Microbiology (2004), **150**, 3889–3897 DOI 10.1099/mic.0.27362-0

Analysis of *Pseudomonas fluorescens* F113 genes implicated in flagellar filament synthesis and their role in competitive root colonization

Silvia Capdevila, Francisco M. Martínez-Granero, María Sánchez-Contreras, Rafael Rivilla and Marta Martín

Departamento de Biología, Universidad Autónoma de Madrid, Campus de Cantoblanco, 28049 Madrid, Spain

Correspondence
Marta Martín
m.martin@uam.es

Received 27 May 2004
Revised 30 June 2004
Accepted 2 July 2004

The ability of plant-associated micro-organisms to colonize and compete in the rhizosphere is specially relevant for the biotechnological application of micro-organisms as inoculants. Pseudomonads are one of the best root colonizers and they are widely used in plant-pathogen biocontrol and in soil bioremediation. This study analyses the motility mechanism of the well-known biocontrol strain *Pseudomonas fluorescens* F113. A 6·5 kb region involved in the flagellar filament synthesis, containing the *fliC*, *flaG*, *fliD*, *fliS*, *fliT* and *fleQ* genes and part of the *fleS* gene, was sequenced and mutants in this region were made. Several non-motile mutants affected in the *fliC*, *fliS* and *fleQ* genes, and a *fliT* mutant with reduced motility properties, were obtained. These mutants were completely displaced from the root tip when competing with the wild-type F113 strain, indicating that the wild-type motility properties are necessary for competitive root colonization. A mutant affected in the *flaG* gene had longer flagella, but the same motility and colonization properties as the wild-type. However, in rich medium or in the absence of iron limitation, it showed a higher motility, suggesting the possibility of improving competitive root colonization by manipulating the motility processes.

INTRODUCTION

The study of rhizosphere colonization by micro-organisms is crucial for the efficient application of bacteria as inoculants, both in agricultural and in environmental biotechnology processes. *Pseudomonas* spp. can colonize the roots of a wide range of plants (Simons *et al.*, 1996; Naseby & Lynch, 1998; Villacieros *et al.*, 2003), being one of the best root colonizers, and are used as a model in root-colonization studies (Bloemberg *et al.*, 2000; Chin-a-Woeng *et al.*, 2000). The rhizosphere is a complex environment that supports a large and metabolically active microbial population, several orders of magnitude higher than the non-rhizospheric soil. Many bacterial genes and traits have been shown to be involved in plant-root colonization (Lugtenberg & Dekkers, 1999; Rainey, 1999; Lugtenberg *et al.*, 2001). However, not only colonization but also the pseudomonads' ability to compete with the indigenous microbial population are essential to improve their biotechnological applications in the rhizosphere environment.

The soil-borne fluorescent pseudomonads are used as biocontrol inoculants because of their ability to produce some antifungal metabolites (Dowling & O'Gara, 1994; Walsh *et al.*, 2001). Other applications of pseudomonads include soil biofertilization and rhizoremediation (Ramos *et al.*, 1991; Brazil *et al.*, 1995; Höflich *et al.*, 1995; Yee *et al.*, 1998).

The strain *Pseudomonas fluorescens* F113 was isolated from the sugarbeet rhizosphere and it is used as a biocontrol agent against the fungal pathogen *Pythium ultimum*, which causes damping-off disease in sugarbeet seedlings. The biocontrol abilities of this strain are due mainly to the production of the antifungal metabolite DAPG (2,4-diacetylphloroglucinol) (Shanahan *et al.*, 1992). *P. fluorescens* F113 has also been genetically modified, by introducing the *bph* genes that encode the biphenyl degradative pathway, to be used in rhizoremediation of polychlorinated biphenyls (Brazil *et al.*, 1995; Karlson *et al.*, 1998). The efficacy of *P. fluorescens* F113 as inoculant clearly depends on its capacity to compete and efficiently colonize the rhizosphere.

Motility seems to be very important in colonization since non-motile mutants of different *P. fluorescens* strains are severely affected in the root colonization. The defect was larger at sites more distant from the inoculation site, in the root systems formed after the bacterial inoculation (De Weger *et al.*, 1987; Dekkers *et al.*, 1998b; Chin-a-Woeng *et al.*, 2000). Furthermore motility-impaired mutants of *Pseudomonas chlororaphis* PCL1391 do not reduce the

The GenBank/EMBL/DDBJ accession number for the 6·5 kb region sequence reported in this paper is AF399739.

disease produced by *Fusarium oxysporum* on tomato plants (Chin-a-Woeng *et al.*, 2000). Therefore, motility is required to colonize growing roots successfully and to maintain the biocontrol capacities.

The objective of this work was to study the phenotype of mutations affecting the flagellar filament synthesis in *P. fluorescens* F113 and their influence on motility and root competitive colonization. Reports to date refer to mutants that are either aflagellate (De Weger *et al.*, 1987) or deficient in chemotaxis (de Weert *et al.*, 2002). The mutants obtained in this work are not affected in chemotaxis but they are affected in motility to different degrees. We show that wild-type motility properties are necessary for competitive rhizosphere colonization.

METHODS

Bacterial strains, plasmids and growth conditions. The strains and plasmids used in this study are described in Table 1. *P. fluorescens* F113 was originally isolated from the sugarbeet rhizosphere (Shanahan *et al.*, 1992). The F113 gene bank was constructed with partially *Eco*RI-digested genomic DNA cloned into plasmid pLAFR3 in *Escherichia coli* LE392. *Pseudomonas* strains were grown on SA medium (Scher & Baker, 1982) at 28 °C; solid growth media contained 1·5 % (w/v) purified agar. When appropriate, kanamycin, gentamicin and rifampicin were supplemented for antibiotic selection to a final concentration of 50, 10 and 100 μg ml^{-1}, respectively. *E. coli* strains were grown at 37 °C in Luria–Bertani (LB) medium (Bertani, 1951), and antibiotics were added at the following concentrations when required: kanamycin, 25 μg ml^{-1}; gentamicin, 10 μg ml^{-1}.

