environmental microbiology

Environmental Microbiology (2010) **12**(12), 3185–3195 doi:10.1111/j.1462-2920.2010.02291.x

Efficient rhizosphere colonization by *Pseudomonas fluorescens* f113 mutants unable to form biofilms on

Emma Barahona,[1†] Ana Navazo,[1†] Fátima Yousef-Coronado,[2] Daniel Aguirre de Cárcer,[1] Francisco Martínez-Granero,[1] Manuel Espinosa-Urgel,[2] Marta Martín[1] and Rafael Rivilla[1*]

[1]*Departamento de Biología, Universidad Autónoma de Madrid, 28049 Madrid, Spain.*

[2]*Departamento de Protección Ambiental, Estación Experimental del Zaidín, CSIC, 18008 Granada, Spain.*

Summary

Motility is a key trait for rhizosphere colonization by *Pseudomonas fluorescens*. Mutants with reduced motility are poor competitors, and hypermotile, more competitive phenotypic variants are selected in the rhizosphere. Flagellar motility is a feature associated to planktonic, free-living single cells, and although it is necessary for the initial steps of biofilm formation, bacteria in biofilm lack flagella. To test the correlation between biofilm formation and rhizosphere colonization, we have used *P. fluorescens* F113 hypermotile derivatives and mutants affected in regulatory genes which in other bacteria modulate biofilm development, namely *gacS* (G), *sadB* (S) and *wspR* (W). Mutants affected in these three genes and a hypermotile variant (V35) isolated from the rhizosphere were impaired in biofilm formation on abiotic surfaces, but colonized the alfalfa root apex as efficiently as the wild-type strain, indicating that biofilm formation on abiotic surfaces and rhizosphere colonization follow different regulatory pathways in *P. fluorescens*. Furthermore, a triple mutant *gacSsadBwspR* (GSW) and V35 were more competitive than the wild-type strain for root-tip colonization, suggesting that motility is more relevant in this environment than the ability to form biofilms on abiotic surfaces. Microscopy showed the same root colonization pattern for *P. fluorescens* F113 and all the derivatives: extensive microcolonies, apparently held to the rhizoplane by a mucigel that seems to be plant produced. Therefore, the ability to form biofilms on abiotic surfaces does not necessarily correlates with efficient rhizosphere colonization or competitive colonization.

Received 10 February, 2010; accepted 25 May, 2010. *For correspondence. E-mail rafael.rivilla@uam.es; Tel. (+34) 914 978 188; Fax (+34) 914 978 344. †E.B and A.N. contributed equally to this work.

Introduction

The rhizosphere is the portion of soil that is influenced by plant roots and is characterized by harbouring a higher number of microorganisms than bulk soil (Hiltner, 1904). Numerous bacteria, generally termed rhizobacteria, are adapted to this ecosystem. Rhizobacteria can affect plant fitness and are also important in biotechnological applications based on integrated plant–bacteria systems. The fluorescent pseudomonads group include several species of rhizobacteria that have been used as model strains for rhizosphere colonization experiments (Lugtenberg and Dekkers, 1999; Lugtenberg *et al.*, 2001) and for applications such as biocontrol (Haas and Defago, 2005) and rhizoremediation (Yee *et al.*, 1998). *Pseudomonas fluorescens* F113 was isolated from the sugar-beet rhizosphere and is able to protect plants against the oomycete *Pythium ultimum* by means of the production of the fungicide 2, 4-diacetylphloroglucinol (DAPG) (Shanahan *et al.*, 1992). It has also been genetically modified for PCB degradation and tested in rhizoremediation experiments (Brazil *et al.*, 1995; Villacieros *et al.*, 2005; Aguirre de Cárcer *et al.*, 2007a,b). *Pseudomonas fluorescens* F113 has been shown to colonize the rhizosphere of a variety of plants including pea (Naseby and Lynch, 1999), tomato (Dekkers *et al.*, 2000), willow (Aguirre de Cárcer *et al.*, 2007a) and alfalfa (Villacieros *et al.*, 2003). Colonization studies on alfalfa have shown that this bacterium establishes on the rhizoplane (root surface) forming extensive microcolonies (Villacieros *et al.*, 2003). This type of colonization pattern has also been observed for other fluorescent pseudomonads on tomato roots (Chin-A-Woeng *et al.*, 1997).

The transition between the sessile lifestyle that biofilms represent and a planktonic, motile lifestyle is controlled in numerous bacteria by the levels of the second messenger cyclic di-GMP. These levels are in turn regulated by the activity of proteins containing diguanilate cyclase (GGDEF domains) and phosphodiesterase (EAL or

HD-GYP domains) activities (for a recent review see Hengge, 2009). Low c-di-GMP levels are associated with a free living, motile phenotype while high c-di-GMP levels lead to biofilm formation and reduced motility.

Although flagella are required for the first steps of biofilm formation (O'Toole and Kolter, 1998a), flagella-driven motility can be viewed as an opposing lifestyle to that of biofilms. In fact, expression of flagellar genes is repressed in biofilms of *P. aeruginosa* or *Bacillus subtilis* (Lazazzera, 2005). Motility has been recognized as one of the most important traits required for rhizosphere colonization by *P. fluorescens* and other bacteria, since non-motile and non-chemotactic mutants are among the most impaired in competitive root colonization (de Weert *et al*., 2002), and a wild-type level of motility is required by *P. fluorescens* for competitive rhizosphere colonization (Capdevila *et al*., 2004). The importance of motility for rhizosphere colonization by *P. fluorescens* is highlighted by the finding that the rhizosphere selects for phenotypic variants (Sanchez-Contreras *et al*., 2002), which harbour mutations in the genes encoding the two-component system GacA/GacS, and in other unidentified genes, resulting in a gradation of hypermotility phenotypes (Martínez-Granero *et al*., 2006).

In this work, we have used *P. fluorescens* F113 hypermotile phenotypic variants isolated from the rhizosphere and hypermotile mutants affected in the *gacS*, *sadB* and *wspR* genes, which have been implicated in biofilm formation, to test its importance in root colonization and competitive root colonization.

