New Phytologist

Review

Tansley insight

Phyllosphere microbiology: at the interface between microbial individuals and the plant host

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Received: *9 November 2017*
Accepted: *12 January 2018*

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Contents

New Phytologist (2018)
doi: 10.1111/nph.15054

Key words: bacterial individuality, fluorescence microscopy, heterogeneity, leaf surface, micrometre resolution, plant–microbe interactions, single-cell microbiology.

Summary

Leaf surfaces are home to diverse bacterial communities. Within these communities, every individual cell perceives its unique environment and responds accordingly. In this insight article, the perspective of the bacterial individual is assumed in an attempt to describe how the spatially heterogeneous leaf surface determines the fate of bacteria. To investigate behaviour at scales relevant to bacteria, single-cell approaches are essential. Single-cell studies provide important lessons about how current 'omics' approaches fail to give an accurate picture of the behaviour of bacterial populations in heterogeneous environments. Upcoming techniques will soon allow us to combine the power of single-cell and omics approaches.

I. Introduction

Plants are colonised by a wide range of microorganisms, such as bacteria, fungi and oomycetes (Agler *et al.*, 2016). The surface of above-ground organs of plants, the phyllosphere, represents a large microbial habitat (Ruinen, 1956). The phyllosphere is dominated by leaves, which feature a heterogeneous topography at the micrometre scale. This topography consists largely of elevations, that is, epidermal cells, and grooves between epidermal cells. These two most abundant features are interspersed with stomata, trichomes, hydathodes and glandular trichomes, whose presence, density and distribution depend on leaf side and plant species. Thereby, leaves offer many different microhabitats.

Leaf surfaces can be densely populated by microorganisms. The most dominant group of microorganisms in the phyllosphere are bacteria, which reach a surprisingly dense population of on average 10^4–10^5 bacteria mm^{-2} of leaf surface or up to 10^8 bacteria g^{-1} leaf material (Remus-Emsermann *et al.*, 2014). This dense population is even more surprising given the microclimatic conditions in this habitat; leaves are light-harvesting organs covered by a waxy cuticle, which results in an environment where epiphytes constantly need to cope with ultraviolet (UV) radiation exposure, low water

and nutrient availability, and high temperature fluctuations throughout the day and variations within a single leaf. If anything, epiphytes should be admired for their ability to cope with this combination of stresses.

What do we know about bacteria living in the phyllosphere? In the last decade, leaves of different plant species were the subject of in-depth sequencing approaches, which generated comprehensive catalogues of microbial life (Redford *et al.*, 2010; Shade *et al.*, 2013; Laforest-Lapointe *et al.*, 2016). These studies demonstrated that the phyllosphere is colonised by a diverse microbiota, which is specific to plant species. At higher phylogenetic ranks, leaf-associated bacterial communities consist of recurring taxa, whereas the composition may differ at the species level.

High-throughput technologies have provided comprehensive datasets portraying microbial life in the phyllosphere by employing transcriptomics (Yu *et al.*, 2013) and metaproteogenomics, a combination of shotgun metagenomics and proteomics (Delmotte *et al.*, 2009). These top-down approaches comprehensively demonstrated the population-level presence, transcriptional activity and protein abundances of bacteria on leaf surfaces. Despite providing important insights, a disadvantage of such top-down 'omics' techniques is that they aggregate results from microbes that were recovered from a larger unit of investigation, that is, a complete leaf or pooled leaf samples, largely ignoring the heterogeneous nature of the investigated system and its microbiota. Here, we discuss how the heterogeneity of leaves impacts on its microbiota and why top-down studies have to be interpreted with great caution.

II. Individuality and the relevance of scales for the investigation of bacteria

Why should we treat bacteria as individuals? Despite being clonal, each bacterial cell occupies a unique three-dimensional site in time and space, containing a distinctive content of molecules, such as metabolites, RNA and proteins. The presence and abundance of these molecules change how bacterial individuals experience and respond to their environment, for example, their response to chemical stimuli (Korobkova *et al.*, 2004). In extreme cases, this leads to clonal populations featuring several subpopulations that exhibit distinct behaviours, such as pathogenicity on leaves and in the mammalian gut, or growth within the mouse spleen (Diard *et al.*, 2013; Claudi *et al.*, 2014; Rufián *et al.*, 2016). Next to endogenous factors, stochasticity also determines the fate of individual cells, for example, during colonisation at random sites on the leaf surface (Remus-Emsermann *et al.*, 2012). As a result of this population heterogeneity, top-down approaches fail to provide an accurate picture of the behaviour and responses within a bacterial population.