DNA techniques. Standard techniques for subcloning procedures, plasmid preparations and agarose gel electrophoresis were used as described by Sambrook *et al.* (1989). Southern blot hybridizations were performed with a non-radioactive detection kit, and a chemiluminiscence method was used to detect hybridization bands according to the manufacturer's instructions (Roche Diagnostics). DNA sequencing was done by the chain-termination method using DyeDeoxy terminator cycle sequencing kit protocol as described by the manufacturer (Applied Biosystems). Homology search and sequence analysis were done using the software from the Genetics Computer Group.

Construction of mutants. Insertional mutagenesis has been used to generate mutants by single homologous recombination. Amplified internal fragments from the different flagellar filament synthesis genes were cloned into the kanamycin-resistant plasmid pVIK112 (Kalogeraki & Winans, 1997) and introduced into wild-type F113 by triparental mating using pRK2013 as the helper plasmid (Figurski & Helinski, 1979). Mutants resulting from single homologous recombination were checked by Southern blotting using probes from the interrupted genes, and by PCR using primers designed from the genes and the pVIK112 plasmid sequences (the primer sequences are available on request). Mutant complementation analysis was done by cloning each intact gene under the control of the *nptII* strong promoter into plasmid pML122 (Labes *et al.*, 1990) and introducing the recombinant plasmid into the corresponding mutant strain by

Table 1. Strains and plasmids used in this study

Strain/plasmid	Description	Reference or source
Strains		
DH5α	General-purpose *Escherichia coli* strain	Gibco-BRL
F113rif	*Pseudomonas fluorescens* wild-type strain, Rif^r	Shanahan *et al.* (1992)
F113-fliC	F113rif *fliC* mutant, Rif^r Km^r	This work
F113-flaG	F113rif *flaG* mutant, Rif^r Km^r	This work
F113-fliS	F113rif *fliS* mutant, Rif^r Km^r	This work
F113-fliT	F113rif *fliT* mutant, Rif^r Km^r	This work
F113-fleQ	F113rif *fleQ* mutant, Rif^r Km^r	This work
F113(pBG1364)	F113rif containing plasmid with *fliC* under the control of the *nptII* promoter, Rif^r Km^r Gm^r	This work
F11(pBG1308)	F113rif containing plasmid with *fliS* under the control of the *nptII* promoter, Rif^r Km^r Gm^r	This work
F113(pBG1307)	F113rif containing plasmid with *fliT* under the control of the *nptII* promoter, Rif^r Km^r Gm^r	This work
F113(pBG1259)	F113rif containing plasmid with *fleQ* under the control of the *nptII* promoter, Rif^r Km^r Gm^r	This work
Plasmids		
pVIK112	Suicide vector, Km^r	Kalogeraki & Winans (1997)
pRK2013	Helper plasmid used in triparental matings, Km^r	Figurski & Helinski (1979)
pML122	RSF1010 derivative expression vector, Gm^r Km^r	Labes *et al.* (1990)
pBG1327	pVIK112 with an internal fragment of *fliC* used to generate mutant F113-fliC, Km^r	This work
pBG1342	pVIK112 with an internal fragment of *flaG* used to generate mutant F113-flaG, Km^r	This work
pBG1291	pVIK112 with an internal fragment of *fliS* used to generate mutant F113-fliS, Km^r	This work
pBG1339	pVIK112 with an internal fragment of *fliT* used to generate mutant F113-fliT, Km^r	This work
pBG1290	pVIK112 with an internal fragment of *fleQ* used to generate mutant F113-fleQ, Km^r	This work

triparental mating. Then, in order to correlate the strain phenotype with the interrupted gene, disappearance of the mutant phenotype was analysed.

Transmission electron microscopy. Formvar-coated grids were placed on the top of a drop of bacterial cells for 30 s to allow bacterial adhesion. Grids were stained for 1 min with a 1 % solution of potassium phosphotungstate and washed for 1 min with a drop of water. Flagellum length was measured with the Q-Win software (Leica).

Swimming assays. SA (Scher & Baker, 1982), LB (Bertani, 1951) and iron-supplemented SA medium plates containing 0·3 % purified agar were used to test the swimming abilities of wild-type F113 and the different mutants. The cells were inoculated in the middle of the plate, in triplicate, using a toothpick, from exponentially growing cultures. Swimming haloes were measured after 18, 24 and 42 h inoculation. Every assay was done at least three times.

Colonization experiments. Alfalfa seeds were sterilized in 70 % ethanol for 2 min and in diluted bleach (1 : 5, v/v) for 15 min and rinsed thoroughly with sterile distilled water. Seeds were germinated 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 using Perlite as the solid substrate and 8 mM KNO_3-supplemented FP (Fahraeus, 1957) as the mineral solution. After 2 days, alfalfa seedlings were inoculated with $\sim 10^8$ cells of the appropriate strain. For the competitive colonization experiments, the tested strain and the competitor were inoculated at a ratio of 1 : 1. Plants were maintained for 3 weeks in a plant growth cabinet in the following controlled conditions: 16 h of light at 25 °C and 8 h of dark at 18 °C. Bacteria were recovered from the last centimetre of the main root by vortexing for 2 min in 5 ml of 0·9 % NaCl and appropriate dilutions were plated in SA supplemented with the selective antibiotics. The mean of recovered bacteria per g of root tip was $2{\cdot}48 \times 10^7$, the range being from $1{\cdot}35 \times 10^6$ to $2{\cdot}96 \times 10^8$. Colonization experiments were done three times in triplicate with at least 20 plants per replica.

