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11 Microbial communities in the phyllosphere

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11.1 Introduction

The term phyllosphere was coined by Last (1955) and Ruinen (1956) to describe the plant leaf surface as an environment that is physically, chemically and biologically distinct from the plant leaf itself or the air surrounding it. The term phylloplane has been used also, either instead of or in addition to the term phyllosphere. Its two-dimensional connotation, however, does not do justice to the three dimensions that characterise the phyllosphere from the perspective of many of its microscopic inhabitants. On a global scale, the phyllosphere is arguably one of the largest biological surfaces colonised by microorganisms. Satellite images have allowed a conservative approximation of 4×10^8 km^2 for the earth's terrestrial surface area covered with foliage (Morris and Kinkel, 2002). It has been estimated that this leaf surface area is home to an astonishing 10^{26} bacteria (Morris and Kinkel, 2002), which are the most abundant colonisers of cuticular surfaces. Thus, the phyllosphere represents a significant refuge and resource of microorganisms on this planet.

Often-used terms in phyllosphere microbiology are epiphyte and epiphytic (Leben, 1965; Hirano and Upper, 1983): here, microbial epiphytes or epiphytic microorganisms, which include bacteria, fungi and yeasts, are defined as being capable of surviving and thriving on plant leaf and fruit surfaces. Several excellent reviews on phyllosphere microbiology have appeared so far (Beattie and Lindow, 1995, 1999; Andrews and Harris, 2000; Hirano and Upper, 2000; Lindow and Leveau, 2002; Lindow and Brandl, 2003). This chapter aims to provide a brief but broad and updated synthesis of research activities and findings related to phyllosphere microbiology. As the next chapter in this volume (Chapter 12) will focus in greater detail on phyllosphere fungi, the present chapter is biased, in some sections at least, towards bacterial colonisers of the leaf surface.

11.2 Methodologies in phyllosphere microbiology

11.2.1 Sampling techniques

Sampling the phyllosphere is at the basis of many observations or experiments in leaf surface microbiology, and thus deserves its own and in-detail section

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at the beginning of this chapter. Several reviews are available that describe the advantages and disadvantages of different methods for sampling and quantification of microorganisms from the phyllosphere (Donegan *et al.*, 1991; Jacques and Morris, 1995; Dandurand and Knudsen, 1996).

One of the simplest techniques is leaf printing (Corpe, 1985), by which a plant leaf is pressed onto the surface of an agar plate for a defined period of time and carefully removed again. The agar plate is then incubated to allow growth of the microorganisms that transferred from the leaf surface. Depending on the medium and the abundance and composition of the microflora, the result is a collection of bacterial and fungal growth foci ('colonies') that often follow the contours of the leaf. Obvious disadvantages of the method are its low resolution of observation and its limited ability to provide a quantitative appreciation for the phyllosphere community composition. However, leaf printing can provide a first indication that the distribution of microorganisms on leaf surfaces is not uniform (Leben, 1998). For example, more growth often occurs where the veins of the leaf touch the agar, suggesting that these structures are more densely populated areas on the leaf (Manceau and Kasempour, 2002).

Furthermore, leaf printing has been an extremely useful and popular laboratory experiment for teaching purposes (Holland *et al.*, 2000). The outcome of a leaf printing experiment will depend in large part on the medium that is used to prepare the agar plate. Medium composition can be varied and exploited to look only at a subset of the microbial community on the leaf. By incorporation of compounds with antifungal (e.g. cycloheximide) or antibacterial (e.g. penicillin) activity, fungal or bacterial colonisers, respectively, can be excluded from the analysis. As microorganisms differ in their nutritional capabilities, it is also possible to include specific nutrient sources in the medium to select for a nutritionally defined subpopulation of the phyllosphere. For example, agar plates that contain methanol as the sole source of carbon have been used to demonstrate the ubiquity of methylotrophic bacteria on plant leaf surfaces (Corpe, 1985).

More quantitative than leaf printing is the method of leaf washing. One or more leaves are placed in a tube or flask containing a specified volume of wash solution (e.g. saline solution or phosphate buffer), and microbes are removed from the cuticular surface by vortexing and/or sonication. The number of bacteria in the wash solution is then determined by the most probable number (MPN) technique (Oblinger and Koburger, 1975) or by plating, directly or after dilution of the wash solution, onto nonselective or selective solid medium to determine the number of colony-forming units (CFUs). The plating can be done using, for example, a Drigalski spatula, glass beads or a spiral plater (Gilchrist *et al.*, 1973). The latter has the advantage that quantitation of CFUs is possible without prior dilution of the leaf wash sample. As with the leaf printing technique, medium composition of the agar plate determines what subpopulation of the phyllosphere will be analysed. This analysis should also take into consideration the fact that different organisms from the leaf surface grow at different rates, so that those bacteria or fungi that grow fast and appear on agar plates first will be more likely to be included in the analysis

than those that grow slowly and appear late or not at all due to overgrowth by fast appearing strains.

From the number of CFUs per plate the number of bacteria that were present on the leaf can be estimated quantitatively by taking into account the volume of the leaf washing solution, the dilution factor and the volume of the aliquot that was spread onto the agar plate or used to inoculate the medium in the MPN method. CFU numbers are usually expressed as the number of bacteria per leaf, per gram of leaf weight or per square centimetre of leaf surface. Often the number is expressed as ^{10}log(CFU), as this allows comparison of population counts from different leaves, which are often distributed not normally but lognormally (Hirano *et al.*, 1982; Kinkel *et al.*, 1995; Woody *et al.*, 2003).

The leaf washing method can be modified to obtain answers to different questions. For example, by not vortexing or sonicating the leaf in a wash solution, bacteria that occupy the leaf surface as aggregates can be removed as intact aggregates and be separated by filtration from those bacteria that live solitary. In this way, Morris *et al.* (1998) were able to estimate the fraction of bacteria that live in bacterial aggregates on the leaf surface. Incidentally, this aggregated lifestyle of bacteria may be a reason for underestimation of total bacterial populations on leaf surfaces: an aggregate of two or more bacterial cells will produce a single CFU on plates, which stresses the need for breaking up of these aggregates as much as possible (Miller *et al.*, 2000).

Another reason for underestimating microbial populations from leaf washing data is the resistance of some microorganisms to be readily removed from the leaf surface by leaf washing (Romantschuk, 1992). A good example is the group of pink-pigmented facultatively methylotrophic bacteria (Holland, 1997). This is probably one of the reasons why these particular bacteria are often missed in phyllosphere composition studies (Holland and Polacco, 1994). Also, plant-pathogenic fungi, such as downy and powdery mildews, produce structures that anchor the fungal hyphae into the plant leaf making them hard to remove (Section 11.3.2, and Chapter 12). To check the efficiency of any leaf washing method, one can use microscopy (Section 11.2.3) to validate the degree of removal from the leaf surface, for example, by staining with the fluorescent DNA stain 4,6-diamidino-2-phenylindole (DAPI). In one instance this was done to check the removal efficiency of a less common sampling method which involves placing a leaf on sterile water, rapidly freezing the water, then carefully removing the leaf and collecting the ice which contains the transferred leaf microflora (Heuser and Zimmer, 2002). Whereas washing by vortexing and sonication usually leaves the cuticular surface covered by residual microorganisms (Jacques and Morris, 1995; Heuser and Zimmer, 2002), the freezing method almost completely removes microorganisms from the leaf (Heuser and Zimmer, 2002).

A method with similar removal efficiency is leaf maceration, by which a single leaf or a collection of leaves is macerated, for example, with a mortar and pestle, in a defined volume of buffer, which is then analysed by plate counting. However, since leaf maceration liberates in addition to those microorganisms that live on

the leaf surface also those that grow inside the plant tissue, phyllosphere population sizes may be overestimated by this method.

One final point of consideration when using methods such as MPN or plate counts is that they provide estimates only for the culturable subset of the microbial population. Microorganisms that resist growing under laboratory conditions are not included in these analyses (also see Section 11.4.1). Furthermore, viable-but-not-culturable-microbial cells (VBNC) that experience prolonged periods of starvation may enter a physiological state which renders them not-culturable, that is, they cannot form a colony on an agar plate, but they are still viable (Bogosian and Bourneuf, 2001). This concept also applies to cuticular surface colonisers. For example, Wilson and Lindow (1992) showed that up to 75% of the bacteria on inoculated bean leaves were VBNC, demonstrating that plate counts can grossly underestimate the viable fraction of bacteria on a leaf surface.

