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A feeling for the micro-organism: structure on a small scale. Biofilms on plant roots

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Biofilms are structured communities of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface; they have clinical, industrial and environmental impacts. Biofilms that are established by bacteria on plants are found on the surfaces of roots, leaves, seeds and internal vascular tissues where the microbes live in commensal, mutualistic or parasitic/pathogenic associations with their host. The study of the structure of plant-associated biofilms has been considerably helped by the development of techniques using fluorescent markers coupled with confocal scanning laser microscopy as well as scanning electron microscopy. We review several of these techniques as well as some of the research that has dealt with plant-associated biofilms. Our investigations focus on biofilm formation in the early stages of the *Rhizobium*–legume symbiosis, in which Gram-negative rhizobia provide fixed nitrogen to a host legume, and in return, the legume provides carbon-containing molecules. Because root colonization is an important early step in the establishment of the nitrogen-fixing symbiosis, we looked at *Sinorhizobium meliloti* attachment and biofilm establishment on the roots of its legume hosts, *Medicago sativa* L. and *Melilotus alba* Desr. We also examined biofilm formation by *Rhizobium leguminosarum* bv. *viciae* on the roots of *Arabidopsis thaliana* (L.) Heynh., a non-legume and non-host. Our ultimate goal is to characterize the rhizobial genes involved in aggregation and attachment to roots because several of these appear to be shared in biofilm formation and rhizobial entry of legume root cells.   © 2006 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2006, **150**, 79–88.

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INTRODUCTION

Until recently, structural studies of bacteria have been primarily carried out on the cellular level and typically on individual or small assemblages of planktonic bacteria. Transmission electron microscopy (TEM) has been the method of choice for examination of individual cells, and scanning electron microscopy (SEM) has been employed for studying the morphology of small clusters of bacteria. In the past, few studies focused on the structure of bacterial populations or on the morphology of the microbial communities established in nature. However, because of an enhanced interest in the establishment and organization of bacterial assemblages as a type of microbial development, more studies on bacterial population structure are being performed. In addition, the development of improved microscopic methods, particularly confocal scanning laser microscopy (CSLM), as well as the utilization of fluorescent antibodies, various reporter genes and fluorescence *in situ* hybridization (FISH) methods (reviewed by Wagner, Horn & Daims, 2003), have been responsible for much of the progress in analysing bacterial structure on a population scale.

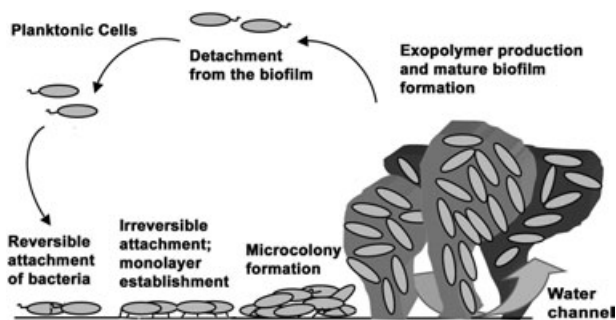
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Figure 1. Stages in biofilm formation. Environmental signals cue the planktonic cells to settle and establish microcolonies on a surface. The mature biofilm is permeated with water channels, and the cells are covered with exopolymer. The colonies assume mushroom, ridge or tower shapes or are configured as streamers. Cells can become motile and return to the planktonic life style.

The amassing of bacteria that results in the colonization of plant organs and tissues has been described under multiple names, including aggregates, microcolonies, symplasmata and biofilms (see review by Morris & Monier, 2003). In this report, we will use the term 'biofilm', which has been defined as a structured community of microbial cells consisting of either a single species or multiple species, adherent to a surface or interface, and according to the canonical definition 'encased in an extracellular matrix' (Costerton & Stewart, 2001) (Fig. 1). Although typically applied to microbes that associate with abiotic surfaces, this term has been used to describe assemblages established by *Agrobacterium tumefaciens* on plant organs (Fuqua & Matthysse, 2001; Ramey *et al.*, 2004a).

Biofilms significantly impact both medicine and industry because of their ability to clog heart valves, catheters and other medical implants, as well as to foul pipes, anaerobic sludge bed reactors, cooling towers and ship hulls. Microbial biofilms on surfaces cost the USA billions of dollars yearly in equipment damage, product contamination, energy losses and medical infections (see Costerton & Stewart, 2001). Some biofilm bacteria are resistant to antibiotics, which results in a compromised host becoming chronically infected (Costerton, Stewart & Greenberg, 1999). Antibiotic resistance may result from an alteration of bacterial metabolism in the biofilm, e.g. such as that caused by oxygen limitation (Borriello *et al.*, 2004). Other strategies of survival in response to antibiotic pressure have also been suggested, including a significant slow down of bacterial growth (Balaban *et al.*, 2004).

In nature, especially in aquatic environments, bacteria live in the biofilm state (Costerton *et al.*, 1995), in contrast to their growth as planktonic organisms in the laboratory. Unlike laboratory cultures, which usu-

ally grow in a rich medium, many bacterial biofilms develop in nutrient-poor environments. Most plant-associated biofilms are found in the phyllosphere or the rhizosphere, both of which are nutrient-poor. However, because of the presence of plant cell exudates, the rhizosphere is relatively nutrient-rich compared with bulk soil and the phyllosphere. Up to 20% of the carbon allocated to the root system can be released into the rhizosphere (Davey & O'Toole, 2000; Knee *et al.*, 2001).

The establishment of a biofilm goes through several developmental stages. In the first phase of biofilm formation, the bacteria swarm, generally by means of flagellae, and subsequently absorb reversibly to a substrate (Fig. 1). Within seconds to minutes, the bacteria become irreversibly attached, often by means of type IV fimbriae (pili), to the substrate where they establish a microcolony. While attached, the bacteria divide and differentiate to form the characteristic three-dimensional shapes (towers, tufts, ridges or mushrooms are frequently used to describe these profiles) that typify a mature biofilm. Water and nutrients bathe the bacterial mass. The bacteria are surrounded by a matrix, which is composed of polysaccharides, especially exopolysaccharide (EPS), many proteins and also DNA. Because of its polyhydroxylated nature, the matrix is very well suited to retain water and help ameliorate the effects of water stress. Lastly, individual bacteria can regain their flagella and exit the biofilm, thereby functioning as dispersal units (Morris & Monier, 2003).