Protein extraction and Western blots. Proteins were extracted from 200 ml cultures grown for 2 days. In order to detach the flagellar filaments, the cultures were agitated by vortexing for 2 min and then centrifuged for 20 min at 12 000 r.p.m. Total proteins were extracted from the pellet with Laemmli buffer (Laemmli, 1970) and extracellular proteins were extracted from the supernatant, by precipitation for 16 h at 4 °C with 10 % (w/v) TCA, followed by two washes with acetone, and were finally resuspended in Laemmli buffer. Proteins were electrophoresed in 12 % acrylamide gels and stained with Coomassie blue. The same electrophoretic conditions were used for Western blotting. Gels were transferred to nitrocellulose membranes and incubated with 1 : 10 000 dilution of an antiflagellin antiserum (Dekkers *et al.*, 1998a) and with a peroxidase-tagged secondary antibody (anti-rabbit immunoglobulin). In the dot-blot experiments, the culture was agitated by vortexing and was centrifuged to separate the flagellar filaments. A drop from the pellets obtained was transferred to the nitrocellulose membrane and incubated with the antiflagellin antiserum in the same conditions as described above.

RESULTS

Characterization of a genetic region containing genes involved in flagellar filament synthesis

Fig. 1 shows the genetic organization of a 6·5 kb DNA region from the *P. fluorescens* F113 genome that was isolated from a cosmid that contained the *fliC* gene, from an F113 gene bank (Sanchez-Contreras *et al.*, 2002). This region also contains another five ORFs and a partial ORF. Two of these complete ORFs and the partial ORF show high homology and synteny with the previously described genes *fliD*, encoding the filament cap protein, *fleQ* (*adnA*), encoding a master transcriptional regulator, and *fleS*, encoding a two-component sensor protein, respectively. These genes have been found in every pseudomonad analysed (Arora *et al.*, 2000; Dasgupta *et al.*, 2002, 2003; Robleto *et al.*, 2003) and have been thoroughly characterized. Downstream of the *fliC* gene, there is a small ORF homologous to ORFs with the same gene context in other pseudomonads. This ORF shows homology with the *flaG* gene of *Vibrio anguillarum* (McGee *et al.*, 1996), which affects filament length through an unknown mechanism. Downstream of the *fliD* gene and after an AT-rich intergenic region there are two other small ORFs that show limited homology with the PA1095 and PA1096 genes in the *Pseudomonas aeruginosa* PAO1 genome (Stover *et al.*, 2000). The first ORF sequence shows high homology (77–79 % identity) with the FliS protein in *P. fluorescens* Pf0-1 and *Pseudomonas syringae*, and lower homology with other pseudomonads including *P. aeruginosa* and *Pseudomonas putida* (58–62 %). It also shows limited but significant homology (37 %) with the enterobacterial FliS proteins. Similarly to the enterobacterial FliS proteins, the F113 FliS has a putative amphipathic alpha-helix in the carboxy-terminus. The second ORF (*fliT*) is similar in size to ORFs with the same location in other genomes, although homology between them is very low (Table 2). This ORF has been assigned to the *orf96* gene in *P. aeruginosa* PAK (Arora *et al.*, 1998), the *fleP* gene in

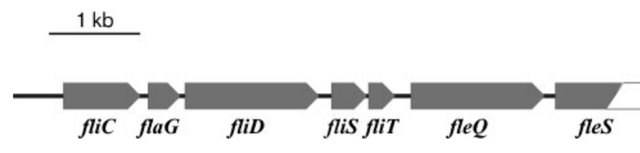

Fig. 1. Physical map of the 6·5 kb DNA region containing the genes implicated in *P. fluorescens* F113 flagellar filament synthesis.

Table 2. FliT protein sequence comparison between *P. fluorescens* F113 and other bacteria

Strain	Identity (%)	Length (aa)*
Pseudomonas fluorescens Pf0-1	83	98
Pseudomonas syringae bv. tomato	69	98
Pseudomonas putida KT2440	56	97
Pseudomonas aeruginosa DG1	46	111
Pseudomonas aeruginosa PAO1	40	98
Pseudomonas aeruginosa PAK	37	96
Salmonella typhimurium	23	122
Escherichia coli	18	121

*In *P. fluorescens* F113 the FliT predicted protein is 98 aa in length.

P. aeruginosa PAO1 strain (Dasgupta *et al.*, 2003), and *fliT* in *Salmonella* (Bennett *et al.*, 2001). Overall, the genetic organization of this region is identical to that of *P. fluorescens* Pf0-1, *P. aeruginosa* PAO1, *P. putida* KT2440 and several pathovars of *P. syringae* (Nelson *et al.*, 2002). However, it differs from the gene order in *P. aeruginosa* strains containing type a flagellin such as PAK (GenBank accession no. L81176) and DG1 (GenBank accession no. L43064), which contain an extra copy of a gene similar to *fliS*, called *fliS′*, located downstream of *fliS*.

Mutants affected in each of these genes were generated by insertional mutagenesis by cloning an internal fragment of the gene in plasmid pVIK112 (Kalogeraki & Winans, 1997) and homologous recombination in the wild-type strain; this resulted in non-polar mutations, as determined by genetic complementation (see below). Despite several efforts, we have been unable to obtain mutants in the *fliD* gene, since besides the insertion, a wild-type copy of the gene was generated.

All the mutants obtained were grown in SA and LB liquid media and showed no differences in growth parameters from the wild-type strain.

The 6·5 kb region sequence has been deposited in GenBank with the accession number AF399739.