All of the sampling techniques described so far are in essence destructive, that is, the population size of a single leaf can be measured only once, at which time the experiment for that leaf ends. This makes it impossible to follow the temporal dynamics of a microbial population for a single leaf, only for leaves picked at different time points. But because the variation among leaves at any time point can be substantial (Hirano *et al.*, 1982; Jacques and Morris, 1995; Hirano and Upper, 2000), sample sizes necessarily have to be large (i.e. many leaves should be analysed for each time point) in order to obtain statistically significant and precise estimates for changes in microbial population sizes over time. To circumvent the collection of large sample sizes, a semi-destructive sampling method can be used such as the one developed by Woody *et al.* (2003) for studying the temporal population dynamics of the phyllosphere yeast *Aureobasidium pullulans*. Its success is based on the prerequisites that (1) sampling of a leaf segment does not affect the yeast population on other parts of the same leaf, and (2) the distribution of yeast cells is similar for different segments of the same leaf. These prerequisites cannot *a priori* be assumed to be true for every other combination of microorganism and plant (Woody *et al.*, 2003).

Microorganisms that are sampled from the phyllosphere may be used not only for the purpose of estimating phyllosphere population sizes; leaf washings have also been used to inoculate BIOLOG EcoPlates to obtain a carbon-metabolism profile for the microbial community as a whole (Yang *et al.*, 2001). Microorganisms washed from a leaf may be examined microscopically (Section 11.2.3), for example, after staining with DAPI or another DNA dye such as acridine orange, after live/dead staining (Monier and Lindow, 2003a) or after hybridisation with rRNA-specific probe(s) using fluorescent in situ hybridisation (FISH) (Joyner and Lindow, 2000; Brandl *et al.*, 2001; Leveau and Lindow, 2001). Microorganisms that have been modified to express the green fluorescent protein (GFP) can be analysed for individual GFP content using fluorescent microscopy and image analysis (Joyner and Lindow, 2000; Brandl *et al.*, 2001; Leveau and Lindow, 2001; Miller *et al.*, 2001) or flow cytometry (Axtell and Beattie, 2002; Marco *et al.*, 2005). Leaf washings may also be examined for the activity of other reporter genes, such as *inaZ* (Miller

et al., 2001). Furthermore, leaf washings may be the starting material for the isolation of microbial DNA for a culture-independent assessment – either qualitative or quantitative – of leaf surface microbiology (Yang *et al.*, 2001; Heuser and Zimmer, 2002, 2003).

11.2.2 Artificial inoculation

Experimental manipulation of phyllosphere composition and abundance has contributed a great deal to our understanding of the cuticular surface as a microbial habitat. It is achieved by artificial inoculation of plants in the field, greenhouse or laboratory with defined suspensions of bacteria, yeasts or fungal spores. The most common purposes of artificial inoculation are:

(1) to determine the ability of the inoculated microorganism(s) to survive and thrive in the phyllosphere;
(2) to follow the growth and/or activity of the introduced strain(s) in relation to factors such as plant and environment;
(3) to assess the effect of the introduced strain(s) on the abundance and activity of other microorganisms in the phyllosphere; or
(4) to exploit the introduced microorganism(s) as a biological indicator of the physical, chemical or biological factors that govern the phyllosphere.

In the greenhouse or laboratory, inoculation of plants is achieved by immersing whole plants in a dilute suspension of microorganisms with a known titer, or by spraying them with such a suspension using a device that produces a fine mist or spray. After inoculation, plants are incubated in one of many ways, depending on experimental design and research question. To create an environment that is conducive to microbial growth, it is most common to incubate under conditions of high relative humidity, for example, by covering the plants in bags or placing them in controlled-environment chambers. Plants have been exposed to diverse conditions in order to see if and how these conditions change microbial population sizes and activities. Some examples are different light intensities (O'Brien and Lindow, 1989), low relative humidity (O'Brien and Lindow, 1989; Andersen *et al.*, 1998), or increased ultraviolet (UV) exposure (Kadivar and Stapleton, 2003; Jacobs *et al.*, 2005). In the field, plants are spray-inoculated using knapsack sprayers or scaled-up devices. Most field inoculations are performed to test or exploit the ability of the microorganism(s) in the inoculum to suppress symptoms of disease or microbe-induced freezing (Section 11.9.3). To some extent, environmental conditions in the field can be manipulated, for example by covering plants with meshes to test the effect of rainfall momentum or exposure to sunlight (Upper and Hirano, 2002).

Inoculation of plants can be done with single strains or with mixtures of strains. By mixing two strains in a 1 : 1 ratio and using this mixture as an inoculum, the two strains in the mixture can be directly compared for their relative fitness in

the phyllosphere (Section 11.6.1). By this approach small but significant differences in behaviour between two species can be revealed better than when the two strains are inoculated separately (Lenski, 1992). Mixtures may also be used to test the effect of strains on each other's performance in the phyllosphere. So-called de Wit replacement series, whereby two strains are mixed in various proportions at a constant total inoculum density, have provided important insights into the relative competitive abilities of microbial epiphytes, niche differentiation and resource utilisation (Wilson and Lindow, 1994a, 1995). Factors that have been shown to contribute significantly to the phyllosphere performance of inoculated microorganisms are preparation of the inoculum and inoculum density (Wilson and Lindow, 1994b). Each of these factors should be taken into consideration during the planning of artificial inoculation experiments and interpretation of their results.

One special type of inoculum involves microbial bioreporters for habitat exploration (Leveau and Lindow, 2002). Bioreporters are whole-cell indicators of a specific microbial activity. They are usually bacteria that have been manipulated to carry a reporter gene such as the one coding for GFP or ice nucleation protein (InaZ) downstream of an inducible promoter. This promoter determines the usefulness and specificity of the bioreporter strain. For example, Leveau and Lindow (2001) introduced into the epiphytic bacterium *Erwinia herbicola* a fusion of a fructose-inducible promoter and the gene for GFP. The resulting strain of *E. herbicola* responds to the availability of fructose by the synthesis of GFP and emission of green fluorescent light. Inoculation of this strain onto plants made it possible to explore the leaf surface for the availability for fructose (see also Section 11.5.2). Several other bioreporters, with different specificities, have been applied to the phyllosphere (Joyner and Lindow, 2000; Miller *et al.*, 2001; Axtell and Beattie, 2002), and each has provided unique insights into the microbial perception of the cuticular surface (Sections 11.5.2 and 11.7.3).

The concept of bioreporting has also been used for the identification of genes that contribute to the fitness of epiphytic bacteria in the phyllosphere (Sections 11.6.1 and 11.6.3). Marco *et al.* (2003) developed a screening method for phyllosphere-inducible promoters of *Pseudomonas syringae* which is based on the insertion of random genomic DNA fragments upstream of a promoterless but essential locus *metXW* for methionine biosynthesis. When a mixture of bacteria, each containing a different gene fusion, is inoculated onto plants only those bacteria that carry a phyllosphere-inducible promoter express the *metXW* genes and thus will survive. In other words, by inoculation onto plant leaves, bacteria with phyllosphere-inducible promoters are enriched. This approach has been very useful in the identification of genes that are involved in the adaptation of bacterial colonisers to the phyllosphere (Marco *et al.*, 2005; also see Section 11.6.3).

11.2.3 Microscopy

The openness of the phyllosphere makes it extremely suitable for direct observation by microscopy. In this respect, the phyllosphere differs dramatically from

the rhizosphere, which essentially is a hidden environment. Microscopical analysis can be done on whole leaves, leaf sections or even isolated leaf cuticles. The highest magnification, that is, 10 000× or more, is achieved by electron microscopy, and both transmission and scanning electron microscopy have revealed interesting details of phyllosphere life at the (sub)micrometre scale (Beattie, 2002). Environmental scanning electron microscopy is a modification of the latter technique which allows 'wet-mode' imaging of phyllosphere samples (Monier and Lindow, 2004). Atomic force microscopy has also been applied to the phyllosphere (Mechaber *et al.*, 1996) to create three-dimensional, high-resolution surface maps of the leaf (see Section 11.5.1).

More accessible to most phyllosphere researchers is light and fluorescence microscopy which generally have a maximum magnification of 1000× and a resolution of about 0.2 micrometres. One of the most exciting developments in phyllosphere research has been the combination of fluorescent microscopy and fluorescent proteins such as GFP (Brandl *et al.*, 2001; Leveau and Lindow, 2001; Axtell and Beattie, 2002; Brandl and Mandrell, 2002; Monier and Lindow, 2003a) but also red-fluorescent protein (Brandl and Mandrell, 2002). Bacteria or fungi that have been modified to express GFP constitutively are easily recognised in situ on the leaf surface by their green fluorescence. Thus, colonisation patterns can be related to leaf surface structures. Furthermore, the activity of GFP-based bioreporters (as discussed in Section 11.2.2) can be interpreted in light of their location on the leaf surface (Leveau and Lindow, 2001). With confocal laser scanning microscopy (CLSM), individually sliced views in the z-axis can be viewed and stacked. CLSM has been used quite successfully to reconstruct the three-dimensional arrangement of microorganisms on the leaf surface (Figure 11.1).

