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Investigations of *Rhizobium* biofilm formation

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Abstract

The development of nitrogen-fixing nodules of the *Rhizobium*-legume symbiosis, especially the early stages of root hair deformation and curling, infection thread formation, and nodule initiation, has been well studied from a genetic standpoint. In contrast, the factors important for the colonization of surfaces by rhizobia, including roots–an important prerequisite for nodule formation–have not been as thoroughly investigated. We developed conditions for analyzing the ability of two fast-growing rhizobia, *Sinorhizobium meliloti* and *Rhizobium leguminosarum* bv. *viciae*, to produce biofilms on abiotic surfaces such as glass, plastic microtiter plates, sand and soil as a prelude to characterizing the genes important for aggregation and attachment. Factors involved in adherence to abiotic surfaces are likely to be used in rhizobial attachment to legume root cells. In this report, we show that *S. meliloti* exopolysaccharide-deficient mutants as well as exopolysaccharide overproducers exhibit reduced biofilm phenotypes that show parallels with their nodulation abilities. We also investigated two flagella-less *S. meliloti* mutants and found them to have reduced biofilming capabilities. To investigate whether there was a symbiotic phenotype, we tested one of the Fla^- mutants on two different *S. meliloti* hosts, alfalfa and white sweetclover, and found that nodule formation was significantly delayed on the latter.

Introduction

Rhizobia are best known for their ability to induce nodules on legume roots, which results in nitrogen self-sufficiency for the host plant. Thus, the vast majority of studies on the legume–rhizobia symbiosis have focused on the development of the nodule and the initiation of nitrogen fixation by bacteroids, the differentiated rhizobial cells that fix atmospheric nitrogen (N_2) into ammonia, within the cells of the root nodule. Legumes develop two types of nodule, determinate and indeterminate. The development of determinate nodules, which are spherical in shape because meristematic activity ceases early, is usually initiated by the so-called 'slow growing' rhizobia, e.g. species of *Bradyrhizobium*. By contrast, indeterminate nodule development is correlated with the presence of fast-growing rhizobia such as *Rhizobium leguminosarum* bv. *viciae* (Rlv) and *Sinorhizobium meliloti* (Sm). Indeterminate nodules are cylindrical due to the presence of a persistent meristem at their distal ends (Hirsch, 1992).

The specificity of the interaction between Rlv and its hosts (species of *Pisum*, *Vicia* and *Lens*) and Sm and its hosts (species of *Medicago*, *Melilotus* and *Trigonella*) is mediated in part by Nod factor, a substituted lipochitooligosaccharide molecule of three to five β 1,4-linked GlcNAc residues, synthesized following the activation of rhizobial nodulation (*nod*) genes by host-produced flavonoids (Long, 1996). However, other rhizobial-produced factors are also critical for nodulation, including protein and polysaccharide components on the bacterial cell surface. For example, *S. meliloti* with mutations in genes important for the synthesis of the exopolysaccharide known as succinoglycan (EPSI) cannot fully invade the root to establish infection threads, and the nodules remain uninfected (Finan *et al.*, 1985; Leigh *et al.*, 1985). These nodules also fail to establish persistent nodule meristems and, hence, remain small and spherical (Yang *et al.*, 1992).

Although cell surface components are involved in the early stages of nodulation elicited by rhizobia, in many bacterial species, surface molecules, especially exopolysaccharide, flagella and lipopolysaccharide, are critical for the formation of a biofilm–a community of bacterial cells, either a single species or a consortia composed of numerous species, adherent to a surface and to each other, and enclosed in a self-produced polymeric matrix (Costerton *et al.*, 1995). Once attached to a surface, bacterial microcolonies are established, and later, three-dimensional

microbial communities of variable depth and architecture, which are frequently permeated by channels through which nutrients and water flow, are elaborated (Stanley & Lazazzera, 2004). The change from planktonic lifestyle to biofilm is mediated by numerous environmental signals, including nutrient availability, osmotic potential, and quorum sensing (Stanley & Lazazzera, 2004). Biofilms are the most common life strategy for bacteria in natural environments, including the rhizosphere.

In this report, we describe the conditions for establishing single-species biofilms of Rlv and two Sm strains–RCR2011 and its derivative, Rm1021, which is the sequenced strain (Galibert *et al.*, 2001) – on plastic, glass, soil and sand. As a proof of concept that the microtiter plate assay is an excellent method for screening for mutants affected in attachment, we also describe the biofilming ability of four Sm mutants with cell surface alterations, two exopolysaccharide (*exoY* and *exoS96*) mutants and the flagella-less (Fla^-) mutants, G910 and G911. We also describe the phenotype of the Fla^- Sm mutants on two legume hosts, alfalfa and white sweetclover. Previous investigations reported that Fla^- Sm mutants elicit nitrogen-fixing nodules on alfalfa (Ames & Bergman, 1981; Finan *et al.*, 1995). We confirm this finding, but also show that nodule development is delayed, especially on white sweetclover.

Materials and methods

Strains

The laboratory strains, Rlv 128C153 and two wild-type Sm strains (Rm1021 and RCR2011), were utilized for this study, as were mutant strains derived from Rm1021 (Table 1). We investigated both Sm wild-type strains because each is widely used by the *Sinorhizobium meliloti* community and because a large number of mutants are available in both genetic backgrounds for further study. Although RCR2011 is the parent of Rm1021, the two Sm strains show differences in their degree of gumminess–Rm1021 is a dry variant of RCR2011 *str3* (H. Meade, pers. comm.) – and also in other traits (Krol & Becker, 2004). For example, recent evidence shows that Rm1021 carries a frame shift mutation in the *pstC* gene, which results in reduced phosphate uptake via the PstSCAB transport system (T. Finan, pers. comm.).

Table 1. Rhizobial strains used in this study

Strain	Relevant characteristic(s)	Source or reference
Rlv128C53	Wild-type *Rhizobium leguminosarum* bv. *viciae*	Laboratory strain
Rm1021	Wild-type *Sinorhizobium meliloti*	Laboratory strain
RCR2011	Wild-type *Sinorhizobium meliloti*	J. Dénarié
Rm7210	*exoY210*::Tn*5*	Leigh *et al.* (1985)
Rm7095	*exoS95*::Tn*5*	Cheng & Walker (1998)
RmG910	1021::*fliP*Ωkan	Finan *et al.* (1995)
RmG911	1021::*flgH*·kanEco	T. Finan (unpublished)

Genes encoding green fluorescence protein (GFP) on the plasmid pHC60 were introduced into Rlv and the assorted Sm strains via a triparental mating using pRK2013 as a helper plasmid (Figurski & Helinski, 1979). pHC60 is a stable IncP plasmid that constitutively expresses *gfp* (Cheng & Walker, 1998). Dr H.-P. Cheng generously provided pHC60 and the GFP-labeled *exoS96*::Tn5 strain. The Sm Fla^- mutants (G910, G911; Table 1) were a gift from Dr T. Finan. The Ω Km insertion in RmG910 disrupts the *fliP* gene, whereas the Ω Km insertion in RmG911, which is in the upstream *Eco*RI site, falls in the *flgH* gene (T. Finan, pers. comm.). This insertion is polar and disrupts the downstream *fliL* and *fliP* genes.