Quorum sensing (QS) has been shown to play a significant role in sensing population density (see review by Henke & Bassler, 2004), in plant-associated microbial communities (Elasri *et al.*, 2001; Quinones, Pujol & Lindow, 2004), and in biofilm formation by both Gram-negative and Gram-positive bacteria (Davies *et al.*, 1998; Parsek & Greenberg, 1999; Huber *et al.*, 2001, 2002; Li *et al.*, 2002; Lynch *et al.*, 2002; Merritt *et al.*, 2003; Yarwood *et al.*, 2004). The use of reporter gene assays sensitive to QS signals has significantly helped analyse bacterial population signalling on plant surfaces (Steidle *et al.*, 2001). Nevertheless, biofilms on plant organs have not been as well studied as medical and industrial biofilms, not only in terms of QS, but also with respect to other parameters that affect biofilms. This is changing, however, as more efforts are being expended to study the colonization of roots, leaves, seeds and vascular tissues by plant-associated bacteria (Ramey *et al.*, 2004a).

In this report, we will review what is currently known regarding the morphology of bacterial biofilms on plants, focusing mostly on roots. Bacteria live as commensals, pathogens/parasites or mutualists/symbionts on their hosts, and these categories often intergrade (Hirsch, 2004). After a brief review of the

techniques used and the types of biofilms formed by different life-styled bacteria that are associated with plants, we will describe our own work on *Rhizobium leguminosarum* bv. *viciae* and *Sinorhizobium meliloti*, two species of soil microbes that have the ability to establish nitrogen-fixing nodules on their legume hosts (Hirsch, 1992).

SURFACE COLONIZATION

MUTUALISTS AND COMMENSALS

Some of the most detailed studies on bacterial communities living on plant organs have been the pioneering studies of the rhizosphere, the up to 1500-μm-thick interface between the root and soil, especially the 50-μm-thick area next to the root surface (the rhizoplane) that is rich in carbohydrate-based root exudates and microbes (Foster, Rovira & Cock, 1983). Many of the microscopic methods that have been developed to examine the rhizosphere were employed because of the difficulties in analysing the microbial community in association with the root. Bacteria in the rhizosphere are often not very metabolically active, making them difficult to culture on bacteriological media. PCR amplification of 16S rDNA helped in the identification of microbial species, but because many soil microbes are slow-growing and accumulate few ribosomes (Assmus *et al.*, 1995), even PCR-based strategies do not always work. For these reasons, other methods have been developed such as FISH (oligonucleotide probes coupled to the use of fluorescent markers) (Assmus *et al.*, 1995; Wagner *et al.*, 2003).

Besides FISH, some of the earliest methods to visualize microbes in the rhizosphere involved the introduction of *lux* genes and detection by luminescence as well as immunological approaches using polyclonal or monoclonal antibodies (see review by Schloter, Assmus & Hartmann, 1995). The latter methodologies were significantly aided by CSLM, which allows not only fluorescence detection but also the reconstruction of three-dimensional images. Moreover, it is possible to use different fluorescently labelled markers, exciting them with specific focused lasers, such that double or even triple labelling can be achieved. Currently, the use of reporter gene fusions, particularly genes expressing green fluorescent protein (GFP) and its various modifications, has improved the visualization of colonization patterns of rhizospheric bacteria.

The majority of studies of the structure of rhizospheric biofilms have focused on *Pseudomonas*, especially *P. fluorescens*, which is used as a biocontrol agent, and on *Azospirillum brasilense*, a plant growth-promoting bacterial (PGPB) species. Both groups of bacteria can be described as mutualists, but they have also been considered to be commensals.

Using CSLM to visualize highly specific polyclonal antibody-labelled cells, Hansen *et al.* (1997) described the assemblages of bacteria on roots as irregular aggregates or 'strings' of cells (Figs 2–4). Differentially labelled *P. fluorescens* strains were detected in young as well as older tissues of the root. Similarly, Normander, Hendriksen & Nybroe (1999) observed GFP-tagged bacteria in the crevices of adjacent barley root epidermal cells and used the term 'microcolonies' to describe their appearance. Using *gusA*- and *luxAB*-marked strains, Wang, Wang & Zhou (2004) observed that *P. fluorescens* strain CS85 cells were not evenly distributed on cotton roots. When roots were examined soon after inoculation, the bacteria were first detected near the root tip or in the root hair zone. Over time, they became non-uniformly dispersed over the root. When examined 14 days post-inoculation (dpi), about two orders of magnitude more bacteria were detected in the older parts of the root. Bloemberg *et al.* (2000), using three different fluorescent reporters, observed mixed colonies of *P. fluorescens* on roots. These colonies were generally quite small and were described as microcolonies by the authors.

Azospirillum forms significantly larger aggregates on root surfaces than do *Pseudomonas* species (Figs 5, 6). Using FISH, Assmus *et al.* (1995) observed large masses of bacteria mainly in the root hair zone of inoculated wheat plants. In a later study, using fluorescently labelled antibodies and FISH simultaneously, *A. brasilense* strain Wa3 was detected in a mixed bacterial community on root hairs of roots grown in non-sterile soil (Assmus *et al.*, 1997). When inoculated singly, strains Sp7 or Wa3 were observed to be in tight clumps on the root hair surface and on the root hairs, whereas no bacteria were observed on the root tips (Assmus *et al.*, 1997).