Therefore, to learn about microbial behaviour in heterogeneous environments, it is necessary to pursue studies at a resolution relevant to the subjects under study, which is the micrometre resolution or single-cell level (Fig. 1). To emphasise differences in bacterial behaviour and give credit to bacterial population and community heterogeneity, the concept of bacterial individuality has been adopted and first used, to our knowledge, by Spudich and Koshland (1976), before it was recently reintroduced (Davidson & Surette, 2008). Several studies provide excellent examples of the importance of this concept for phyllosphere microbiology.

Although technically challenging, the investigation of bacteria at a single-cell resolution on leaves has relied mainly on fluorescence microscopy. Approaches and solutions to common problems, and their advantages and disadvantages are briefly addressed in Box 1. To visualise bacteria on leaves using microscopy, they are usually stained using fluorescent dyes (Table 1). However, the advent of green fluorescent protein (GFP) was pivotal for the study of bacteria at a single-cell resolution and has been adopted by phyllosphere microbiologists shortly after it was introduced as a tool for molecular biology. The first study employing GFP-expressing bacteria on leaf surfaces investigated conjugal gene transfer on leaves and identified the rate and location of plasmid transfer

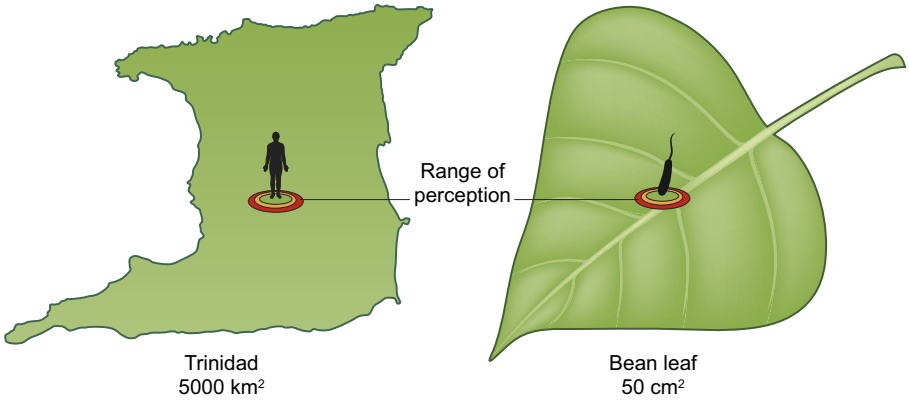


Fig. 1 Why spatial scales matter. To illustrate the situation that most microbes find themselves on leaf surfaces, assume a human subject on the island of Trinidad, which has similar proportions to a human as a bean leaf to a bacterium. Assuming that the human cannot move, has no vision, nor sense of hearing and is left only with its sense of touch and sense of smell, the immediate surrounding becomes vitally important. In other words, that human will not be able to perceive any other part of the island. This is comparable to how individual single-celled microbes perceive a leaf. Without sufficient amounts of water, free movement of bacteria is restricted and they only perceive signals, such as sugars, amino acids or volatiles, diffusing to their occupied site. Thereby, the microhabitat conditions drive the experience and behaviour of individual bacteria.

Box 1 Techniques and solutions

Although single-cell techniques have developed at a rapid pace in recent decades, some aspects remain problematic. Here we briefly discuss problems and solutions to investigate bacteria at single-cell resolution on leaf surfaces.

The biggest issue of (fluorescence) microscopy studies of bacteria on leaf surfaces is the strong uneven background presented by the leaf. The uneven background often leads to false negative and false positive detection of bacteria; for example, stomatal cells are overlaid by a particular wax that exhibits stronger autofluorescence than other epidermal cells (Karabourniotis, 2001), which results in false positive detection of bacteria in stomata. Additionally, bacteria are often redistributed during preparation of microscopy specimens.