Morphological analysis of mutants

Flagellar and cell morphology of *P. fluorescens* F113 and the mutants in the flagellar synthesis region was studied by transmission electron microscopy (Fig. 2). The F113 wild-type strain possesses one or two polar flagella of mean length 2·4 μm (Sanchez-Contreras *et al.*, 2002). The mutations located within the *fliC* and the *fleQ* genes resulted in completely aflagellate bacterial cells. The presence of flagella was restored by the introduction of plasmids containing the wild-type genes under the control of the *nptII* promoter. The mutant affected in the *fliS* gene had a single thin and very short flagellum of about 0·8 μm in length. Introduction of the wild-type gene into this mutant resulted in normal flagellate cells. Conversely, the mutation in the *flaG* gene resulted in bacterial cells with one or two very long (more than 5 μm) flagellar filaments. Finally, the mutation affecting the *fliT* gene had no visible effect, and bacterial cells had flagella with identical morphology to those of the wild-type strain.

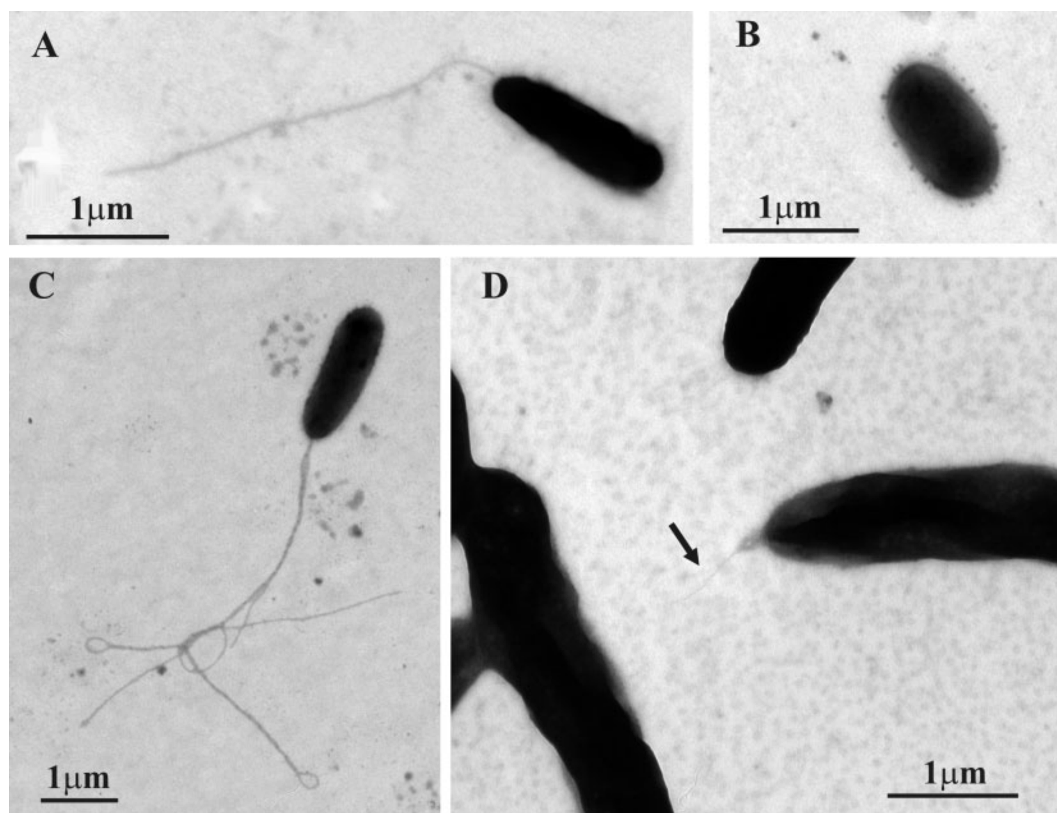

Fig. 2. Electron microscopy images of F113 wild-type and flagellar filament mutants: (A) F113 wild-type; (B) F113-fliC; (C) F113-flaG; (D) F113-fliS. The arrow points to a thin, short flagellar filament produced by the *fliS* mutant. Flagella of the *fliT* mutant are identical to wild-type. The *fleQ* mutant does not produce flagella.

Analysis of the motility phenotype

In order to study the motility characteristics of the mutants, we analysed their ability to swim (Fig. 3). After 18 h the wild-type strain produces a 6–7 mm diameter swimming halo. Mutations located within the *fliC*, *fleQ* and *fliS* genes resulted in non-motile mutants as they did not produce swimming haloes. Complementation of these mutants with the wild-type genes restored motility to 100 %, 85 % and 85 % of the wild-type, respectively. The mutant affected in the *flaG* gene, which had longer flagellar filaments than the F113 wild-type, produced haloes similar to those of the wild-type strain. However, when swimming experiments were performed in richer medium such as LB or iron-supplemented SA, this mutant produced swimming haloes with a diameter 50–100 % wider than those from the wild-type strain. The *fliT* mutant, whose filament morphology was similar to the wild-type strain, produced swimming haloes 50 % smaller than the wild-type. Complementation of the *fliT* mutant restored motility to 83 % of the wild-type. It is important to note that the *fliT* mutant haloes, although smaller, presented clear concentric circles inside them, typical of the chemotactic swimming movement.