For epiphytic microorganisms that occur naturally on leaves or that have not been modified to express fluorescent proteins, different stains or dyes are available

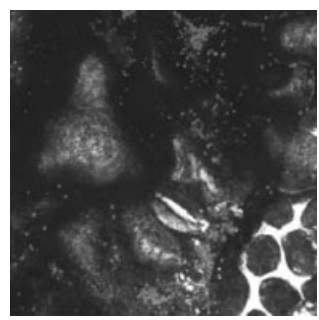

Figure 11.1 View of a colonised bean leaf using confocal laser scanning microscopy. Individual bacterial colonisers producing GFP are visible as green fluorescent dots. The centre shows a stoma; the cuticular top of an epidermal plant cell is visible on the left top corner while at the bottom right the picture slices into the leaf's palisade parenchyma. Chlorophyll is coloured red in this picture. This figure is produced in colour in the colour plate section, which follows page 249.

to facilitate their visualisation. Most practical are those stains that can be used in combination with fluorescence microscopy. These include DNA stains like DAPI and acridine orange, and propidium iodide which stains dead bacteria red (Monier and Lindow, 2003a, 2004). A more specific type of dye is represented by fluorescently labelled oligonucleotides or probes, which are designed to target the rRNA in ribosomes in a procedure that is referred to as FISH (Amann *et al.*, 1995). FISH probes can be labelled with different fluorophores, but carboxytetramethylrhodamine (TAMRA) has been most commonly used in phyllosphere research. TAMRA fluoresces red and its detection is compatible with that of GFP (Joyner and Lindow, 2000; Brandl *et al.*, 2001; Leveau and Lindow, 2001).

11.3 Getting to the phyllosphere (and leaving again)

11.3.1 Immigration

When a leaf appears on the plant, it is generally not sterile, that is, free of microbial inhabitants. Seeds that are naturally or artificially inoculated with bacteria have been shown to develop into plants that carry substantial populations of these bacteria on their leaves (Saettler *et al.*, 1989; Lilley *et al.*, 1997; Upper and Hirano, 2002). Thus, seeds and, indirectly, soil may be potential sources of phyllosphere colonisers. However, most microorganisms in the phyllosphere of naturally occurring plants are considered to arrive only after leaves have emerged by immigration from the air surrounding the leaf (Lindemann and Upper, 1985; Lindow and Andersen, 1996; Kadivar and Stapleton, 2003). The atmosphere is a rich and diverse source of potential microbial colonisers of the phyllosphere. In one study (Lighthart and Shaffer, 1995), the 10-m layer of air over a grass field in Oregon contained an average value of 121 and a peak value of 1369 bacterial CFUs per cubic metre. Plate counts fluctuated greatly between long-term (early, mid and late summer), short-term (morning, afternoon, evening, night) and even very short-term (intervals of 2 min) sampling times. Much of the short- and long-term fluctuation could be attributed to the temporal variation in meteorological conditions (convective wind sweepings, cleansing sea breezes) as they were recorded during the course of the day or season.

Several mechanisms have been proposed for microbial immigration from the air onto the leaf surface (Lindow, 1996), including deposition by aerosol, wind, rain or insects (Lilley *et al.*, 1997). Several studies have produced quantitative data for these processes, which allow a better appreciation for the contribution of immigration to phyllosphere biology. For bean plants in a Wisconsin field, Upper and Hirano (2002) reported an average phyllosphere immigration rate of 11 *P. syringae* bacteria per bean leaf on a rain-free day. Rates of immigration were clearly dependent on the presence of and distance from source plants (i.e. plants carrying high population sizes of *P. syringae*). From airborne insects that were captured in the same field, bacteria could be recovered in numbers that varied from undetectable to 10 000 CFU

per insect (Upper and Hirano, 2002). Dispersal of bacteria by airborne insects appeared to be most effective when leaves were wet and accounted dispersion over up to tens of metres. Leaf-to-leaf migration may also occur through run-off or splash during rains, but this mechanism of dispersal obviously operates at a smaller, local scale within the plant leaf canopy.

Bacterial cells and fungal spores may not arrive on the leaf surface as individuals, but rather in aggregates of more than one individual. The close proximity of these early immigrants to each other on the leaf surface and the interaction that follows between them probably is a first determinant in the fate of these immigrants during the next stages of leaf colonisation.

Much of the success of an immigrant to the phyllosphere also depends on the initial physico-chemical conditions of the cuticular surface (Section 11.5.2; Chapter 4). For example, it has been shown that under conditions of low relative humidity, the chances of survival of *P. syringae* on bean leaves is reduced dramatically compared to conditions of high relative humidity (Monier and Lindow, 2003a). Furthermore, immigrants to a previously colonised leaf surface probably face an environment that is quite different from that of an uncolonised leaf surface. On the one hand, they might enter an environment that, through active modification by the residing microflora, has been made more conducive to their own survival or growth (Section 11.8.1). On the other hand, much of the initially available nutrients on colonised leaves may have been used up by earlier immigrants, leaving less substrate to utilise and to grow on for later immigrants (Section 11.7.3). This principle has been used to explain the relative success of early immigrants in dominating the phyllosphere (Upper and Hirano, 2002) and has been exploited as a biocontrol strategy for the suppression of certain plant pathogens (Section 11.9.3).

11.3.2 Adhesion

Adhesion of epiphytic microorganisms to cuticular surfaces plays several ecological roles. First, it prevents removal from the leaf surface, either immediately after immigration or in the subsequent course of colonisation. It has been suggested, however (Andrews and Buck, 2002), that for many bacteria and yeasts the contribution of adhesion to phyllosphere colonisation is relatively small if the fraction of cells that resists removal and remains on the leaf surface is able to quickly re-populate the leaf surface. A second role of attachment is that it serves as the initial step in the formation of microcolonies, aggregates and biofilms (Andrews and Buck, 2002), which has been implicated in enhanced success of leaf colonisation, for example through niche modification (Section 11.8.1).

The surfaces of leaves and fruits are covered by a hydrophobic cuticle which consists of a complex mix of long-chain aliphatic compounds (Chapters 1 and 4). Many fungi adhere to hydrophobic surfaces, including cuticular surfaces, much better than to hydrophilic surfaces (Clement *et al.*, 1994; Buck and Andrews, 1999; Tucker and Talbot, 2001; Beattie, 2002), although exceptions exist (Buck and Andrews, 1999). Underlying the hydrophobicity mediated adhesion of yeasts, fungi and fungal spores

are hydrophobic interactions between cuticular surface waxes and hydrophobic components in the cell wall such as water-insoluble glycoproteins (Tucker and Talbot, 2001). When leaf waxes are removed, for example, with chloroform, hydrophobicity dependent attachment is reduced (Young and Kauss, 1984). Attachment may also be mediated by fungal-produced components including extracellular polysaccharides (Andrews *et al.*, 1994), hydrophobic mucilage (Hamer *et al.*, 1998) and cutinases and esterases (Deising *et al.*, 1992). The synthesis of such components is often influenced by the availability of water and nutrients (Andrews and Buck, 2002) or temperature (Tucker and Talbot, 2001). Adhesion to the cuticular surface may also be indirect: among phyllosphere yeasts, it is a common trait to be able to attach to fungal spores or hyphae (Allen *et al.*, 2004). Quite a rigorous mode of fungal adhesion to the cuticular surface is through cuticle penetration by specialised hyphae (Chapter 12). Penetration is achieved by secretion of enzymes and/or by sheer physical force. With the subsequent formation of fungal structures inside the leaf (e.g. haustoria), fungal attachment is semi-permanent.

In contrast to fungi, bacteria do not seem to engage in hydrophobic interactions with cuticular surface waxes. Instead, they rely on pili and extracellular polymeric substances for adhesion (Beattie, 2002). Most research has focused on the role of pili (Romantschuk, 1992), which are thin protein tubes originating from the cytoplasmic membrane and which are found in almost all gram-negative bacteria. Mutants of *P. syringae* lacking pili were washed more easily from leaves than the wildtype (Suoniemi *et al.*, 1995), which suggests a role for pili in resisting removal by run-off rain. In field experiments, mutants of *P. syringae* pv. *tomato* lacking type IV pili achieved slightly lower population sizes on leaves of tomato plants than those of the parental wild-type strain DC3000 (Roine *et al.*, 1998). Adhesion of piliated *P. syringae* was not affected by removal of the cuticular surface waxes, suggesting that waxes are not necessary for pili attachment (Romantschuk *et al.*, 1993). Besides pili binding, several other mechanisms have been proposed to explain bacterial adhesion to plant leaf surfaces. Many bacteria produce extracellular polysaccharides on leaves, often as a matrix for biofilms (Romantschuk *et al.*, 1996; Morris *et al.*, 1997; Beattie and Lindow, 1999). Acting as glue, extracellular polysaccharides may help reduce the probability of removal by rain or wind. The same has been suggested for the production of cellulose by *Pseudomonas fluorescens* SBW25 (Gal *et al.*, 2003).