Rm1021, Rm7210 (*exoY*::Tn*5*) and Rm7096 (*exoS96*::Tn*5*) (Table 1) with a constitutively expressed *gusA* gene were also constructed using the plasmid pFAJ31.2 (Van de Broek *et al.*, 1993) via a triparental mating, and used to inoculate *Medicago sativa* var. Iroquois roots.

Media, plant inoculation and growth conditions

Tryptone-yeast extract (TY) medium (Beringer, 1974), *Rhizobium* defined medium (RDM) (Vincent, 1970), and one-quarter strength Hoagland's medium (Machlis & Torrey, 1956) were used for bacterial growth. For the GFP and β-glucoronidase (GUS) plasmids, the medium was supplemented with tetracycline at $10\,\mu g\,mL^{-1}$. The bacterial cultures were grown at 28 °C.

For nodulation studies, alfalfa or white sweetclover (*Melilotus alba* Desr.) seeds were scarified for 30 s and surface-sterilized for 60 min in full-strength commercial bleach after a 5-min pretreatment in 95% ethanol. After copious rinsing with sterile distilled water, the seeds were placed on water-agar (1%) to germinate in the dark. After 72 h, the seedlings from the water-agar plates were transferred to solidified one-quarter strength Hoagland's medium minus nitrogen (Machlis & Torrey, 1956) in square plastic dishes. The plants were flood-inoculated with either wild-type Rm1021 or with the mutant strains. In some experiments, the strains carried a constitutively expressed GUS reporter gene. The bottom half of a stack of plates was covered with aluminum foil, and the plants were incubated in an upright position in a Percival growth cabinet with a 16-h light/8-h dark photoperiod and a 23 °C day/20 °C night temperature. Progress in nodule development was tracked daily, starting 3 days post-inoculation (dpi). The nodules were counted and harvested 3 weeks after inoculation. In some experiments, the nodules were stained for GUS activity (Jefferson *et al.*, 1987).

Buried slide assays

Sterile 50-mL conical centrifuge tubes were filled with moistened soil mix, which had been autoclaved for 40 min at 121 °C, after a sterilized glass slide or sheet of polyvinyl chloride (PVC) had been placed inside the tube. To facilitate drainage, a hole was punched in the bottom of the tube with a hot needle. Two different soil mixes were used. The composted soil is a blend of humus, composted rice hulls and other plant material, and is available as 'Amend' from Kellogg Garden Products (Carson, CA). The greenhouse soil mix (B.D. White Top Soil Co., Torrance, CA), used for routine planting in the UCLA Plant Growth Center, is composed of 1.5 parts Redondo Beach loam, 1.5 parts washed plaster sand, two parts screened peat moss, one part vermiculite, and one part perlite. GFP-labeled Rm1021 or RCR2011 cells were grown to $OD_{600} = 0.2$ (*ca.* 1×10^7 cells mL^{-1}) and added to the sterilized soil after the bacteria had been rinsed several times in sterile distilled water to remove the culture medium. The conical tubes were placed into a 28 °C incubator. The slides were periodically removed from the centrifuge tubes, rinsed five times with sterile water to remove any large adhering particles, and examined using phase and fluorescence microscopy. If the slides were in the soil for more than 7 days, 5 mL of sterile water were added to the centrifuge tube to replenish the soil moisture.

For determination of CFUs, the glass slides were removed from the soil, and rinsed with 1× phosphate-buffered saline (PBS) (Sambrook *et al.*, 1989) until the majority of external debris was removed. The slides were then transferred to 50-mL sterile centrifuge tubes filled with 25 mL of PBS, and the tubes were vortexed for 1 min. An additional 25 mL of PBS were added, and the tubes were re-vortexed. Serial dilutions were plated on RDM with the appropriate antibiotics. The slides were examined under the microscope to verify that all the bacteria had been removed.

Sand assays

We modified a sand attachment assay developed by Hinsa *et al.* (Hinsa *et al.*, 2003). Rhizobial cells were grown to $OD_{600} = 2.0$ (*ca.* 1×10^8 cells mL^{-1}) in RDM containing 2% sucrose. They were then washed and resuspended in the same medium to $OD_{600} = 0.2$ (*ca.* 1×10^7 cells mL^{-1}). Five hundred μL of sterile sand were placed in the wells of a 24-well Costar 3526 polystyrene (PS) dish (Corning Incorporated, Corning, NY), and the sand was inoculated with 500 μL of cells or with the same volume of culture medium only. The dish was then incubated at 28 °C, and the cells were harvested at different time points. Using a P200 pipette fitted with a wide-bore tip, 100 μL of sand and liquid were removed and transferred to a pre-weighed microcentrifuge tube. All traces of liquid culture were removed by pipeting, and only the sand and tube were weighed. One-half of the sand samples were washed once with 10 mM $MgSO_4$. To each sample, washed or otherwise, 150 μL of fresh 10 mM $MgSO_4$ were added, and the tubes were vortexed extensively to dissociate cells from the sand particles. The liquid containing the dislodged cells was serially diluted and plated on selective medium for obtaining CFUs. The CFUs were normalized to the amount of sand weighed.

Microtiter plate assays

We followed the procedure for the microtiter plate assay used by O'Toole *et al.* (1999) with some modifications. The rhizobial cells were grown to $OD_{600} = 2.0$ in RDM containing 2% sucrose. They were then washed and resuspended in the same medium to $OD_{600} = 0.2$ (*ca.* 1×10^7 cells mL^{-1}). One hundred microliters of cells or RDM alone were added to individual wells of a 96-well PVC plate (Falcon 3911, Becton Dickinson, Franklin Lakes, NY). The plates were sealed with sterile rayon adhesive film (AeraSeal, Excel Scientific, Wrightwood, CA) and incubated at 28 °C. At defined times, the OD_{595} was read in a BioRad (Richmond, CA) Microtiter Plate reader (Model No. 680) to verify that there were no differences in growth rate among the wells. At the final time point, the medium was removed, and the biofilms were stained with 0.01% crystal violet for 20 min. Excess dye was washed away with three changes of sterile water. The dye that stained the biofilm was then solubilized with 95% ethanol, and the amount of dye was quantified by measuring the absorbance at 570 nm.

Biofilms were also grown in Costar PS plates. Five hundred μL of Sm cells, which had been grown in RDM with 2% sucrose and diluted to an OD_{600} of 0.2 with the same medium, were added to each well of a Costar plate. Fresh RDM with 2% sucrose was added to the PS wells in the morning and in the evening after the spent medium had been removed. The Costar plates were incubated on a gyrorotary shaker model G2 (New Brunswick Scientific Co., Edison, NJ) at 50 rpm.

