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Biofilm formation in plant–microbe associations

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Bacteria adhere to environmental surfaces in multicellular assemblies described as biofilms. Plant-associated bacteria interact with host tissue surfaces during pathogenesis and symbiosis, and in commensal relationships. Observations of bacteria associated with plants increasingly reveal biofilm-type structures that vary from small clusters of cells to extensive biofilms. The surface properties of the plant tissue, nutrient and water availability, and the proclivities of the colonizing bacteria strongly influence the resulting biofilm structure. Recent studies highlight the importance of these structures in initiating and maintaining contact with the host by examining the extent to which biofilm formation is an intrinsic component of plant–microbe interactions.

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Abbreviations

DSF	diffusible signal factor
EPS	extracellular polymeric substances
GFP	green fluorescent protein
LapA	large adhesion protein A
QS	quorum-sensing
Xcc	*Xanthomonas campestris* pv. *Campestris*
Xf	*Xylella fastidiosa*

Introduction

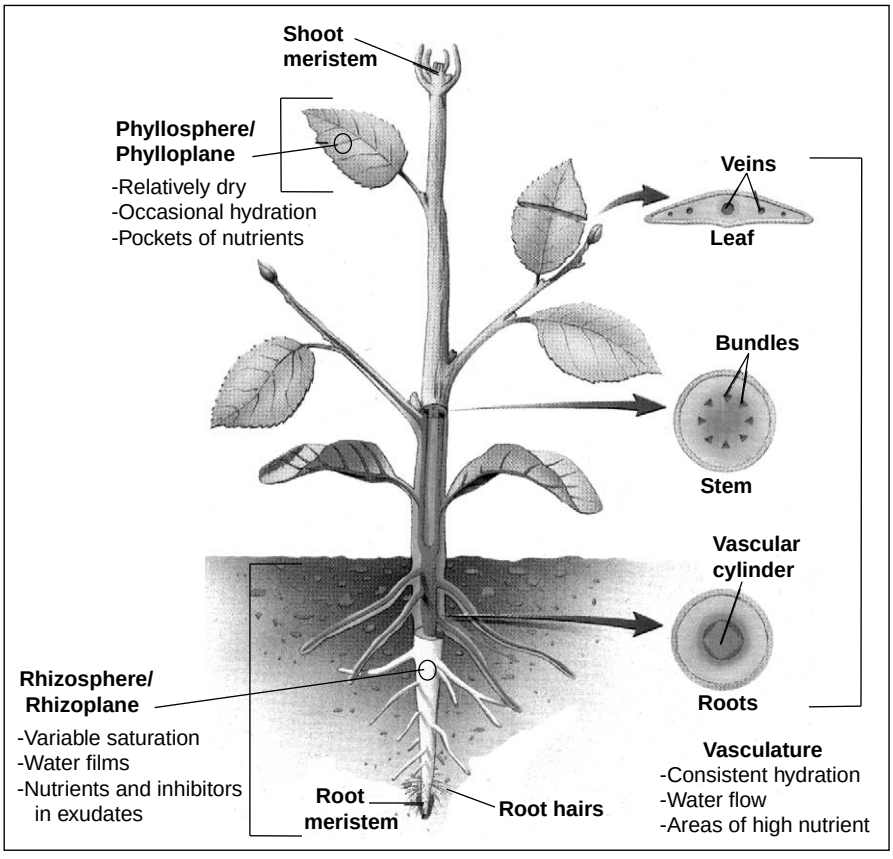
Many microorganisms in the natural environment exist in multicellular aggregates generally described as biofilms, associated with solid surfaces and in intimate contact with other microbial cells [1–3]. Cells adhere to surfaces and each other through a complex matrix comprising a variety of extracellular polymeric substances (EPS) including exopolysaccharides, proteins and DNA. Biofilm configurations range in complexity from flat, relatively featureless films, to tightly clustered aggregates, to complex heterogeneous cellular arrangements such as towers and streamers. Cells within biofilms are physiologically distinct from the same cells grown in dispersed culture [4,5]. Biofilm cells respond to nutrient and waste product diffusion gradients, modulate their metabolism as a function of their position within the biofilm, contact adjacent cells, and engage in cell–cell communication. Adherent populations exhibit elevated antimicrobial tolerance as a consequence of biofilm structure and physiological adaptation [3]. Biofilms have tremendous practical importance in industrial, medical and agricultural settings, exhibiting both beneficial and detrimental activities.