Bacterial attachment to leaves varies with bacterial species and with cuticular surface properties. Immediately after spraying suspensions of *Pantoea agglomerans*, *Clavibacter michiganensis* or *P. syringae* onto maize leaves, all three bacterial species resisted to some extent the removal from the leaf by sonication and vigorous vortexing, but the fraction of non-removable *P. agglomerans* cells was up to ten-fold higher than that of the other two species (Beattie and Marcell, 2002). Furthermore, it was shown with cuticular maize mutants that *P. agglomerans* was less influenced by changes in cuticular wax-dependent surface properties than *C. michiganensis* (Beattie and Marcell, 2002), suggesting that different bacteria use different mechanisms for adhesion. It is unclear whether these differences in mechanisms correlate

with the relative importance of adhesion in the life cycle of any given bacterial epiphyte.

11.3.3 Emigration

Many of the mechanisms by which microorganisms exit the surfaces of leaves are the reverse of those described for immigration (Section 11.3.1). Emigration may account for a substantial reduction in phyllosphere abundance. For example, rainfall may remove as many as 10^5 bacteria from a single bean leaf in the field in a period of 15 min (Lindemann and Upper, 1985). In this process, leaf cuticle properties may play an important role. For example, mutants of corn that differ in wax composition show less retention of sprayed bacteria when cuticular surfaces are more hydrophobic (Beattie, 2002). Microorganisms that run off leaves through rainfall onto the soil usually do not persist there for long. However, they might attach to seeds and end up on the leaves of newly emerging plants (Section 11.3.1).

11.4 Microbial communities in the phyllosphere

11.4.1 Composition

To study the composition of microbial communities in the phyllosphere, the most common procedure is to spread leaf washings onto nonselective or selective solid media (Section 11.2.1), and identify the bacteria, yeasts or fungi that appear on the basis of morphological, physiological or phylogenetic features. Thus, composition analysis is generally biased towards culturable microbial epiphytes only, and in most cases it is not known how representative this subset is for the entire phyllosphere population. The bias towards culturables probably also underlies the common notion that populations of culturable bacteria on leaves are dominated by only a few genera including *Pseudomonas*, *Erwinia* and *Xanthomonas* (Jurkevitch and Shapira, 2000; Lindow and Brandl, 2003). All of these are readily culturable bacteria that grow rapidly on laboratory media. Pink-pigmented facultative methylotrophs of the genus *Methylobacterium* are some of the most abundant bacteria on plants but they are often overlooked due to their comparatively slow growth and special nutrient requirements: they are only found when searched for, and so far, they have been encountered on nearly every plant investigated (Corpe, 1985; Holland and Polacco, 1994).

Very few studies have used culture-independent methods to avoid the bias towards culturables, and their outcome, not surprisingly, shows a much greater diversity in phyllosphere composition (Weidner *et al.*, 2000; Yang *et al.*, 2001; Kadivar and Stapleton, 2003). For example, by denaturing gradient gel electrophoresis (DGGE) of PCR-generated 16S ribosomal RNA gene products, Yang *et al.* (2001) identified 17 unique sequences in DNA collected from the 'Valencia' orange phyllosphere. Only four of these corresponded to bacteria that had been found in

the phyllosphere before, that is, *Acinetobactor* sp., *Bacillus pumilus*, *Enterobacter agglomerans* and a *Cytophagales* species. In contrast, 16S rRNA sequences obtained from leaf washings that were first cultured in BIOLOG EcoPlates showed between 97 and 100% similarity to those of known phyllosphere bacteria such as *Pseudomonas*, *Erwinia* and *Acinetobacter* species, clearly demonstrating that culturing favours the culturable.

Many composition analyses have targeted plants from a single geographical area (Inacio *et al.*, 2002; Pereira *et al.*, 2002) or a single plant species (Austin *et al.*, 1978; Ercolani, 1991; Jurkevitch and Shapira, 2000; de Jager *et al.*, 2001). While such studies may seem rather anecdotal, also given the fact that composition is highly variable (Section 11.4.3), they are instrumental in demonstrating the enormous diversity of microbial life in the phyllosphere. Snap bean plants were shown to carry more than 78 bacterial species representing 37 bacterial genera on their leaves (Beattie and Lindow, 1999). An inventory of phyllosphere fungi and yeasts from Mediterranean plants included a total of 1029 strains of filamentous fungi and 540 strains of yeasts, representing at least 36 and 46 distinct species, respectively (Inacio *et al.*, 2002). Jacques and Morris (1995) presented an overview of bacterial species, representing 29 genera, isolated in 13 independent studies from plants in temperate zones. For at least 3 plants (*Lolium perenne*, *Olea europaea* and *Ranunculus penicillatus*), 3–27% of the bacteria that were isolated and cultured from the phyllosphere could not be identified, showing that our understanding of the microbial composition of the phyllosphere, even for culturable representatives, is still far from complete. Yet, this realisation also comes from culture-independent studies: 5 of the 17 rRNA sequences identified by Yang *et al.* (2001) had a similarity to database entries below 90%, suggesting that they represent previously undescribed taxa.

The degree to which plants can determine the microbial composition in their phyllosphere remains unknown. Using DGGE (Yang *et al.*, 2001), it was shown that the microbial community structure on plants such as green bean, cotton, sugar beet and orange, but not corn, were similar on different individuals of the same plant species, but different from all other plant species. If selection takes place it would probably be on the basis of leaf characteristics such as the presence or absence of protective sites (Section 11.6.2) or the availability of plant-specific nutrients (Section 11.7.3), in combination with environmental factors such as weather conditions or geographical location.

11.4.2 Abundance

In general, microbial abundance in the phyllosphere refers to one of the following: (1) the total average number of bacteria, yeasts or fungi on a leaf, (2) the average number of a specified subset of bacteria, yeasts or fungi on a leaf, (3) the most abundant species of bacteria, yeasts or fungi on a leaf, or (4) the number of bacteria, yeasts or fungi on a dimensional scale smaller than that of a single leaf. As it is the case for composition studies, most estimates for microbial abundances on leaf

surfaces are made on the basis of counts of culturables (Section 11.2.1), although some recent reports have used culture-independent methods such as real-time PCR (Heuser and Zimmer, 2002, 2003) and direct microscopic counting (Andrews and Buck, 2002). To be able to compare between plants, microbial abundances are best described as densities, for example, numbers of microorganisms per square centimetre of leaf surface, but they have also been expressed per leaf or per gram of leaf tissue. Of all microbial epiphytes, bacteria are generally considered to be the most abundant on leaves (Lindow and Brandl, 2003). A typical leaf may contain up to 10^6–10^7 bacteria per square centimetre of surface. Scaled up to human dimensions, this translates into population densities that are 10- to 100-fold higher than the most densely populated countries in the world. The number of yeasts and yeast-like organisms may reach 10^7 CFU per gram (fresh weight) of leaf material on some plant species. The most abundant yeasts and yeast-like fungi on the phylloplane of temperate plants are *Aureobasidium pullulans*, *Sporobolomyces* species, *Rhodotorula* species and *Cryptococcus* species (McCormack *et al.*, 1994).

Microbial abundances in the phyllosphere are characterised by high degrees of variability, a factor 1000 and sometimes more, among leaves from the same plant or field (Hirano *et al.*, 1982; Jacques and Morris, 1995; Hirano and Upper, 2000). Leaf densities of microorganisms are therefore generally described and compared after a ^{10}log transformation. The degree of leaf-to-leaf variation may differ between plant species or change as a result of environmental conditions (Kinkel *et al.*, 2000). It has recently been suggested (Woody *et al.*, 2003) that leaf-to-leaf variability is not due to asynchronous temporal changes in population sizes on individual leaves, but rather due to the variation among individual leaves in their ability to promote survival or sustain microbial growth (Sections 11.6 and 11.7).