PARASITES/PATHOGENS

Agrobacterium tumefaciens is a plant pathogen, but instead of generating a disease that results in necrotic or wilted plants, it causes transformed galls or hyperplasias via virulence functions encoded on a tumour-inducing plasmid (Gelvin, 2003). On certain hosts, such as grape, *Agrobacterium* infection can compromise host survival. As yet, no evidence exists for the importance of biofilm formation as part of the transformation cycle, but studies have demonstrated that this bacterial species can establish biofilms on abiotic substrates (Fig. 7) and also on roots (Ramey, Matthysse & Fuqua, 2004b). GFP-labelled wild-type agrobacteria attached to PVC were examined by CSLM and shown to establish small ridges and towers 2–3 cells in thickness within 48 h. After longer time periods, the agrobacteria were densely packed on the underlying surface. On roots, a dense covering of bacteria was observed (Ramey *et al.*, 2004b).
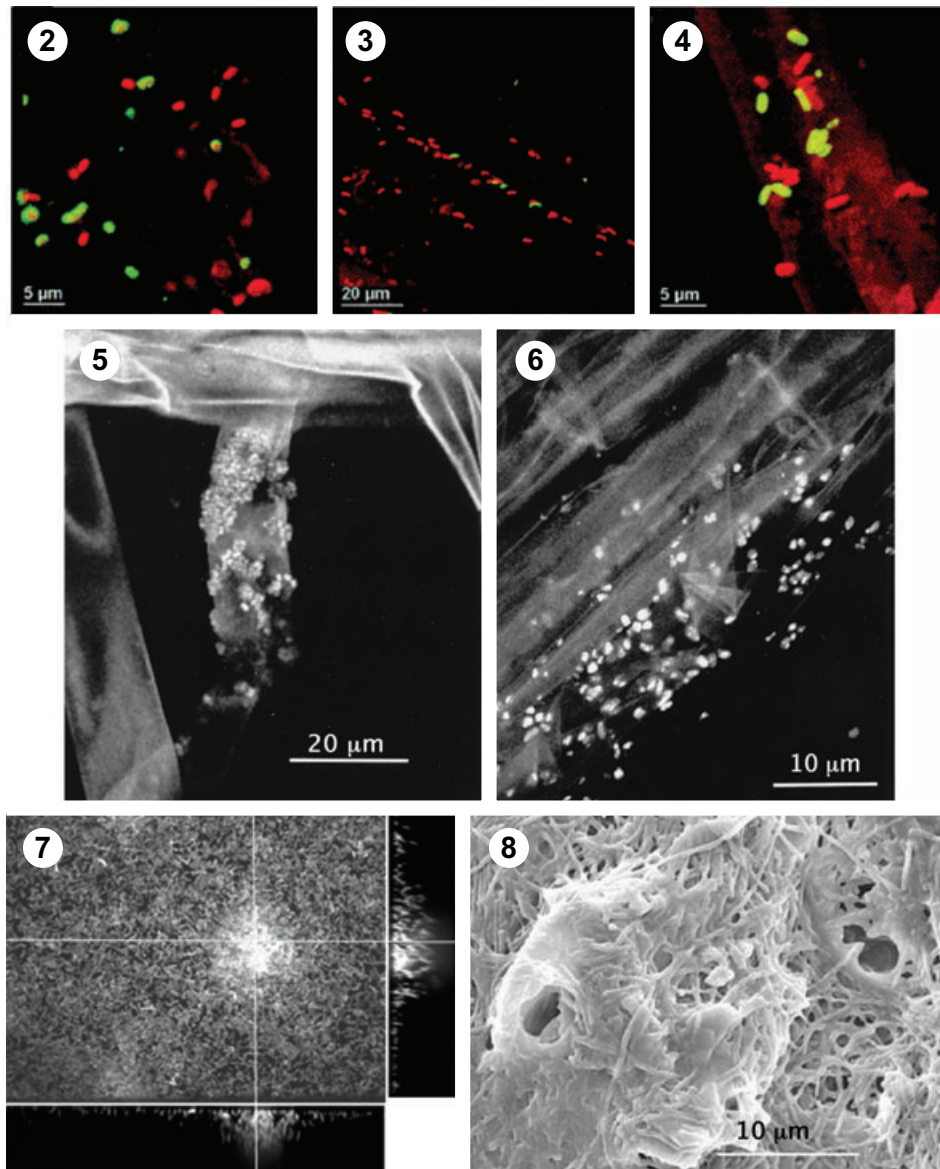
Figures 2–8. Examples of root (2–6) colonization and *in vitro* biofilm formation (7, 8). Figs 2–4. CSLM images of *Pseudomonas fluorescens* strains DF57 (red) and Ag1 (green) colonizing 1-day-old barley roots; 2, the bacteria are associated with a mucilage layer around the root. 3, strings of bacteria between the root epidermal cells. 4, a root hair in the older part of the root. Reprinted with permission from Hansen *et al*. (1997). Early colonization of barley roots by *Pseudomonas fluorescens* studied by immunofluorescence technique and confocal laser scanning microscopy. *FEMS Microbiology Ecology* **23:** 353–360. Figs 5, 6. CLSM images of *Azospirillum brasilense* Sp245 labelled with a fluorescently labelled oligonucleotide probe (TRITC-ALF1b), which is complementary to a region of 16S rDNA of alpha proteobacteria. 5, on the root hair of a soil-grown and 6, root surface of a quartz sand-grown wheat seedling. Reprinted with permission from Assmus *et al*. (1995). *In situ* localization of *Azospirillum brasilense* in the rhizosphere of wheat with fluorescently labelled, rRNA-targeted oligonucleotide probes and scanning confocal laser microscopy. *Applied and Environmental Microbiology* **61:** 1013–1019. Fig. 7. Biofilm phenotype of a GFP-labelled wild-type *A. tumefaciens* strain C58 incubated in a continuous flow cell system for 96 h. The top-down view is an overlap of many section planes and the side views are sagittal sections through the biofilm. Reprinted with permission from Ramey *et al*. (2004b). The FNR-type transcription regulator SinR controls maturation of *Agrobacterium tumefaciens* biofilms. *Molecular Microbiology* **52:** 1495–1511. Fig. 8. *Xylella fastidiosa* strain CCT 6752[T] (grape) biofilm established on the external surface of wood immersed in culture medium. Reprinted with permission from Marques *et al*. (2002). Characterization of biofilm formation by *Xylella fastidiosa in vitro*. *Plant Disease* **86:** 633–638.

Although plant pathogens such as *Pseudomonas syringae* establish biofilms in the phyllosphere, few establish biofilms on roots naturally. The leaf-associated microbes are usually considered commensals because they are below the population threshold necessary to elicit a disease (Rouse *et al.*, 1985). Epiphytic bacteria are found in clusters of 1 to >10^4 cells, frequently near stomata, trichomes and between epidermal cells, especially near veins (Monier & Lindow, 2004). The fluctuating population numbers most likely reflect the harsh conditions found on the leaf surface. For example, desiccation stress decreases bacterial survivability such that only the cells in aggregates remain viable, suggesting that small communities are more resistant to environmental stresses than a single epiphytic bacterial cell (Monier & Lindow, 2003).