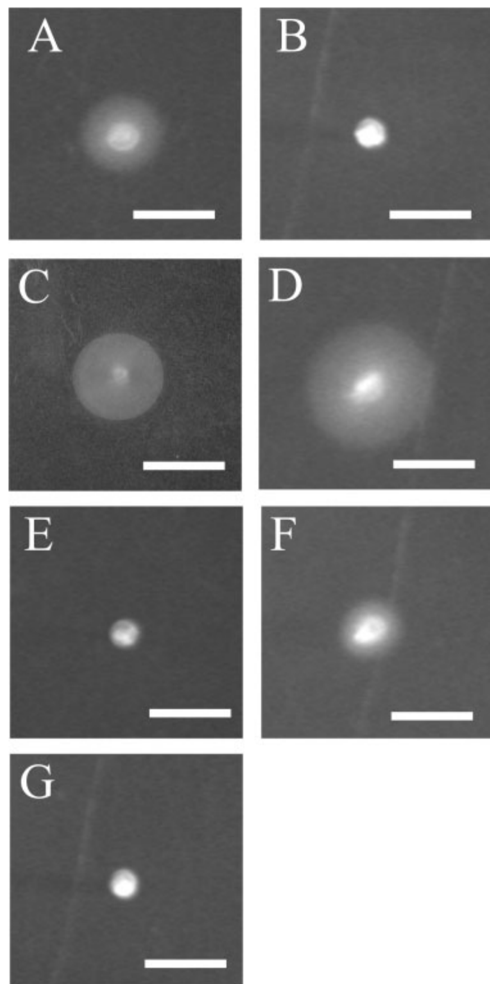

Fig. 3. Swimming haloes produced on 0·3 % agar plates by: F113 on SA (A); F113-fliC on SA (B); F113-flaG on SA (C); F113-flaG on LB (D); F113-fliS on SA (E); F113-fliT on SA (F); and F113-fleQ on SA (G). The bar represents the diameter of the wild-type strain halo in the same swimming conditions.

Flagellin synthesis and export

Total bacterial proteins and exported proteins were analysed by Western blotting with an antiflagellin antiserum (De Weger *et al.*, 1987) (Fig. 4). The F113 wild-type strain gave a band corresponding to the FliC flagellin, both in the total protein and in the exported protein preparations. The same results were obtained with the *fliT* mutant. As expected, the *fliC* mutant did not produce flagellin and no band was detected in either of the protein preparations. The mutation affecting the *fleQ* gene produced very low levels of flagellin in the experiment done with the total proteins; no band appeared in the case of exported proteins. In the *flaG* mutant, the Western blot revealed a normal level of flagellin in the total protein extract and a higher level of exported flagellin, in accordance with its longer flagellar filament morphology. In the case of the *fliS* mutant, we did not observe a band in the total protein Western blot analysis, although a very faint band appeared in the exported proteins extract. In order to understand the results obtained with the *fliS* mutant Western blot experiment, a dot-blot analysis with a whole-cell lysate (soluble and non-soluble proteins) was performed. The antiflagellin antiserum gave a very strong reaction with this bacterial lysate (data not shown). These results indicate that in the *fliS* mutant, most of the FliC protein is probably being accumulated in inclusion bodies formed inside the cell cytoplasm.

Colonization analysis of flagellar mutants

Colonization experiments with each of the mutants inoculated individually showed that all the mutants constructed

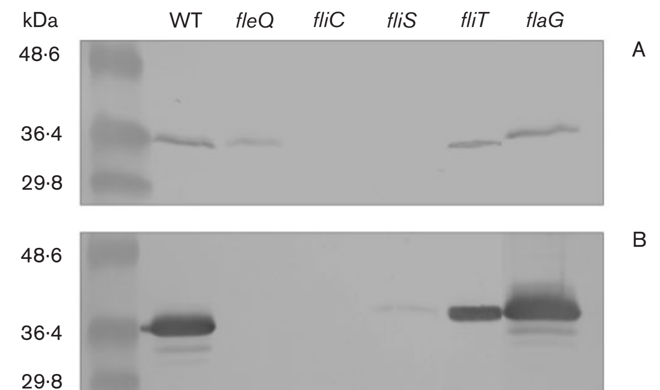

Fig. 4. Western blot analysis of total proteins (A) and external proteins (B) from flagellar filament mutants and wild-type F113, reacted with an antiflagellin antiserum. The observed band is approximately 35 kDa and corresponds to FliC.

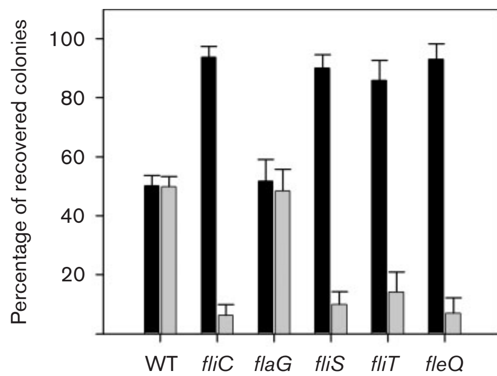

Fig. 5. Competitive root tip colonization analysis of flagellar filament mutants and wild-type F113 competitor strain. Black bars represent percentage of competitor colonies and grey bars represent percentage of colonies from each tested strain recovered from the last centimetre of the main root after competitive colonization assays. Means and standard deviations from three independent assays, performed in triplicate, are shown.

in this work were able to colonize the alfalfa rhizosphere (data not shown). In order to study the importance of bacterial motility in the rhizosphere colonization process, we analysed the competitiveness between the flagellar filament mutants and the wild-type F113 strain. A wild-type F113 strain tagged in a neutral part of the genome with the same integration plasmid (pVIK112) that had been used to generate the mutants was used as the competitor strain. As shown in Fig. 5, all the non-motile mutants, *fliC*, *fliS* and *fleQ*, were very poor competitors and were displaced by the wild-type strain from the last centimetre of the root. In a similar way, the *fliT* mutant that showed reduced motility was displaced by the wild-type F113 strain. No significant differences were observed between the competitive colonization ability of non-motile and reduced-motility mutants. The control strain and the *flaG* mutant competed at the same level as the wild-type strain F113 under our laboratory gnotobiotic competitive assay conditions.

