagenomics, proteogenomics, genome sequencing, and transcriptomics to gain new insights into the structure and function of phyllosphere microbiota and highlight important challenges for future research.

Introduction

The phyllosphere – that is, plant foliage as a microbial habitat – is considered a hostile environment for survival and colonization by microorganisms due to the rapid fluctuation in solar radiation, temperature, humidity, and heterogeneous availability of nutrients (Lindow & Brandl, 2003; Vorholt, 2012). Nonetheless, the phyllosphere is populated by a large and diverse microbiota of bacteria, fungi, yeast, archaea, and other microorganisms that have commensal, pathogenic, and mutualistic interactions with the plant host. Bacteria are estimated to be the most abundant colonists of leaf surfaces with densities reaching as high as 10^8 cells per cm^2 (Leveau, 2006). The majority of epiphytic bacteria are commensal. Some provide specific ecosystem services such as phytoremediation of toxic pollutants (Ali et al., 2012) and biogeochemical cycling of important elements (Fürnkranz et al., 2008). Others contribute to pathogen exclusion (Lindow &

Leveau, 2002) and may be considered 'plant probiotics' (Berlec, 2012).

Previous investigations on phyllosphere microbiota have primarily focused on pathogenic bacteria and fungi to understand their interaction with the host and environment (Montarry et al., 2008). Such studies have provided valuable insights into the population biology and genetics of foliar pathogens leading to more efficient disease management practices (Turechek et al., 2001). Other studies have applied so-called 'first-generation' molecular techniques [e.g. clone library Sanger sequencing, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism] to describe variation in community structure in the context of plant genotype, plant phenotype, and geographical location (Hunter et al., 2010; Vokou et al., 2012; Izhaki et al., 2013). These techniques are low throughput and relatively expensive and allow only a superficial comparison of microbial communities (Rastogi & Sani, 2011). The advent of

next-generation DNA sequencing significantly reduced costs and permitted multiplexing of hundreds of samples in a single sequencing run. This is dramatically changing the landscape of microbial ecology and offering new windows of 'omic exploration'. The 454 pyrosequencing platform was one of the 'first' to be widely implemented in microbial community analysis through rRNA or ITS amplicon sequencing, shotgun metagenomics, whole-genome sequencing, and transcriptional profiling (Delmotte *et al.*, 2009; Rastogi *et al.*, 2012). 'Second' next-generation sequencing technology such as the Illumina platform (Degnan & Ochman, 2012) allows ultra-high-throughput sequencing of microbial communities and yields amounts of sequence data that are several orders of magnitude higher. Convergence of metagenomic with metaproteomic analysis, popularly known as proteogenomics (Delmotte *et al.*, 2009), represents another important technical advancement. Combined, these technological innovations are greatly facilitating comparative ecological analyses and provide new insights into the structure, function, and variability of microbiota in the phyllosphere and other environments.

Application of next-generation molecular technologies in the area of phyllosphere microbiology is rapidly increasing and so is the number of new studies providing further insights into community variation, drivers, functions, and interactions with biotic and abiotic components. In this context, recently published reviews such as those by Vorholt (2012) and Bulgarelli *et al.* (2013) offer an excellent synopsis of many earlier studies on phyllosphere microbiota. In our review, we discuss and connect these with studies that have come out since and which have used next-generation molecular techniques toward a deeper understanding of phyllosphere communities, the biotic and abiotic factors driving spatial and temporal colonization patterns, the cellular adaptations that contribute to epiphytic fitness, and the microorganism–microorganism and microorganism–plant interactions that occur in the phyllosphere (Fig. 1).

