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Fis regulates the competitiveness of *Pseudomonas putida* on barley roots by inducing biofilm formation

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An important link between the environment and the physiological state of bacteria is the regulation of the transcription of a large number of genes by global transcription factors. One of the global regulators, Fis (factor for inversion stimulation), is well studied in *Escherichia coli*, but the role of this protein in pseudomonads has only been examined briefly. According to studies in *Enterobacteriaceae*, Fis regulates positively the flagellar movement of bacteria. In pseudomonads, flagellar movement is an important trait for the colonization of plant roots. Therefore we were interested in the role of the Fis protein in *Pseudomonas putida*, especially the possible regulation of the colonization of plant roots. We observed that Fis reduced the migration of *P. putida* onto the apices of barley roots and thereby the competitiveness of bacteria on the roots. Moreover, we observed that overexpression of Fis drastically reduced swimming motility and facilitated *P. putida* biofilm formation, which could be the reason for the decreased migration of bacteria onto the root apices. It is possible that the elevated expression of Fis is important in the adaptation of *P. putida* during colonization of plant roots by promoting biofilm formation when the migration of bacteria is no longer favoured.

INTRODUCTION

Several bacterial species are able to colonize the rhizosphere and thereafter promote the growth and productivity of crop plants (Barret *et al.*, 2009; Costacurta & Vanderleyden, 1995; Khare *et al.*, 2011; Pandey *et al.*, 2005; Ryu *et al.*, 2004). The positive effects for plants may appear through the production of phytohormones, improved nutrient uptake, and protection from plant pathogens by beneficial bacteria (Bloemberg & Lugtenberg, 2001; Fujishige *et al.*, 2006; Morgan *et al.*, 2005). On the other hand, the plant–bacteria interactions can be beneficial for bacteria as well. For example, bacteria can have access to easily consumable carbon and energy sources in the form of the exudates from root tips and lysates from mature parts of roots (Bais *et al.*, 2006; Dennis *et al.*, 2010). Therefore, effective colonization of and competition on plant roots by soil bacteria are important for their survival and successful proliferation.

One of the important groups of bacteria that are able to colonize the rhizosphere and promote plant growth are the pseudomonads (Lugtenberg *et al.*, 2001; Rudrappa *et al.*, 2008). For instance, *Pseudomonas fluorescens*, *Pseudomonas putida* and *Pseudomonas chlororaphis* have been described as antagonistic towards pathogens of crop plants (Chin-A-Woeng *et al.*, 2000; Espinosa-Urgel *et al.*, 2002; Silby *et al.*, 2011).

Diverse bacterial species, including fluorescent pseudomonads, are able to colonize plant roots by forming microcolonies on different parts of the roots. Such microcolonies eventually develop into mature biofilms (Ramos-González *et al.*, 2005; Rudrappa *et al.*, 2008; Stanley & Lazazzera, 2004; Villacieros *et al.*, 2003). The formation of biofilms is important, as it protects plants against pathogens. For example, it has been demonstrated that *Bacillus subtilis* protects *Arabidopsis* against the pathogen *Pseudomonas syringae* pv. *tomato* DC3000 by forming a biofilm on the roots of the plant (Bais *et al.*, 2004). On the other hand, the movement of bacteria via flagella appears to be an important trait for the recolonization of plant roots (Dennis *et al.*, 2010; Lugtenberg *et al.*, 2001). For instance, non-motile and flagella-less mutants of *P. fluorescens* are less competitive than wild-type bacteria in root apex colonization (De Weger *et al.*, 1987), and the long-term incubation of *P. fluorescens* on the roots of alfalfa selects for enhanced-motility variants of the bacterium (Martínez-Granero *et al.*, 2006). Indeed, although the formation of biofilms may protect plants against pathogens, the colonization of root tips by cells with enhanced biofilm formation ability has been shown to be diminished up to five times compared with wild-type cells.

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Four supplemementary video files, showing the movement of *P. putida* strains on different media, are available with the online version of this paper.

Therefore the formation of biofilm decreases the efficiency of recolonization of plant roots (Barahona *et al.*, 2010).

Since the switch of bacterial lifestyle from planktonic to sessile growth requires alterations in bacterial physiology, the expression of a large number of genes is changed during the process of colonization (Mathesius, 2009; Matilla *et al.*, 2007, 2011; Ramos-González *et al.*, 2005). Matilla *et al.* (2007) identified 93 *P. putida* genes from 11 functional classes which exhibited increased expression when the bacteria grew in the maize rhizosphere, including genes for motility, such as *fliL* and *fliB*, and several transcriptional regulators. Global transcriptional factors can also be an important link between the environment and the physiological state of bacteria (Martínez-Antonio & Collado-Vides, 2003). For example, Fis (factor for inversion stimulation) triggers the fast growth of *Escherichia coli* in the presence of a sudden abundance of nutrients in the environment (Ball *et al.*, 1992; Mallik *et al.*, 2004; Nilsson *et al.*, 1992). Bradley *et al.* (2007) found that apart from the 231 genes that Fis regulates in *E. coli*, it activates the expression of a further 38 flagellar and motility genes as well. This is in accordance with the immobility of the *fis*$^-$ strain of *Salmonella typhimurium* (Osuna *et al.*, 1995).

Fis is a small homodimeric nucleoid-associated protein that is found in the families *Enterobacteriaceae* and *Pseudomonaceae* (Beach & Osuna, 1998; Boswell *et al.*, 2004). Fis is quite well conserved in these families; for example, in *E. coli* and *P. putida*, Fis proteins share 64.8 % identity and 81.7 % similarity at the deduced amino acid sequence level as determined via The Comprehensive Microbial Resource (CMR) website (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi). Although Fis is well studied in *E. coli*, the role of this protein in pseudomonads has been examined only in a few recently published papers (Kugelberg *et al.*, 2005; Teras *et al.*, 2009; Yeung *et al.*, 2009; Yuste *et al.*, 2006).

Interestingly, although the *fis* knockout mutants of *E. coli* and *S. typhimurium* are viable and grow well (Bradley *et al.*, 2007; Johnson *et al.*, 1988; Kelly *et al.*, 2004; Osuna *et al.*, 1995), it seems that pseudomonads do not tolerate the lack of functional Fis protein (Liberati *et al.*, 2006; Teras *et al.*, 2009; Yeung *et al.*, 2009). Liberati *et al.* (2006) generated a library of random transposon insertion mutations in *Pseudomonas aeruginosa* PA14. They obtained the insertion of *MAR2xT7* in 4468 genes which make up 75 % of all the genes in this organism, and suggest that *fis* of *P. aeruginosa* is a candidate essential gene, since only one mutant that carried an insertion in the *fis* gene was detected, and this insertion was located in a stop codon of the *fis* gene (Liberati *et al.*, 2006). Indeed, we have not succeeded in creating a *fis* knockout mutant of *P. putida* in our laboratory (Teras *et al.*, 2009).