Biofilms on plastic tabs

GFP-labeled bacteria were grown as described above and transferred to individual wells of a 24-well Costar PS plate containing a tab of PVC. The plate was placed on top of a gyrorotary shaker oscillating at 50 rpm. The tabs were aseptically removed from the wells, washed once and placed in a depression slide, which was covered with a glass coverslip. The slides were examined under a fluorescence microscope. The Rlv strain was also stained with 0.02% Calcofluor White in HEPES buffer for 1 h and examined under epifluorescence.

Microscopy

A Zeiss Axiovert 200 (Zeiss, Thornwood, NY) motorized, inverted fluorescent microscope and a Zeiss Axiophot light microscope with phase and fluorescent capabilities were used for observing the attachment of the GFP-labeled rhizobia to the glass and PVC substrates in the buried slide experiments. Phase and fluorescence microscopy was also used for examination of the biofilms on plastic tabs. The biofilms established on the Costar PS plates were examined using an Olympus CK40 (Olympus, Melville, NY) inverted microscope at 40× under phase optics, and the nodulated roots were examined with an Olympus SZX12 Stereomicroscope. Images were taken digitally or with Kodak Ektachrome Tungsten 160 slide film (Kodak, Rochester, NY) and prepared with Adobe Photoshop (Adobe, San Jose, CA).

Protein gels

Rm1021 and the Fla$^-$ mutant G911 were grown in 100 mL of filtered RDM containing 10 μM luteolin for 48 h. The OD_{600} of each culture was determined and the bacteria were removed from the medium by centrifugation at 11 300 ***g*** for 30 min at 4 °C. The supernatants were stored at 4 °C for 24–48 h.

The sample volumes were brought to 100 mL in a solution containing 10 mM $Na_2O_5S_2$. Total of 65 g of $(NH_4)_2SO_4$ was added to the mixture at 4 °C and stirred for 12–24 h. The samples were then centrifuged at 20 000 ***g*** for 30 min at 4 °C. After decanting the supernatant, the pellet was rinsed from the walls of the tube with 1 mL of protein resuspension buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10% glycerol) and dialyzed overnight against 50 mM Tris pH 8.0, 300 mM NaCl, 1% PEG-8000. The material was subjected to a second round of dialysis against 50 mM Tris pH 8.0, 300 mM NaCl, 5% PEG-8000 for *ca*. 6 h at 4 °C to concentrate the protein. The samples were then quantified using the Bradford assay.

After precipitation in trichloroacetic acid (TCA), the pellet was resuspended in 50 μL of a 1× SDS loading solution (62.5 mM Tris pH 6.8, 10% glycerol, 2% β-mercaptoethanol, 2% SDS, 1 mg/ml bromophenol blue) and boiled for *ca*. 10 min. Equal amounts of protein were loaded onto two individual SDS gels. Five microliters of BioRad Precision Plus dual color protein standards were also loaded to permit in-gel and on-blot visualization of the protein sizes. One of the gels was stained with Coomassie Brilliant Blue and the other was transferred to a PVDF membrane (Millipore Immobilon, Billerica, MA) at 100 V for 3 h in Western Blot Transfer Buffer (25 mM Tris, 19.2 mM glycine, 10% MeOH, 0.02% SDS) in a BioRad Mini Protein Western Apparatus.

After the transfer, the blot was blocked with 20% bovine calf serum, 4% dried milk, and PBS, 0.1% Tween-20 (PBST) overnight at 4 °C. The blocking solution was removed, and then a 1 : 10 000 dilution of primary rabbit anti-*S. meliloti* flagellin polyclonal antibody (a gift from Dr B. Scharf) was added for *ca*. 1 h with shaking at room temperature. The blot was extensively washed (5 × 5 min PBST rinses) and subsequently, a 1 : 15 000 anti-goat anti-rabbit HRP-conjugated secondary antibody (Calbiochem, La Jolla, CA) was added in blocking solution, and the blot was then incubated on a shaker for 1 h, followed by 5 × 5 min PBST rinses. The antibody-antigen complex was detected by chemiluminescence.

Results

We developed conditions for analyzing biofilms of wild-type Rlv and Sm on abiotic surfaces, using a number of assays, including the buried slide technique (Parkinson *et al*., 1971), a sand attachment assay (Hinsa *et al*., 2003), and a microtiter plate assay (O'Toole *et al*., 1999). We also examined the phenotype of wild-type Rlv and Sm biofilms on plastic tabs and on the bottom of microtiter plate wells. The buried slide and sand attachment assays represent conditions that approximate the rhizosphere, thereby providing potential methods for analyzing the environmental factors that influence biofilm formation. The plate assay, which involves the analysis of attachment to artificial surfaces such as PVC, PS and glass, facilitates the screening of mutants defective in attachment as well as in biofilm formation.

Buried slide assays

Biofilms were established by both wild-type Sm strains Rm1021 and RCR2011 carrying GFP, but overall the latter formed better biofilms. Although biofilms formed equally well on either the glass slides or PVC inserts, we found it more convenient to monitor the glass slides on the microscope because of their rigidity and lack of autofluorescence at the monitored wavelengths.

Figure 1(a) illustrates a fluorescent and a merged, fluorescent-phase photograph of a Rm1021-GFP labeled biofilm established on a sheet of PVC. Most of the bacteria are associated with the dark particles (arrow), presumably soil or organic matter, found in the compost. Bare spots on the PVC or glass slide may reflect the fact that there were either fewer bacteria attached or that fewer soil particles were present. In Fig. 1(b), the fluorescent image, and in Fig. 1(c), the same fluorescent image merged with the phase photograph made of a GFP-labeled Rm1021 biofilm formed on a glass slide that was buried in compost soil is shown. Figure 1(d) was made of a GFP-labeled Rm1021 biofilm formed on a glass slide, which was examined under epifluorescence at a higher magnification. Discrete rhizobial clumps are observed on the glass slide; the arrows point to autofluorescent