Phyllosphere microbiota and interactions with plant pathogens

Historically, disease development in plants has been regarded as a three-party relationship between plant host, pathogen, and the environment (Francl, 2001). Missing from this 'disease triangle' are the other microorganisms that colonize plants, that is, the microbiota and their impact on pathogen establishment. The human microbiome project (Balter, 2012) is revealing that many human diseases can be linked to changes in microbial populations that are naturally associated with different body organs (Albenberg *et al.*, 2012). Evidence is accumulating

that microbial communities associated with plants also play an important role in determining the health and fitness of their hosts (Babalola, 2010; Bulgarelli *et al.*, 2013). For example, in laboratory controlled experiments, strains of *Sphingomonas* were shown to suppress disease symptoms and reduce the growth of pathogenic *Pseudomonas syringae* pv. *syringae* DC3000 on leaves of *Arabidopsis thaliana* (Innerebner *et al.*, 2011). This plant-protective feature seemed confined to *Sphingomonas* strains that were originally isolated from plants and absent in strains recovered from nonplant environments. In another study (Rastogi *et al.*, 2012), high population levels of *Xanthomonas campestris* pv. *vitians*, the causative agent of bacterial leaf spot disease of lettuce, correlated positively with bacteria from the genus *Alkanindiges*, but negatively with *Bacillus*, *Erwinia*, and *Pantoea*. These findings further substantiate the notion that commensal microbiota on leaves can play a role in pathogen exclusion and that they may contribute to plant health and productivity and have practical applications in developing new strategies for disease prevention or prediction.

Phyllosphere microbiota and interactions with human pathogens

Understanding the ecology of phyllosphere microbiota is also important in the context of food safety. There has been a global increase in recent years in the number of outbreaks related to the consumption of fresh produce (Leff & Fierer, 2013). Human pathogens such as *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* are not well adapted for epiphytic survival, yet have been found to persist on leaf surfaces of lettuce and spinach for considerable periods of time (Erickson *et al.*, 2010). Currently, little is known about how these human pathogens interact with native members of the leaf microbial community, although significant progress is being made. For example, the epiphytic bacterium *Enterobacter asburiae* negatively impacted survival of *E. coli* O157:H7 on lettuce leaves by a factor 20–30, while *Wausteria paucula* enhanced survival by a factor 6 (Cooley *et al.*, 2006). Co-inoculation experiments on detached spinach leaves revealed that bacterial isolates belonging to *Firmicutes* and *Enterobacteriaceae* reduced the growth rate of *E. coli* O157:H7, potentially by competing for available nutrients or by acid production (Lopez-Velasco *et al.*, 2012). For crops such as lettuce and tomato, it has been shown that certain cultivars are more prone than others to colonization by *E. coli* and *Salmonella* (Barak *et al.*, 2011; Quilliam *et al.*, 2012). One possibility is that these differences correlate with variation in the microbial community composition that these cultivars support on their surfaces. It has been suggested (Newton *et al.*, 2010) that phyllosphere microbial
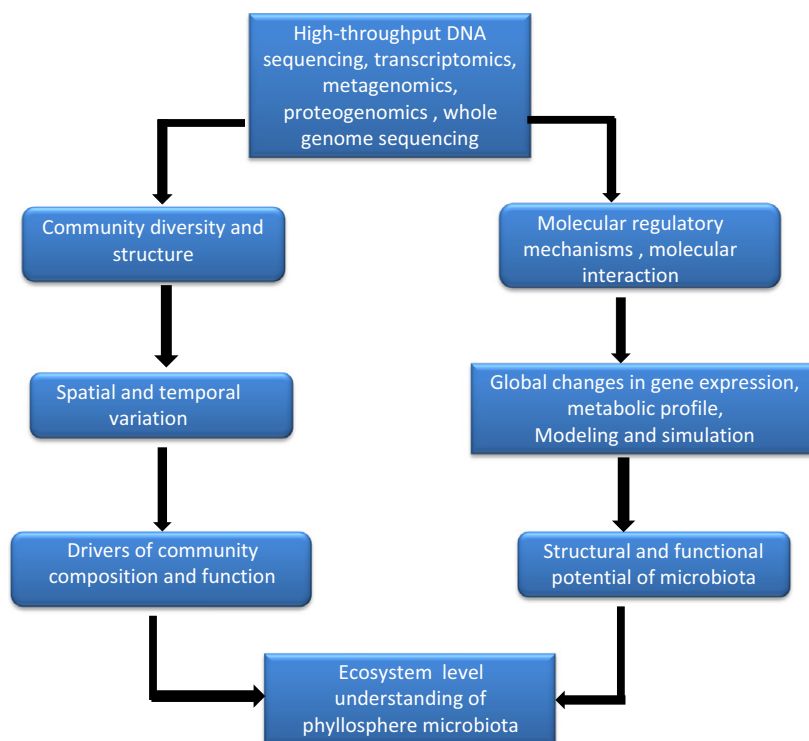
Fig. 1. Application of next-generation molecular techniques in investigating the phyllosphere microbiota.

communities may be manipulated through plant breeding or application of agrochemicals to ensure food safety of fresh produce.

