Phyllosphere microbiology
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Aerial plant surfaces harbor large numbers of microbes, some of which are deleterious to plants whereas others are benign or beneficial. Commercial formulations of bacteria antagonistic to plant pathogenic microbes and ice nucleation active bacteria have been utilized as an environmentally safe method to manage plant disease and to prevent frost damage. Molecular genetic tools, microscopic examination and whole-cell bacterial biosensors have provided extensive information on these microbes, their complex associations and their habitat. The aerial habitat influenced by plants, termed the phyllosphere, is particularly amenable to studies of microbial ecology and the information gained should lead to more effective means of plant protection.

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Abbreviations
GFP green fluorescent protein
UV ultraviolet

Introduction
Aerial plant parts harbor hundreds of species of bacteria, yeast, and fungi. Bacteria are by far the most numerous colonists, often being found at upwards of 10^7 cells/cm^2 of leaf surface. When one considers that a large fraction of the earth’s surface is covered with plants, that leaf surfaces often represent a substantial multiple of the soil surface area, and that leaves and flowers often have complex topographical features on which colonization can occur, the potential population size of microbial associates of plants is indeed impressive. The aerial habitat influenced by plants is termed the phyllosphere and inhabitants are called epiphytes. Much of the interest in phyllosphere microbiology has been driven by the need to better understand the behavior and control of the plant pathogens that are prominent members of this community. Their spread, colonization, survival and pathogenicity mechanisms have been the subject of much research. Plant productivity can be affected by bacteria that incite frost injury [1], whereas others produce phytohormones that have the potential to affect plant development and productivity [2]. Much less is understood about the identity or properties of the numerous non-pathogenic microbes that inhabit the phyllosphere; such colonists apparently play important roles in modulating population sizes of deleterious microbes, and some are being exploited as biological control agents for disease and frost control. New molecular and microscopic tools are being developed to better understand both the identity and behavior of epiphytes as well as the nature of the plant surfaces that they inhabit. Such information will be important for better understanding the process of plant disease and for developing and implementing new methods of control, for example, by interfering with growth, survival or other behaviors of harmful epiphytic microbes. In this review we will emphasize the recent advances made in understanding the epiphytic biology of bacteria since publication of the last reviews on this topic [3–6]. After addressing new studies that focus on the biology of phytopathogens and mechanisms and practice of their biological control, we will illustrate how other fundamental studies of epiphytic bacteria promise to provide the basis for a more comprehensive understanding of the microbial ecology of the phyllosphere.

Biological control of plant disease and frost injury
Potentially devastating diseases such as fire blight of pear and apple are typical of most bacterial diseases in that inoculum of the pathogen, Erwinia amylovora, develops on susceptible plant tissues (flowers in the case of fire blight). Detailed study of the ecology of the pathogen as well as potential antagonists has led to non-chemical means of disease control, thus reducing the need for the frequent applications of antibiotics such as streptomycin and oxytetracycline normally used for disease control. Recent work has shown that prior colonization of the stigmatic surface of flowers with non-pathogenic bacteria such as Pseudomonas fluorescens strain A506 and Pantoea agglomerans C9-1 can greatly inhibit colonization by the pathogen, leading to substantial reductions in disease [7–10] (Figure 1). Lyophilized preparations of P. fluorescens strain A506 are now commercially available for spray application to flowers in the early spring for disease control (Blightban A506®). These antagonists were also shown to move readily from inoculated to non-inoculated flowers, thereby facilitating biocontrol in flowers that otherwise would support relatively few other indigenous bacteria [8,9,11,12]. Non-chemical management of fire blight disease is perhaps the most advanced example of biological disease control, and draws directly from detailed studies of the ecology of both the pathogen and antagonists. Several other recent studies have revealed the in vitro antagonism and/or competitive interactions of potential antagonistic bacteria with bacterial and fungal plant pathogens on plants [13–18]. They illustrate the considerable potential for further development of useful biological control organisms for diverse diseases.
Epiphytic bacterial species with ice nucleation activity (Ice+ bacteria) such as *Pseudomonas syringae* contribute to frost injury of many frost-sensitive plant species by reducing their ability to supercool and avoid damaging ice formation [1,19]. Because the nucleation temperature of these plants increases with increasing population sizes of Ice+ bacteria, pre-emptive competitive exclusion of Ice+ bacteria with naturally occurring non-ice nucleation active bacteria has proven to be an effective and practical means of frost control [1,9]. This model system has received perhaps the most attention as a vehicle to examine interactions of bacteria on plants. Recombinant Ice+ bacteria, the first microorganisms released into the open environment in field experiments, were used to illustrate the specificity with which competitive exclusion of Ice+ bacteria occurred [1,20]. Management of frost injury by reducing Ice+ bacterial populations has become an important new method of frost control. In fact, the adoption of Blightban A506® for disease control by pear and apple growers has been bolstered by the fact that it competitively excludes Ice+ bacteria as well as *E. amylovora* on plants [9].