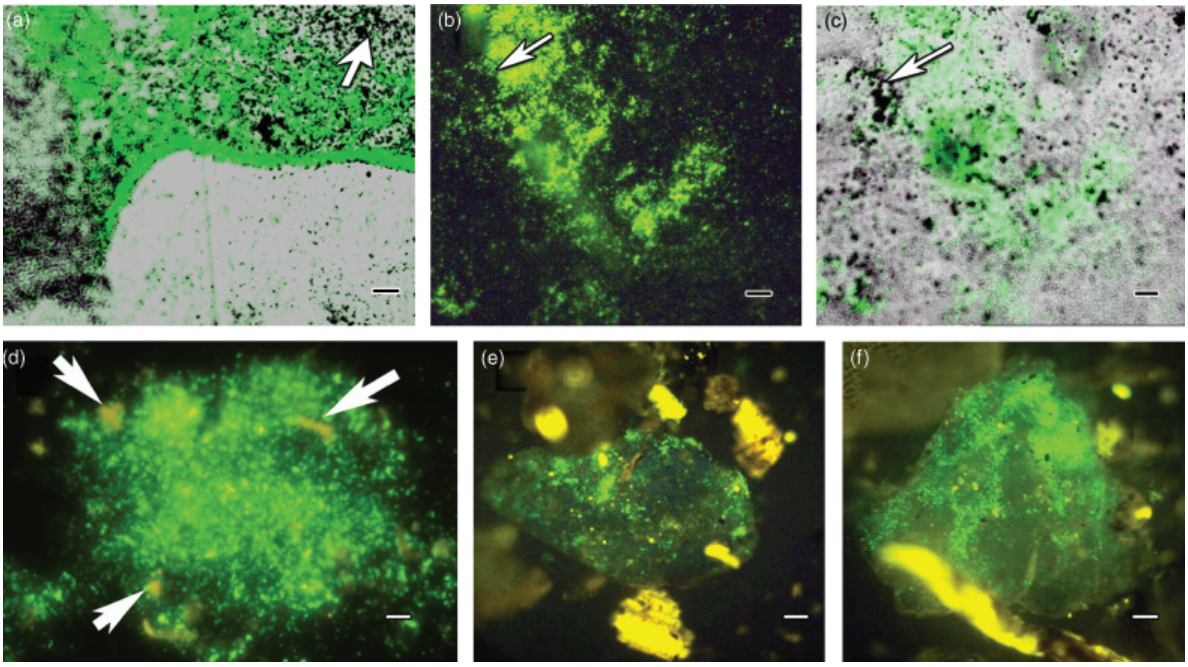


Fig. 1. Sm biofilms formed on buried slides. (a) Rm1021 biofilm after 16 days in composted soil on a sheet of PVC. Merged fluorescent and phase image. The arrow points to soil particles. Bar, 40 μm. (b) Rm1021 biofilm after 16 days on a glass slide buried in compost soil. Fluorescent image. The arrow points to the fluorescent cells in the biofilm. Bar, 40 μm. (c) Merged phase and fluorescent image of (b). The arrow points to soil particles attached to the slide. (d) Higher magnification of Rm1021 biofilm on a glass slide buried in compost soil. Fluorescent image. The arrows point to autofluorescent material. Bar, 10 μm. (e) RCR2011 biofilm on a quartz sand particle from a glass slide buried in greenhouse soil and examined after 48 h. Bar, 40 μm. (f) RCR2011 biofilm on a particle of vermiculite from a glass slide buried in greenhouse soil and examined after 48 h. Bar, 40 μm.

material. The biofilms illustrated in Figs 1(a–d) had been in contact with the buried slide for 16 days.

When we examined the components to which the rhizobia were attaching after 48 h more closely, we found that there was little adherence of the rhizobia to the inorganic components of the greenhouse soil other than to sand and vermiculite particles (Figs 1e and f). There was also minimal adherence of the rhizobial cells to peat remnants (data not shown). After 2 weeks, however, very few green-fluorescing cells were detected in the greenhouse soil (data not shown) in contrast to the compost soil (Figs 1a–d). Compost soil contains significantly more dead plant material, including rice hulls and root fragments, as well as their associated mycorrhizal fungi. GFP-labeled Sm cells were found attached to plant fragments, particularly dead roots (data not shown).

The degree of biofilm formation as measured by the area covered or the height of the aggregated bacteria was difficult to quantify in these experiments because of the irregularity of the soil material, so we measured CFUs 1, 2 and 3 weeks after the start of the experiment. We found that the compost soil supported more CFUs than did than the greenhouse soil mix after weeks 1 and 2 (Fig. 2). After the third week, however, both soil types exhibited a significant decrease in CFUs.

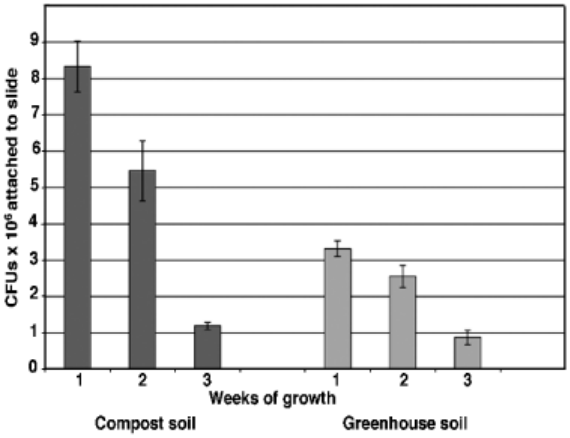


Fig. 2. Quantitative assessment of biofilming activity of RCR2011 based on CFUs from compost vs. greenhouse soil in a buried slide experiment after 1, 2, and 3 weeks after inoculation. The bars indicate the standard deviation from the mean.

Sand assays

We tested the biofilming ability of the Sm wild-type strains on quartz sand because this is a natural environment for

many bacteria and also because it is less complex than soil, thereby allowing us to test potential biofilm mutants acquired in a plate assay in a defined, but still natural substrate. RCR2011 exhibited significantly more attachment to sand particles than Rm1021 24 h-post-inoculation (hpi); $6.5 \times 10^8 \pm 1.1 \times 10^7$ cells g^{-1} of sand were recovered compared to $1.8 \times 10^8 \pm 6.6 \times 10^7$ Rm1021 cells g^{-1} of sand. Although the level of attachment based on CFUs was not as high if the sand had been washed, RCR2011 still adhered significantly more than Rm1021 ($1.6 \times 10^8 \pm 1.5 \times 10^7$ cells g^{-1} of sand vs. $4.4 \times 10^7 \pm 4.6 \times 10^6$ cells g^{-1} of sand).

Plate assays

We analyzed the growth conditions for biofilm formation in a 96-well microtiter plate assay system for both Rlv and the two Sm wild-type laboratory strains, Rm1021 and RCR2011. In preliminary experiments, Rlv exhibited significantly more biofilm formation in the microtiter plate assay than Rm1021 (Fig. 3a). This was confirmed in experiments where the amount of crystal violet staining was measured (cf. Figs 4a and b).

Rlv 128C53 and the Sm strains grow in a number of culture media ranging from tryptone-yeast extract (TY) medium, a relatively nutrient-rich medium, to sucrose-supplemented one quarter-strength Hoagland's medium, which in addition to a carbon source contains only those mineral nutrients essential for plant growth. For testing which nutrients were important for biofilm formation, Rlv was grown in TY, RDM, or one-quarter strength Hoagland's medium supplemented with 1% sucrose for 24 h. The best biofilm formation was observed in Hoagland's medium and the worst in TY (Fig. 3b). Like Rlv, Sm biofilms were more robust when the cells were grown in RDM as compared to TY. However, in sucrose-supplemented Hoagland's medium, Sm did not grow well or make significant biofilms (data not shown). RDM was chosen for all subsequent experiments because it is the standard medium for many rhizobial strains (Vincent, 1970), and yielded consistent biofilm formation.