These issues can be overcome in several ways:

(1) Increasing the signal-to-noise ratio by staining bacteria with strongly fluorescent dyes (Table 1) or tagging bacteria with bright, constitutively expressed fluorescent proteins. This enables the observation of bacterial distributions on plants, but the fluorescence signal is still impacted by the background, making quantification of fluorescence signals error-prone.

(2) Recovery of bacterial cells for *ex situ* analysis. Reliable quantification of fluorescent signals of bacterial bioreporters can be achieved by recovering bacterial cells from leaves prior to microscopy and image acquisition. Despite losing spatial information, this method excels if sensitive fluorescence quantification is needed (Leveau & Lindow, 2001).

(3) Recovery of bacterial cells while retaining spatial information. By using adhesive tape, it is possible to recover the waxy cuticle from leaves, trapping bacteria between tape and cuticle. After performing the cuticle tape lift, background fluorescence is evenly distributed, allowing for background corrections and fluorescence signal quantification while maintaining the spatial distribution of the bacterial cells (Bisha & Brehm-Stecher, 2010; Remus-Emsermann *et al.*, 2014).

(4) Microscopy without the addition of mounting resin. Some microscope objectives of high magnification, numerical aperture and large working distance allow the observation of fluorescent bacteria without the addition of a coverslip or mounting resin. Thereby, bacteria could be observed without disturbing their distribution. However, such objectives are rarely available and the user has to be highly trained to prevent contamination or damage to the objective.

(5) Image processing. A proof-of-concept study has recently shown that after clearing of the leaf and multispectral image acquisition, software-aided 'linear unmixing' allows the identification of fluorophore spectra on leaves to resolve bacteria from the leaf autofluorescence (Peredo & Simmons, 2018). This technique might be the first that enables background-free quantification *in situ* on leaves.

(Normander *et al.*, 1998). Many other studies followed, which will be discussed below.

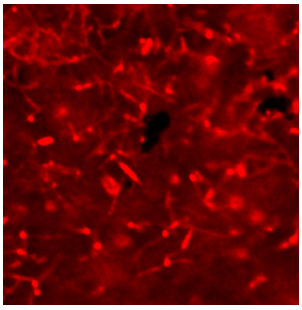
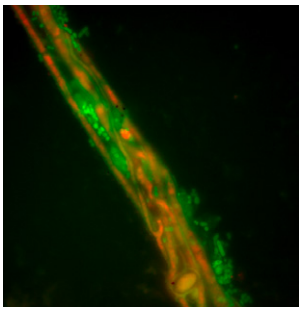
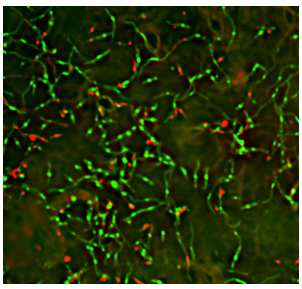
Two seminal back-to-back studies provided insights into the distribution and availability of iron and fructose on leaf surfaces (Joyner & Lindow, 2000; Leveau & Lindow, 2001). The use of GFP-based whole-cell bioreporters to show that leaf-immigrating bacteria experience different abundances of iron and carbohydrates in their local environment was key to providing the first evidence that nutrients are not homogeneously distributed on leaf surfaces and colonists are exposed to variable amounts of nutrients. This study was followed by others, with similar results, to describe the availability of compounds, such as phenol (Sandhu *et al.*, 2007) and water (Axtell & Beattie, 2002), or the distribution of fructose (Remus-Emsermann *et al.*, 2011). Ultimately, the combination of local nutrient availability and biotic and abiotic factors determines the different degrees of habitability for immigrants on leaves. Recent studies have demonstrated that leaf surfaces indeed offer different degrees of micro-habitability, resulting in a measurable probability of colonisation success of immigrant bacteria, or in other words, leaves offer a multitude of habitats with unique carrying capacities (Remus-Emsermann *et al.*, 2012) that are dependent on previous colonisation (Remus-Emsermann *et al.*, 2013).