Variation in population densities is not restricted to the dimensional scale of single leaves. Even on different sections from the same leaf there can exist substantial variation in microbial numbers. For example, among 9-mm^2 sections of potato leaves total bacterial populations varied by over 100-fold (Kinkel *et al.*, 1995), and on bean leaf sections of 44-mm^2, *P. syringae* populations even varied 100 000-fold (Monier and Lindow, 2004). Also at the microscopic level bacteria, yeasts and fungi are not uniformly distributed across the leaf surface (Monier and Lindow, 2004). The most commonly and heavily colonised sites on naturally colonised plant leaves are the bases of trichomes, stomata, epidermal cell wall junctions and the grooves along veins (Beattie and Lindow, 1999). It has been hypothesised that these sites are 'protective' sites where microbial growth is favoured more than on other sites on the leaf surface, for example, because of higher nutrient availability (Section 11.7.3). From microscopy studies, it has also become clear that many bacteria occupy the leaf surface in aggregates (Morris *et al.*, 1997). In fact, aggregates can constitute between 10 and 40% of the total bacterial population on leaves of certain plant species (Morris *et al.*, 1998). On leaves from the field, these aggregates are often mixed populations of different species (Morris *et al.*, 1998). Laboratory studies of aggregate formation by phyllosphere bacteria have revealed that aggregate sizes were distributed with a strong right-hand-skewed frequency (Monier and Lindow, 2004). While large

aggregates on these bean leaves were not frequent, they accounted significantly for the majority of cells present on a leaf.

11.4.3 Dynamics

Many studies have documented differences in microbial abundance and composition on leaves from the same plants or field at different points in time or after a defined treatment (O'Brien and Lindow, 1989; Ercolani, 1991; Ellis *et al.*, 1999; Hirano and Upper, 2000; de Jager *et al.*, 2001; Kadivar and Stapleton, 2003). This demonstrates that microbial populations on cuticular surfaces are not static. Changes in abundance or composition are ultimately a sum of immigration, growth, death and emigration, and each of these is directly or indirectly influenced by external factors such as rain, UV radiation exposure and leaf age. Rains have been shown to trigger rapid multiplication of epiphytic bacteria (Hirano and Upper, 2000), probably due to increased nutrient availability as a result of leaching (Section 11.7.2). Exposure of plants to conditions of low relative humidity usually causes bacterial populations to decrease and/or change in composition (O'Brien and Lindow, 1989; Hirano and Upper, 2000). Weather conditions may also determine the most abundant colonisers of leaves: for example, on bean leaves in the field, PPFMs were most abundant when the weather was hot and dry, whereas *P. syringae* was quite abundant during periods of wet and warm weather (Hirano and Upper, 2000).

Changes in UV exposure have been shown to affect microbial populations on leaf surfaces (Newsham *et al.*, 1997; Sundin and Jacobs, 1999; Jacobs and Sundin, 2001, 2002; Hughes *et al.*, 2003; Jacobs *et al.*, 2005). In one instance, UV-exposed phyllosphere samples showed an increase in bacterial diversity (Kadivar and Stapleton, 2003). Leaf age also has a clear effect on microbial composition (Ercolani, 1991). While bacteria, yeasts and filamentous fungi are often found to colonise the same leaf surface, there can be succession in microbial composition as the leaf gets older. In general, bacteria are the pioneers on young leaves, yeasts may dominate as the leaf becomes older and filamentous fungi up to leaf senescence (Blakeman, 1985). Other factors that may influence the density and diversity of microbial populations in the phyllosphere are plant species (Heuer and Smalla, 1997), air pollution (Brighigna *et al.*, 2000), carbon dioxide levels (Magan and Baxter, 1996), leaf position in the canopy (Andrews *et al.*, 1980; de Jager *et al.*, 2001), acid rain (Helander *et al.*, 1993) and insects (Stadler and Müller, 2000).

11.5 Microbial perception of the phyllosphere

11.5.1 Topography

As the dimensions of humans and microorganisms differ by about six orders of magnitude, only a 1 000 000-fold magnification could offer us the best possible impression of leaf surface topography from the perception of an individual microbial

epiphyte. Electron microscopy commonly achieves a 60 000-fold zoom, while light and fluorescence microscopy maximally reaches 1000-fold, offering (only) a bird's eye view of the habitat. There are relatively few studies that provide quantitative data on the topography of leaf surfaces (Mechaber *et al.*, 1996; Monier and Lindow, 2004). This is surprising, since leaf surface structures have been implicated to have a large influence on the microbial biology of the phyllosphere. Structurally and functionally different features of the leaf such as stomata, veins, trichomes and epidermal cells may vary dramatically in water retention capacity, cuticle thickness or release of plant compounds; so their individual ability to harbour and sustain microbial populations may also differ. Monier and Lindow (2004) scanned adaxial surfaces of bean leaves and found them to consist 74% of undifferentiated epidermal cells, while stomates, veins and trichomes accounted for 17, 7 and 2%, respectively.

Using atomic force microscopy, Mechaber *et al.* (1996) mapped the adaxial surfaces of cranberry leaves in three dimensions. Young leaves showed a regular pattern of broad plateaus on the surface of individual epidermal plant cells with drops in elevation of up to 3 μm between the cells. Old leaf surfaces on the other hand appeared much rougher and with a less regular pattern. These differences between young and old leaves indicate that leaf surface topography and thus physical properties are highly variable with age, and the same is true for leaves of different plant species.

11.5.2 Physico-chemical parameters

The phyllosphere represents a unique habitat in terms of physical and chemical parameters (Burrage, 1971; Chapter 4). Water may be available in the form of rain, fog or dew. In addition, the cuticle may in some cases be covered by aqueous deposits which are a result of the interaction between hygroscopic salt crystals on the cuticular surface and water vapour from the atmosphere and the stomata (Burkhardt *et al.*, 1999). The wetness of individual leaf surfaces can be quantified by measuring electrical conductivity between two electrodes clipped to a living plant leaf surface (Klemm *et al.*, 2002). To assess water availability at a smaller, that is, micrometre scale, bacterial bioreporters have been used (Axtell and Beattie, 2002). Cells of *E. herbicola* carrying a *proU-gfp* gene fusion produce GFP in a quantitative manner in response to low water potential. After inoculation onto bean leaves, GFP expression profiles indicated that cells experienced increased osmolarity, probably as a result of evaporation of the available water. However, there was considerable variation among individual bacteria on the leaf surface: those that were located close to veins were less fluorescent, suggesting that such leaf structures can locally increase water retention.

Other physico-chemical parameters are UV exposure and temperature. UV radiation from the sun reaching the phyllosphere consists on the average 95% of UV-A (320–400 nm) and 5% UV-B (290–320 nm; Jacobs *et al.*, 2005). The effects of these types of radiation on microbial epiphytes differ: UV-A exposure leads

to the (sub)lethal formation of reactive oxygen species, whereas UV-B directly damages the DNA. UV exposure of individual leaves differs with geography, climate and canopy structure, but UV-protective measures are common among many phyllosphere microorganisms (Section 11.6.2). Temperatures can differ dramatically in the course of a single day, but also on a single leaf, for example, from the centre to the edge. Temperature may have a direct effect on the growth of microorganisms, but may also act indirectly, for example, through accelerated or delayed evaporation of available water.

11.5.3 Biological environment

The area of the leaf surface that is actually covered by microorganisms varies depending on many factors. In a laboratory experiment with *P. syringae*, bean leaves were covered for up to 12% of their surface area with bacteria, whereas some 60% of the cells were located in an aggregate of 100 cells or more (Monier and Lindow, 2004). This means (1) that any new bacterial immigrant to these leaves would have a relatively low probability (up to 12%) of landing next to other bacteria, but (2) that any already present inhabitant has a high probability (60% or more) to be surrounded by many others. Bacteria in an aggregate are presented with a biological environment which is very different from that of a solitary cell and which probably sets the scene for fierce competition for food and space, and also opens the possibility for collaboration through density-dependent communication (Section 11.8.4) or for plasmid exchange (Section 11.8.5). In laboratory studies with single bacterial strains, aggregates mostly or exclusively consist of clonal individuals, whereas in nature, aggregates on leaves are most commonly mixes of different bacterial species (Morris *et al.*, 1998).

Surprisingly little is known about if and in what way bacteria on the one hand and fungi or yeasts on the other interact in the phyllosphere. Most of what is known comes from biocontrol studies (Section 11.9.3), which leaves many questions on the biology and ecology of bacteria/fungi interactions in the phyllosphere still unanswered.

11.6 Surviving (or not) in the phyllosphere

11.6.1 Concept of epiphitness

Epiphytic fitness, or 'epiphitness' as it shall be referred to here, can be defined as the ability of a microorganism to survive and thrive on plant cuticular surfaces. Typically, this ability is demonstrated experimentally in the laboratory by inoculation of plants and subsequent monitoring of changes in population sizes. Epiphitness is therefore often expressed as a population size after a defined period of time after inoculation, or as an x-fold increase compared to population sizes immediately after inoculation. Generally, epiphitness is used as a relative measure: if under the same experimental circumstances the population size of one strain is lower than that of another, it is

said that the former has a lower epiphitness than the latter. An example is the comparison of near-isogenic strains that differ in one gene or more – often a mutant versus the wildtype – to assess the role of that gene in phyllosphere competence (Section 11.6.3).