**Microbial food safety**

There is a growing recognition that human pathogenic bacteria can be colonists of food plants. With a trend towards consumption of more fresh (uncooked) fruits and vegetables, and world-wide distribution of such products from diverse production areas with different sanitation schemes, consumers will be at higher risk of exposure to such pathogens. Although most studies of *Escherichia coli* and *Salmonella* contamination of plants are anecdotal reports of their occurrence, there is a growing body of information on features of the plant and/or the environment that dictate their growth and/or survival on plants [21–23]. The extensive information available on the behavior of plant pathogens and other indigenous bacteria on plants (discussed in part below) will undoubtedly aid in developing effective procedures to minimize contamination of plants with human pathogens.

**Studies of the epiphytic biology of plant pathogenic bacteria**

Because plant pathogenic bacteria cause important economic losses, the processes that mediate their epiphytic existence on plants has received much attention. Of particular importance is the question as to whether traits that confer virulence are also required for epiphytic fitness. That such a relation would exist is suggested by recent studies that addressed the location of *P. syringae* and non-pathogenic bacteria on leaves. Although pathogenicity was not required for growth of bacteria in the phyllosphere under conditions of high relative humidity, pathogenicity was involved in the ability to access and/or multiply in certain protected sites in the phyllosphere and in growth on dry leaves [24]. Such results support speculation that there is a broad-spectrum of epiphytic bacteria. Bacteria range from those that employ solely a tolerance strategy of existence during stressful conditions on leaves (such as non-pathogenic bacteria) to those that can also employ an avoidance strategy and establish populations in the internal leaf regions (such as some phytopathogens) [4,5]. Such a phenomenon may also explain why the variation in population size of epiphytic bacteria among leaves changes rapidly upon imposition of stressful conditions; leaves may vary greatly in their ability to shelter bacteria from environmental stresses [25]. The term ‘epiphyte’, by implying a strictly surface location for plant-associated bacteria, may be misleading in the case of certain plant pathogens that might also establish internal populations. The ‘phyllo-sphere’ might thus be somewhat more three-dimensional than one would at first conceive.

Specific genes have been associated with epiphytic fitness in *P. syringae*. Mutants in *hrcC* and *hrpJ* (genes encoding components of the type III secretion system for delivery of virulence effector proteins into plants) as well as *gacS* (global regulator) and *pilD* (type IV pili) all exhibited reduced epiphytic fitness under field conditions [26–28]. Although mutations in *hrcC* and *hrpJ* affect growth of *P. syringae* within the plant, and thus may reduce its ability to avoid stresses on plants, the virulence of *gacS* and *pilD* mutants was similar to that of the parental strains. For *gacS* and *pilD*, the loss of epiphytic fitness was postulated to result from, respectively, a reduced production of a protective alginate capsule and diminished cell–cell aggregation on leaves that may shield some cells from stressful conditions [27,28]. Clearly, the behavior of more mutants with altered expression of fitness traits will be required before we can achieve a comprehensive view of the process of epiphytic colonization.