We tested whether nitrogen was important for biofilm formation in RDM containing 1% sucrose. Figure 3(c) shows that without N there was neither bacterial growth nor biofilm formation. Similarly, we found that without sucrose in the medium, neither growth nor biofilm formation occurred (data not shown). As the sucrose concentration was increased to 4%, better biofilms were established, but there was also a greater possibility of fungal contamination. Thus, for most experiments, we utilized 1.5% or 2% sucrose as the final concentration in RDM to establish rhizobial biofilms.

Figures 4(a–c) illustrate the time courses over a 4–28 h period of biofilm development for Rm1021, RCR2011 and Rlv, respectively, in microtiter plate wells containing RDM with 2% sucrose. After an initial lag period of *ca*. 12 h, the levels of attachment increased significantly for all strains. The intensity of crystal violet staining at 28 h in the PVC plates, as indicated by the absorbance units on the Y-axis, was such that Rm1021 < RCR2011 < Rlv. RCR2011 accumulated more than twice the biofilm mass, evaluated by crystal violet intensity, than Rm1021. Because Rlv exhibited a different growth rate than the Sm strains, the two species cannot be directly compared. However, there was no significant difference in growth rate between Rm1021 and RCR2011 in the microtiter plate wells (data not shown), and thus the difference in biofilming capabilities must be attributed to other features.

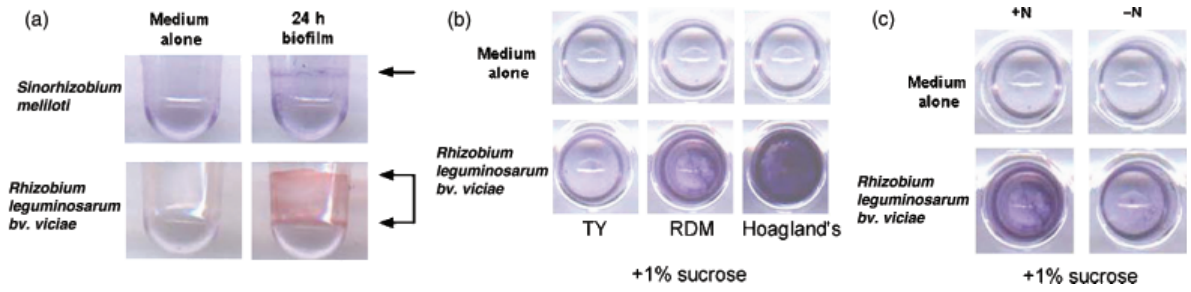


Fig. 3. Microtiter plate assay of Rlv and Sm biofilm formation. (a) Side view of wells from a 96-well microtiter plate comparing the medium control and Sm strain Rm1021 and Rlv after 24 h. The arrow in the top panel points to the narrow band of cells that make up the Rm1021 biofilm, whereas the bracket shows the extent of Rlv biofilm formation on the sides of the wells. The Rm1021 biofilm is stained with crystal violet and that of Rlv with safranin. (b) Top view of wells from a 96-well microtiter plate comparing the medium controls (top panel) and Rlv grown in tryptone-yeast extract (TY) *Rhizobium* defined medium (RDM) and one-quarter complete Hoagland's medium (bottom panel). All media were supplemented with 1% sucrose. (c) Top view of wells from a 96-well microtiter plate comparing the RDM controls with and without N (top panel) and Rlv grown in the same medium (bottom panel). All media were supplemented with 1% sucrose.

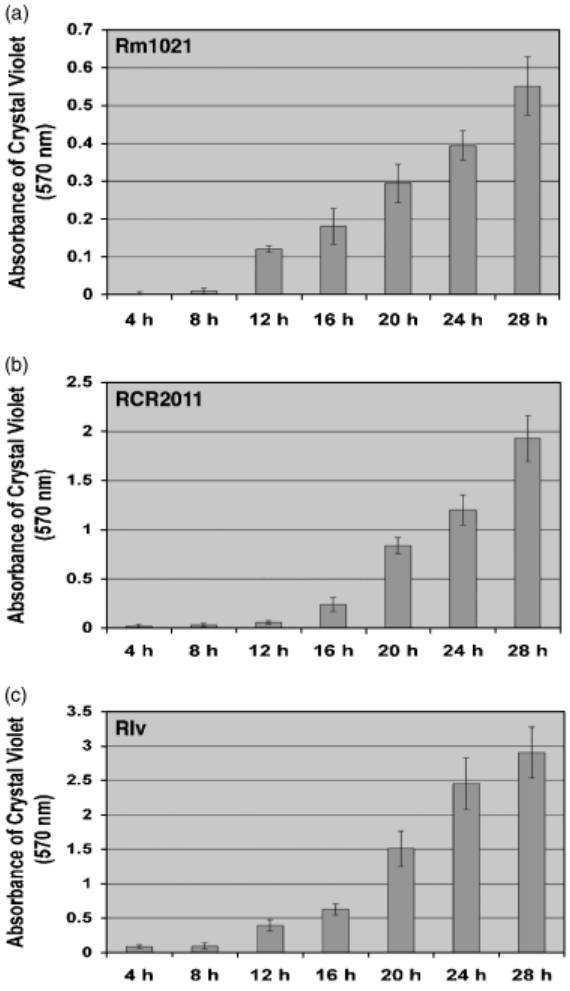


Fig. 4. Quantitative assessment of biofilm activity evaluated by crystal violet staining of rhizobial biofilms over a 28-h period. The bar indicates the standard deviation of the mean. Note the difference in the Y-axes for the three strains. (a) *Sinorhizobium meliloti* wild-type Rm1021. (b) *S. meliloti* wild-type RCR2011. (F) Wild-type *Rhizobium leguminosarum* bv. *viciae*, Rlv.

Visualization of biofilms on plastic

We examined the three-dimensional architecture of Sm and Rlv biofilms by growing the biofilms either on the bottom of a Costar PS plate and then viewing the bottom of the wells with an inverted phase microscope, or on a PVC tab and then examining the biofilm on the edge of the tab under phase and epifluorescence microscopy. Figures 5(a–d) illustrate different stages of Rm1021 biofilm formation on Costar PS plates. Microcolonies (arrows, Figs 5a and b) are established 24 to 28 hpi, and by 48 hpi, distinct ridges and small towers were observed (arrows, Figs 5c and d). Although larger towers were visible by 72 hpi, these were difficult to photograph using the inverted microscope, so we switched to growing the biofilms on plastic tabs. The earlier time points were reminiscent of what was observed in the Costar PS plates. At 7 dpi, a few towers of cells were observed, but these appeared loose and flocculent (Fig. 5e). At 10 dpi, the towers were taller and appeared more compact (Figs 5f and g).