11.6.2 Adaptive strategies

The cuticular surface is considered a harsh environment not only because of the relative extremes to which epiphytic microorganisms are exposed (e.g. water stress, nutrient availability, UV radiation), but perhaps even more so because of the rapidity with which such conditions change, for example, even within the time it takes a bacterium to double. Beattie and Lindow (1999) proposed two not mutually exclusive strategies that allow epiphytic bacteria to survive and thrive on plant cuticular surfaces. The first is a strategy of tolerance, which is based on traits that protect microorganisms against and help them to deal with the harsh environment of the phyllosphere. A good example is UV-protective pigmentation which is quite common among phyllosphere bacteria (Goodfellow *et al.*, 1976; Dickinson, 1986; Lindow and Brandl, 2003; Jacobs *et al.*, 2005), as are repair mechanisms for UV-induced damage to the DNA (Kim and Sundin, 2000; Sundin *et al.*, 2000; Zhang and Sundin, 2004).

In response to nutrient limitation in the phyllosphere, the bacterium *P. syringae* reduces its size (Björklöf *et al.*, 2000; Monier and Lindow, 2003b), which is an active process, probably to optimise surface-to-volume ratio and nutrient uptake capacity (Monier and Lindow, 2003a). The phyllosphere fungus *Epicoccum nigrum* accumulates solutes such as glycerol and arabitol in response to water stress, which may assist in its survival and establishment on leaf surfaces (Pascual *et al.*, 2003).

The ever-changing conditions in the phyllosphere demand a high degree of plasticity from microbial epiphytic colonisers. There are several lines of evidence that suggest such plasticity in phyllosphere bacteria. For example, cells of *P. syringae* that were recovered from bean leaf surfaces and re-applied to uninoculated leaves showed a higher epiphitness than cells that were applied to leaves from a plate or broth culture (Wilson and Lindow, 1993). It has been proposed that nutrient limitation, in particular starvation for carbon, makes bacterial cells more adapted to the stressful conditions of the phyllosphere (Monier and Lindow, 2003a). Bacterial plasticity is probably correlated to the ability to go from a solitary lifestyle to life in an aggregate (see later), and vice versa (Boureau *et al.*, 2004). Inability to tolerate the phyllosphere probably results in death or transition into a VBNC state. This may be the fate of many new microbial immigrants to the leaf or to those that are not part of a protective environment such as described later. It is unknown whether VBNCs can recover from their state, and under what conditions such recovery would occur.

The second strategy proposed by Beattie and Lindow (1999) is a strategy of avoidance, which is based on so-called protective sites on the leaf surface. Some foliar pathogens avoid many of the leaf surface stresses by escaping to the interior of the leaf. This niche poses its own challenges for survival (Manceau and

Kasempour, 2002), but many bacteria have learned to cope with these. The abaxial side of a leaf may protect better against the damaging effects of UV than the adaxial side (Sundin and Jacobs, 1999). Trichomes or other leaf structures may also represent protective sites as they offer shading from UV radiation, or retain water. Bacteria living in aggregates or biofilms in fact also employ a strategy of avoidance, as they create a local environment that is more conducive to growth and survival (see Section 11.8.1).

11.6.3 Epiphitness genes

The common definition of epiphitness genes is that they confer a measurable advantage to a bacterium, yeast or fungus in colonising a leaf surface. Most epiphitness genes have been identified in knock-out studies: the underlying hypothesis is that mutations in epiphytic genes would reduce the epiphitness of its carrier. Lindow (1993) identified 82 transposon mutants of *P. syringae* with altered behaviour on leaf surfaces. Several of these were not able to withstand desiccation stress (Lindow *et al.*, 1993a). None of the mutants were disrupted in their utilisation of 31 different carbon compounds, suggesting that *P. syringae* has a broad spectrum of carbon sources it can use or has redundant genes for utilisation of the most important carbon sources. Several mutants showed decreased motility, osmotolerance and extracellular polysaccharide production suggesting that these are important traits for establishment in the phyllosphere. Mutants of *P. syringae* lacking the *gac* regulon were less fit on bean plants in the field (Hirano *et al.*, 1997). The *gac* regulon consists of the two-component regulatory genes *gacA* and *gacS*, which in *P. syringae* regulate, among other things, swarming, the synthesis of quorum sensing signal molecules called *N*-acyl homoserine lactones (AHLs; see Section 11.8.4) and production of the extracellular polysaccharide alginate, all of which have been considered to play a role in the epiphytic lifestyle of this and other epiphytic bacteria (Yu *et al.*, 1999). Genes of the *hrp* regulon also have been shown to contribute significantly to epiphitness in field situations (Hirano *et al.*, 1999). Both *gac* and *hrp* genes were initially identified as being involved in pathogenicity, suggesting a close link between epiphitness and being a successful pathogen (Section 11.9.2). Several other examples of epiphytic genes that have been identified through mutation analysis are available (e.g. Andersen *et al.*, 1998; Brandl and Lindow, 1998; Roine *et al.*, 1998; Sundin, 1999); they code for such traits as pili formation, alginate synthesis, production of the plant hormone indole-3-acetic acid and AHL production.

A well-recognised problem with knock-out approaches is their limited effectiveness in identifying genes with incremental contribution to leaf surface fitness (Marco *et al.*, 2003). An alternative approach is the use of reporter gene technology, which aims to answer what genes are specifically expressed during colonisation of the phyllosphere. Cirvilleri and Lindow (1994) used random insertion of a promoterless *lux* gene into the genome of *P. syringae* to identify transposon mutants that were bioluminescent on leaf surfaces. This analysis revealed that about 3% of all *P. syringae* genes are expressed on the leaf surface. Independently,

Marco *et al.* (2003) came to a similar estimate using *in vitro* expression technology by which random fragments of the *P. syringae* genome were screened for their ability to complement a conditionally lethal phenotype on bean leaves. Using this strategy, called HIRS for habitat-inducible rescue of survival, Marco *et al.* (2005) were then able to describe a number of phyllosphere-induced loci with anticipated or presumed epiphytic functions such as water stress tolerance or utilisation of organic sulfur. Interestingly, several virulence-associated genes were also identified by the HIRS method, indicating that *P. syringae* expresses virulence factors during leaf colonisation. To some loci, no function could be assigned on the basis of sequence homology of DNA or protein, suggesting that yet-to-identify epiphitness traits may exist.

11.7 Microbial growth in the phyllosphere

11.7.1 Growth requirements

Individual epiphytic microorganisms have different nutritional requirements. For example, it does not take much for a bacterium to duplicate: it was calculated that the epiphytic bacterium *E. herbicola* needs 0.3 pg of sugar to double (Leveau and Lindow, 2001). But growth yield, that is, the efficiency with which food is converted into biomass, is not the only factor that determines the success of a leaf coloniser. There are three additional factors to be considered, namely versatility, affinity and growth rate. Microorganisms differ in the range of nutrients, for example, carbon sources that they can use for growth (Ji and Wilson, 2002). Comparison of the nutrient utilisation spectrum of microbial species with the presence or absence of specific nutrients on leaf surfaces could, in theory, predict whether a bacterium or fungus has the potential to grow in the phyllosphere. By comparing nutrient utilisation spectra of two or more different microbial species, it is possible to derive a so-called nutritional niche overlap index (Wilson and Lindow, 1994a; Ji and Wilson, 2002). It may be used as a predictor of co-existence or competition. A low index value, for instance, indicates that species vary greatly in their nutritional spectrum, so that there is a low probability for competition for the same nutrients and a high probability for co-existence. This has also been referred to as nutritional resource partitioning (Wilson and Lindow, 1994c). It should be noted that niche overlap indices may be influenced by external factors including water availability and temperature (Lee and Magan, 1999). Another factor determining growth in the phyllosphere is affinity, which divides microorganisms into those that can use nutrients at very low concentrations and those that cannot. This dichotomy probably overlaps for a large part with the separation of microbial epiphytes into so-called K- and r-strategists (Andrews, 1984). The K-strategists reproduce more slowly and tend to be successful in resource-limited situations, whereas r-strategists are characterised by a high growth rate and dominate in situations where nutrient resources are abundant.