In this study we show that Fis (encoded by PP4821) is an important factor for the regulation of the colonization of barley roots by *P. putida*, and that it is involved in the persistence of pseudomonads in plant roots. Although the overexpression of *P. putida* Fis per se does not decrease the persistence of *P. putida* on barley roots, it dramatically decreases the persistence of *P. putida* on barley roots in the presence of a competitor *P. putida* strain that expresses Fis at a wild-type level. The cells of *P. putida* with an elevated level of Fis form up to three times more biofilm than the cells with a wild-type level of Fis. Therefore, the overexpression of Fis facilitates biofilm formation and severely reduces the migration of bacteria onto the root apex.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this study are described in Table 1. Bacteria were grown in complete Luria–Bertani (LB) medium (Miller, 1992) or barley seedling (BS) medium [10 % barley seedling extract in M9 buffer (Adams, 1959)]. BS was used as a medium that imitates the compounds and energy sources around barley roots. To obtain barley seedling extract for the BS medium, 5 kg barley seeds were germinated for 4 days until the seeds had 3–4 cm long roots without developed coleoptiles. The seeds were watered with sterile distilled water. The extracts from the whole barley seedlings with the remaining water were separated with a Stollar BJE520 juice extractor. For better extraction, sterile distilled water was added to the seedlings, resulting in 1 l extract. The seedling extract was filtered several times with Whatman filter paper and 0.22 μm pore-size Millipore filters until the solution was clear.

Solid media for routine use contained 1.5 % Difco agar. Antibiotics were added at the following concentrations: ampicillin, 100 μg ml^{-1}; gentamicin, 10 μg ml^{-1}; streptomycin, 200 μg ml^{-1}; penicillin, 1500–3000 μg ml^{-1}. *E. coli* was incubated at 37 °C and *P. putida* at 30 °C. Bacteria were electrotransformed as described by Sharma & Schimke (1996). *E. coli* strains DH5α (Invitrogen) and CC118*λpir* (Herrero *et al.*, 1990) were used for DNA cloning procedures; BL21(DE3) (Studier & Moffatt, 1986) was used for the overexpression of *P. putida* Fis by a previously published protocol (Teras *et al.*, 2009).

DNA manipulation and strain construction. To assess the effect of Fis overexpression on *P. putida* colonization of barley roots, strain F15 was constructed from *P. putida* PaW85 (Bayley *et al.*, 1977), which is isogenic to strain KT2440. The mini-Tn*7* containing the overexpression cassette for the *fis* gene (PP4821) was inserted into the chromosome of *P. putida* using suicide vector pGP-miniTn*7*-fis (Table 1) by employing the mini-Tn*7* transposition method described by Højberg *et al.* (1999).

For the construction of pGP-miniTn*7*-fis, first, a suicide vector, pGP-miniTn*7*-ΩGm, was constructed. The 1.8 kb *Xba*I–*Sac*I fragment from pBK-miniTn*7*-ΩGm (Koch *et al.*, 2001) carrying a mini-Tn*7*-ΩGm cassette was inserted into plasmid pGP704 L (Pavel *et al.*, 1994) cut with *Xba*I and *Sac*I. Then, the overexpression cassette of *P. putida fis* was cloned into pJB785TT (Santos *et al.*, 2001). The *luc* gene in pJB785TT was replaced with the 2 kb *Bam*HI–*Xba*I fragment from pBR-lacItac (Ojangu *et al.*, 2000) carrying a *lacI*^{q}-P$_{tac}$ cassette to obtain pJB-*lacI*^{q}-P$_{tac}$. Then, the *fis* gene (PP4821) was amplified from the chromosomal DNA of *P. putida* PaW85 by PCR using oligonucleotides fisRBSPstI (5′-CACTGCAGAAGGGGTGGCCGCA-TGAC-3′) and fisBamHI (5′-ATGGATCCTTACAACAAGTCGTAC-TGC-3′). The PCR fragment cut with *Pst*I and *Bam*HI was inserted into plasmid pJB-*lacI*^{q}-P$_{tac}$ cut with *Pst*I and *Bgl*II to obtain pJB-PF. In the overexpression cassette the *fis* gene was placed under the control of the P$_{tac}$ promoter and the *lacI* repressor gene, the product of which represses transcription from the P$_{tac}$ promoter in the absence

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
***E. coli* strains**		
DH5α	*supE44* Δ*lacU169*(*ϕ*80 *lacZ*ΔM15) *recA1 endA1 hsdR17 thi-1 gyrA96 relA1*	Invitrogen
BL21(DE3)	*hsdS gal* (*λcIts857 ind1 Sam7 nin5 lac*UV5-T7 gene *1*)	Studier & Moffatt (1986)
CC118*λpir*	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1 λpir* phage lysogen	Herrero *et al.* (1990)
***P. putida* strains**		
PaW85	Isogenic to KT2440	Bayley *et al.* (1977)
PSm	PaW85; chromosomal mini-Tn*7*-ΩSm1 (Sm^r)	This study
F15	PaW85; chromosomal mini-Tn*7*-ΩGm-term-*lacI*^{q}-P_{tac}-*fis*-T1T2 (Gm^r)	This study
Plasmids		
pBluescript KS	*E. coli* cloning vector (Ap^r)	Stratagene
pET19b-fisNcoI	His_6-head tagged *P. putida fis* cloned into pET19b (Ap^r)	Teras *et al.* (2009)
pJB785TT	Contains *luc* gene flanked by transcriptional terminators *rrnB*T1T2 and term (Ap^r)	Santos *et al.* (2001)
pBR-lacItac	*lacI*^{q} repressor and P_{tac} promoter in pBR322 (Ap^r)	Ojangu *et al.* (2000)
pJB-*lacI*^{q}-P_{tac}	*luc* gene replaced with *Bam*HI–*Xba*I fragment of *lacI*^{q}-P_{tac} from pBR-lacItac (Ap^r)	This study
pJB-PF	PCR fragment of *P. putida fis* inserted under control of P_{tac} promoter in pJB-*lacI*^{q}-P_{tac} (Ap^r)	This study
pBK-miniTn*7*-ΩGm	pUC19-based delivery plasmid for mini-Tn*7*-ΩGm (Ap^r, Gm^r)	Koch *et al.* (2001)
pBK-miniTn*7*-ΩSm1	pUC19-based delivery plasmid for mini-Tn*7*-ΩSm1, *mob*$^+$ (Ap^r, Sm^r)	Koch *et al.* (2001)
pUX-BF13	R6K replication-based helper plasmid, providing the Tn*7* transposition function *in trans*, *mob*$^+$ (Ap^r)	Bao *et al.* (1991)
pGP704 L	*Pir*-dependent R6K replicon suicide vector (Ap^r)	Pavel *et al.* (1994)
pGP-miniTn*7*-ΩGm	pGP704 L carrying a *Sac*I–*Xba*I mini-Tn*7*-ΩGm cassette from pBK-miniTn*7*-ΩGm (Ap^r, Gm^r)	This study
pGP-miniTn*7*-fis	pGP-miniTn*7*-ΩGm carrying a blunted *Nde*I–*Eco*RI term-*lacI*^{q}-P_{tac}-*fis*-T1T2 fragment from pJB-PF in blunted *Acc*65I site (Ap^r, Gm^r)	This study

of the inducer molecule IPTG. Finally, for construction of pGP-miniTn*7*-fis, the 3.6 kb *Eco*RI–*Nde*I fragment from pJB-PF carrying the term-*lacI*^{q}-P_{tac}-*fis*-T1T2 cassette was blunted with Klenow fragment and cloned into the blunted *Not*I site of pGP-miniTn*7*-ΩGm.