A number of opportunistic human pathogens can cause disease on plant leaves and roots (Walker *et al.*, 2004; Jha, Bais & Vivanco, 2005). For example, *Pseudomonas aeruginosa*, which frequently colonizes the lungs of people with cystic fibrosis, has been shown to establish biofilms on roots of *Arabidopsis* and sweet basil (Walker *et al.*, 2004). SEM revealed that the bacteria attached both perpendicularly and horizontally to the roots of each plant species. A phase-bright area, suggestive of an extracellular matrix, surrounded the biofilm.

INTERNAL COLONIZATION

PARASITES/PATHOGENS

Only a few plant pathogens have been thoroughly studied enough to discover whether they establish biofilms on their plant hosts as part of the disease syndrome. Those that do establish biofilms cause disease by blocking xylem elements, resulting in plant water loss, severe wilting and subsequent tissue necrosis. The pathogens include: *Xylella fastidiosa*, the causative agent of Pierce's disease of grapevine and variegated chlorosis of citrus; *Clavibacter michigenesis* subsp. *sependonicus*, which elicits potato ring rot; and *Pantoea stewartii* subsp. *stewartii*, the source of Stewart's wilt disease (Purcell & Hopkins, 1996).

The first studies of *Xylella fastidiosa* biofilms were of biofilms established on abiotic surfaces, namely wood and glass, and used SEM, which, like CSLM, generates a three-dimensional view of a biofilm. However, artefacts may arise due to SEM processing, which includes fixation and dehydration as well as critical point drying. Each of these steps can lead to a loss of attached cells and shrinkage of water-containing components such as exopolysaccharide and mucigel. Nevertheless, the SEM methodology is very useful for comparative purposes. Under the same environmental conditions, different strains of *X. fastidiosa*

developed morphologically distinct biofilms: blunt, consisting of short cells; or filamentous, composed of elongated cells or filaments of cells (Marques *et al.*, 2002). Citrus and coffee strains attached poorly to glass substrates and formed blunt biofilms on wood, whereas elm and grape *X. fastidiosa* strains attached to glass and established filamentous biofilms on wood (Fig. 8). A polysaccharide matrix was evident on both types of biofilms (Marques *et al.*, 2002).

As a pathogen, *X. fastidiosa* clogs the xylem cells of its hosts, and also lives within the foregut of the insect host that serves as the vector for the disease (Purcell & Hopkins, 1996). SEM analyses of the grapevine–sharpshooter leafhopper–*Xylella fastidiosa* interaction show a different biofilm phenotype depending on the host (Newman *et al.*, 2004). In the insect, the bacteria are polarly attached to a section of the foregut via a mat-like structure, which may represent a matrix. By contrast, in the plant cell, the bacteria are randomly organized into a mass, which ultimately occludes the xylem cell. Strands, presumably composed of polysaccharide, extend from the bacteria to the xylem cell wall (Newman *et al.*, 2004).

SEM analysis showed that a cell-signalling (*rfpF*) mutant of *X. fastidiosa* makes a significantly reduced biofilm in its insect host compared with wild-type bacteria; very few bacterial cells were observed attached to the insect foregut (Newman *et al.*, 2004). By contrast, the biofilms established by mutant and wild-type bacteria in the plant xylem cell were found to be very similar to each other, suggesting that the biofilms established in the two hosts employ different mechanisms of attachment.

MUTUALISTS

Azoarcus strain BH72 was originally isolated as an endorhizospheric organism from Kallar grass (*Leptochloa fusca* (L.) Kunth). Using BH72-specific antibodies to probe sections of roots in gnotobiotic culture, this bacterial strain was localized to xylem vessels (Hurek *et al.*, 1994). BH72 also associates with rice and can colonize both non-living root cortical cells as well as xylem cells (Hurek & Reinhold-Hurek, 2003). Similar colonization patterns are observed for a number of endophytic nitrogen fixers, including *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae* and certain *Azospirillum* strains (Hurek & Reinhold-Hurek, 2003).

MATERIAL AND METHODS

STRAINS

Rhizobium leguminosarum bv. *viciae* (Rlv) 128C153 and two wild-type *Sinorhizobium meliloti* (Sm) strains

(Rm1021 and RCR2011) were utilized for this study. Although Rm1021 was derived from RCR2011, the two strains show some significant differences in the degree of gumminess as well as other traits (Krol & Becker, 2004). GFP on the plasmid pHC60 was introduced into Rlv and Sm via a triparental mating using pRK2013 as a helper plasmid (Figurski & Helinski, 1979). pHC60 is a stable IncP plasmid that constitutively expresses the *gfp* gene (Cheng & Walker, 1998).

PLANT MATERIAL, INOCULATION, GROWTH, AND HARVESTING

Seeds of *Medicago sativa* L. (alfalfa) and *Melilotus alba* Desr. (white sweetclover), the legume hosts for *S. meliloti*, were surface-sterilized in 95% ethanol for 10 min and full-strength bleach for 30 min. Seeds were washed six times with sterile water, and were germinated in the dark on Whatman paper moistened with sterile water. The plants were maintained in a growth chamber at 22 °C with an 18-h light/6-h dark photoperiod.

To prepare the inoculum, the rhizobial stains were grown to early stationary phase in *Rhizobium* Defined Medium (RDM) (Vincent, 1970) containing tetracycline at 10 μg mL^{-1}. Cells were washed twice and resuspended in quarter-strength Hoagland's medium (Machlis & Torrey, 1956) without nitrogen. The final concentration of rhizobia was 10^6 cells mL^{-1}.