Although most fundamental work on microbial biofilms has focused on abiotic surfaces, it is clear that biofilms can and do form on biotic surfaces during host–microbe interactions [2]. Most plant–bacterial associations rely upon the physical interaction between bacteria and plant tissues. Direct observations of bacteria adhered to plant surfaces have revealed multicellular assemblies variably described as microcolonies, aggregates and cell clusters [6,7•,8]. These multicellular structures exhibit many of the defining attributes of biofilms — groups of cells enmeshed within an EPS matrix on a solid surface. For the purposes of this review, we will therefore refer to these different multicellular structures as biofilms, bearing in mind their differences from the paradigmatic microbial biofilm, and highlighting their unique properties. Our focus will be on recent work regarding the structure, formation and activity of microbial biofilms associated with terrestrial plants.

Plant surfaces: complex and dynamic environments

The terrestrial environment harbors abundant and diverse microbial populations that can compete for and modify resource pools. In this complex and competitive environment, plants offer protective oases of nutrient-rich tissues. Plants are colonized by bacteria on their leaves, roots, seeds and internal vasculature (Figure 1). Each tissue type has unique chemical and physical properties that represent challenges and opportunities for microbial colonists. Biofilms may form upon association or at later stages, with significant potential to direct or modulate the plant–microbe interaction. Additional temporal and spatial complexity arises as many microbes actively modify the colonized plant environment.

Figure 1



Sites of microbial colonization on terrestrial plants. Stylized dicotyledonous plant are depicted. Phyllosphere/phylloplane on leaves, rhizosphere/ rhizoplane on roots, and internal vascular system are highlighted. Figure adapted and reproduced with permission from William C Brown Publishers (McGraw-Hill). (Mader S. *Inquiry into Life*, 6th edition, 1991; Chapter 7, p142).

Water availability and saturation levels in terrestrial environments vary considerably. Plant-associated bacteria experience different levels of hydration depending on the colonization site, prevailing climate conditions and soil composition. The phyllosphere and phylloplane (leaves and leaf surfaces) are relatively dry but can be wetted by rainfall and dew. The rhizosphere and rhizoplane (root microenvironment and root surfaces) and soil-borne seeds are more consistently hydrated with a surface water film that is highly dependent on soil saturation. Bacteria that can invade the internal plant vasculature experience the most consistent levels of saturation. Water limitation has dramatic effects on biofilm structure and, therefore, the saturation level of a particular environment and a specific tissue will profoundly affect biofilm growth [9–11•].

Among each major tissue type are a variety of microenvironments. For example, the surface characteristics vary along the length of the root [12]. Actively growing root tissues typically exhibit higher exudation rates into the soil, and root cap cells at the growing tip can be sloughed away (Figure 1). Biofilms can be dramatically influenced by nutrient release and exudation at different sites. Leaf tissues often have a waxy cuticle that differs between the upper and lower portions of the leaf, interspersed with veins and petioles, trichomes and stomata. Phloem and xylem vessels are distinct tissue types within the vasculature that differ in fluid composition, architecture and spatial arrangement within leaves, stems and roots (Figure 1). Bacteria have adapted to each of these microenvironments, and the biofilms thus formed reflect the nature of their colonization sites.