**Understanding phyllosphere bacterial ecology and the habitats the leaf provides to its bacterial residents**

Molecular tools have proven exceptionally useful in describing the composition and interactions of members of phyllosphere communities as well as the nature of the habitat that they occupy. Like most other habitats, the identity of microbes in the phyllosphere has until recently been limited to those that could be cultured. Although a great diversity of culturable bacteria has been described in the phyllosphere [6,29], the pioneering study of Yang et al. [30**] has shown that phyllosphere microbial communities are more complex than previously thought and that many members have not yet been cultured. This study of 16S rRNA sequences revealed that a majority of sequences were from species not previously recognized as phyllosphere bacteria [30**]. Although these results are perhaps not surprising given that such findings have been made in other habitats, it does suggest that there are many phyllosphere inhabitants that have never been investigated and which may harbor unique traits enabling them to thrive on leaves. Given that the leaf surface is considered to be a hostile location for bacterial colonization owing to frequent changes in water availability, incident irradiation, and low nutrient availability (see below), such strains may serve as
sources of genes encoding stress tolerance traits that may be of considerable biotechnological value.

Several reports have documented remarkably high rates of plasmid transfer among phyllosphere bacteria. The transfer of plasmid RP1 from donor to recipient *P. syringae* cells on leaves occurred in frequencies as high as $10^5$ cells/flower, primarily on the nutrient-rich stigmatic surface. As *E. amylovora* is often one of the initial immigrants to flowers, being vectored by visiting insects and bees, it is often a dominant member of the flower’s microflora. Movement of this large stigmatic inoculum along the surface of the pistil to the hypanthium by water allows infection to occur. Spray inoculation of flowers upon opening with suspensions of *P. fluorescens* A506 (10⁸ cells/mL) establishes initial populations of the antagonist of about 10⁶ cells/flower. Rapid multiplication of strain A506 to 10⁶ cells/flower effectively prevents multiplication of the small numbers of *E. amylovora* that might subsequently be vectored to flowers, thereby preventing infection. Note that stigma, bacteria, and bee are not drawn to scale.

Although flowers emerge from buds nearly axenic, they are rapidly colonized by immigrant bacteria and populations reach as high as $10^6$ cells/flower, primarily on the nutrient-rich stigmatic surface. As *E. amylovora* is often one of the initial immigrants to flowers, being vectored by visiting insects and bees, it is often a dominant member of the flower’s microflora. Movement of this large stigmatic inoculum along the surface of the pistil to the hypanthium by water allows infection to occur. Spray inoculation of flowers upon opening with suspensions of *P. fluorescens* A506 (10⁸ cells/mL) establishes initial populations of the antagonist of about 10⁶ cells/flower. Rapid multiplication of strain A506 to 10⁶ cells/flower effectively prevents multiplication of the small numbers of *E. amylovora* that might subsequently be vectored to flowers, thereby preventing infection. Note that stigma, bacteria, and bee are not drawn to scale.
conjugal efficiency and the 30-fold higher rate of plasmid transfer on leaves compared with membrane surfaces was ascribed to the aggregation of cells that occurred between epidermal cells, thus facilitating exchange [32•]. Such laboratory studies help explain the very high rates of acquisition of indigenous mercury-resistance plasmids by a genetically marked strain of *P. fluorescens* after it was introduced onto plant surfaces [33]. The abundance of phage reported on plants suggests that transduction may also be prevalent [34,35]. Although chemical analysis showed that about 0.2–10 µg of sugars (enough to support the growth of 10^7 to 10^8 cells/leaf) could be washed from uncolonized bean leaves, a portion of this sugar remained on leaves after bacterial colonization, suggesting that nutrient resources were patchy and that some nutrients were spatially sequestered from epiphytes [37]. Data in support of this conjecture were obtained using whole-cell bacterial biosensors responsive to fructose and sucrose, which consisted of *Erwinia herbicola* cells harboring fructose/sucrose-responsive promoters fused to a *gfp* gene. Molecular biosensors have revealed a great deal about the chemical and physical nature of the phyllosphere at the spatial scales of relevance to microbes. Although chemical analysis showed that about 0.2–10 µg of sugars (enough to support the growth of 10^7 to 10^8 cells/leaf) could be washed from uncolonized bean leaves, a portion of this sugar remained on leaves after bacterial colonization, suggesting that nutrient resources were patchy and that some nutrients were spatially sequestered from epiphytes [37].
reporter gene [38**,](#) (Figure 2). Although nearly all bioreporter cells were engaged in consumption of fructose (as evidenced by GFP fluorescence) within 1 h after inoculation, this fraction dropped to less than 1% within 24 h, suggesting a highly heterogeneous availability of nutrients to individual cells [38**]. The use of short half-life variants of the gfp reporter gene in these studies provided unparalleled information on the process of nutrient consumption on plants. A similar variability in available Fe**3** on leaves was observed using an iron biosensor strain of P. syringae [39]. Variation in sucrose abundance on leaves was also reported [40]. Such heterogeneity in the phyllosphere environment places constraints on the patterns of competition and other interactions that can occur among phyllosphere bacteria. Microbes can be exposed to high fluxes of ultraviolet (UV) irradiation on leaves, and most bacteria recovered from leaves exhibit high levels of UV tolerance [41]. In P. syringae this tolerance was associated with UV-inducible plasmid-borne rulAB genes conferring mutagenic DNA repair in most strains, as naturally occurring or induced rulA mutants exhibited less UV survival in culture and on plants [42,43,44**,45]. Clearly, epiphytes have evolved effective mechanisms for coping with UV damage. The presence of such adaptive traits on plasmids may be one means by which epiphytes maintain such elements and other conditionally beneficial genes [46]. The further examination of traits harbored on plasmids should shed light on traits important for an epiphytic lifestyle.