Three different methods of growing plant material for root harvest and inoculating roots with rhizobia were used. (1) The roots were grown a modified Fahraeus slide assembly (Fàhraeus, 1957), which consisted of a slide and a coverslip held together with aquarium cement. The slide was immersed in a vial containing filtered Jensen's medium (Vincent, 1970) that bathed the root. (2) Three-day-old seedlings were transferred to square Petri dishes containing agar-solidified quarter-strength Hoagland's solution without nitrogen and inoculated 24 h later. (3) One-week-old seedlings were inoculated by dipping the roots into a *Rhizobium* suspension of 10^6 cells mL^{-1} for 5 min. The seedlings were then blotted on sterile Whatman paper to remove the excess liquid, and the plants were grown on Whatman paper wetted with quarter-strength Hoagland's medium without nitrogen.

Two days after inoculation, some of the roots were washed overnight with sterile water containing 0.05% Tween-20 on a rocking platform shaker to remove loosely associated cells. The roots were blotted on sterile Whatman paper to remove the excess liquid and were then weighed. To count the number of attached rhizobia, the roots were vortexed and homogenized, and the root homogenate was plated on RDM plates for colony counting. For each Sm strain tested, we counted the attachment to at least ten roots. The experiment was repeated twice.

Seeds of *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia) were sterilized in 50% ethanol for 5 min and 50% bleach for 15 min. The seeds were washed three times with sterile water, and then germinated on Petri dishes containing half-strength solidified Murashige–Skoog medium. The plants were grown in a growth chamber at 22 °C with an 18-h light/6-h dark photoperiod.

To prepare the inoculum for the *Arabidopsis* roots, Rlv128C53 (pHC60) was grown to early stationary phase in TY broth containing 10 μg mL^{-1} tetracycline. The cells were washed twice and resuspended in quarter-strength Hoagland's complete medium to a density of 10^7 cells mL^{-1}. Three-week-old seedlings were inoculated by dipping the roots into the *Rhizobium* cell suspension for 5 min. The seedlings were plated on Whatman paper that had been moistened with quarter-strength Hoagland's complete medium and were returned to the growth chamber. At varying times after inoculation, the roots were thoroughly rinsed with fresh quarter-strength Hoagland's medium to remove any unattached cells. The experiment was repeated three times.

MICROSCOPY

Confocal images were taken with a Bio-Rad MRC1024ES (krypton/argon) confocal laser scanning microscope associated with a Nikon Eclipse E800 light microscope using settings for fluorescein isothiocyanate (FITC) (488 nm) for observation of the GFP-stained rhizobia. A Zeiss Axiophot light microscope in conjunction with fluorescence microscopy was used for observation of the attachment of GFP-labelled rhizobia.

RESULTS AND DISCUSSION

RHIZOBIA FORM BIOFILMS ON ROOTS

Rhizobia are best known for their ability to induce nodules on legume roots, an event of considerable agronomic and ecological importance. Within these nodules, the rhizobia develop into bacteroids, differentiated forms that fix atmospheric nitrogen into ammonia, thereby freeing the legume host from its dependence on exogenous sources of nitrogen (N).

Rhizobia also frequently heavily colonize roots of both legumes and non-legumes, although generally rhizobia colonize roots of legume hosts better than non-legume roots (Schloter *et al*., 1997). We examined the attachment of two Sm strains on two of its hosts, alfalfa and white sweetclover. We found that the Sm strains heavily colonized the root, especially the root hair zone. There was extensive colonization of the
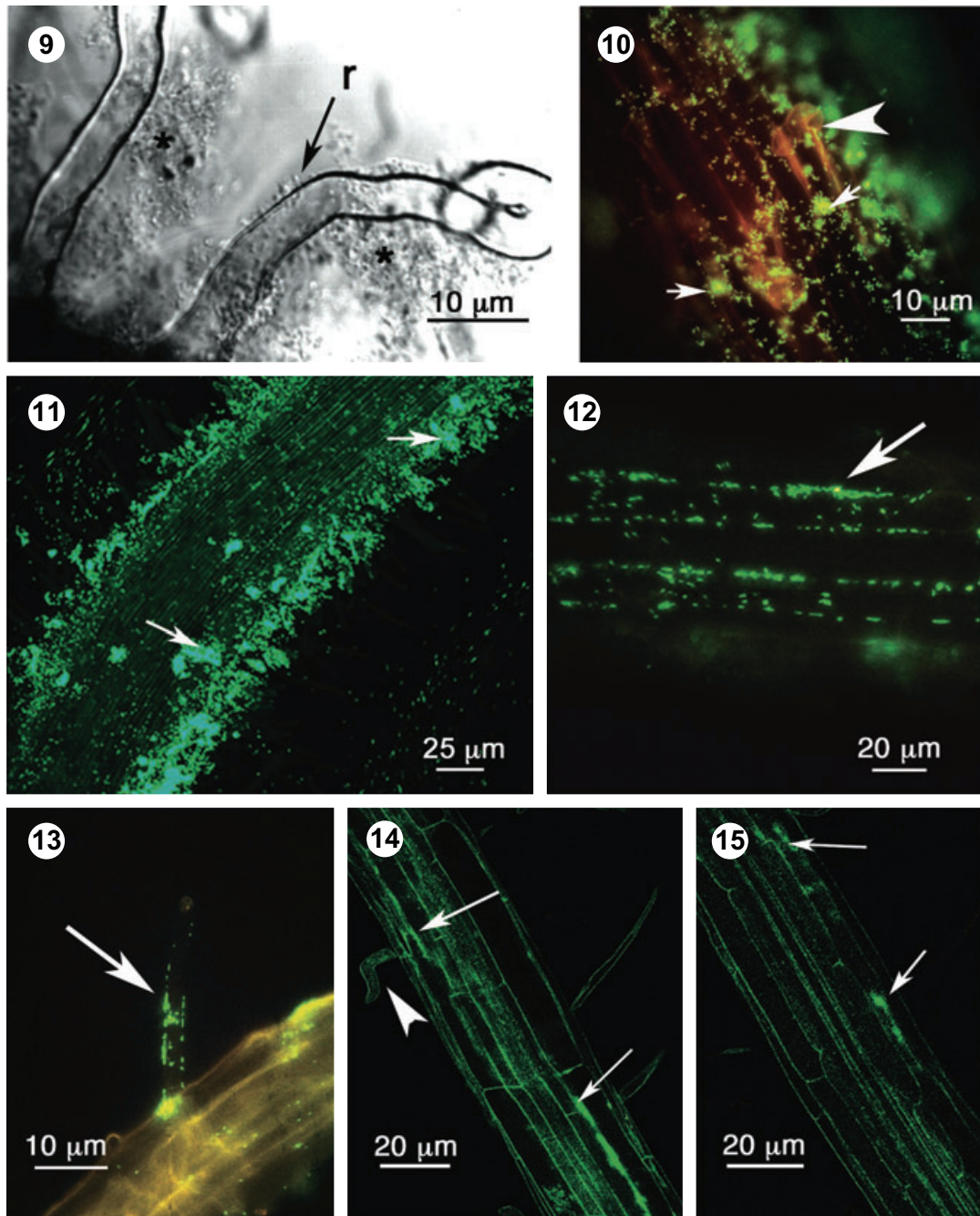
Figures 9–15. Legume roots (9–11) inoculated with *Sinorhizobium meliloti* strain Rm1021 and Rm1021-GFP. Fig. 9. Rhizobia (r) aggregate and attach perpendicularly to the deformed root hairs of *Medicago sativa*. Clusters of more loosely attached rhizobia (*) are adjacent to the deformed root hairs. Fig. 10. Alfalfa root inoculated with GFP-labelled Rm1021 and viewed 48 hpi under a fluorescent microscope. The red colour is due to the autofluorescence of the root. The large arrowhead points to a shepherd's crook and the arrows point to clumps of rhizobia. Fig. 11. Wild-type white sweetclover root inoculated with GFP-labelled Rm1021 and viewed under a confocal microscope 72 hpi. The arrows point to typical clumps of cells on the root surface towards the base of the root hair. Figs 12–14. *Arabidopsis* roots inoculated with GFP-labelled Rlv128C53. Fig. 12. Clusters of rhizobia are evident in the crevices between epidermal cells (arrow). 72 hpi. Epifluorescent micrograph. Fig. 13. Rhizobia are also firmly attached to the root hairs at this time (arrow). Epifluorescent micrograph. Figs 14, 15. Two different *Arabidopsis* roots 15 dpi were viewed with CSLM. The GFP-labelled rhizobia are detected on top of most epidermal cells and clustered in the crevices between epidermal cells (arrows). They are also associated with root hairs (large arrowhead).