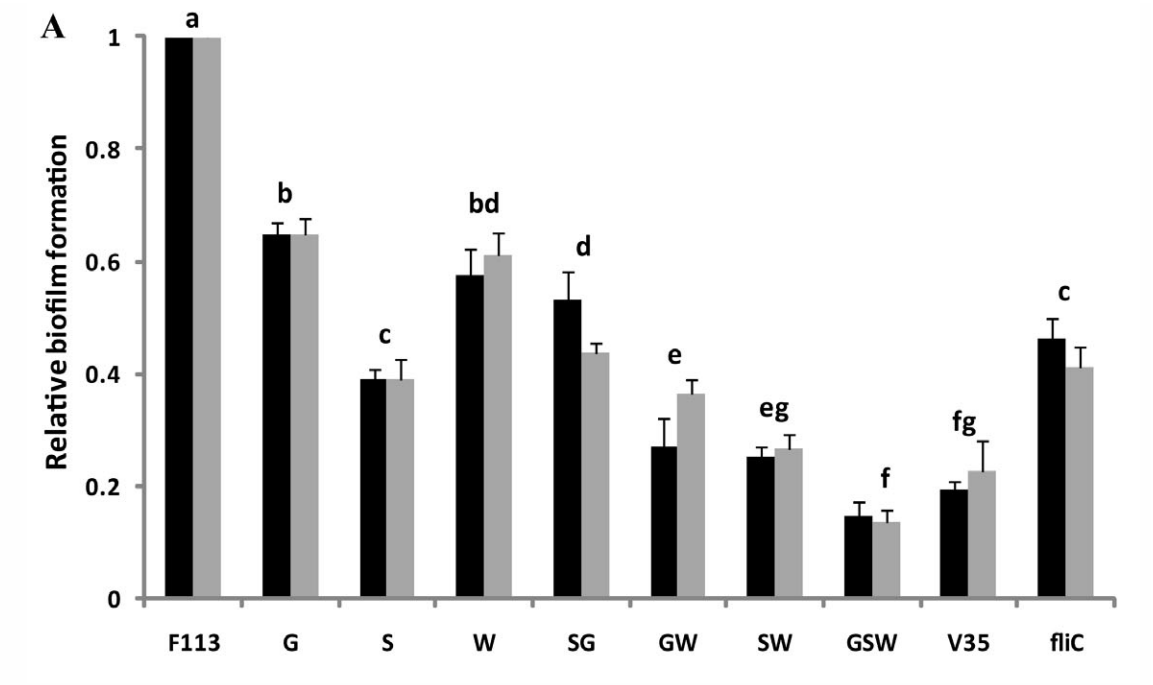
Results

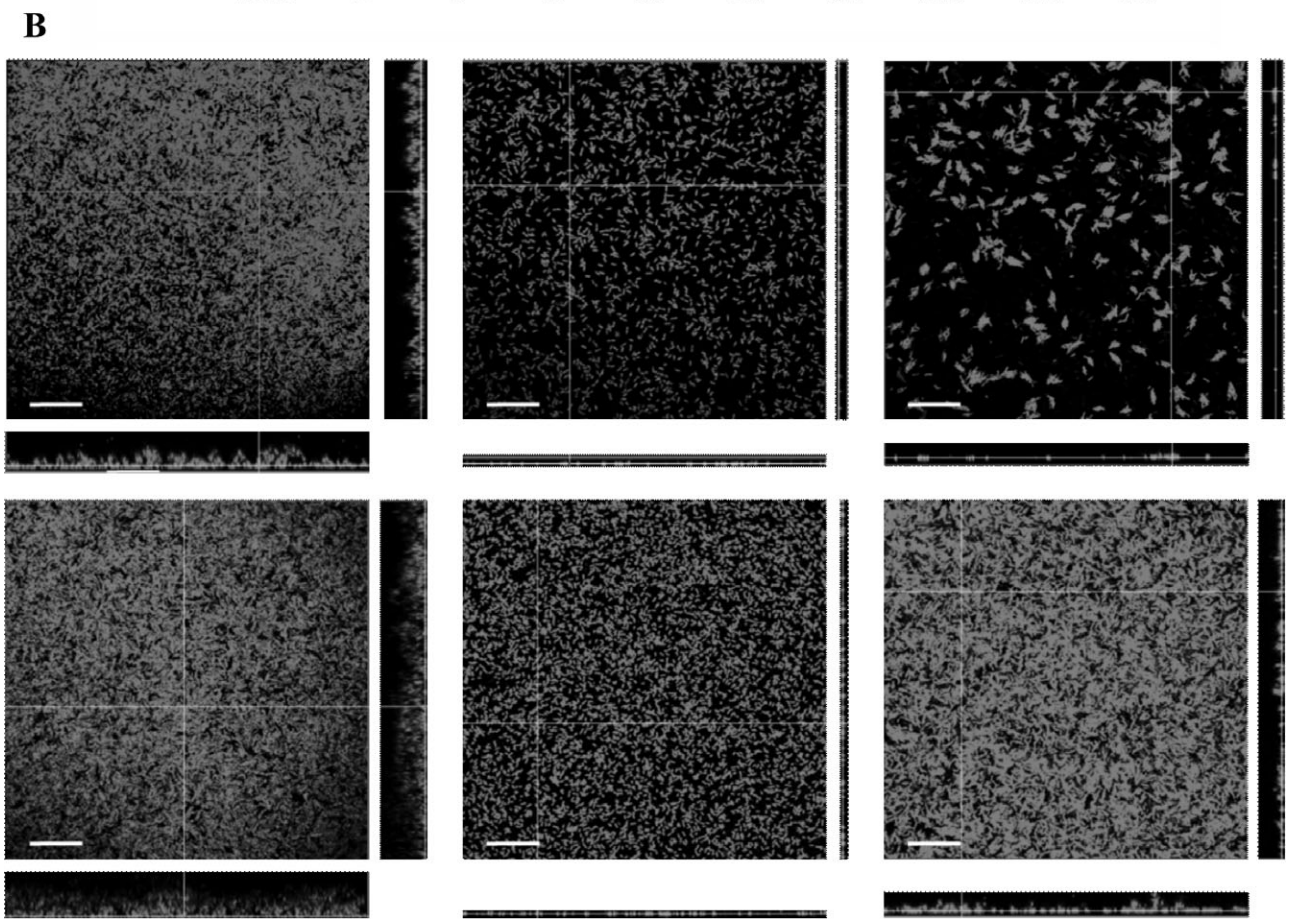
Biofilm formation by hypermotile mutants and phenotypic variants

In a previous study investigating motility repressors we found that several of the hypermotile mutants isolated were affected in genes that had been described as implicated in biofilm formation in different pseudomonads (Navazo *et al*., 2009). To investigate whether they had a similar role in *P. fluorescens* F113, the *gacS*, *wspR* and *sadB* mutants were tested for biofilm formation on abiotic surfaces using a standard crystal violet test on two different plastic surfaces. As shown in Fig. 1A, the three mutants are impaired in biofilm formation on both surfaces, showing a substantial reduction on crystal violet staining when compared with the wild-type strain. Double and triple mutants were also tested for biofilm formation and an additive phenotype was observed (Fig. 1A), except in the case of the double *sadBgacS* (SG) mutant, which shows an intermediate phenotype. However, the additive phenotype of all other mutants, including the triple *gacSsadBwspR* (GSW) mutant, suggests that the three mutants were affected in different pathways regulating biofilm formation. The GSW triple mutant was almost unable to form biofilms (five times reduction, as compared with the wild-type strain) on these surfaces and presented a swimming phenotype very similar to variant 35 (V35) (Fig. 2A), a hypermotile phenotypic variant of F113 isolated from the alfalfa rhizosphere (Martinez-Granero *et al*., 2006). For this reason we also tested V35 for biofilm formation in the same systems resulting in an almost complete inability to form biofilms (five times reduction). It is interesting to note that according to this test, GSW and V35 are more affected in biofilm formation than the non-motile, aflagellated mutant *fliC* (Capdevila *et al*., 2004), a mutant affected in initial attachment because of the lack of flagella.