Active and passive deposition on plant surfaces

Passive mechanisms of microbial deposition are common throughout the terrestrial environment, including wind and rain splash in the phyllosphere, and water flow in the rhizosphere [13]. Chemotaxis and motility are active mechanisms for establishment of biofilm communities [14–16]. Motility of several different pseudomonads appears to be important for competitive root colonization and long-term survival in soils [17–19]. Seed-associated

bacteria may also colonize the developing rhizosphere via chemotaxis and motility [17,18]. On leaf surfaces, passive deposition is important, as bacteria are removed and aerially transported to new locations [13]. Once deposited in the phyllosphere, motility may aid the bacteria in accessing a specific niche, but more often they congregate at their landing site [20]. In moist and nutrient-rich sites the bacteria can grow into aggregates and biofilms [20].

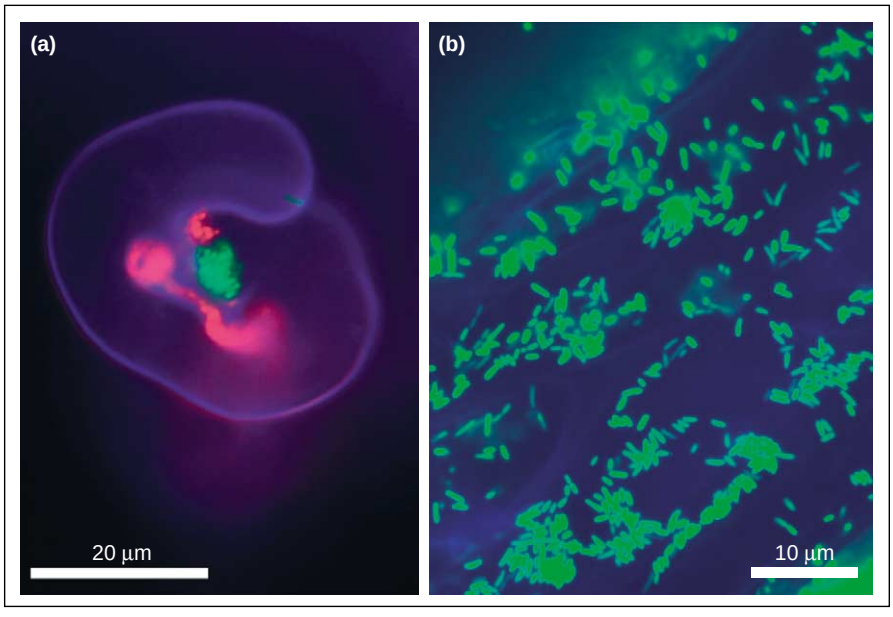
Biofilms in the rhizosphere

Root-associated pseudomonads have been studied extensively, and many of these promote the growth of host plants or are used as biocontrol agents [21]. Species of *Pseudomonas* form dense biofilms on both abiotic and biotic surfaces, and are a primary model in biofilm research [2]. *Pseudomonas putida* can respond rapidly to the presence of root exudates in soils, converging at root colonization sites and establishing stable biofilms [22•]. The plant-growth-promoting pseudomonads have been reported to discontinuously colonize the root surface, developing as small biofilms along epidermal fissures [23]. By contrast, recent studies analyzing pathogenic pseudomonads revealed dense, confluent biofilms on root surfaces [24•,25••]. Although the underlying cause for these different observations is unclear, it seems that pseudomonal root biofilms can range from relatively small multicellular clusters to extensive biofilm networks.

Azospirillum brasilense and related species are motile, heterotrophic proteobacteria that interact with roots of a variety of cereals such as wheat and maize, and often promote the growth of their host plants [26]. Although *A. brasilense* is a free-living nitrogen fixer, its ability to promote plant growth seems to be related to stimulation of root proliferation, rather than providing fixed nitrogen to the plant. The bacteria colonize root elongation zones and root hairs, forming dense biofilms [27]. Species of *Agrobacterium* and genera of symbiotic rhizobia not only cause neoplasia and symbiotic nodules on roots but are also effective root colonizers. Rhizobia preferentially associate with legume root hairs, stimulate root hair curling, infection thread elongation, and nodule formation on the appropriate host plant [28,29]. Microscopy of rhizobial cells within curled root hairs reveals small biofilm-type aggregates that provide the inocula for root invasion; the rhizobial cells migrate down infection threads as biofilm-like filaments towards the root interior (Figure 2a) [30•]. *Agrobacterium tumefaciens* and rhizobia can form dense, structurally complex biofilms on root surfaces, extensively coating the epidermis and root hairs, and these bacteria also form elaborate biofilms on abiotic surfaces (Figure 2b; AM Hirsch, personal communication) [31•,32].