The studies above highlight the relevance of considering leaves as a multitude of microsites with unique, highly localised conditions and not as a reference unit of investigation. Thereby, these studies gathered evidence for the view of bacterial perception introduced in Fig. 1 in the phyllosphere. This can be explained by a model of the leaf surface that is interspersed by localised hotspots of bidirectional diffusion, that is, potential sites of host–microbe communication (Fig. 2).

III. Bacterial aggregation and community patterning at the single-cell resolution

What is the scale of bacterial interactions on leaf surfaces? Microscopy studies revealed that bacteria are prevalent in epidermal cell grooves, around trichomes and in stomata, and less prevalent on the elevated surface of epidermal cells (Fig. 2) (Esser *et al.*, 2015). A recent study has shown how individual bacterial colonisers exhibit clonal growth and form clusters on leaves. However, these clusters were smaller than predicted based on measurements employing a bioreporter for reproductive success. This was interpreted to be a result of individual cells that emigrated from the cluster (Tecon & Leveau, 2012). Few studies have focused on the interplay between different bacterial strains on leaves *in situ*. A pioneering study investigated the interplay of binary strain combinations on leaves and how these strains are co-colonising the environment (Monier & Lindow, 2005). Only very recently this approach was followed by a study that used spatial statistical methods to understand the interplay between colonisers. Using fluorescence *in situ* hybridisation and spatial statistics on environmentally grown *Arabidopsis thaliana* leaves, the study revealed that different taxa exhibit short-distance aggregation of up to 7 μm and intraspecific taxa aggregation of up to 10 μm (Remus-Emsermann *et al.*, 2014). This study has provided the first estimates of the spatial scales at which bacterial species aggregate on *A. thaliana* leaves under environmental

Table 1 Fluorescent dyes used in microscopy approaches to visualise and analyse bacteria on leaves

Dye name	Use	Image
Acridine orange/DAPI/Hoechst 33342 and 33258/Syto dyes	DNA intercalating dyes that pass the cell membrane and enable microscopy of microbes on leaves. The picture shows acridine orange-stained bacterial cells on an environmentally grown silver beech leaf (*Lophozonia menziesii*)	
Fluorescence *in situ* hybridisation (Remus-Emsermann *et al.*, 2014)	Fluorescently labelled oligonucleotides that discriminate bacterial taxa. The intensity of fluorescence *in situ* hybridisation signals also reflects the ribosomal content of cells. The picture shows fungal hyphae (orange) recovered from an *Arabidopsis thaliana* leaf colonised with bacteria (green)	
Live/dead stain (e.g. BacLight)	Contains DNA intercalating red and green fluorescent dyes. The red fluorescent dye propidium iodide cannot penetrate the cell membranes of living cells. The green fluorescent dye Syto 9 can penetrate living cells. Live–dead staining reports on the physiological state of bacteria. The picture shows bacteria found on an environmentally grown silver beech leaf (*Lophozonia menziesii*)	

conditions. A study performed under laboratory conditions provided further evidence to the observed scale of aggregation (Esser *et al.*, 2015). Both studies provided the first indication of the scale of bacterial interactions on leaves (Fig. 3). It is worth noting that the sphere of influence might be larger along epidermal cell grooves, as these sites may contain residual water that constitutes the leaf surface waterscape, the phyllotelma (Doan & Leveau, 2015).

IV. What are the effects on the plant host?

Nonpathogenic phyllosphere-colonising bacteria are known for their ability to modify their microenvironment. By producing plant hormone mimics, for example auxins and cytokinins, or secreting biosurfactants, microbes actively impact on the plant and gain fitness advantages, although the ramifications of these modifications for the host are often unclear (Ali *et al.*, 2009; Zhang *et al.*, 2009; Meena *et al.*, 2012; Burch *et al.*, 2014; Radhika *et al.*, 2015). First insights into the effects of nonpathogenic leaf surface colonisers on host fitness suggest that the microbiota supports the host against microbial infections. This could be through priming of the plant immune system (Vogel *et al.*, 2016) or through competition between invasive pathogenic and resident microbes (Pusey *et al.*, 2011).