DISCUSSION

The study of bacterial motility and its influence on root colonization and competition in the rhizosphere may eventually result in improved efficacy of biotechnological applications. It is already known that bacterial motility is important in the colonization of the rhizosphere, since different non-motile mutants from *P. fluorescens* strain WCS374 were severely impaired in colonization (Dekkers *et al.*, 1998a). In fact, the non-motile mutants belong to the most defective competitive class of colonization mutants (Dekkers *et al.*, 1998b; Chin-a-Woeng *et al.*, 2000).

In this work we have characterized a genetic region of *P. fluorescens* F113 implicated in the synthesis of the flagellar filament and we have shown that the genetic organization is similar to other pseudomonads but differs from the *P. aeruginosa* PAK and DG1 strains because they have two copies of the *fliS* gene. The first gene in the region, *fliC*, encodes a type b flagellin, the main structural protein of the flagellar filament. As expected, the mutation of this gene yields completely non-motile and aflagellate bacteria. In the mutation affecting the *fleQ* gene we have found the same morphological and non-motile phenotype. These results are in agreement with the already described function for the FleQ protein, which is the major flagellar regulator in *P. aeruginosa* (Dasgupta *et al.*, 2002; Jyot *et al.*, 2002). In *P. fluorescens* Pf0-1, a *fleQ* homologue gene called *adnA* encodes a transcriptional factor that affects persistence and spread, also being required for bacterial adhesion and motility (Casaz *et al.*, 2001; Marshall *et al.*, 2001). From the F113 *fleQ* mutant phenotype (Figs 2 and 3) and the results from the Western blot analysis (Fig. 4), it can be concluded that in *P. fluorescens* F113 the *fleQ* gene is necessary for the production and secretion of the flagellin FliC.

Another mutation causing non-motile cells affects the *fliS* gene. In pseudomonads the role of the FliS protein remains unknown. Its distant homologue in enterobacteria has been described as a substrate-specific cytosolic chaperone that facilitates FliC secretion and contributes to the stabilization of the flagellin subunits during polymerization (Auvray *et al.*, 2001; Ozin *et al.*, 2003). The F113 *fliS* mutant has a very short and thin flagellum (Fig. 2), probably because FliC is not well stabilized and is undergoing wrong polymerization and limited secretion, thus impairing the formation of a normal flagellar filament. To our knowledge, this is the first description of such a phenotype. The results from the *fliS* mutant Western blot analysis confirm the FliS putative function as a FliC chaperone. These results indicate that, instead of being secreted, the FliC flagellin is accumulated in the cytoplasm and as these proteins cannot be extracted together with the total soluble bacterial proteins, the flagellin might be accumulated inside the cytoplasm inclusion bodies. Moreover, the structural analysis of the *P. fluorescens* F113 FliS protein shows that it is homologous to other FliS proteins, being a small peptide, with an acidic isoelectric point (5·18) and having an amphipathic alpha-helix in the C-terminal domain, typical characteristics for most cytoplasmic chaperones (Wattiau *et al.*, 1996; Fraser *et al.*, 1999).

Downstream of *fliS*, the pseudomonads contain a small ORF showing very low homology with the *fliT* genes of enterobacteria (Table 2). In these bacteria, the FliT protein has been described as the FliD substrate-specific chaperone (Fraser *et al.*, 1999), although motility studies done with the *Salmonella typhimurium fliT* mutant concluded that there were no differences in the swimming ability compared with the wild-type strain (Bennett *et al.*, 2001). In *P. fluorescens* F113, the morphological phenotype of the *fliT* mutant was identical to the wild-type strain and the Western blot analysis revealed that the FliC protein is exported to form the flagellar filament. Therefore the putative protein FliT

cannot be acting as a FliD cytosolic chaperone. Furthermore, the structural characteristics of the FliT protein are different to those described for cytoplasmic chaperones. In *P. aeruginosa* PAK, an ORF similar in size and gene location to *fliT* has been designed as *fleP*, encoding a hypothetical protein FleP. The swimming haloes produced by a mutation affecting the *fleP* gene are much smaller than those produced by the *P. aeruginosa* wild-type strain (Dasgupta *et al.*, 2003). This *fleP* mutant motility phenotype is similar to the *P. fluorescens* F113 *fliT* mutant phenotype, which produced swimming haloes 50 % smaller than the wild-type strain. However, electron microscopy studies of the mutant affected in the *fleP* gene in *P. aeruginosa* PAK revealed that its flagella were mostly detached from the cells and the length of polar type IV pili was significantly longer than those from the wild-type strain (Dasgupta *et al.*, 2003). Based on these results, the authors concluded that *fleP* represents a novel flagellar gene specific for *Pseudomonas*, responsible for maintaining the length of type IV pili and stable flagellar attachment to the bacterial pole. These results do not correlate with the normal flagellar morphology observed for our *P. fluorescens* F113 *fliT* mutant (Fig. 2). Such differences, together with the lack of homology between them (Table 2), indicate that the *fleP* gene in *P. aeruginosa* and the *fliT* gene in *P. fluorescens* are different and possess different functions.

A mutant affected in the *flaG* gene had longer flagella than the wild-type F113 (Fig. 2) and flagellin was clearly exported in a higher quantity than in the wild-type strain as observed by Western blot analysis (Fig. 4). These characteristics did not result in higher motility properties in an iron-limited minimal medium, and the *flaG* mutant produced swimming haloes with a diameter similar to that of the wild-type strain (Fig. 3). However, in rich medium or in the absence of iron limitation, this mutant showed a substantially higher motility (Fig. 3). In *V. anguillarum*, an ORF 3 showing a 57 % identity to FlaG from *Vibrio parahaemolyticus* and 34 % identity to FlaG from *P. fluorescens* F113 has been described, and a mutant affected in this gene had elongated flagella, the same morphological phenotype as in the *P. fluorescens* F113 *flaG* mutant, although it showed an 11 % decrease compared with the wild-type motility (McGee *et al.*, 1996). These results indicate that in addition to filament length, FlaG could influence swimming speed, at least under certain conditions.