To exclude possible effects of the presence of mini-Tn*7* in the chromosome of *P. putida* strain F15 on the colonization of barley roots by *P. putida*, the mini-Tn*7* transposon without the *fis* gene overexpression cassette was inserted into the same site of the wild-type *P. putida* PaW85 chromosome, yielding strain PSm. The suicide vector pBK-miniTn*7*-ΩSm1 (Koch *et al.*, 2001) was used to insert the mini-Tn*7* transposon into the chromosome of *P. putida* strain PSm, and this strain was subsequently used as the wild-type strain in our experiments.

In order to mark PSm and F15 with different antibiotic-resistance genes for reisolation, a streptomycin-resistance gene was inserted into the mini-Tn*7* transposon in the chromosome of *P. putida* strain PSm and a gentamicin-resistance gene in the chromosome of strain F15.

Overexpression and purification of *P. putida* Fis, production of polyclonal antibodies against Fis, and Fis immunoblotting. For the production of polyclonal mouse anti-Fis antibody, *P. putida* Fis(His) was overexpressed and purified with Ni-NTA agarose matrix (Qiagen), as described previously (Teras *et al.*, 2009). The polyclonal antibodies against Fis (PP4821) were produced and purified by LabAs.

Western immunoblot analysis was carried out to detect the amount of Fis from crude lysates of *P. putida*. Bacteria were grown in LB and BS broths to mid-exponential or stationary phase in the presence of different concentrations of IPTG (0.5, 1 and 2 mM) or without IPTG. After 2 h of IPTG supplementation, cells were collected by centrifugation and sonicated in Fis buffer (100 mM Tris/HCl, pH 7.5, 0.3 M NaCl, 5 %, v/v, glycerol). The cell lysates were centrifuged at 12 000 ***g*** for 30 min at 4 °C. The total amount of protein in the cleared supernatant was measured by Folin reagent (Lowry *et al.*, 1951). Proteins were separated by Tricine-SDS-PAGE (10 %) electrophoresis (Schägger, 2006) and transferred to a PVDF membrane (Hybond-P, Amersham GE Healthcare). For Western blotting, the membrane was probed with mouse anti-Fis purified polyclonal antibodies at a final dilution of 1 : 500, followed by alkaline phosphatase-linked goat anti-mouse IgG diluted 1 : 5000 (LabAs). The blots were developed using 5-bromo-4-chloro-indolyl phosphate/nitro blue tetrazolium.

Characterization of growth of PSm and F15 strains in liquid media. Cells of *P. putida* strains PSm and F15 were grown overnight in LB or BS medium. These cultures were used to inoculate fresh media in 96-well microtitre plates (200 µl medium per well) so that the OD_{580} of the cultures was 0.05. The optical density of cultures was measured at 20 min intervals using a Tecan Sunrise-Basic microplate reader (Tecan Austria). Approximately 25 viable count data points were produced for each growth curve. Growth rate (µ; h^{-1}) values of bacteria were estimated from the growth curves by the Richards model (Dalgaard & Koutsoumanis, 2001). The mean growth rate of strains derived from separate colonies was calculated from 24 parallel experiments.

Surface sterilization, seed germination, and root colonization assay. Barley seeds were surface-sterilized in diluted bleach (1 : 10) for 10 min and in 75 % ethanol for 2 min, and rinsed thoroughly with sterile distilled water. Surface-sterilized seeds were germinated on wetted filter paper at room temperature for 4 days, resulting in ~3 cm

long roots. Barley seedlings pre-germinated for 4 days were inoculated with overnight cultures of *P. putida* strains grown in LB medium either supplemented with 0.5 mM IPTG or not supplemented. Bacteria were washed once with M9 buffer (Adams, 1959) and resuspended in MS medium (Murashige & Skoog, 1962) to OD_{580} ~1. Germinated barley seeds were inoculated with the appropriate *P. putida* strains by submerging (30 min) in a 100 ml suspension of bacteria without shaking at room temperature. Inoculated seeds were washed in MS medium and planted in tubes containing sterilized vermiculite or were used to determine the number of bacteria attached to the seedling. Planted seedlings were maintained in a growth chamber at 22 °C and 40 to 55 % humidity with a daily light period of 14 h for 21 days. The plants were watered every 2 days with MS medium or MS medium supplemented with 0.5 mM IPTG. To recover the bacteria from the roots, 0, 1, 3, 9 and 21 day-old plants were removed, and the roots were cut and then ground in 1 ml sterile M9 buffer. Serial dilutions were plated onto LB agar amended with gentamicin or streptomycin. The lowest dilution was 10^{-2}. The number of c.f.u. was calculated per gram of roots. To obtain the 0 day c.f.u. value, barley roots were cut immediately after inoculation and lavage of pre-germinated seeds with bacteria. *P. putida* colonies were verified by PCR with Tn*4652* transposon-specific oligonucleotides PRH10 (5′-GCTGAGCTCGGCCGGTAGCTGACTGGG-3′) and Tnots (5′-GGGGTCATGCCGAGATAAG-3′), and were further checked for the presence of the term-$lacI^q$-P_{tac}-*fis*-T1T2 cassette in their genome using Prtac (5′-AATTAATCATCGGCTCGTATAA-3′) and fisBamHI oligonucleotides.

For the root apex migration experiments, barley and bacteria were grown as described above, but the 2 cm-length root apex region and the 2 cm-length basal root region were separated from barley roots in 3 and 9 day-old plants.

Bacterial motility. LB or BS medium plates containing 0.3 % agar were used to test *P. putida* swimming motility, and 1 % agar plates were used to test twitching motility. The plates used in the motility experiments were either supplemented with different concentrations of IPTG (0.5, 1 and 2 mM IPTG) or no IPTG was added. Motility medium plates were inoculated with *P. putida* grown overnight in liquid LB or BS medium by poking the agar with pipette tips. The haloes due to swimming motility were measured after 24 h of inoculation.

For the swimming competition assay, the same medium was used. Plates were inoculated either with PSm or F15 alone or with a mixture of both strains (PSm + F15) and were incubated for 48 h. To determine the numbers of living cells in the centre of the halo and in the edge of the halo, 20 µl semisolid medium with bacteria was obtained from each position. Then serial dilutions were plated onto LB agar amended with gentamicin or streptomycin. Surface-associated twitching motility was measured as the maximum diameter of the halo of bacteria that moved between the surface of the Petri dish and the agar after 24 h of inoculation (Déziel *et al.*, 2001).

Biofilm formation assay in multi-well plates. A modified version of Fletcher's method was used (Fletcher, 1977). A 100 µl volume of LB or BS medium amended with 0.5, 1 and 2 mM IPTG or without IPTG supplementation was placed on a well of a polystyrene multi-well plate and inoculated with 5 µl overnight-grown bacterial culture. Bacteria were incubated for 24 h at 30 °C. Then 25 µl 1 % crystal violet was added to each well for 15 min to allow the staining of adhered cells. The wells were washed twice intensively with distilled water, and then 180 µl 96 % ethanol was added twice to each well in order to extract crystal violet from cells. After that, 100 µl crystal violet in ethanol was diluted into 200 µl water in another multi-well plate and the A_{540} of the solutions was measured with a microtitre plate reader (Tecan Sunrise-Basic). Each assay was performed at least three times with 16 parallel experiments for each variant.