Biofilm formation ability of the wild-type strain and the most affected strains (V35 and the GSW mutant) was also tested under flow conditions. Table 1 shows the parameters of the biofilms formed after 3 and 6 h by the three strains. Similarly to the static biofilm experiments, flow-cell biofilms formed by V35 and the triple mutant presented a more than 10 times reduction in biomass, average thickness and maximum thickness, compared with the wild-type strain, indicating again a severe defect on biofilm formation. According to these parameters, V35 was the most affected strain, with values between 19- and 49-folds lower than the wild-type strain, after 6 h. Confocal microscopy observation also showed that the wild-type strain (Fig. 1B) formed thick three-dimensional biofilms after 6 h. No three-dimensional structures, but only isolated attached cells were observed for V35, while the triple mutant attached forming small clusters of cells. Differences were also evident at later time points. After 24 h, the wild-type maintained a thicker, structured biofilm, whereas V35 was the most affected strain and did not progress beyond a monolayer of cells on the surface.

Fig. 1. Biofilm forming ability of *P. fluorescens* F113 and derivatives on abiotic surfaces.
A. Static biofilm assay. Biofilms were measured as the amount of crystal violet absorbed by the biofilm formed on multi-well plates and determined by absorbance at 590 nm after de-staining with ethanol (see methods). Absorbance of the wild-type strain was arbitrarily given a value of 1. Averages and standard deviations of eight replicas per strain corresponding to three independent experiments are shown. Different letters indicate a significant difference ($P < 0.05$). Black bars correspond to experiments performed on polystyrene plates and grey bars to experiments on polypropylene.
B. Flow-cell assay. Upper row from left to right F113, after 6 h; V35, after 6 h; Triple mutant GSW, after 6 h. Lower row, from left to right F113, after 24 h.; V35, after 24 h.; GSW, after 24 h. Bar represents 50 μm. Mature, three-dimensional structures were only observed in the case of the wild-type strain F113.


A
Relative biofilm formation
1
0.8
0.6
0.4
0.2
0
a
b
c
bd
d
e
eg
f
fg
c
F113
G
S
W
SG
GW
SW
GSW
V35
fliC


B

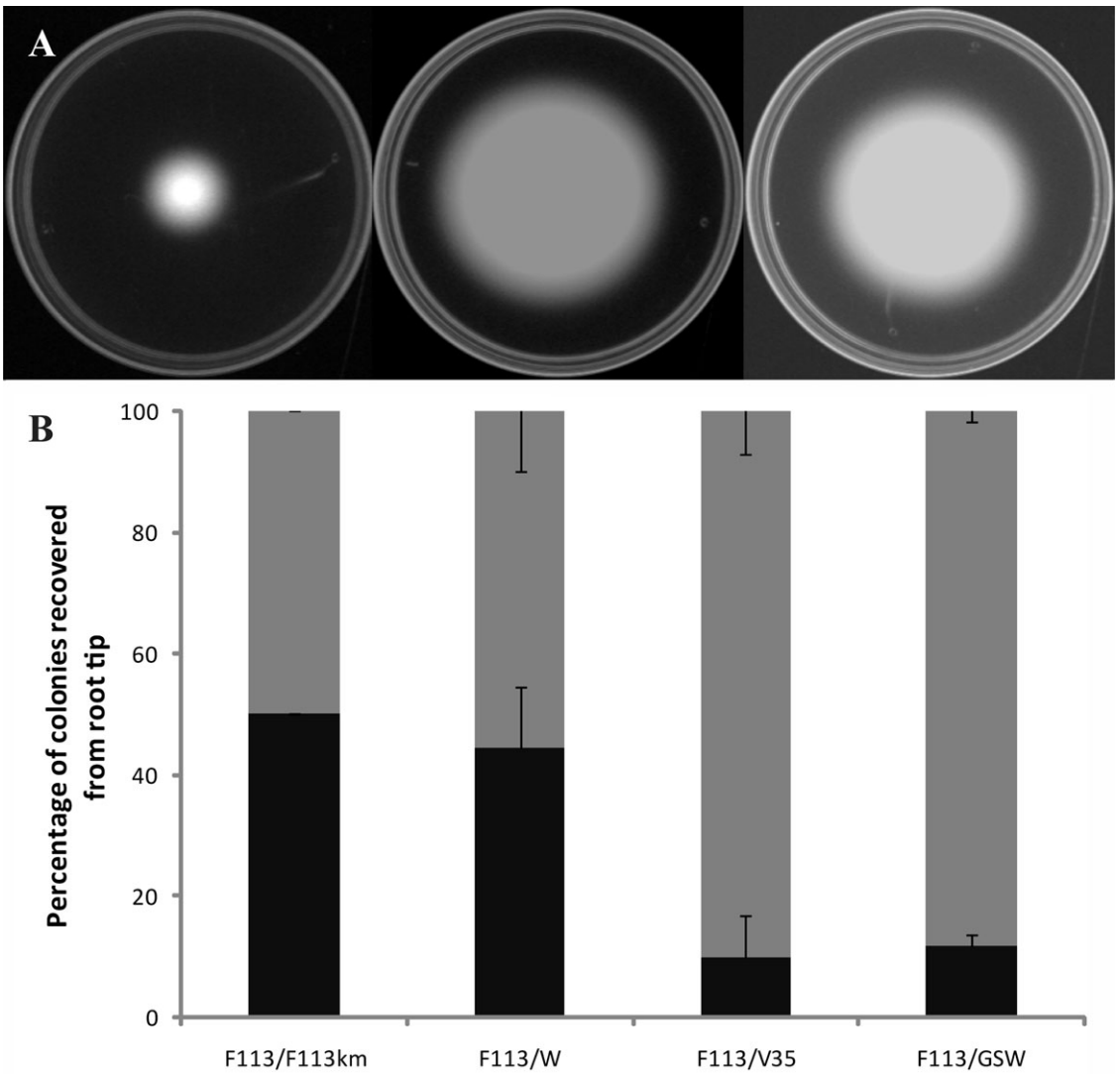


Fig. 2. Swimming motility phenotype and competitive alfalfa rhizosphere colonization by F113 derivatives.
A. Swimming motility. Haloes produced by the wild-type strain, the GSW mutant and V35 after 18 h. Bacterial cells were inoculated with a sterile toothpick just below the surface of a plate containing SA medium supplemented with 0.3% purified agar. Plates were incubated 18 h at 28°C. Average halo diameter was 14, 40 and 42 mm for the wild-type strain, the GSW mutant and V35 respectively.
B. Competitive rhizosphere colonization. The wild-type strain was used as the competitor in all the experiments. Plants were inoculated 1:1 with the test strain and the competitor and after 2 weeks root tips were collected and the bacteria present plated. Grey bars represent the percentage of colonies recovered from the tested strains; black bars represent the percentage of colonies recovered from the competitor (wild-type) strain. Arithmetic means and standard deviation are presented. The *gacS* and *sadB* mutants gave results similar to the *wspR* mutant.

Table 1. Biofilm parameters.