Although plants systemically integrate environmental signals and cells communicate via plasmodesmata and second messengers (Seybold *et al.*, 2014; Lim *et al.*, 2016), it may be necessary to consider single plant cells as individual entities, which has been discussed by Libault *et al.* (2017). Considering the patchiness of microbial populations on the leaf surface, it is tempting to assume that their impact on the plant host would be equally heterogeneous (Fig. 2). Variation in the occupancy, density or composition of these communities on the leaf surface might be reflected in differential local host responses. In leaf pathosystems, plant epidermal cells react by deploying localised immune responses upon perception of pathogenic microbes, which is characterised by an accumulation of reactive oxygen species, cell-wall appositions and programmed-cell death (Melotto *et al.*, 2006; Voigt, 2014; Cui *et al.*, 2015). However, it remains unclear if individual epidermal cells respond differently to nonpathogenic microbes, and whether this response remains local or is systemically integrated.

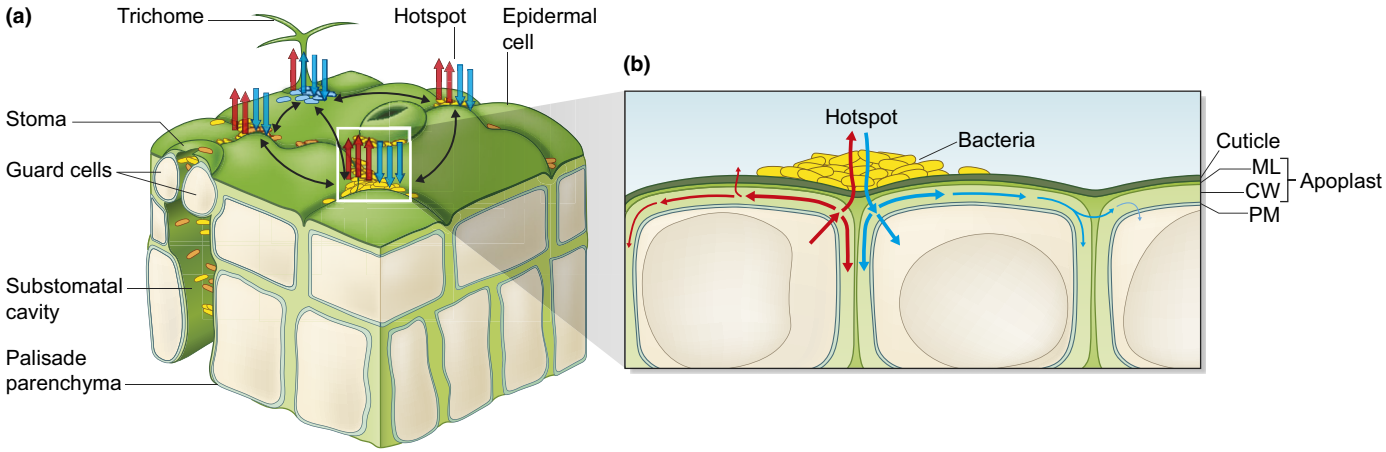


Fig. 2 Interaction hotspots on the leaf surface. (a) Patchy distribution of localised microsites, where compounds are diffusing at higher rates across the leaf surface. Red and blue arrows represent the direction of diffusion from the apoplast to the surface and vice versa, respectively. At these sites, bacterial populations (yellow, orange and blue) are able to grow due to a higher availability of nutrients. At the same time, the bacteria secrete compounds (e.g. phytohormones, biosurfactants), which permeate into the leaf tissues. The spatial distribution of bacterial communities is also determined by bacteria–bacteria interactions (black two-headed arrows), such as competition and cross-feeding. (b) Model of diffusion pathways and formation of interaction hotspots. Resources from plant cells diffuse through the apoplast and leach onto the leaf surface (red arrows). Diffusion rates are higher at the depressions formed between epidermal cells junctions. In turn, bacteria thriving in hotspots secrete compounds that diffuse into the leaf apoplast and epidermal cells (blue arrows). Localised responses are expected, as a gradient of secreted compounds is established, leading to differential responses between adjacent epidermal cells. ML = middle lamella; CW = cell wall; PM = plasma membrane.