11.7.2 Types and sources of nutrients

One of the most obvious sources of nutrients on the plant leaf and fruit surface is the cuticle. It consists of the cutin polymer and associated waxes with high carbon and energy contents, and so would seem a logical growth substrate. However, there is no evidence to support the theory that cuticle components are used by microorganisms for growth (Beattie, 2002). Exogenous nutrients may be available fortuitously in the form of pollen, honeydew, dust, air pollution or microbial debris (Stadler and Müller, 2000; Leveau, 2004). However, the major source of nutrients to microbial colonisers is represented by plant metabolites that leach from the leaf's interior to the surface. Leaching is a passive process, and is stimulated by the presence of water on the leaf, for example, in the form of rain drops or fog. Leaf leachates contain a variety of compounds (Tukey, 1970), but the most abundant are photosynthates such as glucose, fructose and sucrose (Fiala *et al.*, 1990; Mercier and Lindow, 2000; Leveau, 2004). Uninoculated bean plants in the greenhouse carry 0.2–10 micrograms of sugars per leaf (Mercier and Lindow, 2000), enough to support bacterial populations of 10^7 per leaf. The carrying capacity, that is, the maximum microbial population which a given leaf can support, is often correlated to the availability of sugars. This is in agreement with the observation that the availability of carbon, not nitrogen or phosphate, is generally limiting the sizes of bacterial populations in the phyllosphere (Wilson and Lindow, 1994a; Wilson *et al.*, 1995; Mercier and Lindow, 2000). Other perhaps less known examples of carbon sources in the phyllosphere are methanol and methylamine which are plant waste products that are preferentially used by PPFMs (Holland and Polacco, 1994), and the plant hormone indole-3-acetic acid which also has been shown to be a substrate for growth by phyllosphere bacteria (Leveau and Lindow, 2005).

11.7.3 Nutrient bioavailability

There are several lines of evidence to suggest that nutrients are not equally available on different leaves from the same plant or field, nor that they are evenly distributed across the surface of a single leaf. Uninoculated bean leaves contain on average 2.5 μg of surface sugar per gram of leaf (Mercier and Lindow, 2000), but this may vary by about 25-fold from leaf to leaf. The variation has been explained due to differences between leaves in, for example, cuticle leakiness (Mercier and Lindow, 2000), photosynthesis rates, for example, as a result of positional effects (Fiala *et al.*, 1990), leaf age or plant nutrition. After inoculation and incubation of bean plants with the bacterium *P. fluorescens*, original sugar abundances were decimated, suggesting (1) that bacteria utilise sugars and (2) that any new immigrants would face nutritional conditions different from those the first immigrants did. Interestingly, a residual amount of sugars remained on the leaf surface, even after the bacterial populations reached carrying capacity (Mercier and Lindow, 2000). This has been explained by the heterogeneous distribution of sugars, that is, some sugars remain unavailable to the bacteria. By using the size of individual cells as an

indicator for trophic status, it has been shown that *P. syringae* bacteria on a single bean leaf experience very different nutrient bioavailabilities (Monier and Lindow, 2003b). Bacteria near glandular trichomes or veins were larger than those located elsewhere, suggesting that such sites offer more nutrients. Glandular trichomes have been shown to secrete a number of plant compounds such as sugars, proteins, oils, secondary metabolites and mucilage, all of which may contribute to microbial growth. In addition, their ability to retain water would favour local leaching of nutrients.

A pattern of high heterogeneity in sugar availability has been demonstrated with bacterial bioreporters for fructose. It was estimated that newly arrived *E. herbicola* bacteria on a bean leaf were exposed to local initial fructose abundances ranging from less than 0.15 pg to more than 4.6 pg fructose (Leveau and Lindow, 2001). Uneven leaf surface distribution applies not only to fructose but also to other nutrients such as for example sucrose (Miller *et al.*, 2001) and iron (Joyner and Lindow, 2000). One of the ways that epiphytic bacteria deal with low iron availability is by the production of siderophores (Loper and Buyer, 1991). These low-molecular-weight molecules chelate ferric ion and after recognition by specialised receptors are taken up by the bacteria. The molecular biology of siderophore production and recognition has been exploited to construct iron-responsive bioreporter strains of *Pseudomonas* species (Loper and Lindow, 1994; Joyner and Lindow, 2000). Using the *inaZ* reporter gene, it was possible to estimate that the average bacterial cell in the phyllosphere experienced low-iron conditions (Loper and Lindow, 1994). A similar result was obtained with a *gfp*-based iron bioreporter (Joyner and Lindow, 2000), but because GFP allows for the interpretation of reporter activity in individual cells, it was possible to show that actually there existed substantial microscale heterogeneity in iron availability in the phyllosphere.

11.8 Microbial interactivities in the phyllosphere

11.8.1 Niche modification

Niche modification represents one of the microbial strategies to change local conditions away from the harsh environment that characterises the phyllosphere. The production of the plant hormone indole-3-acetic acid (Brandl *et al.*, 2001) or the phytotoxin syringomycin (Lindow and Brandl, 2003) by bacterial leaf colonisers is thought to stimulate the localised release of nutrients by the plant. Another strategy to increase nutrient availability is by altering cuticular surface permeability (Schreiber *et al.*, 2005), or by decreasing the contact angle of sessile droplets on the cuticular surface (Knoll and Schreiber, 2000). The latter stimulates leaf wetting, for example, by the production of surfactants (Bunster *et al.*, 1989), thereby facilitating the leaching of nutrients (Knoll and Schreiber, 2000; Chapters 7 and 8).

Extracellular polysaccharides are quite commonly used by phyllosphere bacteria to firmly attach to the cuticular surface. They may in addition serve to improve

living conditions, for example, to protect from desiccation, trap nutrients, as a barrier against chemical, biological or environmental stresses or as a matrix for the communication via small diffusible molecules such as AHLs (see Section 11.8.4).

11.8.2 Competition

Competition for nutrients is thought to be most fierce when nutrients are scarce. Evidence for nutrient competition in the pyllosphere comes from studies that demonstrate the principle of pre-emptive competitive exclusion (Lindow and Leveau, 2002), that is, the ability of an established microbial epiphyte to inhibit the development of a population of a second strain on leaves (Kinkel and Lindow, 1993). This principle is based on nutrient depletion and has been applied as a biocontrol strategy (Section 11.9.3). So-called r-strategists (Section 11.7.1) are more affected by competition than K-strategists, and strategies based on pre-emptive competitive exclusion are thought to be more effective in controlling the former than the latter (Marois and Coleman, 1995).

11.8.3 Antibiosis

Several epiphytic yeasts have been shown to produce antibacterial compounds (McCormack *et al.*, 1994), and antifungal activities have been attributed to epiphytic bacteria (Giesler and Yuen, 1998; Nair *et al.*, 2002; Collins *et al.*, 2003; Daayf *et al.*, 2003). Antibiosis, however, has not been explicitly demonstrated to be a major mechanism in the interaction between bacteria on leaf surfaces, despite observations that antibiosis of bacterial epiphytes can be demonstrated in the laboratory (Lindow, 1988). It could well be that under such conditions production of and sensitivity towards antibiotics is different than under phyllosphere conditions.

11.8.4 Communication

Many plant-associated bacteria produce quorum sensing signals, such as AHLs (Cha *et al.*, 1998). These allow for the indirect sensing of population density and density-dependent control of gene expression (Juhas *et al.*, 2005). In the epiphytic bacterium *P. syringae*, the production of AHLs is regulated in a complex and hierarchical manner (Quiñones *et al.*, 2004), involving the GacS/GacA two-component system (Section 11.6.3). In the model proposed by Quiñones *et al.* (2004), the *ahlI* gene, which codes for the synthesis of 3-oxo-C6-homoserine lactone, is positively regulated by the AhlR protein in combination with 3-oxo-C6-homoserine lactone, resulting in a typical positive feedback (auto-induction). The enhanced survival of bacterial cells in densely packed aggregates on leaf surfaces seems to suggest that many epiphitness traits may be controlled in a cell-density-dependent manner. An *ahlI*$^{-}$ *ahlR*$^{-}$ double mutant of *P. syringae* had a reduced epiphitness on dry leaves compared to the wildtype (Quiñones *et al.*, 2004), suggesting a role for quorum sensing in withstanding desiccation stress in the phyllosphere.

11.8.5 Gene exchange

Many bacterial epiphytes have been shown to carry plasmids (Kobayashi and Bailey, 1994; Sundin *et al.*, 2004). Together with transposons, these constitute the so-called horizontal gene pool (Bailey *et al.*, 2002). This gene pool often confers traits that promote survival in the phyllosphere, including virulence factors (Sundin *et al.*, 2004) and resistance to antibiotics such as tetracycline which are sprayed in apple orchards (Schnabel and Jones, 1999). Cuticular surfaces are hotspots for gene exchange and have been called 'breeding grounds for microbial diversity' (Lindow and Leveau, 2002). The aggregated nature of bacterial cells on the cuticular surface is believed to play a key role in the efficiency of plasmid transfer. Rates of plasmid transfer on bean leaf surfaces were 30-fold higher than on membrane surfaces (Normander *et al.*, 1998). Using a reporter gene system that is based on de-repression of GFP expression in plasmid recipients, plasmid transfer has been observed in situ on leaf surfaces (Normander *et al.*, 1998). With this bioreporter, it became clear that plasmid exchange occurs not randomly, but primarily in junctures between epidermal cells and in substomatal cavities. Apparently, plasmid exchange did not require the bacteria to be metabolically active (Normander *et al.*, 1998), although other environmental factors such as water availability do seem to matter (Björklöf *et al.*, 2000).