Root colonization is a complex and crucial process for the use of micro-organisms for agricultural and environmental biotechnology applications, since an improvement in colonization could result in an improvement in the efficacy of these applications, as has been shown for biocontrol (Chin-a-Woeng *et al.*, 2000). Most of the already described non-motile mutants are severely affected in root colonization, especially at sites most distant from the inoculation site (De Weger *et al.*, 1987). We have also observed the predominance of flagellar variants with enhanced surface motility, in the distal parts of the rhizosphere that are not easily reached by the wild-type strain (Sanchez-Contreras *et al.*, 2002).

All the non-motile *P. fluorescens* F113 mutants studied in this work – *fliC*, *fleQ*, *fliS* – although able to colonize when inoculated independently, were very poor competitors, compared with the wild-type F113. These results are in agreement with previously published work that shows that aflagellate (De Weger *et al.*, 1987) or non-chemotactic mutants (de Weert *et al.*, 2002) are very poor competitors. We have isolated and tested a mutant affected in the *fliT* gene that, although still motile and chemotactic, showed a reduced motility phenotype. This mutant was as poor a competitor as aflagellate mutants, showing that not only flagella and chemotactic motility (de Weert *et al.*, 2002), but also a wild-type level of motility are necessary for competitive rhizosphere colonization. Furthermore, although no differences in motility or colonization were observed for a *flaG* mutant under the standard conditions used, the fact that this mutant showed higher motility under certain conditions and the preferred location of hypermotile variants in distal parts of the root (Sanchez-Contreras *et al.*, 2002) suggest the possibility of improving competitive root colonization by manipulating the motility processes.

ACKNOWLEDGEMENTS

We are grateful to Ine Mulders and Ben Lugtenberg for the antiflagellin antiserum.

This work was supported by grants from the European Union (GM-RHIZOREMEDIATION QLK3-CT-2001-00101), Comunidad Autónoma de Madrid (07 M-0062-2000) and Spanish Ministry of Science and Technology BIO 2003-03412. M. M. is the recipient of a Ramón y Cajal contract from the Spanish Ministry of Science and Technology.

REFERENCES

Arora, S. K., Ritchings, B. W., Almira, E. C., Lory, S. & Ramphal, R. (1998). The *Pseudomonas aeruginosa* flagellar cap protein, FliD, is responsible for mucin adhesion. *Infect Immun* **66**, 1000–1007.

Arora, S. K., Dasgupta, N., Lory, S. & Ramphal, R. (2000). Identification of two distinct types of flagellar cap proteins, FliD, in *Pseudomonas aeruginosa*. *Infect Immun* **68**, 1474–1479.

Auvray, F., Thomas, J., Fraser, G. M. & Hughes, C. (2001). Flagellin polymerization control by a cytosolic export chaperone. *J Mol Biol* **308**, 221–229.

Bennett, J. C. Q., Thomas, J., Fraser, G. M. & Hughes, C. (2001). Substrate complexes and domain organization of the *Salmonella* flagellar export chaperones FlgN and FliT. *Mol Microbiol* **39**, 781–791.

Bertani, G. (1951). Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol* **62**, 293–300.

Bloemberg, G. V., Wijfjes, A. H. M., Lamers, G. E. M., Stuurman, N. & Lugtenberg, B. J. J. (2000). Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent proteins in the rhizosphere: new perspectives for studying microbial communities. *Mol Plant–Microbe Interact* **13**, 1170–1176.

Brazil, G. M., Kenefick, L., Callanan, M., Haro, A., de Lorenzo, V., Dowling, D. N. & O'Gara, F. (1995). Construction of a rhizosphere pseudomonad with potential to degrade polychlorinated-biphenyls and detection of *bph* gene-expression in the rhizosphere. *Appl Environ Microbiol* **61**, 1946–1952.

Casaz, P., Happel, A., Keithan, J., Read, D. L., Strain, S. R. & Levy, S. B. (2001). The *Pseudomonas fluorescens* transcription activator AdnA is required for adhesion and motility. *Microbiology* **147**, 355–361.

Chin-a-Woeng, T. F. C., Bloemberg, G. V., Mulders, I. H. M., Dekkers, L. C. & Lugtenberg, B. J. J. (2000). Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. *Mol Plant–Microbe Interact* **13**, 1340–1345.

Dasgupta, N., Ferrell, E. P., Kanack, K. J., West, S. E. H. & Ramphal, R. (2002). *fleQ*, the gene encoding the major flagellar regulator of *Pseudomonas aeruginosa*, is sigma(70) dependent and is downregulated by Vfr, a homolog of *Escherichia coli* cyclic AMP receptor protein. *J Bacteriol* **184**, 5240–5250.

Dasgupta, N., Wolfgang, M. C., Goodman, A. L., Arora, S. K., Jyot, J., Lory, S. & Ramphal, R. (2003). A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in *Pseudomonas aeruginosa*. *Mol Microbiol* **50**, 809–824.

Dekkers, L. C., Phoelich, C. C., Van der Fits, L. & Lugtenberg, B. J. J. (1998a). A site-specific recombinase is required for competitive root colonization by *Pseudomonas fluorescens* WCS365. *Proc Natl Acad Sci U S A* **95**, 7051–7056.