Statistical analysis. To assess the persistence of *P. putida* on barley roots, each obtained c.f.u. value was normalized to the c.f.u. value measured on day 0. To calculate the inoculation efficiency of *P. putida* strains, the c.f.u. value per gram of barley roots on day 0 was normalized to the c.f.u. value of a 1 ml bacterial suspension used for the inoculation of seeds (c.f.u. g^{-1}/c.f.u. ml^{-1}). To obtain a normal distribution, data were transformed to $\log_{10}$ values when necessary. To avoid taking a $\log_{10}$ of zero, zero was replaced with '1'. The factorial analysis of variance (ANOVA) method was used to assess the variability of experimental data. For all variables, multiple comparisons of means were conducted with Bonferroni's test for unequal *n*. For statistical tests, the significance level was set at 0.05. The calculations were performed using Statistica 7 software.

RESULTS

Construction and initial characterization of *P. putida* Fis-overexpressing strain F15

In order to study the involvement of Fis in plant root colonization by *P. putida*, we constructed strain F15, where the cellular level of Fis can be artificially changed by adding IPTG to induce transcription of the *fis* gene from the P_{tac} promoter (for more details, see Methods). First, the ability of F15 cells to overproduce Fis protein was assessed by Western blot analysis. Crude cell lysates were prepared from exponentially growing and stationary phase F15 cells. The addition of IPTG to the growth medium caused a significant overexpression of the Fis protein in both exponentially growing and stationary phase cells of F15 in LB and BS media (Fig. 1), showing that it is possible to

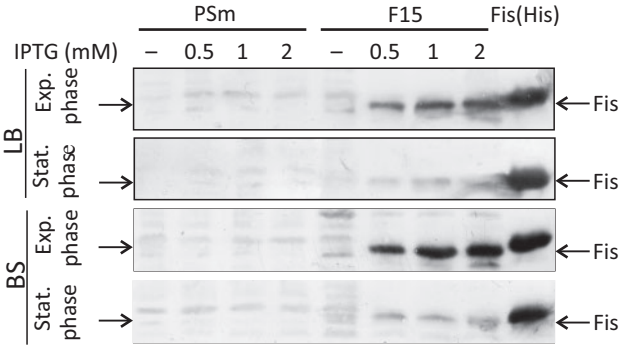

Fig. 1. Overexpression of Fis in *P. putida* cells determined by immunoblot analysis using polyclonal anti-Fis antibodies. Immunoblot analysis was performed with cryde cell lysates prepared from *P. putida* strains PSm and F15 grown in LB and BS media. The crude cell lysates of *P. putida* were prepared from exponentially growing bacteria (Exp. phase) and stationary phase bacteria (Stat. phase). The concentration of IPTG used for the overexpression of Fis is shown at the top of the figure. Twenty micrograms of crude cell lysates of PSm and F15 without IPTG supplementation and 5 µg of crude cell lysates of F15 in the presence of 0.5, 1 and 2 mM IPTG were analysed. Four nanograms of purified Fis(–6His) was used as a positive control. Arrows show the location of Fis.

overproduce Fis protein in different physiological states of *P. putida*.

We also examined whether the overexpression of Fis could reduce the growth rate of *P. putida* in LB and BS media. First, the growth rates of wild-type cells of *P. putida* (PSm) were measured. In general, the growth rate of PSm was not affected by IPTG in either medium studied. However, the growth rate of the wild-type PSm was slightly higher in BS medium than in LB broth (Table 2) at up to 1.1-fold higher ($P<0.001$). At the same time, the overexpression of Fis had a marked influence on the growth rate of *P. putida*. Compared with cells with a wild-type level of Fis (PSm), the overexpression of Fis in the presence of 2 mM IPTG reduced the growth rate of *P. putida* F15 up to 2.4-fold in LB broth ($P<0.001$). However, in contrast to growth in LB medium, the overexpression of Fis did not reduce the growth rate of *P. putida* in BS medium; instead, it slightly enhanced the growth rate of bacteria in BS medium (Table 2). Compared with the absence of IPTG, the supplementation of the BS medium with 0.5 and 1 mM IPTG raised the growth rate of F15 by up to 1.1-fold ($P<0.001$).

Overexpression of Fis in *P. putida* F15 results in a dramatic loss of swimming motility in semisolid medium

Since Fis enhances the flagellar motility of *E. coli* and *S. typhimurium* (Bradley *et al.*, 2007; Osuna *et al.*, 1995), we were inspired to study whether Fis could affect the flagellar motility of *P. putida*. Our results showed that the swimming motility of strain PSm expressing Fis at a wild-type level was not affected by the presence of IPTG in either growth medium studied (Table 2). Surprisingly, the overexpression of Fis significantly decreased the swimming motility of F15 cells (Table 2). For example, the presence of 0.5 mM IPTG in LB medium reduced the swimming motility of F15 cells 1.8-fold compared with F15 the absence of IPTG supplementation ($P<0.001$). However, the 1.3-fold smaller haloes of F15 in BS plates in the presence of 0.5 mM IPTG were not statistically significantly different from those observed when Fis was not overexpressed (Table 2). Notably, the migration of F15 cells in LB and BS media was linearly dependent on increasing concentrations of IPTG, resulting in 4.2-and 3.3-fold smaller haloes on media in the presence of 2 mM IPTG than without IPTG. In sum, the results of our experiments demonstrated that overexpression of Fis reduced the swimming motility of *P. putida* on LB as well as BS medium. Our results suggest that the reduced swimming motility of bacteria under Fis overexpression conditions was not caused by a slower growth rate, as on BS medium the growth rate was not reduced in comparison with that of bacteria expressing Fis at a wild-type level.

The twitching motility of *P. putida* strains PSm and F15 was also measured. Considering the maximum diameter of the halo, the twitching motility of *P. putida* appeared to be dependent on the growth medium but not on the Fis expression level. LB medium was a less suitable environment for *P. putida* twitching motility than BS medium. The mean maximum diameters of the haloes of PSm and F15 in LB medium Petri dishes were 6.3 ± 2.3 mm and 6.1 ± 2.3 mm, and with BS medium 10.4 ± 3.9 mm and 11.5 ± 3.9 mm, respectively. At the same time, a closer inspection of the haloes on BS medium revealed the erratic movement of a subpopulation of bacteria. Some bacteria did not twitch as a permanent front, but like solitary cells moved away from a centre of the initial cell population and then started to spread and form separately located colonies. However, the wild-type strain PSm also showed a similar erratic motility on BS medium. The erratic movement of bacteria was not influenced by the presence of IPTG or the overexpression of Fis (data not shown).

Adherence of *P. putida* to barley roots depends on the amount of Fis

Since the motility of bacteria is one of the most important traits for rhizosphere colonization (Dennis *et al.*, 2010;

Table 2. Swimming motility and growth rate of *P. putida* strains PSm and F15 in LB and BS media.