	F113		V35		GSW	
	3 h	6 h	3 h	6 h	3 h	6 h
Biomass (volume cells per surface)	4.40 ± 0.9	8.11 ± 3.20	0.35 ± 0.15	0.30 ± 0.08	0.09 ± 0.06	0.34 ± 0.08
Average thickness (μm)	3.56 ± 1.01	8.36 ± 2.79	0.21 ± 0.12	0.17 ± 0.06	0.06 ± 0.05	0.27 ± 0.06
Maximum thickness (μm)	25.33 ± 14.97	35.92 ± 16.85	1.69 ± 0.48	1.90 ± 0.32	5.88 ± 1.24	7.88 ± 1.73

Strain GSW showed a somewhat intermediate phenotype, showing some structure, but not forming a mature biofilm. Figures S1 and S2 show the three-dimensional reconstruction of the biofilm formed on flow-cells by the wild-type and V35. These results clearly show that the *gacA*, *sadB* and *wspR* genes are required for full biofilm formation, being implicated in independent pathways.

Rhizosphere colonization and competitive colonization

Rhizosphere colonization experiments showed that none of the mutants or the phenotypic variants was impaired in root-tip colonization (*c.* 10^7 cfu g^{-1} root-tip), and no differences with the wild-type strain were observed. No differences were observed in whole root colonization assays (data not shown). It is important to note that under these laboratory conditions even the most affected mutants in competitive colonization are able to colonize the rhizosphere when individually inoculated. This has been previously shown for non-motile (Capdevila *et al*., 2004) and non-chemotactic mutants (de Weert *et al*., 2002).

Competitive colonization experiments (Fig. 2B) between the wild-type strain and any of the single mutants showed no differences, and strains were recovered from the root tip at a 1:1 proportion. However, differences were obtained when either V35 or the GSW triple mutant were tested against the wild-type strain. As shown in Fig. 2B, these strains were able to displace the wild-type strain from the root-tip, showing that the strains that were more affected in biofilm formation on abiotic surfaces were even more competitive than the wild-type strain in root-tip colonization. These data indicate that motility, rather than the ability to form mature biofilms on abiotic surfaces, is a key trait for the competitive efficiency of *P. fluorescens* F113 with respect to root tip colonization.

To analyse if the hypermotile, biofilm-defective phenotype causes an altered colonization pattern, the wild-type strain and the most affected strain in biofilm formation (V35) were tagged with plasmids producing constitutive expression of *gfp*. Rhizosphere colonizing cells were observed by epifluorescence microscopy. Figure 3 shows that both strains form the same structures on the rhizoplane and no differences can be observed between them either in colonization sites or in the shape, size or abundance of the microcolonies. In order to analyse colonization at a higher detail, scanning electron microscopy was also used. Figure 4 shows that both strains extensively colonized the rhizoplane and no differences were observed between them. Interestingly, bacterial cells appear tightly packed in microcolonies, and no extracellular matrix, typical of a mature biofilms could be observed between bacterial cells. This extracellular matrix is usually visualized as fibrils interconnecting bacteria. No such fibrils were observed in microcolonies formed by either strain. Instead, bacteria appear to be covered and held to the plant surface by what seems to be a mucigel layer of plant origin (Chin-A-Woeng *et al*., 1997).

Discussion

Fluorescent pseudomonads have been studied as model microorganisms in their interaction with the roots (Lugtenberg and Dekkers, 1999; Lugtenberg *et al*., 2001) and have been found forming dense biofilm-like structures that occupy the rhizoplane, especially at junctions between epidermal root cells (Chin-A-Woeng *et al*., 1997; Normander *et al*., 1999; Cassidy *et al*., 2000; Ramos *et al*., 2000; Villacieros *et al*., 2003). Biofilm formation is a complex process and different strains develop different biofilm architectures, depending also on environmental conditions. Preliminary studies by O'Toole and Kolter (1998b) showed that several *P. fluorescens* mutants were unable to form fully developed biofilms because they were affected in the initial attachment step. These mutants were termed sad (surface attachment defective) and included *sadB*. The *sadB* gene encodes a protein proposed to participate in c-di-GMP sensing (Kuchma *et al*., 2007; Merrit *et al*., 2007), and it has been shown that *sadB* mutants are impaired in the transition from reversible to irreversible attachment during biofilm formation (Caiazza and O'Toole, 2004). We have shown here that in *P. fluorescens* the hypermotile *sadB* mutant (Navazo *et al*., 2009) shows reduced biofilm formation on abiotic surfaces. However, this mutant is not defective in competitive root tip colonization, and no differences in colonization were observed when compared with the wild-type strain. Similarly, Hickman and colleagues (2005) reported that mutations in the *wspR* gene, which encodes a diguanilate cyclase, resulted in reduced biofilm formation by *P. aeruginosa*. In *P. fluorescens* F113 the *wspR* mutant is hypermotile (Navazo *et al*., 2009) and we have shown here that it is also negatively affected in biofilm formation but not in rhizosphere colonization. A *sadBwspR* double mutant displays a significant reduction of its ability to form biofilms on abiotic surfaces. The fact that the phenotype of the double mutant is additive suggests that *sadB* and *wspR* influence biofilm formation by two independent pathways. A similar result was found for swimming and swarming motility (Navazo *et al*., 2009).

Besides c-di-GMP regulation of biofilm formation, other signalling pathways have been proposed to be involved in the transition to this lifestyle. The two-component system formed by GacS and GacA post-transcriptionally regulates multiple traits related to secondary metabolism. The regulatory mechanism is complex and involves the titration of RNA binding proteins (RsmAE) by small RNAs transcriptionally induced by GacA. In the absence of these small RNAs, the Rsm proteins block the translation