11.9 Biocontrol in the phyllosphere

11.9.1 Phyllosphere diseases

Examples of phyllosphere microorganisms that harm plants are plenty. Some, such as *Pseudomonas* and *Erwinia* species, cause frost injury through biological ice nucleation (Lindow, 1983). Bacterial ice nucleation has been shown to be mediated by bacterially produced proteins that serve as nucleators for ice formation at subzero temperatures. Genes encoding ice nucleation activity (*ina*) have been isolated from *P. syringae*, *P. fluorescens*, *E. herbicola*, *E. ananas* and *X. campestris* among others. Sequence analyses suggest that *ina* genes have a common ancestor, but the selective advantage of ice formation to bacteria is still unknown; perhaps they benefit for some unidentified reason from frost injury to plants (Hirano and Upper, 2000). Several bacteria and fungi can produce phytohormones which may disrupt normal plant functioning and cause growth deformation, such as leafy gall on sweet pea caused by *Rhodococcus fascians* (Vandeputte *et al.*, 2005). Other bacteria affect plant productivity by the formation of leaf spots or lesions, or by inducing leaf blight or curling (Agrios, 1997). Some examples of foliar fungal diseases (and their causative agents) are rice blast disease (*Magnaporthe grisea*), downy mildew of grape (*Plasmopara viticola*) and powdery mildew affecting all kinds of plants (different fungal species). Interestingly, no yeasts are known to cause foliar disease (Agrios, 1997). Most common bacterial pathogens of the phyllosphere include members of the genera *Erwinia*, *Pseudomonas* and *Xanthomonas*. Different pathovars of

P. syringae cause leaf spots and blights on tobacco, cucumber, bean, lilac or tomato, while *X. campestris* pathovars affect bean, cotton, rice cereals, tomato or pepper.

A growing concern is the occurrence of microorganisms such as *Salmonella* and *Campylobacter* species in the phyllosphere of produce. By definition, these bacterial residents of cuticular surfaces are not epiphytic, as they are usually not capable of multiplying, yet they are apparently quite able to survive for prolonged periods of time on leafy vegetables (Brandl and Mandrell, 2002), posing a health danger to human consumers.

11.9.2 What makes a plant pathogen?

There are many different ways in which pathogenic microorganisms attack plants. Common themes, however, are invasion of the plant tissue, nutrient acquisition and counteracting plant defence reactions (Agrios, 1997). The mechanisms that underlie pathogenicity involve, among others, degradation of plant structural components (e.g. cuticle, cell walls) by enzymatic activity (e.g. cutinase, pectinase, cellulase), the production of phytotoxins (e.g. tabtoxin, syringomycin), injection of effector molecules (e.g. type III secretion in certain bacteria) and modulation of phytohormone levels (e.g. indole-3-acetic acid) to exploit plant physiology. In bacteria, pathogenicity factors such as production of plant growth hormones, phytotoxins and other virulence genes are often plasmid-borne (Bailey *et al.*, 2002), suggesting a role of the horizontal gene pool in the evolution and spreading of disease.

Many bacterial plant pathogens differ from non-pathogenic epiphytes in having the ability to colonise also the interior of leaves, thereby avoiding the stresses associated with the cuticular surface. These internal populations are generally believed to be responsible for disease induction, and the larger the internal population size, the more likely it is that disease symptoms occur (Beattie and Lindow, 1999). The bacterium *Xanthomonas campestris* avoids the leaf surface and actively seeks the interior of the leaf (Hugouvieux *et al.*, 1998) where it reaches high population sizes that may egress to the leaf surface. Other bacterial pathogens, such as *P. syringae* generally establish large surface populations before they ingress into the leaf's interior and proliferate there (Hirano and Upper, 2000). For the latter type of pathogens, leaf surface population sizes are predictive of the probability of disease occurrence (Beattie and Lindow, 1999). For both types of pathogens, these external populations are probably important sources of inoculum for emigration to other plant leaf surfaces (Upper and Hirano, 2002).

11.9.3 Strategies for biocontrol

Strategies for biocontrol of foliar diseases are often based on the prevention of establishment of the pathogen in the phyllosphere. Spraying leaves with spores of common phyllosphere fungi such as *Alternaria*, *Cochliobolus*, *Septoria* and *Phoma* has been shown to reduce fungal foliar diseases (Agrios, 1997). Yeasts have been successfully used as antagonists of pathogenic fungi (Fiss *et al.*, 2000; Avis and

Bélanger, 2002; Buck, 2002; Urquhart and Punja, 2002) and bacteria (Assis *et al.*, 1999), while bacteria have been shown to control disease symptoms caused by fungi (Kucheryava *et al.*, 1999; Zhang and Yuen, 1999; Nair *et al.*, 2002; Collins *et al.*, 2003) and bacteria (Volksch and May, 2001; Stromberg *et al.*, 2004). Some mixtures of biocontrol agents show more anti-fungal activity than the single strains alone (Guetsky *et al.*, 2002). The mechanisms underlying some of these strategies are not always clear. Mycoparasitism, that is, fungal attack on fungi, is an effective biocontrol mechanism which is best described for *Trichoderma* species (Bélanger and Avis, 2002). Some biocontrol agents have been shown to induce systemic resistance in plants (Bargabus *et al.*, 2002). Others produce antibiotics (Giddens *et al.*, 2003), but antibiosis in the laboratory is not necessarily a guarantee for success in the field (Lindow, 1988).

Another strategy for biocontrol is based on the principle of pre-emptive competitive exclusion which assumes that growth in the phyllosphere is limited by the availability of nutrients. When plants are deliberately inoculated with a non-pathogen (the biocontrol agent) which will use up most of the nutrients, any immigrating pathogen will find itself unable to grow and form sufficiently large population sizes to cause damage. Naturally occurring non-ice-nucleating (Ice^-) strains of *P. syringae* and *E. herbicola* applied pre-emptively to plants prevented successful colonisation by Ice^+ *P. syringae* strains and reduced the severity of frost injury (Wilson and Lindow, 1994a). Crucial to success of this strategy is the fact that the nutrient overlap indices of the pathogen and the biocontrol agent are quite similar. However, nutrient-overlap indices are not always predictive for the ability of a non-pathogen to control disease in the laboratory or in the field (Ji and Wilson, 2002).

11.10 Future directions of phyllosphere microbiology

Historically, much of the research on microbial communities in the phyllosphere has been driven by the desire to understand the biology and ecology of microorganisms that are harmful to aerial plant parts. This trend is obvious from the bias, even in this chapter, towards our knowledge and understanding about plant pathogens. Clearly, the attention is slowly shifting in favour of non-pathogenic inhabitants of primary plant surfaces, not only because they have the potential to affect the function of phyllosphere pathogens, but also because they hold many undiscovered traits of adaptation to life on cuticular surfaces. The use of reporter genes such as *gfp* for the first time has opened up the possibility to study microbial epiphytes as individuals, and has fuelled the realisation that microorganisms operate at micrometre scales, and that the cuticular surface at that level of magnification is a highly heterogeneous environment. The ability of studying epiphytic individuality needs to be exploited much more than it has been already: it may help to explain phyllosphere-related phenomena for which there are currently no good explanations if one continues to stick to the traditional, that is, macroscale, view of single leaves as operational units.

Furthermore, as more and more microbial interactivities in the phyllosphere are revealed and in ever more detail, their interconnected complexity will soon reach (or has already reached) a level where the interpretation of experimental results through linear thinking is no longer realistic. Instead, phyllosphere microbiologists will need to rely more on predictive and interpretive modelling, for which at this point much more quantitative data is needed, both on leaf surface structures and properties and on microbial (inter)activities in the phyllosphere. Also, the cautious embrace by phyllosphere microbiologists of culture-independent methods will need to mature into a full-scale exploitation of the molecular toolbox, including the application of such exciting new technologies as metagenomics analysis.

As a final point, phyllosphere microbiology has entered the genomic era with the sequencing of whole-genomes of several plant-pathogenic, epiphytic lifestyle microorganisms. Many more genomes of phyllosphere microorganisms will become available in the next decade, and it will be a huge and incredibly exciting challenge to explain this enormous wealth of sequence information in the light of the experimental data from the 50 years of pre-genomic phyllosphere microbiology research.

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