IPTG concn (mM)	PSm				F15			
	LB medium		BS medium		LB medium		BS medium	
	Motility*	Growth rate†	Motility	Growth rate	Motility	Growth rate	Motility	Growth rate
0	30.3 (5.5)	1.255 (0.051)	28.3 (5)	1.356 (0.08)	27.8 (3.4)	0.999 (0.042)	16.9 (3.8)	1.225 (0.037)
0.5	30.0 (4.1)	1.254 (0.010)	26.0 (7.2)	1.341 (0.035)	15.2 (2.5)	0.893 (0.008)	13.4 (5.7)	1.352 (0.067)
1	28.6 (3.9)	1.231 (0.01)	26.6 (5.1)	1.359 (0.037)	10.0 (1.5)	0.767 (0.05)	6.8 (0.7)	1.399 (0.031)
2	29.4 (4.3)	1.187 (0.023)	26.3 (3.7)	1.335 (0.062)	6.6 (1)	0.530 (0.046)	5.1 (0.6)	1.215 (0.087)

*Swimming motility was measured as the halo of bacteria after 24 h induction at 30 °C. The diameters of haloes are given in millimetres. SDs are shown in parentheses.

†Growth rates (μ) are represented as generations of bacteria per hour (h^{-1}). SDs are shown in parentheses.

Lugtenberg *et al.*, 2001), we were inspired to study whether Fis could affect the colonization of barley roots by *P. putida*. We chose barley (*Hordeum vulgare*) for colonization experiments, as barley is an agriculturally important cereal plant in Northern Europe, where the cold climate makes it difficult to grow other cereal plants such as maize and wheat.

We observed that in the absence of Fis overexpression, strain F15 adhered to barley roots in a manner similar to the wild-type strain PSm (Table 3, see columns without competitor strain). However, the overexpression of *fis* diminished the adherence of *P. putida* to the barley roots. Comparing the adherence of strain F15 precultivated without IPTG with that of cells grown in the presence of IPTG revealed 11-fold lower adherence by the cells overexpressing Fis ($P<0.001$). To exclude the possibility that IPTG itself could influence the adherence of bacteria to barley roots, we compared the adherence of the wild-type strain PSm in the presence and absence of IPTG. The results showed that IPTG did not have any significant effect on the inoculation efficiency of strain PSm.

To assess the influence of a competitor strain on adherence, we counted cells of strains F15 and PSm from barley roots inoculated with a mixture of *P. putida* strains (PSm + F15), where PSm was the competitor strain for F15 and vice versa. We did not discover any influence of the competitor strain (PSm) on the adherence of F15, as the F15 cells adhered similarly in the presence and absence of competitor cells (Table 3). However, as seen in the absence of the competitor strain, IPTG decreased sevenfold ($P<0.001$) the number of adhered F15 cells on barley roots. These results indicate that Fis influences root colonization by *P. putida* at a very early stage, when the bacteria adhere to the plant roots and have not yet started to proliferate in order to persist on the roots.

Surprisingly, we discovered that colonization by the wild-type cells (PSm) was moderately decreased by the presence of cells of the competitor strain (F15). The number of c.f.u. of PSm on barley roots was decreased threefold by the presence of cells of the competitor strain (F15), irrespective of the absence of IPTG ($P=0.011$) or the presence of IPTG ($P=0.014$) in the medium (Table 3). At the same time, IPTG had no effect on the adherence of PSm in the presence of cells of the competitor strain F15 (Table 3).

Persistence of *P. putida* on barley roots depends on the expression level of *fis* and on the presence of the competitor strain

The persistence of *P. putida* on barley roots was studied in the presence or absence of the competitor strain and with the overexpression of the *fis* gene during a 3 week period. In general, the number of *P. putida* cells calculated per gram of barley roots decreased during the incubation period in all cases studied (Fig. 2). However, we observed a statistically significant increase in c.f.u. at the beginning of the incubation period (on day 3) when the persistence of F15 cells on roots in the presence of IPTG was measured (Fig. 2a). In this case, the overexpression of Fis increased the persistence of *P. putida* on barley roots by 1.5 orders of magnitude ($P=0.001$). Thus, in the absence of the competitor strain the overexpression of Fis did not have a negative effect on the persistence of *P. putida* on barley roots. At the same time, the overexpression of Fis caused a dramatic decrease in the persistence of *P. putida* on barley roots in the presence of the competitor strain (Fig. 2b). Already on day 1, the fraction of F15 cells overexpressing Fis started to decrease. This process led to 1.9 and 2.3 orders of magnitude reduced c.f.u. on days 9 and 21 when compared with that of F15 without Fis overexpression, when no IPTG was added ($P<0.001$). Neither the presence of IPTG nor that of cells of the competitor strain affected the persistence of strain PSm on barley roots (Fig. 2b). These results indicated that *P. putida* cells that overexpress Fis are less competitive than those expressing Fis at a wild-type level. However, since the overexpression of Fis had positive effects on the persistence of *P. putida* on barley roots during the first few days of colonization when the competitor strain was absent (Fig. 2a), the overexpression of Fis seems not to be energy-expensive for *P. putida* and does not hinder its growth and proliferation per se.

Table 3. Adherence of *P. putida* strains PSm and F15 to barley roots

Values shown are based on at least 21 measurements; SD values are shown in parentheses. Adherence of bacteria to barley roots is presented as c.f.u. per gram of barley roots.

P. putida strain	Without competitor strain* (c.f.u. g^{-1})		With competitor strain† (c.f.u. g^{-1})	
	Without IPTG	0.5 mM IPTG	Without IPTG	0.5 mM IPTG
PSm	1.28×10^8 ($\pm2.97\times10^7$)	1.62×10^8 ($\pm7.27\times10^7$)	4.62×10^7 ($\pm3.03\times10^7$)	5.72×10^7 ($\pm3.76\times10^7$)
F15	1.25×10^8 ($\pm4.00\times10^7$)	1.15×10^7 ($\pm1.61\times10^7$)	9.61×10^7 ($\pm5.00\times10^7$)	1.39×10^7 ($\pm1.10\times10^7$)

*Barley roots were inoculated with only one strain, PSm or F15.
†Barley roots were inoculated with a mixture of strains PSm and F15, where PSm is the competitor strain for F15 and vice versa.

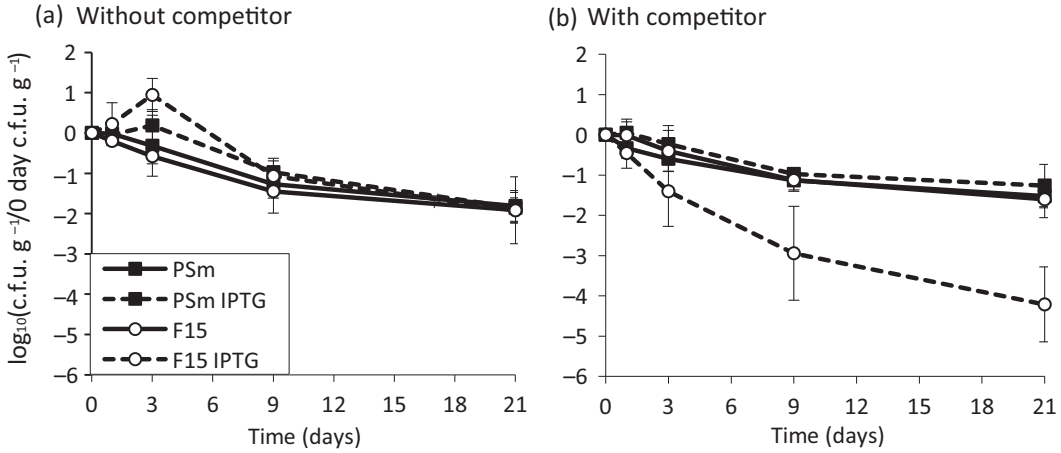

Fig. 2. Persistence of *P. putida* strains PSm and F15 on barley roots. Log_{10} values of the normalized c.f.u. of *P. putida* calculated per gram of barley roots are shown for cells sampled on days 0, 1, 3, 9 and 21; error bars, 95 % confidence intervals. Each c.f.u. value is normalized to the number of c.f.u. on day 0. Mean c.f.u. values are calculated from log_{10} values of raw data. The persistence of *P. putida* cells on barley roots without a competitor strain (a) and in the presence of the competitor strain (b) is shown. The persistence of *P. putida* on barley roots without and with IPTG treatment is shown by solid and dashed lines, respectively. Data from at least nine measurements are shown.