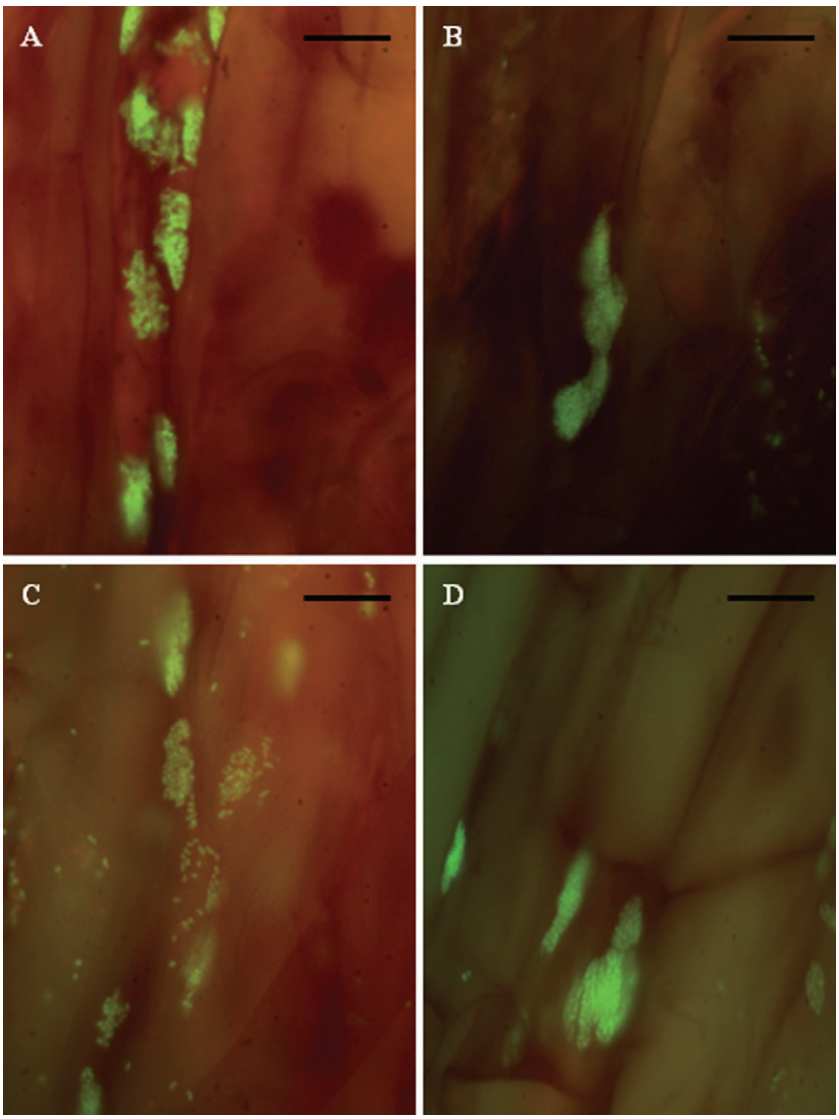


Fig. 3. Epifluorescence microscopy analysis of the colonization of the alfalfa rhizosphere by gfp-tagged F113 and V35. Roots were visualized 1 week after inoculation.
A and B. F113.
C and D. V35.
No differences in shape, size, location or number of microcolonies were observed. Bar represents 5 μm.

of specific m-RNAs. Mutants in the *gacA* and/or *gacS* genes are impaired in biofilm formation by *P. aeruginosa* (Parkins *et al*., 2001; Davies *et al*., 2007), *Pseudomonas* sp. (Choi *et al*., 2007) and a clinical strain of *P. fluorescens* (Rossignol *et al*., 2009). Here we have shown that in *P. fluorescens* F113 the Gac system is necessary for full biofilm formation. Furthermore, since a *gacSsadBwspR* triple mutant shows an additive phenotype for biofilm formation under all tested conditions, we can affirm that regulation through these three genes is independent, as we previously observed for swimming and swarming phenotypes (Navazo *et al*., 2009). We have also shown that in F113 *gacS* is not necessary for rhizosphere colonization or competitive colonization, as was previously reported for *P. chlororaphis* (Schmidt-Eisenlohr *et al*., 2003).

In a previous work (Martinez-Granero *et al*., 2006), we showed that when overexpressing the site-specific recombinases Sss and XerD and after rhizosphere passage, hypermotile phenotypic variants were selected. Here we have tested one of these variants (V35) for biofilm formation. V35 harbours a mutation on the *gacS* gene and additional un-identified mutations repressing motility, since the cloned *gacS* gene complements the swimming phenotype only partially (Martínez-Granero *et al*., 2006). We have shown here that V35 is affected in its ability to develop a fully structured mature biofilm on abiotic surfaces but not in rhizosphere colonization, either in the absence or in the presence of a wild-type competitor. Furthermore, both the triple GSW mutant and V35 were more competitive than the wild-type strain for root tip colonization. These results show that hypermotility constitutes an advantage for bacterial establishment on the root tip, an environment where the ability to form biofilms on abiotic surfaces seems not to be a relevant trait. Thus, the

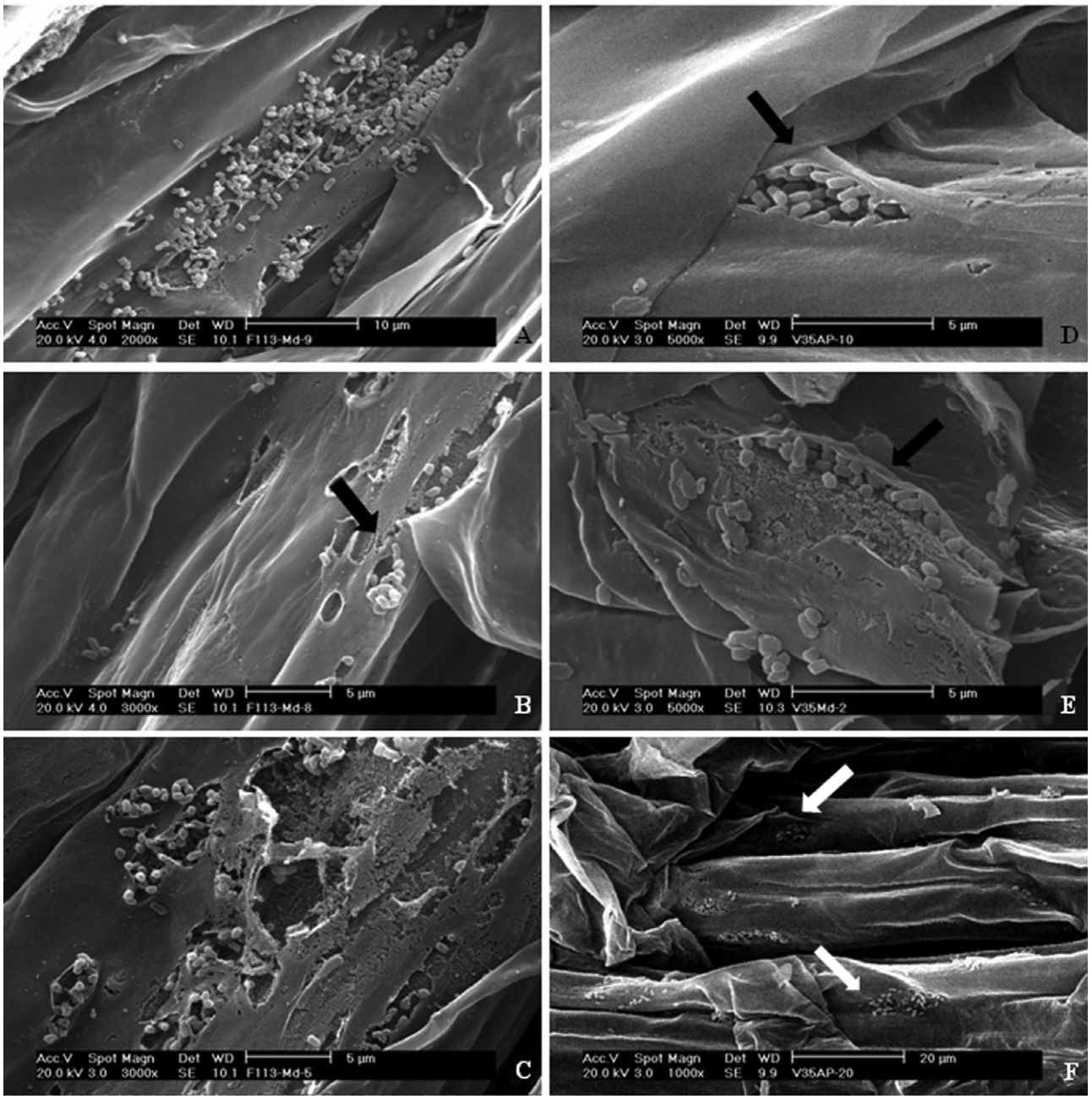


Fig. 4. Scanning microscopy analysis of the colonization of the alfalfa rhizosphere by F113 and V35.
A–C. F113.
D–F. V35.
No differences were observed in the colonization pattern of both strains. Note the mucigel layer holding the bacteria in the rhizoplane. Black arrows point to breaks in the mucigel, which allow the observation of bacterial cells. Cells are also observed when the mucigel is displaced (white arrows). Bacteria appear densely packed and no exopolysaccharide fibrils linking the bacterial cells were observed.

genetic determinants necessary to colonize abiotic surfaces and the plant rhizoplane by *P. fluorescens* appear to be different. In this sense, it has been proposed that colonization of plant and abiotic surfaces by *P. putida* follow distinct, although partially overlapping pathways (Yousef-Coronado *et al*., 2008). In *P. putida*, flagellar motility determines the distribution of cells along the root, but does not seem to have a significant impact on its overall fitness in the rhizosphere.