Rlv also established elaborate three-dimensional biofilm structures as evidenced by the GFP-fluorescing cells in Fig. 5h (top). The lower panel shows the same biofilm stained with Calcofluor White. The positive staining reaction provides evidence for the presence of a biofilm matrix because Calcofluor White is known to stain rhizobial exopolysaccharide (Leigh *et al*., 1985; Yang *et al*., 1992).

Exopolysaccharide and flagella mutants

We next utilized the microtiter plate assay method to examine biofilms of *Sinorhizobium meliloti* exopolysaccharide and Fla$^-$ mutants because such mutants have often been shown to be impaired in biofilm formation in other bacterial species (Yildiz & Schoolnik, 1999; Danese *et al*., 2000; Whiteley *et al*., 2001; Matsukawa & Greenberg, 2004). Previous research demonstrated that Sm Exo$^-$ mutants elicit on alfalfa roots ineffective (Fix$^-$), meristemless nodules that are not colonized internally by rhizobia (Finan *et al*., 1985; Yang *et al*., 1992) (Fig. 6b). The *exoY* gene encodes a galactosyl-1-P transferase that carries out the first step in the synthesis of EPSI in Sm (Reuber & Walker, 1993).

In an examination of the biofilming ability of the *exoY* strain Rm7210, we found that biofilm formation was *ca*. 57–60% reduced compared to wild-type Rm1021 strain after 24 h and *ca*. 50% 40 h after the start of the experiment (Fig. 6e). There was no difference in growth rate between the wild-type and *exoY* strain during the course of the experiment.

The most striking change between the *exoY* and wild-type biofilms was in the three-dimensional architecture as examined under the microscope. Prior to washing, the biofilms of the *exoY* mutant appeared to be robust because they were compact. However, *exoY* cells were readily removed with each successive washing step, whereas Rm1021 remained attached. Whereas distinct towers were formed by wild-type biofilms at the termination of the experiment (Fig. 6c), the *exoY* mutant did not establish a mature biofilm (Fig. 6d). Only small, tightly appressed mounds of cells, four or five cells deep, were evident.

We then tested the biofilming ability of the Sm *exoS96* mutant because it overproduces succinoglycan at levels eight-fold higher than Rm1021 (Cheng & Walker, 1998). Recently, this mutant was discovered to exhibit a decreased cell motility phenotype due to a loss of flagella (Yao *et al*.,

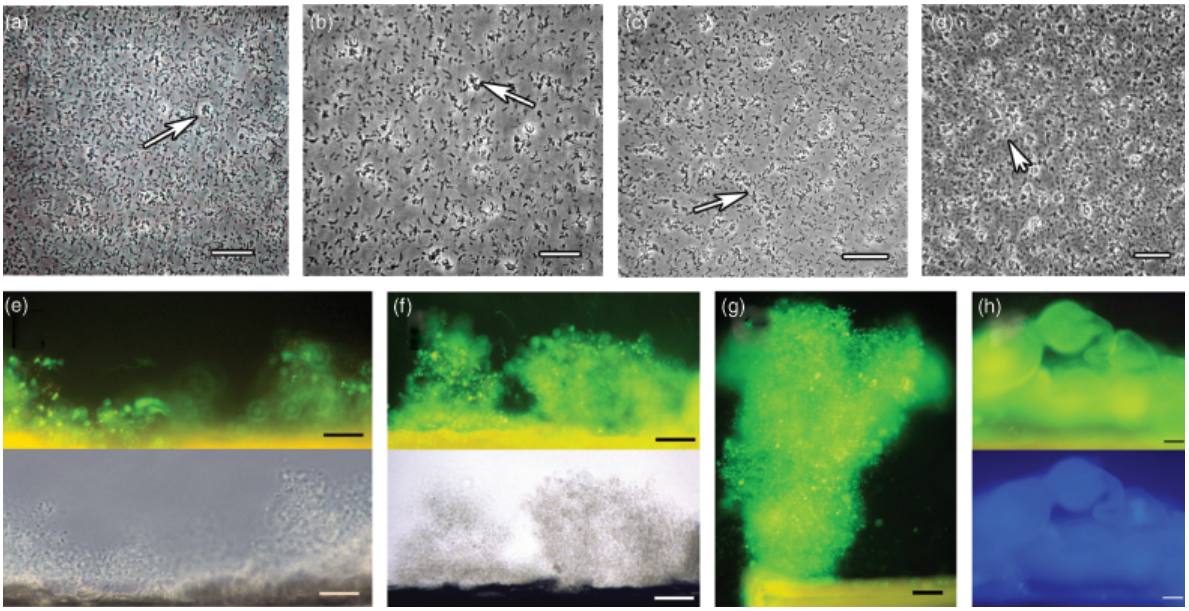


Fig. 5. (a–d) Views of Rm1021 biofilms on the bottom of wells of Costar PS plates from two separate experiments. Bars, 10 μm. (a) View of biofilm 24 hpi; the arrow points to a microcolony. (b) View of biofilm 28 hpi; the arrow points to a microcolony. (c) and (d) Two views of biofilms 48 hpi; the arrows point to ridges of cells developing on the well bottom. (e) Rm1021 biofilm formed on the edge of a polyvinyl chloride tab under phase (bottom panel) and epifluorescence (top panel) 7 dpi. Bar, 20 μm. (f) Bright field (bottom) and fluorescent (top panel) images of Rm1021 towers 10 dpi. Bar, 20 μm. (g) A large tower of Rm1021 at the edge of a fluorescent tab. Bar, 40 μm. (h) Fluorescent images of green fluorescence protein-labeled (top panel) and Calcofluor White-stained (bottom panel) Rlv. Bar, 40 μm.