shepherd's crook, a characteristic type of root hair deformation, which is frequently apparent as early as 24 h post-inoculation (hpi) and is elicited by a compatible rhizobial strain (Figs 9–11). After thorough washing, the rhizobia remained firmly attached to the root surface, to the root hairs and to the detached root cap cells known as border cells (Hawes *et al.*, 1998). Often, the rhizobia clustered in discrete clumps on the root surface (arrows, Figs 10, 11) or were closely associated with root hairs. Because these roots were harvested 48–72 hpi, nodules were not yet apparent.

White sweetclover roots were harvested and macerated for colony counts as described in the Material and Methods. For strain Rm1021, 5650 ± 187 rhizobia cells per 2.76 ± 0.47 mg root fresh weight were attached to the root. A similar number, 5799 ± 231 of RCR2011 cells per 2.70 ± 0.68 mg of root fresh weight, were attached.

We next monitored rhizobial biofilm formation on the roots of a non-legume, *Arabidopsis*. We chose Rlv128C53 for these studies because Sm strain Rm1021 did not attach to *Arabidopsis* roots in repeated trials. By contrast, by 72 hpi, Rlv attached to *Arabidopsis* roots, with the strongest contact in the crevices between the epidermal cells and on the root hairs (Figs 12, 13). This type of attachment is very similar to that described earlier for *Pseudomonas* and *Azospirillum* species on roots (cf. Figs 2–6 with Figs 12–15). By 15 dpi, a similar number of Rlv cells appeared to be associated with the *Arabidopsis* roots, but those that were attached were found mostly in the root hair zone, again clustered on root hairs and especially on and in between root epidermal cells (Figs 14, 15). These micrographs clearly show that Rlv cells associated with *Arabidopsis* roots as a biofilm.

Several studies have shown that rhizobia can colonize the outer surface of non-legume roots. Reddy *et al.* (1997) reported that a number of rhizobial strains attach to rice roots, and a few strains, particularly *Azorhizobium caulinodans* ORS571, are able to invade the crevices surrounding the emerging lateral roots. Our results differ from those of Yanni *et al.* (1997), who found that *R. leguminosarum* bv. *trifolii* invaded the internal root cells of rice. However, the *R. leguminosarum* bv. *trifolii* strain in the aforementioned study was isolated from soils in Egypt where rice has been rotated with berseem clover since antiquity. Our results on *Arabidopsis* also differ from those of Stone *et al.* (2001), who reported internal colonization of non-legume roots. We did not detect GFP fluorescence inside any of the *Arabidopsis* roots inoculated with Rlv, using either light or confocal microscopy whereas Stone *et al.* (2001) performed their studies with *A. caulinodans* ORS571, which invades its host as well as non-host roots by 'crack entry'. This mode of invasion is not as frequent for Rlv

or Sm, which exhibit more stringent modes of host infection (Hirsch, 1992).

Most researchers working with root association bacteria do not describe this interaction as a biofilm. However, O'Toole & Kolter (1998) described *P. fluorescens* WCS365 as forming a bona fide biofilm based on its phenotype on an abiotic surface. Similarly, Hinsa *et al.* (2003) described a gene, *lapA*, in *P. fluorescens* WCS365, which when disrupted results in a lack of bacterial adherence to an abiotic surface. The same gene, when mutated in *P. putida*, yields bacteria that cannot adhere to either biotic (seed) or abiotic (quartz sand) surfaces. Some of the concerns as to whether root-colonizing bacteria establish bona fide biofilms stems from the uncertainty of whether or not these bacteria are 'encased in an extracellular matrix'. Although the previously mentioned studies on *P. fluorescens* or *Azospirillum* do not refer to the presence of an exopolymeric matrix on the root-associated biofilms, Walker *et al.* (2004) presented data for *P. aeroginosa* indicating the presence of EPS on root-associated biofilms. Moreover, the overlap in genes important for adherence to abiotic and biotic surfaces argues strongly that bacterial root colonization is equivalent to biofilm formation. We are currently testing whether a polysaccharide matrix is present on rhizobial biofilms formed on legume and non-legume roots. Preliminary TEM analysis indicates the presence of fibrillar material around rhizobia attached to the root surface. Demonstrating that this is composed of polysaccharide will lend further support to the proposal that root-colonizing bacteria establish a bona fide biofilm.