The results presented here also have biotechnological implications. In the selection of strains for biological control or plant growth promotion, the ability to form biofilms on abiotic surfaces is frequently tested, on the assumption that good biofilm producers will also be good

plant colonizers (Bais *et al*., 2004; Haggag and Timmusk, 2008; Rudrappa *et al*., 2008). Based on our results, this assumption is not necessarily correct, since strains unable to form biofilms on abiotic surfaces can displace isogenic, biofilm forming strains from a key colonization site as is the root tip.

A comparison between the colonization pattern of the wild-type strain and V35 did not reveal any differences. Both strains formed microcolonies on the rhizoplane, preferentially between epidermic cells, as reported before for the wild-type strain (Villacieros *et al*., 2003). Using scanning electron microscopy, we observed that bacterial cells were tightly packed in microcolonies and that the extracellular material typical of biofilms appeared to be absent. Instead, microcolonies were surrounded by a mucigel layer that holds the microcolony on the rhizoplane. Similar observations were made by Chin-A-Woeng and colleagues (1997). In that study, microcolonies surrounded by a plant derived mucigel were observed not only for *P. fluorescens*, but also for *P. mendocina*, *P. putida*, *Xantomonas oryzae* and *Acidovorax facilis.* The results presented here provide genetic evidence to these observations. However, these authors also noted that other rhizobacteria such as *Rhizobium* spp. and *Acinetobacter radioresistens* present different colonization patterns, being unable to form microcolonies on the rhizoplane. It has also been recently observed (Pliego *et al*., 2008) that two biocontrol pseudomonads show different colonization patterns on the same plant. These data highlight the differences in strategies followed by bacteria for rhizosphere colonization.

As a conclusion, the results reported here indicate that biofilms formed by *P. fluorescens* on abiotic surfaces and on the rhizoplane are not equivalent structures, since each seems to follow a different genetic programme. Therefore, the root-colonizing capacity of a bacterial strain cannot be generally inferred from its ability to form biofilms on abiotic surfaces under standard laboratory conditions.

Experimental procedures

Bacterial strains and growth conditions

Wild-type *P. fluorescens* F113 (Shanahan *et al*., 1992), F113 mutants (Navazo *et al*., 2009) and phenotypic variant 35 (V35) (Martinez-Granero *et al*., 2006) have been previously described. *Pseudomonas fluorescens* strains were grown with shaking in SA medium (Scher and Baker, 1982) overnight at 28°C, except for biofilm formation assays, which were grown in Luria–Bertani (LB) medium. *Escherichia coli* strains were grown in LB medium with shaking overnight at 37°C. When required, the followings antibiotics were added: rifampicin 100 μg ml^{-1}, spectinomycin 100 μg ml^{-1}, tetracycline 10 μg ml^{-1} (for *E. coli*) or 70 μg ml^{-1} (for *P. fluorescens*) and kanamycin 25 μg ml^{-1} (for *E. coli*) or 50 μg ml^{-1} (for *P. fluorescens*). The pDSK-GFPuv (Wang *et al*., 2007) and the pHC60 (Cheng and Walker, 1998) plasmids, expressing the green fluorescent protein (gfp), were mobilized to *P. fluorescens* by triparental mating, using pRK600 as the helper plasmid (Finan *et al*., 1986).

Biofilm formation assays in multi-well plates

A modification of the method of Fletcher (1977) was used. Exponentially growing cultures on LB medium were diluted to an OD_{600} of 0.04. Approximately 100 μl from each diluted culture was placed on a well of polyestirene or polypropylene multi-well plates that were incubated 8 h at 28°C. Approximately 25 μl of a crystal violet solution was added to each well for 15 min to allow the staining of adhered cells. Excess stain was eliminated by rinsing with water. Plates were air dried and 200 μl of 95% ethanol was added to each well in order to extract crystal violet from cells. Destaining was performed overnight with shaking. After that, colour on plates was measured at OD_{590} with a microtiter plate reader. Every assay was performed eight times for each strain.

Biofilm formation in flow cells

Plasmids pDSK-GFPuv (Wang *et al*., 2007) or pHC60 (Cheng and Walker, 1998) were introduced in *P. fluorescens* strains by triparental mating and green fluorescence due to the presence of *gfp* was visualized by epifluorescence microscopy. A modification of the method of Sternberg and Tolker-Nielsen (Gjermansen *et al*., 2005) was used for biofilm formation under flow conditions, using LB diluted 1:10 as growth medium. Biofilms were grown at 30°C in three-chanel flow chambers (BioCentrum-DTU, Technical University of Denmark), using a Watson Marlow 205S peristaltic pump (Watson Marlow, Wilmington, MA, USA). Fresh overnight cultures were diluted to an OD_{600} of 0.5, and 300 μl was injected in the flow chamber. During the first hour, the flow was turned off in order to allow cells attach to the chamber; then, the flow was turned on and kept at a constant flow rate of 3.0 ml h^{-1}, which corresponds to a Reynolds number of 0.02 (laminar flow conditions). Biofilm structures were visualized after 3, 6 and 24 h of growth with a Nikon C1 confocal laser scanning microscope. Images were analysed with the Imaris software (Bitplane), and biofilm parameters (biovolume, distribution, average thickness and maximum thickness) were calculated using COMSTAT (Heydorn *et al*., 2000). All data were compared using the statistics application software SPSS.

Rhizosphere colonization and competition assays

Alfalfa seeds were surface sterilized in 70% ethanol for 2 min and then in diluted bleach (1:5) for 15 min and rinsed thoroughly with sterile distilled water. Seed vernalization was performed at 4°C for 16 h followed by incubation in darkness at 28°C for 1 day. Germinated alfalfa seeds were sown in Leonard jar gnotobiotic systems (Villacieros *et al*., 2003) using perlite as the solid substrate and 8 mM KNO_3 supplemented FP (Fahraeus, 1957) as the mineral solution. After 2 days, alfalfa seeds were inoculated with *c.* 10^8 cells of the appropriate strains. In competition experiments, strains were

inoculated at a 1:1 ratio. Plants were maintained under controlled conditions (16 h in the light at 25°C and 8 h in the dark at 18°C) for 2 weeks. Whole plants were carefully extracted from the perlite and roots were excised and thoroughly washed with water to eliminate any perlite particle remaining attached to the root, or loosely attached bacteria. Bacteria were recovered from the rhizosphere by vortexing the roots tips (last centimetre of the main root) for 2 min in a tube containing FP medium and plating the appropriate dilutions on SA plates with the appropriate antibiotics. Every experiment was performed three times with three replicates each times, and every replicate contained at least 20 pooled plants.