The reduced swimming motility is not suppressed during the persistence of Fis-overexpressing cells on barley roots for 3 weeks

Since the overexpression of Fis negatively affected the motility of *P. putida*, we examined the motility phenotype of the clones reisolated from the F15 population during the 3 weeks of persistence of this strain on barley roots. Strain F15 was incubated on the roots in the presence of the wild-type competitor strain PSm, and the Fis protein was overexpressed by the addition of 0.5 mM IPTG to the growth medium. Because of a strong selective pressure against F15 under such conditions we expected to isolate mutant clones of this strain with recovered swimming motility. About 96 colonies of F15 were isolated at each time point, except on days 9 and 21, when 192 colonies were isolated. From all 672 colonies studied none of the isolates expressed a recovery of swimming motility.

To further confirm the stability of Fis overexpression in F15 cells during long-term persistence on barley roots, the *fis*-expression cassette region in the chromosome of 50 randomly picked F15 isolates was sequenced. No mutations were found in the *fis*-expression cassette, including the promoter region. Moreover, the retained ability of isolates to overexpress Fis after the addition of IPTG to the growth medium was confirmed by Western blot analysis of 10 randomly picked colonies from the 50 colonies whose *fis* cassettes were sequenced (data not shown).

Fis promotes *P. putida* biofilm production

The motility of *P. putida* strains in semisolid LB agar (0.3 %) was monitored by light microscopy. We found that in addition to the reduced swimming motility of the bacteria overexpressing Fis, the cells were aggregated, unlike cells of PSm, which remained separate and were not adhered. The motile bacteria were found at the edges of the haloes of both strains. Whereas the majority of PSm cells were motile, only a few motile F15 cells were found at the edges of the haloes (see Supplementary videos S1–S4). At the same time, overexpression of Fis did not lead to aggregation of *P. putida* cells when liquid medium was used as a growth environment for bacteria (data not shown).

It has been noted that fluorescent pseudomonads are able to form biofilm-like structures that occupy the surface of plant roots (Ramos *et al.*, 2000; Villacieros *et al.*, 2003). Therefore, we examined the effect of the overexpression of Fis in *P. putida* on the formation of biofilm in LB and BS media. The overexpression of *fis* caused the adherence of F15 cells to the walls of multi-well plates in all media used (Fig. 3). The most significant effect of Fis overexpression was observed in LB medium. In this case, the over-expression of Fis facilitated biofilm formation up to 2.9-fold compared with F15 without IPTG supplementation. At the same time, the effect of the adherence caused by Fis overexpression was less conspicuous in BS medium, at 1.9-fold (Fig. 3c).

Overexpression of Fis causes migration deficiency of *P. putida* cells when mixed with cells expressing Fis at the wild-type level

To get a better understanding of the behaviour of bacteria on plant roots, we carried out several migration competition

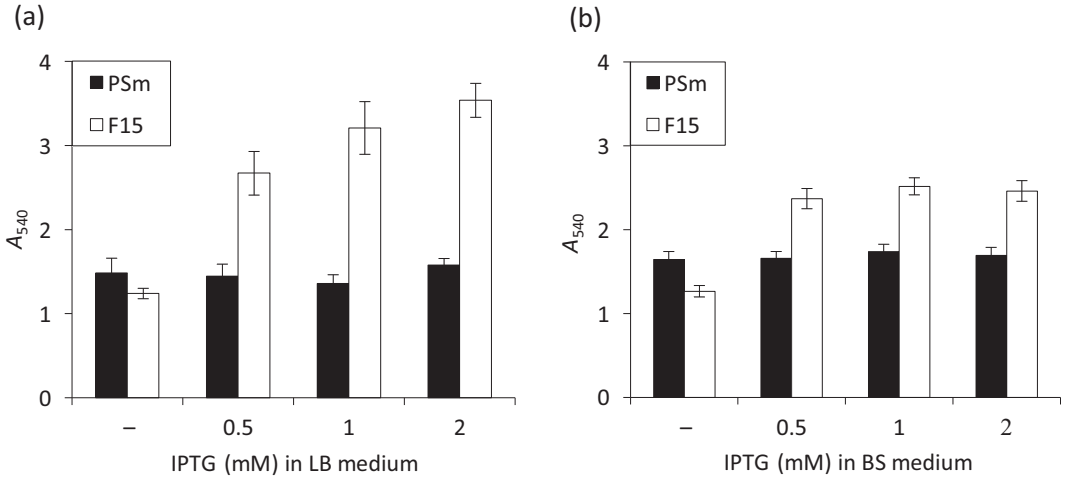

Fig. 3. Biofilm formation by *P. putida* strains PSm and F15 in LB (a) and BS media (b). Biofilm formation was measured as the A_{540} of the crystal violet solution rinsed from cells that had adhered to the walls of multi-well plates after 24 h of inoculation. Media were supplemented with 0.5, 1 or 2 mM IPTG, or no IPTG was added. Data from at least 13 measurements are shown.

experiments. Since the results presented in Fig. 3 indicated that the overexpression of Fis could facilitate biofilm formation by *P. putida*, the possible crosstalk of the wild-type strain and the strain overexpressing Fis was examined by a migration competition experiment on semisolid agar.

We observed that in the centre of a halo, the effect of Fis overexpression on the competitiveness of F15 cells depended on the medium, probably due to different growth rates of F15 cells. Notably, the overexpression of Fis even had a mild positive effect on the growth rate of F15 in BS medium (Table 2). Therefore, the competitiveness of F15 cells was not affected by the overexpression of Fis in a mixture of populations (PSm + F15) in BS medium (Fig. 4c). At the same time, the overexpression of Fis decreased the growth rate of F15 2.4-fold in LB medium (Table 2). These results are corroborated by the results obtained from competition experiments in semisolid agar, where the overexpression of Fis decreased the competitiveness of F15 cells in the centre of the halo in the presence of the wild-type strain PSm (Fig. 4a). However, the c.f.u. value for strain F15 was decreased only slightly by Fis overexpression when the competitor strain was absent on semisolid agar. Since the centre of the halo was 'overcrowded' by bacteria, the overexpression of Fis probably influenced the uptake of nutrients by *P. putida* and not the proliferation itself. These results suggest that the reduced competitiveness of *P. putida* cells in mixed populations due to Fis overexpression appears only under certain growth conditions. Moreover, it seems that overexpression of Fis does not reduce the fitness of *P. putida* in BS medium, a medium which better reflects the environmental conditions on barley roots.