Genomic approaches have also provided mechanistic insights through which human pathogens attach to the leaf surfaces of fresh produce. Recently, transcriptional profiling was used to identify genes that are differentially expressed during attachment, interaction, and survival of *E. coli* O157:H7 on lettuce leaf surfaces (Fink *et al.*, 2012). These findings suggest that *E. coli* O157:H7 attaches to leaf surfaces using curli fibers, slows down its metabolism to adapt to the nutrient-deficient environment of the phyllosphere, and suppresses the formation of a permanent biofilm.

Ecology of phyllosphere microbiota

Recent advances in culture-independent molecular techniques have exposed the phyllosphere as host to a complex microbial community. At the phylum level, bacterial communities across a wide range of agricultural crops (e.g. wheat, rice, apple, lettuce, and spinach) and naturally growing plants/trees are largely composed of *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (Bulgarelli *et al.*, 2013). Further analysis of community composition at the genus level suggests that *Pseudomonas*, *Sphingomonas*, *Methylobacterium*, *Bacillus*, *Massilia*, *Arthrobacter*, and *Pantoea* are consistently found as part

of the phyllosphere microbiota across a wide range of plant species. In Table 1, we list several recent examples of phyllosphere studies that employed high-throughput molecular approaches. In the following sections, we will discuss in more detail how these studies have broadened our understanding related to spatial and temporal variation in microbial communities and the various factors that underlie this variation.

Major drivers of phyllosphere microbiota composition

Geographical location, climatic factors, and plant genetics are three recognized drivers of bacterial community composition on leaves. Teasing out the contribution of each of these mechanisms is not an easy task, which hampers the formulation of a general conclusion. In the section below, we will discuss some recent findings offering important, but sometimes contradicting insights.

In a study on phyllosphere microbiota associated with the salt excreting desert tree *Tamarix* (Finkel *et al.*, 2011), geographical location rather than plant species was found to be the major determinant of microbial community composition, evidenced by the fact that different species of *Tamarix* (*T. aphylla*, *T nilotica*, *T. tetragina*) grown in the same geographical location supported highly similar bacterial communities. The effect of geographical distance in shaping the community structure was scrutinized

Table 1. List of studies applying high-throughput molecular approaches to phyllosphere communities

Plant	Molecular approach	Major findings	Reference
Soybean, clover, *Arabidopsis*,	16S rRNA gene pyrosequencing, metaproteogenomics	Unique metabolic adaptations contribute to the epiphytic fitness of *Sphingomonas* and *Methylobacterium*	Delmotte *et al.* (2009)
Oak	ITS pyrosequencing	Urban and rural management practices affect fungal communities in the Oak phyllosphere	Jumpponen & Jones (2009)
Pine and other trees	16S rRNA gene pyrosequencing	Tree species, not the location, is the major determinant of phyllosphere bacteria community composition	Redford *et al.* (2010)
Tamarix	16/18S rRNA gene pyrosequencing	Geographical location, not the tree species, is a major determinant of phyllosphere bacterial communities	Finkel *et al.* (2011)
Spinach	16S rRNA gene pyrosequencing	*Proteobacteria* and *Firmicutes* were the most commonly associated bacteria on field-grown spinach leaves. At genus level, communities were largely composed of *Pseudomonas*.	Lopez-Velasco *et al.* (2011)
Grape	16S rRNA gene pyrosequencing	Bacterial communities were significantly different on the surface of leaves and berries from the same grapevine.	Leveau & Tech (2011)
Lettuce	16S rRNA gene pyrosequencing	A 'core' community composed of *Pseudomonas*, *Bacillus*, *Massilia*, *Arthrobacter*, and *Pantoea* was found associated with lettuce foliage. Geographical location was an important determinant of community composition on lettuce foliage.	Rastogi *et al.* (2012)
Rice	Metaproteogenomics	Phyllosphere communities were largely composed of *Rhizobium*, *Methylobacterium*, and *Microbacterium*. Several methylotrophic enzymes were assigned to *Methylobacterium*, suggesting their role in the carbon cycle.	Knief *et al.* (2012)
Beech	ITS pyrosequencing	Fungal communities showed variation even at the smallest spatial scale of individual leaf surfaces. Plant genotype was identified as a major driver of the fungal community composition.	Cordier *et al.* (2012)
Balsam poplar	ITS pyrosequencing	Plant species was found the major determinant of fungal community composition	Bálint *et al.* (2013)
Lettuce	16S rRNA gene pyrosequencing	Planting season and irrigation practices (sprinkler/drip) together explained majority of the variation in phyllosphere microbiota composition. *E. coli* O157:H7 inoculation resulted in lower population sizes and induced minor, but lasting changes in microbiota composition.	Williams *et al.* (2013)