Fluorescence microscopy

pDSK-GFPuv plasmid (Wang *et al*., 2007), harbouring a gene expressing green fluorescent protein, was introduced into *P. fluorescens* F113 and V35 by triparental mating. Alfalfa seeds were independently inoculated with *c*. 10^8 of either strain, and were grown as described above. After 7 days, plants were collected, the root system excised and stained with 0.5% crystal violet during 1 min. Antifading (Vectashield, Vector Laboratories) was added and slides were assembled. Slides were observed with an optical epifluorescence microscope (OLYMPUS BH2-RFCA) with a Leica-MTV-3 camera. Rhizosphere colonization fluorescence images of both *pseudomonas* strains were captured with 40× or 100× objectives. Images of GFP-labelled bacterial cells were obtained by using a filter set consisting of a 400 to 490 nm (BP490) bandpass exciter, a 505 nm dicroic filter and a 530 nm longpass emitter (EO530).

Scanning electron microscopy

Alfalfa plants were independently inoculated with the wild-type strain and the phenotypic variant V35 and grown for 7 days as indicated above. Plants were collected and the roots were excised. The main root was divided in three sections, proximal, middle and root-tip and were fixed in 2.5 % glutaraldehyde for 2 h, washed twice with 0.2 M sodium cocodylate (pH = 7.1) during 30 min and dehydrated through increasing ethanol concentrations (10%, 30%, 50%, 70%, 90% and 100%). The fixed roots were subjected to critical point, mounted on stubs and gold coated. Samples were visualized using a scanning electron microscope Philips XL30.

Acknowledgements

We are grateful to Juan de Dios Alché for assistance with confocal laser microscopy and to Unidad de Microscopía Electrónica de Barrido (SiDI-UAM) for assistance with scanning microscopy. A.N. and F.Y.C. were recipients of a FPI fellowship from Ministerio de Ciencia e Innovación (MICINN, Spain). E.B and D.A.C. were recipients of a postgraduate research contract from Comunidad de Madrid. Research was funded by grants BIO2006-08596, BIO2009-08254 and BFU2007-64270 from MICINN and the research program MICROAMBIENTE-CM from Comunidad de Madrid.

References

Aguirre de Cárcer, D., Martín, M., Mackova, M., Macek, T., Karlson, U., and Rivilla, R. (2007a) The introduction of genetically modified microorganisms designed for rhizoremediation induces changes on native bacteria in the rhizosphere but not in the surrounding soil. *ISME J* **1:** 215–223.

Aguirre de Cárcer, D., Martín, M., Karlson, U., and Rivilla, R. (2007b) Changes in bacterial populations and in biphenyl dioxygenase gene diversity in a polychlorinated biphenyl-polluted soil after introduction of willow trees for rhizoremediation. *Appl Environ Microbiol* **73:** 6224–6232.

Bais, H.P., Fall, R., and Vivanco, J.M. (2004) Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. *Plant Physiol* **134:** 307–319.

Brazil, G.M., Kenefick, L., Callanan, M., Haro, A., de Lorenzo, V., Dowling, D.N., and O'Gara, F. (1995) Construction of a rhizosphere pseudomonad with potential to degrade polychlorinated-byphenyls and detection of BPH gene-expression in the rhizosphere. *Appl Environ Microbiol* **61:** 1946–1951.

Caiazza, N.C., and O'Toole, G.A. (2004) SadB is required for the transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas aeruginosa* PA14. *J Bacteriol* **186:** 4476–4485.

Capdevila, S., Martínez-Granero, F., Sánchez-Contreras, M., Rivilla, R., and Martín, M. (2004) Analysis of *Pseudomonas fluorescens* F113 genes implicated in flagellar filament synthesis and their role in competitive root colonization. *Microbiology* **150:** 3889–3897.

Cassidy, M.B., Leung, K.T., Lee, H., and Treyors, J.T. (2000) A comparison of enumeration methods for culturable *Pseudomonas fluorescens* cells marked with green fluorescent protein. *Microbiol Methods* **40:** 135–145.

Cheng, H.P., and Walker, G.C. (1998) Succinoglycan is required for initiation and elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*. *J Bacteriol* **180:** 5183–5191.

Chin-A-Woeng, T.F.C., de Priester, W., van der Bij, A.J., and Lugtenberg, B.J. (1997) Description of the colonization of a gnotobiotic tomato rhizosphere by *Pseudomonas fluorescens* biocontrol strain WCS365, using scanning electron microscopy. *Mol Plant Microbe Interact* **10:** 79–86.

Choi, K.S., Veeraraqouda, Y., Cho, K.M., Lee, S.O., Jo, G.R., Cho, K., and Lee, K. (2007) Effect of *gacS* and *gacA* mutations on colony architecture, surface motility, biofilm formation and chemical toxicity in *Pseudomonas* sp. KL 28. *J Microbiol* **45:** 492–498.

Davies, J.A., Harrison, J.J., Marques, L.L., Foglia, G.R., Stremick, C.A., Storey, D.G., *et al*. (2007) The GacS sensor kinase controls phenotypic reversion of small colony variants isolated from biofilms of *Pseudomonas aeruginosa* PA14. *FEMS Microbiol Ecol* **59:** 32–46.

Dekkers, L.C., Mulders, I.H.M., Phoelich, C.C., Chin-A-Woeng, T.F.C., Wijfjes, A.H.M., and Lugtenberg, B.J.J. (2000) The *sss* colonization gene of the tomato-*Fusarium oxysporum F*. sp *radicis-lycopersici* biocontrol strain *Pseudomonas fluorescens* WCS365 can improve root colonization of other wild-type *Pseudomonas* spp. bacteria. *Mol Plant Microbe Interact* **13:** 1177–1183.

Fahraeus, G. (1957) The infection of clover root hairs by nodule bacteria studied by simple glass technique. *J Genet Microbiol* **16:** 374–381.

Finan, T.M., Kunkel, B., de Vos, G.F., and Signer, E.R. (1986) Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. *J Bacteriol* **167:** 66–72.

Fletcher, A. (1977) The effects of culture concentration and age, time and temperature on bacterial attachment to polystyrene. *Can J Microbiol* **23:** 1–6.

Gjermansen, M., Ragas, P., Sternberg, C., Molin, S., and Tolker-Nielsen, T. (2005) Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms. *Environ Microbiol* **7:** 894–906.

Haas, D., and Defago, G. (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat Rev Microbiol* **4:** 307–319.

Haggag, W.M., and Timmusk, S. (2008) Colonization of peanut roots by biofilm-forming Paenibacillus polymyxa initiates biocontrol against crown rot disease. *J Appl Microbiol* **104:** 961–969.

Hengge, R. (2009) Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* **7:** 263–273.

Heydorn, A., Nielsen, A.T., Hentzer, M., Sternberg, C., Givskov, M., Ersbøll, B.K., and Molin, S. (2000) Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* **146:** 2395–2407.

Hickman, J.W., Tifrea, D.F., and Harwood, C.S. (2005) A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. *Proc Natl Acad Sci USA* **102:** 14422–14427.