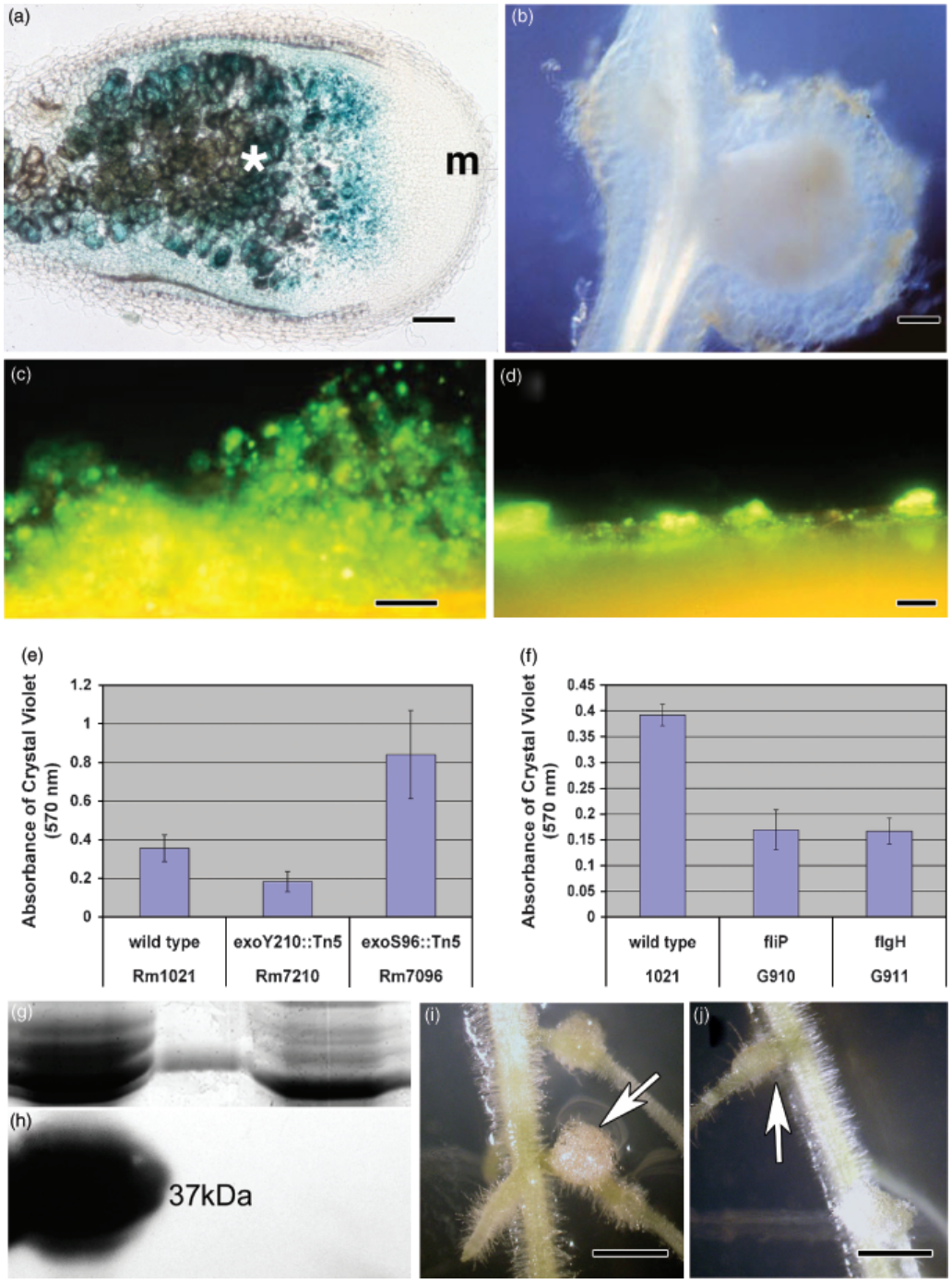
2004). Although *exoR95* is also a succinoglycan overproducer, we analyzed the *exoS96* mutant because it elicits normal, nitrogen-fixing nodules on alfalfa (Cheng & Walker, 1998). By contrast, the *exoR95* mutant induces a mixture of Fix^+ and Fix^- nodules (Cheng & Walker, 1998). Moreover, the *exoR* gene encodes a protein with no significant homology in the database, whereas the *exoS* gene encodes the membrane-bound sensor of the ExoS/ChvI two-component regulatory system.

We confirmed the results of Cheng & Walker (1998) and found that the *exoS96* mutant elicited the formation of nitrogen-fixing nodules on alfalfa. GUS-stained rhizobia were distributed in the same regions of the nodule (Fig. 6a) as observed in Rm1021-elicited nodules. In the microtiter plate assay, the *exoS96* mutant exhibited significantly more adherence to the PVC wells than either the wild-type Rm1021 or the *exoY* mutant (Fig. 6e). However, there was a large standard deviation in the measurement of the *exoS96* biofilming activity. This resulted from large clumps of rhizobia becoming detached from the wells after extensive washing. This loose biofilm phenotype was observed repeatedly.

To address the issue as to whether the *exoS96* phenotype was due to the overproduction of exopolysaccharide or to the lack of flagella, we examined the Sm Fla^- mutants, G910 and G911, which are non-motile, but reported to induce normal, nitrogen-fixing nodules on alfalfa (Finan *et al.*, 1995). First, we confirmed that G911 was lacking flagella by

Fig. 6. Analysis of *exo* and Fla^- mutants. (a) Longitudinal section of an alfalfa nodule elicited by the β-glucoronidase (GUS)-labeled *exoS96* mutant Rm7096. Blue color indicating the presence of bacteria (*) is observed within the nodule except at the distal end, where the meristem (m) is located. The nodule is essentially identical to the one elicited by wild-type Rm1021 (data not shown). Bar, 120 μm. (b) Alfalfa nodule elicited by GUS-labeled Rm7210 (*exoY*). No blue color and hence no bacteria are observed within the center of the nodule. Bar, 40 m. (c) Wild-type Rm1021 biofilm on a plastic tab 10 dpi. Bar, 10 μm. (d) Rm7210 (*exoY*) biofilm. Microcolonies are apparent, but no large aggregations of rhizobia are observed. Bar, 20 μm. (e) Quantitative assessment by crystal violet staining of the biofilming ability of wild-type Rm1021, *exoY*, and *exoS96* mutants. (f) Quantitative assessment by crystal violet staining of the biofilming ability of wild-type Rm1021 and Fla^- mutants, G910 and G911. (g) Coomassie stained protein gel of bacterial supernatants. From left to right, Rm1021; 37 kDa protein standard; G911. (h) Western blot using the Sm flagellin antibody. From left to right, Rm1021; 37 kDa protein standard; G911. The antibody recognizes a band in Rm1021 only. (i) Nodules induced by wild-type Rm1021 on a white sweetclover root 2 weeks after inoculation. The arrow points to a well-developed, pink-colored nodule. Bar, 1 mm. (j) Nodule induced by the *flgH* mutant G911 on a white sweetclover root 2 weeks after inoculation. The arrow points to a small, white nodule. Bar, 1 mm.

performing a western blot with an antibody prepared against *S. meliloti* flagella. In contrast to Rm1021, no protein cross-reacting to the antibody was observed (Figs 6g and h). Then we inoculated both alfalfa and white sweetclover roots with the Fla$^-$ mutant, G911. Although nodules developed on the roots of the two legume hosts, they were slower to develop than on the roots inoculated with Rm1021. For sweetclover, the first nodule appeared 48 h after the first one developed on the Rm1021-inoculated roots. For alfalfa, nodulation was delayed *ca.* 24 h. When examined on the



same day, white sweetclover nodules elicited by mutant G911 were smaller, confirming the delay in nodule development (Figs 6i and j).