Gram-positive microbes also effectively colonize the rhizoplane and are well represented in soil populations [33]. Biocontrol agents such as *Bacillus cereus* develop dense surface-associated populations, and one recent study has

Figure 2



Colonization of plants by the *Rhizobiaceae*. **(a)** Curled root hair of alfalfa with red (DsRed) and green (GFP)-expressing *Sinorhizobium meliloti* in a mixed microcolony occupying the interior bend of the curl. The DsRed-labeled cells have initiated an infection thread. (Image courtesy of DJ Gage, [30•]). **(b)** Epifluoresence micrograph (Nikon E80040 X objective) of *Agrobacterium tumefaciens* C58 (P_{tac}–*gfp*) adhered to *Arabidopsis thaliana* Landsberg root segment. Overlay of *gfp* fluorescence and autofluorescence of plant tissue (T Danhorn and C Fuqua, unpublished).

linked biocontrol with the ability to form biofilms [25••]. Several functions known to influence biocontrol activity are also likely to play a role in biofilm formation [34].

A variety of specific functions are relevant to colonization and biofilm formation on plant roots. Motility via flagella or type IV pili is required for competitive colonization of roots by pseudomonads [16,19,35]. Motility mutants typically demonstrate only modest deficiencies in non-competitive root colonization, emphasizing the efficacy of passive rhizodeposition. Surface structures such as lipopolysaccharide and outer membrane proteins are also important in biofilm formation on roots. Hinsa *et al.* [36•] recently identified a 900-kDa cell surface protein called LapA (large adhesion protein A), that affects *P. fluorescens* colonization of glass, plastic and quartz sand, and is speculated to be a general adhesin. The LapA homologue in *P. putida* KT2440 is also required for competitive root colonization and seed adhesion [37]. LapA has domains that resemble adhesins involved in biofilm formation of Gram-positive bacteria, and a domain similar to Ca^{2+}-binding proteins and haemolysins, often involved in host–cell interactions [36•]. For *Rhizobium leguminosarum* biovar *trifolii* a set of secreted agglutinins also thought to bind Ca^{2+} called Rap (*Rhizobium*-adhering) proteins localize to cell poles and are hypothesized to play a role in binding of rhizobial cells to plant tissues [38].

Production of exopolysaccharide is generally important in biofilm formation, and likewise can effect the interaction of bacteria with roots and root appendages [39,40]. Recent findings suggest that multiple polysaccharides modulate the chemical and physical attributes of the *P. aeruginosa* biofilm matrix on abiotic surfaces [41]. Such complexity may explain variable observations regarding the requirement for specific exopolysaccharides in biofilm formation and root association. For example, cellulose production in *A. tumefaciens* facilitates normal root adherence, and cellulose overproduction results in extremely dense biofilms ([42]; AG Matthysse, personal communication). By contrast, *A. tumefaciens* mutants that cannot synthesize the abundant exopolysaccharide succinoglycan (SCG) interact normally with roots, while mutants that overproduce SCG exhibit severely diminished adhesion (BE Ramey *et al.* unpublished data).

Later stages of biofilm maturation can also influence the structure of bacterial populations on roots. Recent findings suggest that *A. tumefaciens* biofilm formation on abiotic surfaces is regulated by the SinR transcription factor [31•]. SinR is a member of the FNR (fumerate and nitrate reductase) family of proteins, oxygen-responsive regulators that often control the transition to oxygen-limited conditions. Although it is unlikely that SinR senses oxygen directly, its expression is activated under oxygen limitation. *A. tumefaciens* with a *sinR* disruption formed a sparse, heterogeneous biofilm on abiotic surfaces, whereas strong *sinR* expression resulted in much thicker and less structured biofilms. The *sinR* biofilm phenotypes were recapitulated when examined on plant roots. Surface boundary layers and biofilms are generally oxygen-limited, and we speculate that SinR functions to respond to this feature of surface-associated growth. Other bacteria also appear to experience oxygen limitation during plant association [43,44].