To examine the migration efficiency of the cells in the mixed populations of PSm and F15, samples for c.f.u. measurement were taken from the edges of the haloes after 48 h of incubation. Since the diameters of the haloes of the mixtures of the strains and of strain PSm inoculated alone were generally the same (~60 mm), we assumed that the migration of PSm was not affected by the presence of F15. Contrary to the wild-type strain PSm, the movement of F15 decreased linearly with the overexpression of Fis in both LB and BS semisolid agar (Table 2). Since it is known that bacteria can communicate via signal molecules in the environment (Harmsen *et al.*, 2010; Mikkelsen *et al.*, 2011), the question arose of whether the presence of cells of the wild-type strain would help F15 cells to overcome the retardation of their motility. However, the migration of F15 cells was severely restrained in comparison with the migration of PSm on both growth media used (Fig. 4b, d). The number of c.f.u. of strain F15 in the edges of haloes was five to six orders of magnitude smaller than that of PSm (Fig. 4b, d). The migration of F15 cells was similarly retarded in LB and BS media by cells of the wild-type strain PSm, indicating that the migration of F15 cells depended on the amount of Fis and not on the growth rate of the bacteria.

We assumed that the results obtained from the studies of bacterial migration in mixed populations of PSm and F15 on semisolid agar plates would mimic the growth environment of bacteria on plant roots. Therefore, next we wanted to find out whether the overexpression of Fis could decrease the migration of *P. putida* on barley roots. The inoculation procedure and the growth of barley plants were performed as described above, except that bacterial c.f.u. were measured separately in 2 cm-long base and apex regions of the barley roots. The roots were not separated on day 0, because at that time point the bacteria might have been adhered homogeneously across the roots. The migration of *P. putida* was examined only during the first

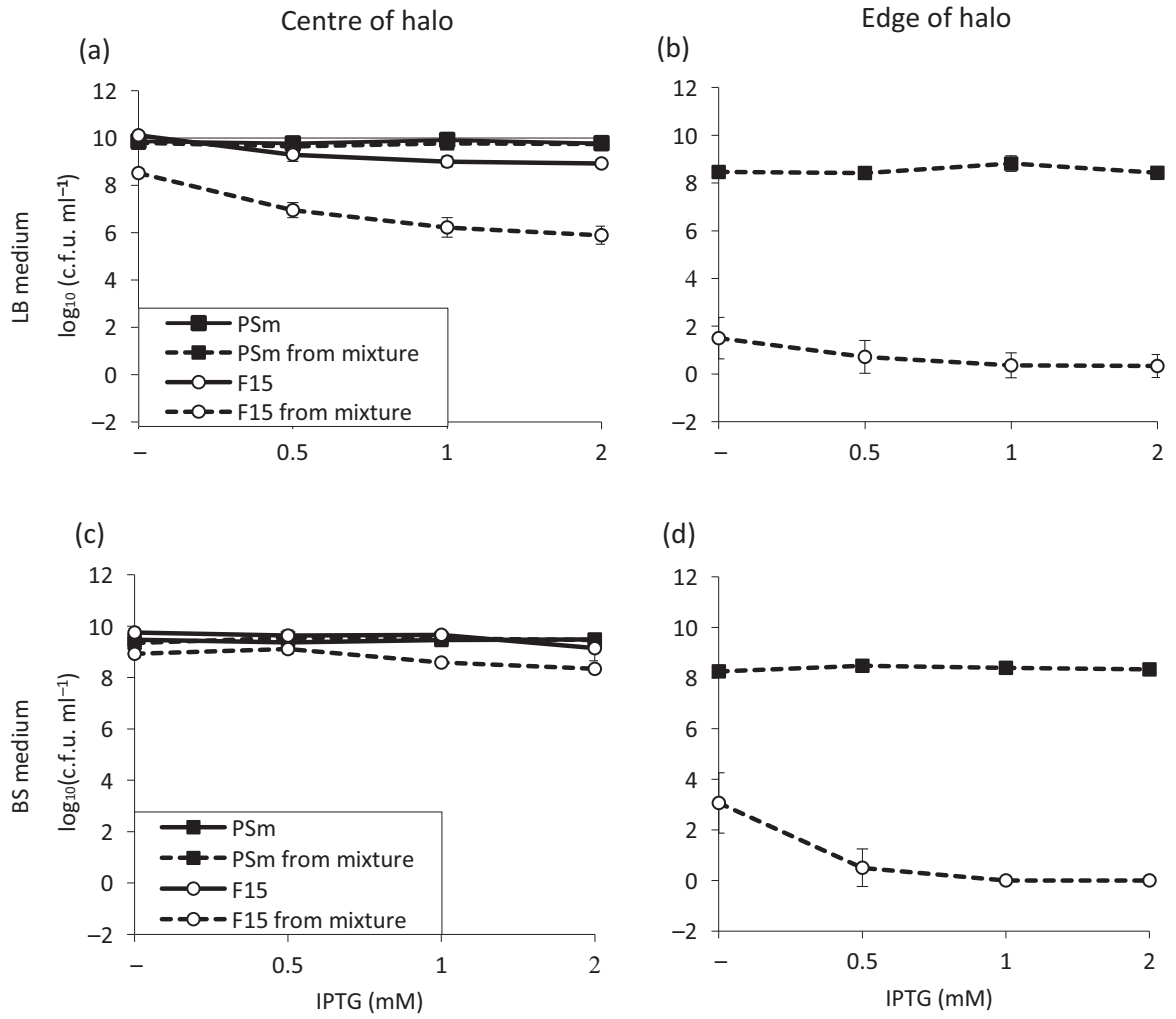

Fig. 4. Proliferation and migration of cells of *P. putida* PSm and F15 on semisolid LB (a, b) and BS media (c, d). Log_{10} values of normalized c.f.u. of *P. putida* are presented; error bars, 95 % confidence intervals. The c.f.u. ml^{-1} was calculated from serial dilutions of samples taken from inoculation sites at the centres of haloes (a, c) and from the edges of haloes (b, d). IPTG concentration is shown on the *x* axis. Solid lines, cultures inoculated with PSm or F15 alone; dashed lines, cultures inoculated with a mixture of PSm and F15 cells. Data from at least three measurements are shown.

9 days after inoculation, because after that barley roots began to grow in a circle on the bottom of the tube and started to form a knot of roots.

Indeed, we observed that Fis overexpression reduced the migration of *P. putida* cells to the apex of barley roots (Fig. 5b). On day 9 the number of c.f.u. of F15 was reduced by approximately six orders of magnitude on the apices of the roots but only by one order of magnitude on the bases of the barley roots when Fis was overexpressed (Fig. 5).