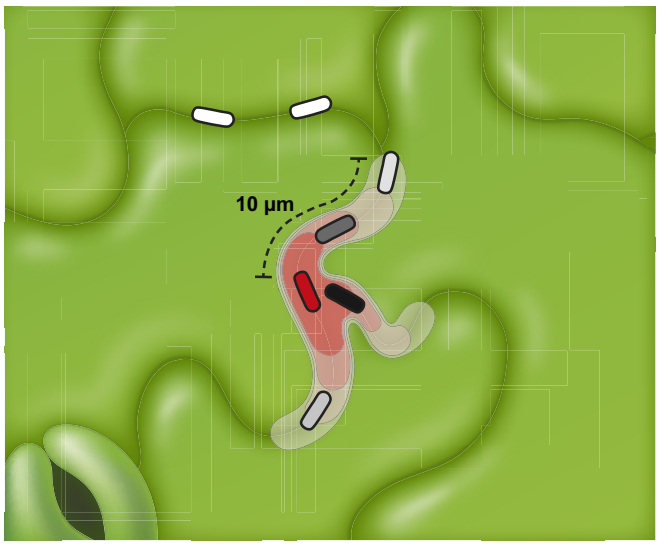


Fig. 3 Bacterial sphere of influence on leaf surfaces. Studies applying spatially explicit statistical approaches revealed that bacterial interactions on leaf surfaces are limited to *c.* 10 µm. This indicates that the sphere of influence (shades of red) of signal donors (in red) on recipients (shades of grey) may be limited to this distance. Within epidermal cell grooves, the radius of influence may be further compared to the top of epidermal cells due to residual water in grooves.

V. Future directions and current questions

Environmentally grown plants and plants grown in gnotobiotic systems are colonised in a reproducible manner, that is, colonists contribute to communities similarly between experiments (Bodenhausen *et al.*, 2014; Bai *et al.*, 2015). However, how bacterial taxa coexist on plants remains unclear. We propose that a key to understanding important aspects of the development and coexistence of bacterial leaf surface communities lies in single-cell approaches. Well-characterised, full genome-sequenced phyllosphere bacteria equipped with fluorescent proteins or other fluorescent probes will allow the bottom-up study of the spatial composition of synthetic bacterial communities on leaves. The observed patterns may relate to the genomic makeup of the community members, permitting identification of the genetic factors involved.

To appreciate the variety of responses within a population and to understand the ecology of bacteria, particularly phytopathogens (Zhang *et al.*, 2009; Rufián *et al.*, 2016), new approaches are dearly needed. The nature of microbial life in the phyllosphere requires the adoption of approaches that go beyond the current single-cell fluorescent bioreporter and aggregative 'omics' approaches. Among them, techniques that combine and account for bacterial individuality and spatial information, and high-throughput techniques will help to capture the mechanisms shaping leaf–microbe and microbe–microbe interactions with fine detail. A recent proof of concept study showed the potential of spatially resolved metabolite analysis by matrix-assisted laser desorption ionisation imaging MS on leaf surfaces colonised by bacteria (Ryffel *et al.*, 2016). This technique is still in its infancy and we are likely to see improvements in spatial resolution and limit of metabolite detection in the near future. Additionally, single-cell manipulation techniques combined with 'omics' approaches offer unique opportunities for analysing, for example, the metabolome of individual leaf cells through live single-cell MS (Fujii *et al.*, 2015) or the transcriptomic profiles of individual bacteria and plant cells through single-cell sequencing techniques. The combination of fluorescent bioreporter approaches and cell sorting techniques allows us to selectively analyse subpopulations of bacteria and plant host cells (Coker *et al.*, 2015; Rufián *et al.*, 2016). If the scientific community adopts those and similar approaches, we are likely

to experience a rapid change in our understanding of plant–microbe and microbe–microbe interactions in the phyllosphere.

Acknowledgements

We thank Matthew Turnbull for his mentorship. Daniela Remus and Ian Dickie are acknowledged for critically reading the manuscript. M.R.E. was supported by the New Zealand Royal Society Te Apārangi (Marsden Fund grant UOC1704) and a College of Science Doctoral Research Scholarship, University of Canterbury, to support R.O.S. R.O.S. was supported by a New Zealand International Doctoral Research Scholarship (NZIDRS).

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