Biofilms on seeds and sprouts

Bacterial adherence to seeds is a process that strongly influences rhizosphere colonization. Suppliers often deliberately coat their seed stocks with microbial biofilms to inoculate the developing rhizosphere. Additionally, biofilms on seeds and sprouts used for human consumption are common sources of infection. *P. putida* adheres effectively to seeds and will subsequently colonize the rhizosphere [37]. Several *P. putida* mutants, including one in the *lapA* homologue of *P. fluorescens*, are deficient in seed adherence and biofilm formation on inert surfaces, emphasizing the overlap between these activities. Recently, Coombs and Franco [45] identified endophytic populations of nonpathogenic actinobacteria in wheat tissues and determined that these were derived from interior colonization of surface-sterilized seeds. Endophytic seed populations help ensure future rhizosphere colonization.

Other studies of seed colonization have observed rod-shaped and coccal bacteria embedded within EPS in scanning electron micrographs of alfalfa seeds and sprouts [46,47]. Biofilms are notoriously resistant to washing and other common antibacterial treatments. Fett *et al.* found that both *Escherichia coli* O157:H7 and *Salmonella* populations on alfalfa sprouts required treatments much harsher than simple water washing to reduce the numbers of adherent microbes, and full removal was never achieved [48,49]. It seems likely that the surviving bacteria resided within biofilms, although this was not addressed.

Biofilm formation by vascular pathogens

Vascular pathogens inhabit the xylem or phloem of plant hosts and generally depend on insect vectors or wounding for dissemination. Several xylem-localized pathogens have received significant attention, while investigations of biofilm formation by phloem-restricted pathogens have focused primarily on spiroplasma in insects [50].

Xylella fastidiosa (*Xf*) is deposited into the xylem of plants by sap-feeding leafhoppers, and can induce Pierce's disease of grapevine and citrus variegated chlorosis [51]. Biofilms of *Xf* in the insect are composed of cells that are polarly attached to insect foregut tissue [52,53,54••]. In the plant host, most xylem vessels are sparsely colonized and asymptomatic, whereas densely populated vessels with biofilms are more rare, but symptomatic [54••]. Colonization depends on the *rpf* quorum-sensing (QS) system and the diffusible signal factor (DSF) [55•]. DSF

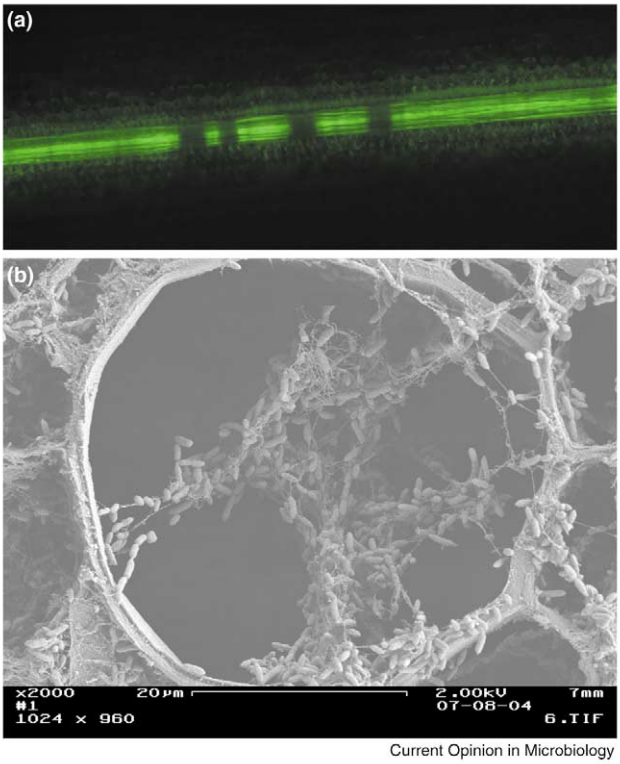
appears to govern expression of insect-specific adhesion factors [55•]. Insects exposed to a mutant in *rpfF*, encoding the DSF synthase, are not colonized by the bacteria and remain deficient for disease transmission. Interestingly, the *rpfF* mutant is hypervirulent when manually introduced into the plant host. Wild type *Xf* produces pit-membrane-degrading enzymes thought to aid movement into neighboring xylem vessels. It is plausible that expression of these enzymes is DSF-dependent. If so, a DSF mutant might densely populate xylem vessels, causing enhanced symptoms because it is unable to traverse the pit membrane [55•]. Fastidium exopolysaccharide is an important virulence factor for *Xf*. Bacteria attach to xylem vessels in the absence of the exopolysaccharide, while matrix-encased bacteria appear mainly in densely colonized vessels. Leite and co-workers proposed an adhesion model in which cell surface-exposed thiol groups associated with membrane features, impart a net negative cell surface charge, promoting divalent ion bridging for bacteria-to-bacteria and bacteria-to-host cell adhesion [56•]. The same group found that artificial media based on xylem chemistry stimulates *Xf* biofilm formation [57].