further through sampling of *T. aphylla* leaves from trees growing in a transect across California and Arizona (Finkel *et al.*, 2012). Community differences were strongly correlated with geographical distances between the sampling sites. A similar finding was reported for lettuce leaf microbiota (Rastogi *et al.*, 2012). Samples that were collected from the same field were generally also more similar in bacterial community structure and composition. In addition, with increasing distance between lettuce production fields, community composition became more dissimilar, indicating that variation in community composition was dependent on the physical distance between the fields.

In another recent study (Redford *et al.*, 2010), it was suggested that not the environment, but the plant drives the bacterial community composition on leaf surfaces. Phyllosphere bacterial communities associated with *Pinus*

ponderosa were fairly similar to each other irrespective of the geographical location from which leaf samples were collected. Also, leaves from 56 different tree species from the same location carried plant species-specific bacterial communities. A significant correlation between bacterial community composition and plant species strongly implies a role of plant genetic factors (Whipps *et al.*, 2008). A microbial survey of different cultivars of lettuce grown in the same field showed that they supported different bacterial communities on their foliage (Hunter *et al.*, 2010). It was suggested that these differences correlated with plant genetic components that regulate leaf texture and the leaching of metabolites to the leaf surface. Strong evidence for the role of plant genotype in selecting specific microbial populations came from a study of a recombinant inbred population of maize (Balint-Kurti *et al.*, 2010). Using QTL mapping techniques,

specific chromosomal regions that control epiphytic microbial population were identified. Interestingly, these bacterial chromosomal loci displayed significant overlap with the loci controlling the susceptibility to Southern leaf blight (SLB) fungal pathogen in the field. A greater understanding of the effect of plant genetics on the leaf microbiota would facilitate development of disease-resistant cultivars supporting microbial communities on their leaves that would be refractory to the colonization by pathogens.

Microbial populations on foliage in agricultural settings are influenced by management practices such as organic vs. conventional farming (Ottesen *et al.*, 2009), application of antibiotics (Balint-Kurti & Stapleton, 2011) and pesticides (Zhang *et al.*, 2009), as well as nitrogen fertilization (Ikeda *et al.*, 2011). As an example, we highlight a recent study (Balint-Kurti & Stapleton, 2011) that demonstrated that application of streptomycin on leaves of maize changed the microbial community in such a way that it provided greater resistance against the foliar fungus *Cochliobolus heterostrophus*, causative agent of SLB.