Microscopic examinations of colonized leaves have revealed that many epiphytes occur in large aggregates on plant surfaces [47,48**]. While large numbers of solitary bacterial cells occur on plants, a few large masses of apparently mixed bacterial species can also be found. Initial results suggest that, although uncommon, such aggregates could constitute 10 and 40% of the total bacterial population on certain plant species [48**]. Given the new appreciation for cell-density-dependent gene expression and the different behavior of bacterial cells in biofilms that has been demonstrated in other habitats, such biofilms on leaves have great implications for not only the behavior of epiphytic bacteria, but also for plant disease management. If cell-cell signaling via small molecules proves to be an important factor in regulating genes involved in epiphytic fitness, as in other habitats, then many new avenues for managing bacterial colonization of plants might be developed.

**Conclusions**

The phyllosphere is both scientifically and economically an important habitat in which to study microbial ecology. Because of the importance of many phyllosphere microbial inhabitants to plant health, there will probably be many practical applications that result from a better understanding of the interactions of microbes with the plant and with themselves. While the microbiology of roots has received quite a lot of attention, the microbiology of aerial plant parts is much less well-studied, although it is arguably of even more importance than the soil environment. The phyllosphere also has many features that make it a far better habitat in which to study microbial ecology than most other habitats. Microbes can be directly observed on leaves, enabling the use of powerful new microscopic techniques to measure microbial identity, activity, and gene expression. Plants can be readily genetically altered to change habitat conditions to test models of microbial behavior. Phyllosphere communities can be readily manipulated and can be made as simple or complex as needed by simple inoculation. Important microbial processes such as immigration and models such as island biogeography can be readily explored in plant systems. Thus, phyllosphere microbiology has much to offer to the field of microbial ecology and promises more effective and less environmentally damaging means of plant protection.

**References and recommended reading**

Papers of particular interest, published within the annual period of review, have been highlighted as:

• of special interest
•• of outstanding interest


44. Sundin GW: The major role of plasmid-encoded rulAB genes in allowing epiphytic strains of P. syringae to tolerate UV irradiation typical of that on leaves is demonstrated using mutant analysis and complementation. The common presence of this UV tolerance locus on a ubiquitous plasmid in P. syringae provided evidence that such determinants may help maintain these plasmids in the bacterial population. Furthermore, rulAB determinants in P. syringae strains may distinguish them from the many plant-associated bacteria that are not good phyllosphere inhabitants.


A clear illustration that bacterial communities on field-grown leaves can include large aggregates, often of mixed species. The magnitude of the size of such aggregates is illustrated well here for the first time.