Xanthomonas campestris pv. *campestris* (*Xcc*) causes black rot on cruciferous plants, accessing the vasculature through wound sites in roots. Virulence involves degradative exoenzymes and the exopolysaccharide xanthan gum, both governed by *rpf*-encoded regulatory proteins and a DSF signal synthase [58•]. *Xcc* DSF was recently characterized as *cis*-11-methyl 2-dodecenoic acid, a novel α,β-unsaturated fatty acid QS signal [59]. DSF-dependent exopolysaccharide synthesis is necessary for biofilm formation and virulence, but not for bacterial adhesion. Candidate adhesion factors include pili and non-fimbrial adhesins. Dow *et al*. [58•] report that *Xcc* extracellular enzyme preparations induce dispersion of bacterial aggregates. The dispersion factor is an endo-β-(1,4)-mannanase (ManA) that expresses in an *rpf*/DSF-dependent manner and appears to facilitate spread of the pathogen through the plant vasculature.

Pantoea stewartii subsp. *stewartii* causes Stewart's wilt disease in maize and is transmitted by the corn flea beetle [60]. The bacteria reside primarily in the host xylem and produce large amounts of exopolysaccharide, controlled as a function of cell density through the EsaI/EsaR QS regulatory system [61,62]. Mutants of *esaI* and/or *esaR* alter bacterial adhesion, swarming motility, and biofilm formation [63]. Seedling infection assays using green fluorescent protein (GFP)-tagged strains show that the wild type strain colonizes the xylem vessels in discontinuous biofilms, while electron microscopic imaging shows bacterial aggregates covered with fibrous material associated with the xylem walls (Figure 3a,b).

Ralstonia solanacearum is a soil-borne pathogen that causes lethal wilt on many plants. Virulence depends on EPS and cell-wall-degrading enzymes controlled by a complex regulatory network [64•]. Denny and co-workers showed that the bacterium uses type IV pili for surface adhesion and twitching motility [64•]. Polar adhesion to plant cells is mediated by the PilA protein. A *pilA* mutant is less virulent and fails to form three-dimensional aggregates [64•]. Allen and colleagues [19] demonstrated a link between swimming motility and *Ralstonia* virulence and reported the observation of structures consistent with xylem biofilms.

Figure 3



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Colonization of the vasculature by *Pantoea stewartii* subsp. *stewartii*. **(a)** GFP-tagged wild-type strain DC283 colonizing leaf xylem vessels of a susceptible maize cultivar. The discontinuous colonization pattern might be indicative of successive cycles of biofilm formation and dispersal as a strategy for systemic infection. Obtained using an Olympus IX70 inverted epifluorescence microscope, 40X magnification. **(b)** Scanning electron micrographs depicting colonization of sweetcorn xylem vessels. Image obtained on a LEO/Zeiss DSM 982 digital field emission scanning electron microscope (M Koutsoudis and SB von Bodman).