Sources of phyllosphere microbiota

Plant leaf surfaces are colonized in large part through immigration of bacteria, fungi, and other microorganisms from air, soil, water, seed, or through animal-borne sources (Vorholt, 2012). The relative contribution of each of these microbial sources is unclear, representing a crucial gap in our knowledge of phyllosphere community assembly. While a variety of microorganisms can be associated with phyllosphere as transient residents, the harsh environmental conditions that typify the phyllosphere select only few that persist, multiply and represent the 'true' residents of the phyllosphere. While soil appears to be one of the most likely sources of phyllosphere microbiota, comparison of bacterial communities on leaf surface and surrounding soil through pyrosequencing revealed that a very small fraction (0.5%) of operational taxonomic units were shared between the two environments (Kim *et al.*, 2012). A similar conclusion was reached through pyrosequencing of 16S rRNA genes to compare bacterial communities in the phyllosphere and rhizosphere of *Arabidopsis thaliana*. *Actinomycetales* and *Actinoplanes* were more abundantly represented in rhizosphere communities, while phyllosphere communities were largely represented by *Pseudomonas* (Bodenhausen *et al.*, 2013). In another study, the sink–source relationship between air and leaf surfaces was investigated on perennial trees in a Mediterranean ecosystem (Vokou *et al.*, 2012). DGGE analysis showed strikingly different microbial patterns in the airborne communities compared to phyllosphere samples, suggesting that plant leaf surfaces offer different degrees of survival to different bacteria arriving from the air. A more detailed spatial and temporal study using next-generation sequencing and application of a tool such as 'SourceTracker' (Knights *et al.*, 2011) is warranted in this area.

Genetic and metabolic adaptations in phyllosphere microbiota

Bacterial colonization of the phyllosphere is limited by several factors including the availability of nutrients. A recent study (Delmotte *et al.*, 2009) offered insights into the metabolic adaptations in the phyllosphere using a metaproteogenomic approach. Bacterial populations associated with the phyllosphere of soybean, clover, and *Arabidopsis* plants were found to be largely composed of *Sphingomonas* and *Methylobacterium.* Both genera abundantly occupy the phyllosphere of other plant species and are generally considered core members of the phyllosphere microbiota (Kim *et al.*, 1998; Green, 2006). Metaproteogenomic analysis of leaf surface communities identified porins and transporter-related proteins (TonB transporters), which allow transport of substrates (e.g. sugars, vitamins, siderophore) by *Sphingomonas*. These adaptations allow *Sphingomonas* to be competitive with other colonizers.

Methanol is abundantly present on plant leaf surfaces as a byproduct of pectin demethylation during plant cell-wall metabolism (Galbally & Kirstine, 2002). Proteome profiling showed that proteins belonging to *Methylobacterium* spp. and involved in the assimilation of methanol were found in high abundance in the phyllosphere (Delmotte *et al.*, 2009). The identification of proteins involved in methylotrophy and the assignment of these proteins to *Methylobacterium* spp. seem to explain to a large extent the epiphytic fitness of this bacterium in the phyllosphere of several plants.

In another study (Knief *et al.*, 2012), foliar microbiota were compared with rhizosphere microbiota to identify unique metabolic processes that are specific to microbiota from the two compartments. At the genus level, phyllosphere communities of rice supported large populations of *Rhizobium, Methylobacterium*, and *Microbacterium*. Proteome analysis indicated the occurrence of many methylotrophic enzymes (e.g. methanol dehydrogenase, formaldehyde-activating enzyme) that were assigned to *Methylobacterium*. Genes associated with nitrogen fixation were found in both phyllosphere and rhizosphere samples. However, proteomic analysis revealed that gene expression was confined to the rhizosphere. These findings further highlight the use of functional techniques to understand the *in situ* metabolic process that cannot be inferred by the use of genomic techniques alone.

Proteomic profiling was also applied to *Methylobacterium extorquens* colonizing leaves or roots or growing on synthetic medium (Gourion *et al.*, 2006). Proteins involved in methylotrophic metabolism (e.g. MxaF and Fae) and stress response (e.g. PhaA) were specifically up-regulated during epiphytic growth. This study also provided insights into the two-component regulatory mechanisms involving response regulator PhyR, which play a key role in controlling many proteins (e.g. SodA, KatE) that contribute to phyllosphere colonization.