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REFERENCES

Assmus B, Hutzler P, Kirchhof G, Amann R, Lawrence JR, Hartmann A. 1995. In situ localization of *Azospirillum brasilense* in the rhizosphere of wheat with fluorescently labeled, rRNA-targeted oligonucleotide probes and scanning confocal laser microscopy. *Applied and Environmental Microbiology* **61:** 1013–1019.

Assmus B, Schloter M, Kirchof G, Hutzler P, Hartmann

A. 1997. Improved in situ tracking of rhizosphere bacteria using dual staining with fluorescence-labeled antibodies and rRNA-targeted oligonucleotides. *Microbial Ecology* **33:** 32–40.

Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S. 2004. Bacterial persistence as a phenotypic switch. *Science* **305:** 1622–1625.

Bloemberg GV, Wijfjes AHM, Lamers GEM, Stuurman N, Lugtenberg BJJ. 2000. Simultaneous images of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent proteins in the rhizosphere: new perspectives for studying microbial communities. *Molecular Plant–Microbe Interactions* **13:** 1170–1176.

Borriello G, Werner E, Roe F, Kim AM, Ehrlich GD, Stewart PS. 2004. Oxygen limitation contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms. *Antimicrobial Agents and Chemotherapy* **48:** 2659–2664.

Cheng H-P, Walker GC. 1998. Succinoglycan is required for initiation and elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*. *Journal of Bacteriology* **180:** 5183–5191.

Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. 1995. Microbial biofilms. *Annual Review of Microbiology* **49:** 711–745.

Costerton JW, Stewart PS. 2001. Battling biofilms. *Scientific American* **285:** 74–81.

Costerton JW, Stewart PS, Greenberg EP. 1999. Bacterial biofilms: a common cause of persistent infections. *Science* **284:** 1318–1322.

Davey ME, O'Toole GA. 2000. Microbial biofilms: from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews* **64:** 847–867.

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **280:** 295–298.

Elasri M, Delorme S, Lemanceau P, Stewart G, Laue B, Glickmann E, Oger PM, Dessaux Y. 2001. Acyl-homoserine lactone production is more common among plant-associated *Pseudomonas* spp. than among soilborne *Pseudomonas* spp. *Applied and Environmental Microbiology* **67:** 1198–1209.

Fàhraeus G. 1957. The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. *Journal of General Microbiology* **32:** 374–381.

Figurski DH, Helinski DR. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proceedings of the National Academy of Sciences USA* **73:** 1648–1652.

Foster RC, Rovira AD, Cock TW. 1983. *Ultrastructure of the root–soil interface*. St. Paul, MN: The American Phytopathological Society, pp. 1–8.

Fuqua C, Matthysse AG. 2001. Methods for studying bacterial biofilms associated with plants. *Methods in Enzymology* **337:** 3–18.

Gelvin SB. 2003. *Agrobacterium*-mediated plant transformation: the biology behind the 'gene-jockeying' tool. *Microbiology and Molecular Biology Reviews* **67:** 16–37.

Hansen M, Kragelund L, Nybroe O, Sørensen J. 1997. Early colonization of barley roots by *Pseudomonas fluorescens* studied by immunofluorescence technique and confocal laser scanning microscopy. *FEMS Microbiology Ecology* **23:** 353–360.

Hawes MC, Brigham LA, Wen F, Woo HH, Zhu Y. 1998. Function of root border cells in plant health: pioneers in the rhizosphere. *Annual Review of Phytopathology* **36:** 311–327.

Henke JM, Bassler BL. 2004. Bacterial social engagements. *Trends in Cell Biology* **14:** 648–656.

Hinsa SM, Espinosa-Urgel M, Ramos JL, O'Toole GA. 2003. Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescens* WCS365 requires an ABC transporter and a large secreted protein. *Molecular Microbiology* **49:** 905–918.

Hirsch AM. 1992. Developmental biology of legume nodulation. *New Phytologist* **112:** 211–237.

Hirsch AM. 2004. Plant–microbe symbioses: a continuum from commensalism to parasitism. *Symbiosis* **37:** 345–363.

Huber B, Riedel K, Hentzer M, Heydorn A, Gotschlich A, Givskov M, Molin S, Eberl L. 2001. The *cep* quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming ability. *Microbiology* **147:** 2517–2528.

Huber B, Riedel K, Kothe M, Givskov M, Molin S, Eberl L. 2002. Genetic analysis of functions involved in the late stages of biofilm development in *Burkholderia cepacia* H111. *Molecular Microbiology* **46:** 411–426.

Hurek T, Reinhold-Hurek B. 2003. *Azoarcus* sp. strain BH72 as a model for nitrogen-fixing grass endophytes. *Journal of Biotechnology* **106:** 169–178.

Hurek T, Reinhold-Hurek B, van Montagu M, Kellenberger E. 1994. Root colonization and systemic spreading of *Azoarcus* strain BH72 in grasses. *Journal of Bacteriology* **176:** 1913–1923.

Jha AK, Bais HP, Vivanco JM. 2005. *Enterococcus faecalis* mammalian virulence-related vectors exhibit potent pathogenicity in the *Arabidopsis thaliana* plant model. *Infection and Immunity* **73:** 464–475.

Knee EM, Gong F-C, Gao M, Teplitski M, Jones AR, Foxworthy A, Mort AJ, Bauer WD. 2001. Root mucilage from pea and its utilization by rhizosphere bacteria as a sole carbon source. *Molecular Plant–Microbe Interactions* **14:** 775–784.

Krol E, Becker A. 2004. Global transcriptional analysis of the phosphate starvation response in *Sinorhizobium meliloti* strains 1021 and 2011. *Molecular Genetics and Genomics* **272:** 1–17.

Li YH, Tang N, Aspiras MB, Lau PCY, Lee JH, Ellen RP, Cvitkovitch DG. 2002. A quorum-sensing signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation. *Journal of Bacteriology* **184:** 2699–2708.