When we examined the two Fla$^-$ mutants, G910 and G911 in the microtiter plate assay, we found that both mutants were more than 50% reduced in biofilming capability compared to wild-type Rm1021 (Fig. 6f). In addition, we observed fewer Fla$^-$ cells in the microtiter plate wells after staining with crystal violet and before the rinsing stages than in the wells containing *exoY* mutants, strongly suggesting that G910 and G911 are defective in their initial attachment to a surface.

Discussion

We have described conditions for analyzing biofilm formation in two different fast-growing rhizobia, *Rhizobium leguminosarum* bv. *viciae* and *Sinorhizobium meliloti*. We modified two methods – the buried slide procedure and the sand attachment assay – for analyzing biofilms in natural substrates. We found that RCR2011 formed thicker biofilms than Rm1021, and that both wild-type strains established better biofilms in the compost vs. inorganic soil, possibly as a consequence of the greater amount of organic material in the compost soil. However, after two weeks, the numbers of CFUs dropped in both soils. This may have resulted from a depletion of carbon in the soil substrates or alternatively, from a decrease in cultivability of the bacterial cells over time. Rhizospheres, although rich in carbon compounds from the activity of roots and associated microbes (Foster *et al*., 1983), often contain slow-growing microbes that accumulate few ribosomes (Assmus *et al*., 1995). Because no plant tissues or sources of carbon were included in the buried slide assays, bacteria inoculated into these conditions are not likely to be actively dividing over the course of the experiment, and hence could have become recalcitrant to being cultured.

Both wild-type Sm strains formed effective biofilms on the natural substrate, quartz sand; however, washing removed most of the attached cells. Our result differs from that of Hinsa *et al*. (2003) for *Pseudomonas fluorescens*, who found that washing did not have as drastic an effect. Three possible explanations could account for this: (1) *P. fluorescens* attaches to sand better than Sm; (2) the difference in inoculum size (1×10^9 vs. 1×10^8 for Sm); or (3) *P. fluorescens* was grown in a nutrient-rich medium (LB) before transfer to sand, whereas *S. meliloti* was grown in minimal medium (RDM). In any case, in a preliminary testing of Sm mutants affected in biofilm formation on PVC, the sand attachment assay is useful for validating their decreased adherence to a more natural substrate (data not shown).

Developing the microtiter plate assay was difficult at first because we initiated our experiments with Rm1021, which compared to Rlv, is a poor biofilmer. Including RCR2011 in the experiments enabled us to improve the parameters for both Sm strains. For example, we found that a minimal medium gave better results than TY or LB, and that higher levels of sucrose had to be added to the medium to get better biofilm formation. In addition, we found that using a rayon seal over the microtiter plate instead of the PVC cover yielded more consistent results, most likely due to better gas exchange. When the PVC cover was used, the outer wells had different numbers of cells compared to the inner wells, thereby increasing the standard deviation.

As a proof of concept that we can use the microtiter plate assay as a method for discovering biofilm mutants in rhizobia as has been done for many other bacteria, we examined the responses of rhizobia with mutations in selected candidate genes. We choose two Sm *exo* mutants, an EPSI-deficient mutant and an EPSI overproducer, as well as two motility mutants. The *exoY* mutant elicits the formation of uninfected nodules, whereas *exoS96* induces normal nitrogen-fixing nodules on alfalfa, although less efficiently than Rm1021 (Yao *et al*., 2004).

The Sm *exoY* exhibited reduced biofilming ability as measured by crystal violet staining. This is not unexpected as exopolysaccharide mutants in other bacterial species, namely, *Vibrio cholera* El Tor (Yildiz & Schoolnik, 1999), *Escherichia coli* K-12 (Danese *et al*., 2000), and *Pseudomonas aeruginosa* PAO1 (Friedman & Kolter, 2004; Jackson *et al*., 2004; Matsukawa & Greenberg, 2004), establish poor biofilms. The most striking difference from the wild-type Sm strain was the architecture of the *exoY* biofilm, which was only a few cells thick and more compact than the wild-type, which established well-developed towers. This phenotype is similar to that described for *V. cholera* El Tor and for *P. aeruginosa exo* mutants. Exopolysaccharide has been proposed to be important for stabilizing the three-dimensional structure of the biofilm (Watnick & Kolter, 1999) or as a scaffold for other molecules that hold the biofilm together (Matsukawa & Greenberg, 2004).

The *exoS96* mutant exhibited significantly greater biofilm formation than its wild-type parent, most likely because it is a succinoglycan overproducer. Surprisingly, large cell clumps easily came off the wells after the washing steps resulting in a variable absorbance reading, which generated a large standard deviation. We do not know the reason for this result, but it may be due to the secondary defect in motility (Yao *et al*., 2004). Presumably, the large cell aggregates formed as a result of the overabundance of succinoglycan, which holds the cells together. However, despite the ability of the *exoS96* cells to attach to each other, they do not appear to attach well to substrates. For this reason, we tested two Fla$^-$ mutants, and found as expected,

based on results from other bacteria, that they are reduced in their ability to make a biofilm due to poor adherence. This would suggest that the increase in biofilm formation by *exoS96* is due to the presence of excess EPSI. We are currently screening for Tn*5* mutants that are superior biofilmers, but that are Fla^+, to see if these are EPSI overproducers.

In *P. aeruginosa* and many other bacteria, flagella are necessary for the initial attachment to the surface for biofilm establishment (O'Toole & Kolter, 1998). We have confirmed that the motility mutants, G910 (*fliP*) and G911 (*flgH*), are impaired in biofilm formation. Furthermore, we have validated the finding that G911 lacks flagella, based on the lack of reaction to an antibody to flagellin (Figs 6g and h). Both *fliP* and *flgH* are important for Sm motility. FliP is part of the flagellar export apparatus in *E. coli* and *Salmonella typhimurium* (Ohnishi *et al*., 1997), and is absolutely essential for bacterial motility in these species. It is not known for certain whether FliP is part of the flagellar basal body in Sm. However, the Sm FliP and a homologous gene in *Agrobacterium tumefaciens* belong to the same superfamily as the *E. coli* and *Salmonella* proteins (Deakin *et al*., 1997), strongly suggesting that the FliP proteins in these plant-associated bacteria have the same function. In many bacteria, *flgH* genes are important for flagella synthesis; they encode the L-ring protein of the basal body.

Nevertheless, neither FlgH nor FliP appears to be absolutely essential for nodulation because both alfalfa and white sweetclover establish nodules, although they develop later, the earliest 24 and 48 h later, respectively, than those elicited by the wild-type Rm1021. The developmental time lag may explain why previous studies have found Sm Fla^- mutants to have a competitive disadvantage compared to the motile, wild-type strains (Ames & Bergman, 1981).

With these methods, we are able to examine the effects of other symbiotic mutations, such as nodulation mutants, as well as to screen mutant libraries for as-yet uncharacterized genes that affect the establishment of rhizobial biofilms, and ultimately, rhizobial attachment to plant root cells.

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