Plant leaves offer two very different habitats to colonize: (1) the surface and (2) the apoplast or leaf interior. Surface bacteria are exposed to many external environmental factors such as solar radiation, water availability, and temperature, while apoplast bacteria are challenged with plant defense reactions. The foliar pathogen *P. syringae* pv. *syringae* B728a can colonize the leaf surface of bean plants as well as the intracellular spaces of the apoplast. Recently, transcriptional profiling of B728a cells showed that genes involved in motility, chemosensing, sequestration of phosphate, uptake of sulfur compounds, and utilization of plant-derived indole as a source of tryptophan were more highly expressed on the surface than in the apoplast (Yu *et al.*, 2013). In contrast, genes involved in the metabolism and transport of gamma-aminobutyric acid, production of secondary metabolites, and phytotoxins (syringomycin, syringopeptin) were induced to greater levels in the apoplast. This study sheds light on the role of local environmental conditions (epiphytic vs. apoplastic stages) in selecting specific colonization features during the life cycle of a bacterial phytopathogen.

Stress adaptation in phyllosphere microbiota

Epiphytic bacteria need to adapt to different stresses that are commonly present in the phyllosphere. Noticeable among these are exposure to harmful UV light, high temperatures, low humidity, and osmotic stress that vary throughout the day and have a strong impact on the community composition of phyllosphere microbiota. Many bacteria in the phyllosphere such as *Methylobacterium, Sphingomonas*, and *Pseudomonas* possess pigmentation, which confers protection against UV radiation (Lindow & Brandl, 2003). Phyllosphere bacteria also often feature special DNA repair systems that fix damage caused by UV exposure (Sundin, 2002). Production of extracellular polysaccharide is another shared feature among foliar bacteria; it allows the formation of cell aggregates and protects from desiccation and osmotic stress (Monier & Lindow, 2004).

The metaproteogenomic survey of microbial proteins in the phyllosphere of soybean, clover, and *Arabidopsis* identified several proteins with a role in stress resistance (Delmotte *et al.*, 2009). Among these proteins were chaperones, superoxide dismutases, catalases, and DNA-binding proteins. Also detected were regulators of stress-related responses such as PhyR and EcfG that were assigned to *Methylobacterium* and *Sphingomonas*. Motility-related proteins such as flagellin were detected in high abundance and mostly assigned to *Pseudomonas*. These proteins enable *Pseudomonas* to actively explore nutrients on the leaf surface and contribute to the epiphytic fitness of this bacterium (Yu *et al.*, 2013).

Whole-genome sequencing of phyllosphere bacteria

The advent of cost-effective and high-speed next-generation sequencing has also accelerated the scale and pace of genome analysis. Comparative genomic analyses of closely related strains as well as comparing commensal to pathogenic isolates have refined our understanding of the molecular mechanisms that confer specific metabolic adaptations.

Pseudomonas syringae pv. *syringae* B728a is a common epiphyte that under certain conditions can cause bacterial brown spot disease of bean. The genome of this bacterium was first sequenced using Sanger capillary sequencing (Feil *et al.*, 2005), but it has been resequenced using paired-end Illumina sequencing with a 25X coverage (Farrer *et al.*, 2009). Analysis of the sequence data suggested that genes encoding for DNA repair, UV resistance, reactive oxygen species, siderophores, and indole-3-acetic acid may contribute to epiphytic growth during colonization. As an adaptive strategy to deal with osmotic stress on leaf surface, B728a genome also contained many biosynthetic pathways that code for osmoprotectants (e.g. trehalose, betaine, ectoine). Thus, whole-genome analysis of model epiphytic bacteria offers mechanistic insights into the ecological fitness of phyllosphere microbiota.

Recently, high-throughput Illumina sequencing was applied to discover the genes associated with phyllospheric fitness of *Pantoea agglomerans* 299R (Remus-Emsermann *et al.*, 2013). This strain has been used widely as a model bacterium to understand the physiological adaptation in epiphytes to the phyllosphere environment. Genome sequence analysis of *P. agglomerans* 299R revealed many adaptations that are considered typical in plant leaf-associated bacteria, such as sugar utilization, DNA repair systems, and production of osmoprotectants. The genome of 299R also features a gene for bacterial rhodopsin (S. Belkin, pers. commun.), which permits this bacterium and other colonizers of the phyllosphere to extract energy from sun light (Atamna-Ismaeel *et al.*, 2012).