Clavibacter michiganensis subsp. *sepedonicus* is a Gram-positive phytopathogen that causes bacterial ring rot in potato. Marques and colleagues [65] showed large bacterial, matrix-encased aggregates attached to the xylem vessels. Nonpathogenic Gram-positive filamentous actinobacteria were recently reported as endophytes of wheat [66•]. The bacteria were observed to form aggregates and

microcolonies in the intercellular spaces of healthy plant tissues, although it is not yet clear if they are disseminated through the vasculature.

Biofilms in epiphytic plant colonization

Aerial plant surfaces (i.e. the phylloplane) support large populations of bacterial epiphytes, including plant pathogens that multiply on the leaf surface before initiating disease [7•,67]. The leaf surface is partitioned into preferred microhabitats along veins, near trichomes and stomates.

Pseudomonas syringae pv. *syringae* (*Pss*), the cause of brown spot disease on bean, colonizes the leaf surface sparsely in solitary small groups (fewer than ten cells), while larger populations (more than 1000 cells) primarily develop near trichomes or veins with higher nutrient availability. Large aggregates survive desiccation stress better than solitary cells [68]. Lindow and co-workers [11] report that the epiphytic fitness of *Pss* is governed by the AhlI/AhlR QS system and the AefR regulator. AHL-null mutants are less tolerant to desiccations on leaves. Morris and colleagues assessed the epiphytic population structure of field-grown endive and cantaloupe and found that fluorescent pseudomonads were equally distributed in solitary or biofilm-associated populations, while Gram-positive epiphytes on cantaloupe tended to be in biofilms [6]. Leaf colonization by fluorescent pseudomonads may involve the deposition at new sites by solitary bacteria [48]. Large and small aggregates may vary as a function of nutrient availability at a given site [10].

Erwinia chrysanthemi (*Ech*) causes soft-rot disease through rapid maceration of plant tissue. Collmer and colleagues [69•] reported that *Ech* mutants in the filamentous hemagglutinin HecA have reduced leaf surface attachment and aggregate formation, fail to express macerating enzymes and are avirulent. The production of pectic enzymes may be QS-regulated, and therefore the inability to form bacterial aggregates may preclude pectinolytic enzyme secretion, illustrating how interference with an early phase of infection can dramatically impact successive steps.

Conclusions

Bacteria physically interact with plants in diverse ways. A common feature of this interaction is surface colonization, in which the microbes adhere to external and internal plant tissues as individual cells and in clusters. The adherent populations we define as biofilms exhibit a range of dimensions, locations and compositions. Each microenvironment of the plant has characteristic saturation levels, nutrient availabilities and surface chemistries, all of which strongly influence the form and activity of biofilms.

We have reviewed current examples of plant-associated biofilms, and some of the bacterial functions influencing the establishment of these structures. A fundamental question in this regard is whether the process of biofilm formation *per se* drives or significantly impacts the dynamics of plant–microbe interactions and the effect of pathogens, symbionts and commensals on their hosts. *A priori*, the answer appears to be 'yes'. The number, conformation and viability of the associated bacteria must be important. A handful of recent studies such as those on *A. tumefaciens* and *X. fastidiosa* provide direct evidence to support this conjecture, but a great deal of research in different systems remains to determine how biofilm formation mechanisms are integrated with productive plant association.

Acknowledgements

We wish to thank our many colleagues who study plant-associated biofilms and apologize for those studies we were unable to discuss in this necessarily brief review. We also wish to thank AM Hirsch and DJ Gage for sharing unpublished results and biofilm images. Biofilm research is supported by the United States Department of Agriculture in the Fuqua laboratory (NRI 2002-35319-12636) and in the von Bodman laboratory (NRI 2002-35319-1237, and USDA Agricultural Experiment Station grant CNSOO712).

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