Lynch MJ, Swift S, Kirke DF, Keevil CW, Dodd CE, Williams P. 2002. The regulation of biofilm development by quorum sensing in *Aeromonas hydrophila*. *Environmental Microbiology* **4:** 18–28.

Machlis L, Torrey JG. 1956. *Plants in action. A laboratory manual of plant physiology*. New York: W.H. Freeman.

Marques LLR, Ceri H, Manfio GP, Reid DM, Olson ME. 2002. Characterization of biofilm formation by *Xylella fastidiosa* in vitro. *Plant Disease* **86:** 633–638.

Merritt J, Qi S, Goodman D, Anderson MH, Shi W. 2003. Mutation of *luxS* affects biofilm formation in *Streptococcus mutans*. *Infection and Immunity* **71:** 1972–1979.

Monier J-M, Lindow SE. 2003. Differential survival of solitary and aggregated bacterial cells promotes aggregate formation on leaf surfaces. *Proceedings of the National Academy of Science USA* **100:** 15977–15982.

Monier J-M, Lindow SE. 2004. Frequency, size, and localization of bacterial aggregates on bean leaf surfaces. *Applied and Environmental Microbiology* **70:** 346–355.

Morris CE, Monier J-M. 2003. The ecological significance of biofilm formation by plant-associated bacteria. *Annual Review of Phytopathology* **41:** 429–453.

Newman KL, Almeida RPP, Purcell AH, Lindow SE. 2004. Cell–cell signaling controls *Xylella fastidiosa* interactions with both insects and plants. *Proceedings of the National Academy of Science USA* **101:** 1737–1742.

Normander B, Hendriksen NB, Nybroe O. 1999. Green fluorescent protein-marked *Pseudomonas fluorescens*: localization, viability, and activity in the natural barley rhizosphere. *Applied and Environmental Microbiology* **65:** 4646–4651.

O'Toole GA, Kolter R. 1998. Initiation of biofilm in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis. *Molecular Microbiology* **28:** 449–461.

Parsek MR, Greenberg EP. 1999. Quorum sensing signals in development of *Pseudomonas aeruginosa* biofilms. *Methods in Enzymology* **310:** 43–55.

Purcell AH, Hopkins DL. 1996. Fastidious xylem-limited bacterial plant pathogens. *Annual Review of Phytopathology* **34:** 131–151.

Quinones B, Pujol CJ, Lindow SE. 2004. Regulation of AHL production and its contribution to epiphytic fitness in *Pseudomonas syringae*. *Molecular Plant–Microbe Interactions* **17:** 521–531.

Ramey BE, Koutsoudis M, von Bodman SB, Fuqua C. 2004a. Biofilm formation in plant–microbe associations. *Current Opinion in Microbiology* **7:** 602–609.

Ramey BE, Matthysse AG, Fuqua C. 2004b. The FNR-type transcription regulator SinR controls maturation of *Agrobacterium tumefaciens* biofilms. *Molecular Microbiology* **52:** 1495–1511.

Reddy PM, Ladha JK, So RB, Hernandez RJ, Ramos MC, Angeles OR, Dazzo FB, de Bruijn FJ. 1997. Rhizobial communication with rice roots: induction of phenotypic changes, mode of invasion and extent of colonization. *Plant and Soil* **194:** 81–98.

Rouse DI, Nordheim EV, Hirano SS, Upper CD. 1985. A model relating the probability of foliar disease incidence to the population frequencies of bacterial plant pathogens. *Phytopathology* **75:** 505–509.

Schloter M, Assmus B, Hartmann A. 1995. The use of immunological methods to detect and identify bacteria in the environment. *Biotechnology Advances* **13:** 75–90.

Schloter M, Wiehe W, Assmus B, Steindl H, Becke H, Höflich G, Hartmann A. 1997. Root colonization of different plants by plant-growth-promoting *Rhizobium leguminosarum* bv. *trifolii* R39 studied with monospecific polyclonal antisera. *Applied and Environmental Microbiology* **63:** 2038–2046.

Steidle A, Sigl K, Schuhegger R, Ihring A, Schmid M, Gantner S, Stoffels M, Riedel K, Givskov M, Hartmann A, Langebartels C, Eberl L. 2001. Visualization of N-acylhomoserine lactone-mediated cell–cell communication between bacteria colonizing the tomato rhizosphere. *Applied and Environmental Microbiology* **67:** 5761–5770.

Stone PJ, O'Callaghan KJ, Davey MR, Cocking EC. 2001. *Azorhizobium caulinodans* ORS571 colonizes the xylem of *Arabidopsis thaliana*. *Molecular Plant–Microbe Interactions* **14:** 93–97.

Vincent JM. 1970. *A manual for the practical study of root-nodule bacteria*. London: Blackwell Scientific Publications.

Wagner M, Horn M, Daims H. 2003. Fluorescence *in situ* hybridisation for the identification and characterisation of prokaryotes. *Current Opinion in Microbiology* **6:** 302–309.

Walker TS, Bais HP, Déziel E, Schweizer HP, Rahme LG, Fall R, Vivanco JM. 2004. *Pseudomonas aeruginosa*–plant root interactions. Pathogenicity, biofilm formation, and root exudation. *Plant Physiology* **134:** 320–331.

Wang C, Wang D, Zhou Q. 2004. Colonization and persistence of a plant growth-promoting bacterium *Pseudomonas fluorescens* strain CS85, on roots of cotton seedlings. *Canadian Journal of Microbiology* **50:** 475–481.

Yanni YG, Rizk RY, Corich V, Squartini A, Ninke K, Philip-Hollingsworth S, Orgambide G, de Bruijn F, Stoltzfus J, Buckley D, Schmidt TM, Mateos PF, Ladha JK, Dazzo FB. 1997. Natural endophytic association between *Rhizobium leguminosarum* bv. *trifolii* and rice roots and assessment of its potential to promote rice growth. *Plant and Soil* **194:** 99–114.

Yarwood JM, Bartels DJ, Volper EM, Greenberg EP. 2004. Quorum sensing in *Staphylococcus aureus* biofilms. *Journal of Bacteriology* **186:** 1838–1850.