Fungal communities in the phyllosphere

So far, the focus of this minireview has been on bacterial colonizers of the phyllosphere. Foliar surfaces also support a diverse community of fungi; however, their population sizes are typically lower than their bacterial counterparts. Phyllosphere fungal communities are known to impact the fitness of their host plant (Herre *et al.*, 2007; Sunshine *et al.*, 2009). Compared with bacterial communities, relatively little is known about fungal community structure and function on leaf surfaces or how fungi impact bacterial community composition. Suda *et al.* (2009) demonstrated a larger population size, greater functional diversity, and increased species richness in bacterial communities in the phyllosphere of powdery mildew fungus-infected leaves.

Jumpponen & Jones (2009) investigated the fungal communities in the phyllosphere of oak trees (*Quercus macrocarpa*) under rural or urban management practices. High-throughput pyrosequencing of fungal internal transcribed spacer 1 (ITS1) exposed differences in the foliar fungal community composition and identified specific taxa that were responsive to these two management practices. *Devriesia, Mycosphaerella, Ramularia, Stenella, Dioszegia, Paraphaeosphaeria, Phaeosphaeria,* and *Sphaceloma* were more common in the oak trees in the rural environments, while *Aureobasidium, Davidiella, Didymella,* and *Microsphaeropsis* occurred more frequently in urban environments. Oak trees in the rural environment supported greater species richness and diversity than trees in the urban environment. Overall, this study showed that landuse was a major driver in determining the fungal community composition, diversity, and richness on the foliage of oak trees.

Spatial variation in fungal communities on leaf surfaces of European beech (*Fagus sylvatica*) was investigated using 454 pyrosequencing of the ITS1 region (Cordier *et al.*, 2012). Variation was assessed at the level of the whole tree, branches, group of leaves, and individual leaves. Sequence analysis of fungal communities showed a high degree of diversity, and communities were largely composed of generalist fungal species: *Lalaria, Woollsia,* and *Taphrina*, all of which have a cosmopolitan distribution. Greatest variability in community composition was observed at very small spatial scales, that is, at the leaf level. Spatial distance between leaves within a canopy or trees within a forest could not account for the differences in community composition. However, a strong correlation was observed between genetic distance of beech trees and differences in fungal community composition, indicating that host genetics is a determinant of fungal community assembly on beech leaves. Plant genotype was also identified as a driver of foliar fungal community composition in a recent study that used ITS pyrosequencing analysis of balsam poplar (*Populus balsamifera* L.) phyllosphere (Bálint *et al.*, 2013). Many of the genera that were identified as genotype specific were represented by very low numbers of sequences in fungal communities. This observation underscores the importance of high-throughput sequencing in providing sufficient phylogenetic depth, which allows detection of these 'rare' signature genera that are specific to host genotype.

Concluding remarks

The application of next-generation molecular techniques holds great promise in phyllosphere microbiology. These techniques have provided answers to several long-standing questions such as who lives on the leaf surfaces, what do they do, and how does their community composition change across various spatial and temporal scales? The large size data set generated by high-throughput DNA sequencing has provided new insights into the major drivers of community composition, which opens up avenues for the development and validation of models that predict community composition based on location, weather, and plant genotype (Fig. 1). Metaproteomic techniques have identified microbial proteins in the phyllosphere, thus unraveling new biochemical mechanisms that contribute to the fitness of common phyllosphere inhabitants such as *Methylobacterium* and *Sphingomonas*. Whole-genome sequencing and transcriptional profiling of model epiphytic bacteria (e.g. *P. agglomerans* and *Pseudomonas syringae*) and human pathogens (e.g. *E. coli* O157: H7) have provided detailed insights into their interactions with plant hosts, but also with other bacterial residents of the phyllosphere. This information will be extremely valuable for combining and complementing culture-dependent and culture-independent experiments. Knowledge gathered through a combination of metagenomics and proteomics will uncover the role of phyllosphere bacteria in global biogeochemical cycles. The combination of multiple omics technologies will lead us to a system-level understanding of the phyllosphere microbial communities and their physiological potential.

Acknowledgements

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