DISCUSSION

Global regulators are important in changing bacterial physiology (Martínez-Antonio & Collado-Vides, 2003). For example, it is known in *E. coli* that Fis is involved in the regulation of genes from 15 different categories, including metabolism of several carbon sources, and motility (Bradley *et al.*, 2007). In some cases the inactivation of particular global regulator(s) can be complemented by a protein with a similar function [for example, integration host factor (IHF) can replace HU in the Sin recombinase synapse on the *resH* site (Rowland *et al.*, 2006)], but in other organisms the inactivation of the same regulators can be lethal, as seen in an *hupN* and *hupB* double mutant of *P. putida* (Bartels *et al.*, 2001) and an *hbs* mutant of *B. subtilis* (Micka & Marahiel, 1992). Similarly, although Fis-minus mutants of *E. coli* and *S. typhimurium* are viable (Bradley *et al.*, 2007; Johnson *et al.*, 1988; Kelly *et al.*, 2004; Osuna *et al.*, 1995), Fis is essential to *P. putida*. It should be noted,

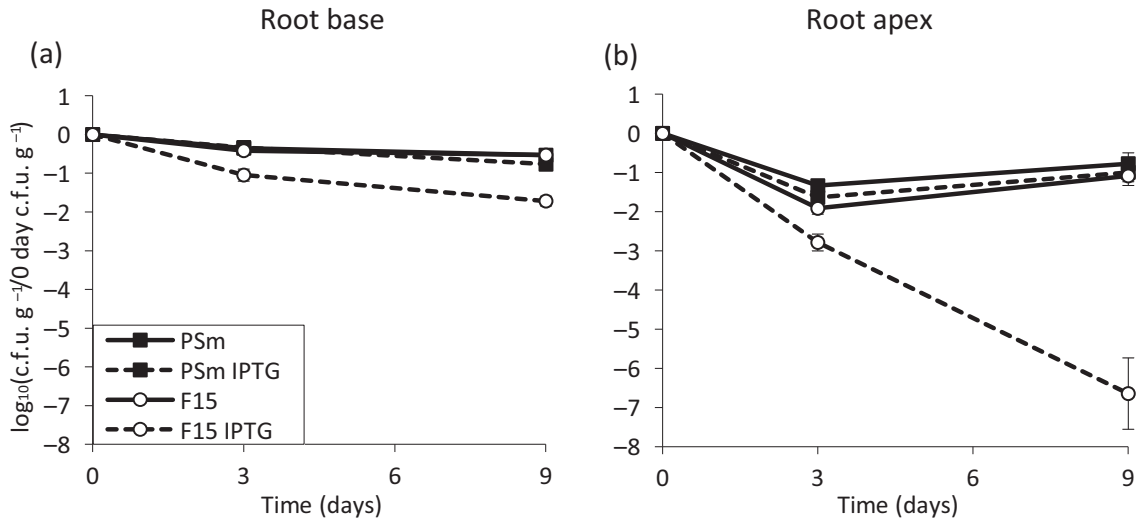

Fig. 5. Migration of *P. putida* cells onto apices of barley roots. The figure shows the persistence of *P. putida* strains F15 and PSm on the root bases of barley (a) and on the apices of barley roots (b). Barley seedlings pre-germinated for 4 days were inoculated with a mixture of cells of *P. putida* strains F15 and PSm. To overexpress Fis in strain F15, plants were watered with MS medium supplemented with 0.5 mM IPTG. Log_{10} values of normalized c.f.u. of *P. putida* strains F15 and PSm calculated per gram of barley roots are presented for each experimental combination at every time point; error bars, 95 % confidence intervals. Each c.f.u. value is normalized to the number of c.f.u. determined on day 0. Mean c.f.u. values were calculated from log_{10} values of raw data. Data from six measurements are shown.

however, that a Fis-defective mutant of *P. putida* strain UW4 has recently been reported (Cheng *et al.*, 2009). This strain carries the disrupted gene of a transcriptional regulator that belongs to the Fis family (EU514688; http://www.ncbi.nlm.nih.gov/nuccore/169123282), although this gene does not encode Fis. Although the disrupted gene was misleadingly named as a *fis* gene, it has 85 % identity to the sequence of the gene for a two-component transcriptional regulator (PP0888) of *P. putida* KT2440, and lacks significant similarity to the *fis* gene (PP4821) of *P. putida* KT2440.

All our attempts to reduce the amount of Fis in *P. putida* (knockout by interrupting or deleting the *P. putida fis* gene; gene deletion from bacteria that contained an additional *fis* gene under the control of an inducible promoter, etc.) were unsuccessful (data not shown). Therefore, in order to study the role of Fis in the regulatory network of *P. putida*, we were only able to study the effects of overexpression of this global regulator. The essential nature of Fis for pseudomonads might be the main reason why the involvement of Fis in several bacterial processes has not been discovered until now.

The results of our study revealed that the overexpression of Fis in *P. putida* strain F15 decreased the adhesion of bacteria to barley roots (Table 3) and dramatically reduced the persistence of Fis-overexpressing cells on roots when mixed with cells of strain PSm, which expresses Fis at a wild-type level (Fig. 2). However, our results indicate that overexpression of *fis* does not decrease the competitiveness of *P. putida* on barley roots via a slower growth rate (Table 2, see growth rate in BS medium), but rather via converting bacteria to a sessile lifestyle, and repressing colonization of new areas of roots by enhanced formation of biofilm (Fig. 3) and reduced swimming motility (Table 2). The presence of a solid substrate, such as barley roots, may cause adhesion of *P. putida*, resulting in enhanced formation of biofilm on roots. Indeed, the positive effect of Fis on biofilm formation was evident on the walls of microtitre plates, where the overexpression of Fis facilitated biofilm formation by up to 1.5-fold in BS medium compared with cells of the wild-type strain (Fig. 3). This could explain the negative effects of overexpression of Fis on the expansion of F15 cells on plant roots during the later stages of colonization, especially when mixed with cells expressing Fis at a wild-type level (Fig. 2). Since the meristematic zone of plant roots continuously elongates, which results in shifted root apices, the reduced migration of the *P. putida* cells means that they are less competitive in colonizing new root areas, so that roots become covered by *P. putida* cells with a wild-type Fis level (Fig. 5).

The adhesion, biofilm formation and motility of pseudomonads are modulated by several factors, including the intracellular signal molecule bis-(3′-5′)-cyclic diguanylic guanosine monophosphate (cyclic-di-GMP) (Kuchma *et al.*, 2007; Merritt *et al.*, 2010; Navazo *et al.*, 2009; Römling *et al.*, 2005; Simm *et al.*, 2004), two-component regulatory systems (Goodman *et al.*, 2004; Navazo *et al.*, 2009; Petrova & Sauer, 2009; Ventre *et al.*, 2006), and outer-membrane

proteins (Hinsa *et al.*, 2003; Hinsa & O'Toole, 2006). The results of our study indicate that Fis also modulates *P. putida* biofilm formation and motility. Notably, we observed that Fis-overexpressing cells retained twitching motility (data not shown), which is an ATP energy-wasteful process (Turner *et al.*, 1993). Therefore the influence of overexpressed Fis seems to be more specific for biofilm formation than indirect regulation via energy deficiency. The involvement of Fis in biofilm formation by *P. putida* is intriguing and will be one of our topics of further research.

In conclusion, our results indicate that Fis is directly or indirectly involved in the regulation of several processes in *P. putida*, such as swimming motility and the formation of biofilms, which in turn affect the competitiveness of bacteria on plant roots. We have observed that the overexpression of Fis facilitates *P. putida* biofilm formation, and this may be a reason for the decreased migration of bacteria onto the apices of barley roots. At the same time, on the base of barley roots, the competitiveness of *P. putida* cells overexpressing Fis is comparable with that of wild-type cells, implying that a high amount of Fis is not a stress factor for *P. putida* on the base of barley roots. Hence, it is possible that the elevated expression of Fis is important for the adaptation of *P. putida* during the colonization of plant roots by encouraging the formation of biofilm when the migration of bacteria is no longer favoured.

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