Dekkers, L. C., Van der Bij, A. J., Mulders, I. H. M., Phoelich, C. C., Wentwoord, R. A. R., Glandorf, D. C. M., Wijffelman, C. A. & Lugtenberg, B. J. J. (1998b). Role of the O-antigen of lipopolysaccharide, and possible roles of growth rate and of NADH : ubiquinone oxidoreductase (Nuo) in competitive tomato root-tip colonization by *Pseudomonas fluorescens* WCS365. *Mol Plant–Microbe Interact* **11**, 763–771.

de Weert, S., Vermeiren, H., Mulders, I. H. M., Kuiper, I., Hendrickx, N., Bloemberg, G. V., Vanderleyden, J., De Mot, R. & Lugtenberg, B. J. J. (2002). Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens*. *Mol Plant–Microbe Interact* **15**, 1173–1180.

De Weger, L. A., Van der Vlugt, C. I., Wijfjes, A. H., Bakker, P. A., Schippers, B. & Lugtenberg, B. J. J. (1987). Flagella of a plant-growth-stimulating *Pseudomonas fluorescens* strain are required for colonization of potato roots. *J Bacteriol* **169**, 2769–2773.

Dowling, D. N. & O'Gara, F. (1994). Metabolites of *Pseudomonas* involved in the biocontrol of plant-disease. *Trends Biotechnol* **12**, 133–141.

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

Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. *Proc Natl Acad Sci U S A* **76**, 1648–1652.

Fraser, G. M., Bennett, J. C. Q. & Hughes, C. (1999). Substrate-specific binding of hook-associated proteins by FlgN and FliT, putative chaperones for flagellum assembly. *Mol Microbiol* **32**, 569–580.

Höflich, G., Wiehe, W. & Hecht-Buchholz, C. (1995). Rhizosphere colonization of different crops with growth promoting *Pseudomonas* and *Rhizobium* bacteria. *Microbiol Res* **150**, 139–147.

Jyot, J., Dasgupta, N. & Ramphal, R. (2002). *fleQ*, the major flagellar gene regulator in *Pseudomonas aeruginosa*, binds to enhancer sites located either upstream or atypically downstream of the RpoN binding site. *J Bacteriol* **184**, 5251–5260.

Kalogeraki, V. S. & Winans, S. C. (1997). Suicide plasmids containing promoterless reporter genes can simultaneously disrupt and create fusions to target genes of diverse bacteria. *Gene* **188**, 69–75.

Karlson, U., Dowling, D., O'Gara, F., Rivilla, R., Bittens, M., Francesconi, S., Pritchard, H. & Pedersen, H. C. (1998). Development of self-contained plant/GMM systems for soil bioremediation, In *Past, Present and Future Risk Assessment when using GMOs*, pp. 23–31. Edited by G. E. de Vries. Overschild, NL: ProBio Partners.

Labes, M., Pühler, A. & Simon, R. (1990). A new family of RSF1010-derived expression and *lac*-fusion broad-host-range vectors for Gram-negative bacteria. *Gene* **89**, 37–46.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **277**, 680–685.

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

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

Marshall, B., Robleto, E. A., Wetzler, R., Kulle, P., Casaz, P. & Levy, S. B. (2001). The *adnA* transcriptional factor affects persistence and spread of *Pseudomonas fluorescens* under natural field conditions. *Appl Environ Microbiol* **67**, 852–857.

McGee, K., Hörstedt, P. & Milton, D. L. (1996). Identification and characterization of additional flagellin genes from *Vibrio anguillarum*. *J Bacteriol* **178**, 5188–5198.

Naseby, D. C. & 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.

Nelson, K. E., Weinel, C., Paulsen, I. T. & 40 other authors (2002). Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ Microbiol* **4**, 799–808.

Ozin, A. J., Claret, L., Auvray, F. & Hughes, C. (2003). The FliS chaperone selectively binds the disordered flagellin C-terminal domain central to polymerisation. *FEMS Microbiol Lett* **219**, 219–224.

Rainey, P. B. (1999). Adaptation of *Pseudomonas fluorescens* to the plant rhizosphere. *Environ Microbiol* **1**, 243–257.

Ramos, J. L., Duque, E. & Ramos-González, M. I. (1991). Survival in soils of an herbicide-resistant *Pseudomonas putida* strain bearing a recombinant Tol plasmid. *Appl Environ Microbiol* **57**, 260–266.

Robleto, E. A., López-Hernández, I., Silby, M. W. & Levy, S. B. (2003). Genetic analysis of the AdnA regulon in *Pseudomonas fluorescens*: nonessential role of flagella in adhesion to sand and biofilm formation. *J Bacteriol* **185**, 453–460.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory.

Sanchez-Contreras, M., Martin, M. Villacieros M., O'Gara, F., Bonilla, I. & 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. & 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.

Shanahan, P., Borro, A., O'Gara, F. & 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.

Simons, M., Vanderbij, A. J., Brand, I., Deweger, L. A., Wijffelman, C. A. & Lugtenberg, B. J. J. (1996). Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting *Pseudomonas* bacteria. *Mol Plant–Microbe Interact* **9**, 600–607.

Stover, K. C., Pham, X. Q., Erwin, A. L. & 28 other authors (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1: an opportunistic pathogen. *Nature* **406**, 959–964.

Villacieros, M., Power, B., Sanchez-Contreras, M. & 8 other authors (2003). Colonization behaviour of *Pseudomonas fluorescens* and *Sinorhizobium meliloti* in the alfalfa (*Medicago sativa*) rhizosphere. *Plant Soil* **251**, 47–54.

Walsh, U. F. J. P., Morrissey, J. P. & O'Gara, F. (2001). Pseudomonas for biocontrol of phytopathogens: from functional genomics to commercial exploitation. *Curr Opin Biotechnol* **12**, 289–295.

Wattiau, P., Woestyn, S. & Cornelis, G. R. (1996). Customized secretion chaperones in pathogenic bacteria. *Mol Microbiol* **20**, 255–262.

Yee, D. C., Maynard, J. A. & 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.