Hiltner, L. (1904) Über neuere Erfahrungen und Probleme auf dem Gebiete der Bodenbakteriologie unter besonderer Berücksichtigung der Gründüngung und der Brache. *Arb DLG* **98:** 59–78.

Kuchma, S.L., Brothers, K.M., Merritt, J.H., Liberati, N.T., Ausubel, F.M., and O'Toole, G.A. (2007) BifA a cyclic-Di-GMP phosphodiesterase, inversely regulates biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14. *J Bacteriol* **189:** 8165–8178.

Lazazzera, B. (2005) Lessons from DNA microarray analysis: the gene expression profile of biofilms. *Curr Opin Microbiol* **8:** 222–227.

Lugtenberg, B.J., and Dekkers, L.C. (1999) What makes *Pseudomonas* bacteria rhizosphere competent? *Environ Microbiol* **1:** 9–13.

Lugtenberg, B.J., Dekkers, L.C., and Bloemberg, G.V. (2001) Molecular determinants of rhizosphere colonization by *Pseudomonas*. *Ann Rev Phytopathol* **39:** 461–490.

Martínez-Granero, F., Rivilla, R., and Martín, M. (2006) Rhizosphere selection of highly motile phenotypic variants of *Pseudomonas fluorescens* with enhanced competitive colonization ability. *Appl Environ Microbiol* **72:** 3429–3434.

Merritt, J.H., Brothers, K.M., Kuchma, S.L., and O'Toole, G.A. (2007) SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. *J Bacteriol* **189:** 8154–8164.

Naseby, D.C., and Lynch, J.M. (1998) Impact of wild-type and genetically modified *Pseudomonas fluorescens* on soil enzyme activities and microbial population structure in the rhizosphere of pea. *Mol Ecol* **7:** 617–625.

Navazo, A., Barahona, E., Redondo-Nieto, M., Martínez-Granero, F., Rivilla, R., and Martín, M. (2009) Three independent signalling pathways repress motility in *Pseudomonas fluorescens* F113. *Microb Biotechnol* **2:** 489–498.

Normander, B., Hendriksen, N.B., and Nybroe, O. (1999) Green fluorescent protein-marked *Pseudomonas fluorescens*: localization, viability, and activity in the natural barley rhizosphere. *Appl Environ Microbiol* **65:** 4646–4651.

O'Toole, G.A., and Kolter, R. (1998a) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* **30:** 295–304.

O'Toole, G.A., and Kolter, R. (1998b) Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol* **28:** 449–461.

Parkins, M.D., Ceri, H., and Storey, D.G. (2001) *Pseudomonas aeruginosa* GacA, a factor in multihost virulence, is also essential for biofilm formation. *Mol Microbiol* **40:** 1215–1226.

Pliego, C., de Weert, S., Lamers, G., de Vicente, A., Bloemberg, G., Cazorla, F.M., and Ramos, C. (2008) Two similar enhanced root-colonizing *Pseudomonas* strains differ largely in their colonization strategies of avocado roots and *Rosellinia necatrix* hyphae. *Environ Microbiol* **10:** 3295–3304.

Ramos, C., Molina, L., Mølbak, L., Ramos, J.L., and Molin, S. (2000) A bioluminescent derivative of *Pseudomonas putida* KT2440 for delibérate release into the environment. *FEMS Microbiol Ecol* **34:** 91–102.

Rossignol, G., Sperandio, D., Guerrillon, J., Duclairoir Poc, C., Soum-Soutera, E., Orange, N., *et al*. (2009) Phenotypic variation in the *Pseudomonas fluorescens* clinical strain MFN1032. *Res Microbiol* **160:** 337–344.

Rudrappa, T., Biedrzycki, M.L., and Bais, H.P. (2008) Causes and consequences of plant associated biofilms. *FEMS Microbiol Ecol* **64:** 153–166.

Sánchez-Contreras, M., Martín, M., Villacieros, M., O'Gara, F., Bonilla, I., and Rivilla, R. (2002) Phenotypic selection and phase variation occur during alfalfa root colonization by *Pseudomonas fluorescens* F113. *J Bacteriol* **184:** 1587–1596.

Scher, F.M., and Baker, R. (1982) Effects of *Pseudomonas putida* and a synthetic iron chelator on induction of soil supressiveness to *Fusarium* wilt pathogens. *Phytopathology* **72:** 1567–1573.

Schmidt-Eisenlhr, H., Gast, A., and Baron, C. (2003) Inactivation of *gacS* does not affect the competitiveness of *Pseudomonas chlororaphis* in the *Arabidopsis thaliana* rhizosphere. *Appl Environ Microbiol* **69:** 1817–1826.

Shanahan, P., Borro, A., O'Gara, F., and Glennon, J.D. (1992) Isolation, trace enrichment and liquid-chromatographic analysis of diacetylphloroglucinol in culture and soil samples using UV and amperometric detection. *J Chromatogr* **606:** 171–177.

Villacieros, M., Power, B., Sánchez-Contreras, M., Lloret, J., Oruezábal, R.I., Martín, M., *et al*. (2003) Colonization behaviour of *Pseudomonas fluorescens* and *Sinorhizobium meliloti* in the alfalfa (*Medicago sativa*) rhizosphere. *Plant Soil* **251:** 47–54.

Villacieros, M., Whelan, C., Mackova, M., Molgaard, J., Sánchez-Contreras, M., Lloret, J., *et al*. (2005) Polychlorinated biphenyl rhizoremediation by *Pseudomonas fluorescens* F113 derivatives, using a *Sinorhizobium meliloti* nod system to drive *bph* gene expression. *Appl Environ Microbiol* **71:** 2687–2694.

Wang, K., Kang, L., Anand, A., Lazarowits, G., and Mysore, K.S. (2007) Monitoring in planta bacterial infection at both cellular and whole-plant levels using the green fluorescent protein variant GFPuv. *New Phytol* **174:** 212–223.

de Weert, S., Vermeiren, H., Mulders, I.H.M., Kuiper, I., Hendrickx, N., Bloemberg, G.V., *et al*. (2002) Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Psuedomonas fluorescens*. *Mol Plant Microbe Interact* **15:** 1173–1180.

Yee, D.C., Maynard, J.A., and Wood, T.K. (1998) Rhizoremediation of trichloroethylene by a recombinant, root-colonizing *Pseudomonas fluorescens* strain expressing toluene ortho-monooxygenase constitutively. *Appl Environ Microbiol* **64:** 112–118.

Yousef-Coronado, F., Travieso, M.L., and Espinosa-Urgel, M. (2008) Different, overlapping mechanisms for colonization of abiotic and plant surfaces by *Pseudomonas putida*. *FEMS Microbiol Lett* **288:** 118–124.

Supporting information

Additional Supporting information may be found in the online version of this article:

Fig. S1. 3-D reconstruction of the biofilm formed by *Pseudomonas fluorescens* F113 in flow cells after 24 h observed by confocal laser microscopy.
Fig. S2. 3-D reconstruction of the biofilm formed by *Pseudomonas fluorescens* F113 Variant 35 in flow cells after 24 h observed by confocal laser microscopy.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.