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Three independent signalling pathways repress motility in *Pseudomonas fluorescens* F113

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Summary

Motility is one of the most important traits for rhizosphere colonization by pseudomonads. Despite this importance, motility is severely repressed in the rhizosphere-colonizing strain *Pseudomonas fluorescens* F113. This bacterium is unable to swarm under laboratory conditions and produce relatively small swimming haloes. However, phenotypic variants with the ability to swarm and producing swimming haloes up to 300% larger than the wild-type strain, arise during rhizosphere colonization. These variants harbour mutations in the genes encoding the GacA/GacS two-component system and in other genes. In order to identify genes and pathways implicated in motility repression, we have used generalized mutagenesis with transposons. Analysis of the mutants has shown that besides the Gac system, the Wsp system and the *sadB* gene, which have been previously implicated in cyclic di-GMP turnover, are implicated in motility repression: mutants in the *gacS*, *sadB* or *wspR* genes can swarm and produce swimming haloes larger than the wild-type strain. Epistasis analysis has shown that the pathways defined by each of these genes are independent, because double and triple mutants show an additive phenotype. Furthermore, GacS, SadB and WspR act at different levels. Expression of the *fleQ* gene, encoding the master regulator of flagella synthesis is higher in the *gacS*$^-$ and *sadB*$^-$ backgrounds than in the wild-type strain and this differential expression is reflected by a higher secretion of the flagellin protein FliC. Conversely, no differences in *fleQ* expression or FliC secretion were observed between the wild-type strain and the *wspR*$^-$ mutant.

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Introduction

Pseudomonas fluorescens F113 is a biocontrol strain, originally isolated from the sugar-beet rhizosphere (Shanahan *et al*., 1992). This bacterium is capable of controlling damping-off caused by the oomycete *Pythium ultimum* due, at least partially, to the production of diacetylphloroglucinol (Fenton *et al*., 1992). This strain has also been genetically modified (Brazil *et al*., 1995; Villacieros *et al*., 2005) and tested for the rhizoremediation of polychlorinated biphenyls (de Carcer *et al*., 2007).

The biotechnological applications of *P. fluorescens* F113 depend on its ability to colonize the plant rhizosphere. F113 has been shown to be able to colonize the rhizosphere of a variety of plants including pea (Naseby and Lynch, 1999), alfalfa (Villacieros *et al*., 2003), willow (de Carcer *et al*., 2007), tomato (Barea *et al*., 1998), potato (Cronin *et al*., 1997) among others.

Motility is one of the most important traits for rhizosphere colonization by pseudomonads. It has been shown that non-motile (Simons *et al*., 1996; Capdevila *et al*., 2004), non-chemotactic (de Weert *et al*., 2002) or reduced motility (Capdevila *et al*., 2004) mutants are among those more affected in competitive rhizosphere colonization. Three types of movement have been described in pseudomonads. (i) Swimming, dependent on flagella, is a movement on interstitial liquid that is generally tested in soft agar media. It requires chemotaxis and produces concentric circles forming a halo. (ii) Swarming is a coordinated movement over surfaces that depends on flagella and the production of surfactants. In a variety of bacteria, it has been shown to be inversely regulated with biofilm formation through the levels of the second messenger c-di-GMP. Swarming produces generally a dendritic pattern on agar surface. (iii) Twitching is also a movement on surfaces and depends on the contraction of type IV pili.

Despite this importance, motility is severely repressed in F113. This strain is unable to swarm under laboratory conditions (Sánchez-Contreras *et al*., 2002). However, rhizosphere selects phenotypic variants that swarm *in vitro*, indicating that swarming motility is repressed in this strain. Furthermore, the rhizosphere selects also for phenotypic variants that show enhanced swimming motility and are more competitive than the wild type for rhizosphere colonization (Martínez-Granero *et al*., 2006). The

swimming motility of these variants ranges from 150% to 300% of the motility of the wild-type strain, indicating that several pathways might be repressing the swimming potential of F113. Variants showing 150% motility were shown to contain mutations in the *gacAS* genes (Martínez-Granero *et al.*, 2006) which encode a two-component system that regulates secondary metabolism in pseudomonads (Laville *et al.*, 1992; Reimmann *et al.*, 1997), indicating that this system is involved in the repression of swimming motility. However, Gac repression cannot explain the phenotype of the rest of the highly motile variants. Although these variants also harbour *gac* mutations, cloned *gacA* or *gacS* genes expressed *in trans* complemented the swimming phenotype only partially. These observations suggest that several signalling pathways independent of the Gac system are repressing swimming motility in F113.

The aim of this work was to identify such pathways and relevant genes implicated in the repression of motility in *P. fluorescens*. To find these genes, we have performed a generalized transposon mutagenesis of *P. fluorescens* F113 that has allowed us to identify two genes, *sadB* and *wspR*, which define two new independent signalling pathways that repress swimming and swarming motility in this biotechnological important bacterium.

Results

Isolation of 'transposon tagged' mutants involved in the repression of swimming motility

In order to identify genes involved in motility repression, we mutagenized *P. fluorescens* F113 with transposons Tn5*luxAB* (Wolk *et al.*, 1991) and miniTn5*gusA* (Wilson *et al.*, 1995) that contain a replication origin functional in *Escherichia coli*. A total of 3000 transposon mutants were screened in duplicate to identify mutants that produced a swimming halo larger than the wild-type strain. Putative mutants were isolated and retested for halo formation. Those that showed hypermotility were tested for single transposon insertion by southern blot. Pyoverdine and exoprotease production was monitored at this stage to discriminate mutants affected in the *gacAS* genes, because we have previously shown that this system repress motility (Martínez-Granero *et al.*, 2005).

Twenty-one mutants that produced haloes larger than the wild type, did not show differences in growth rate with the wild type, were not affected in secondary metabolism and had a single transposon insertion were selected and genomic DNA was extracted. The DNA was digested with restriction enzymes that did not cut within the transposon, ligated and transformed into *E. coli*. Plasmids recovered were used to determine the transposon insertion site by sequencing, using primers annealing with the transposon ends. Significant sequences were obtained for 17 mutants, which corresponded with eight different genes (Table 1).

Table 1. Isolated hypermotile mutants.

Mutant	Identification	Putative function
LA154	SadB	Signal transduction
LA157	KinB	Signal transduction
LA163	DUF1329 family	Unknown
GC282	Pfl_2797	Amidohydrolase 2
GN286	LysR family protein	Transcriptional regulator
GN513	WspR	Signal transduction
GN603	WspE	Signal transduction
GN777	WspC	Signal transduction

Five of these mutants had insertions in genes that encoded proteins with homology to signal transduction proteins: KinB, SadB and WspCER. KinB is the sensor protein of a two-component signal transduction system implicated in the regulation of alginate production (Ma *et al.*, 1997). SadB is a multidomain protein that has been implicated in biofilm formation (Caiazza and O'Toole, 2004) and swarming motility (Caiazza *et al.*, 2005). This protein possesses an N-terminal Yba-like domain, of unknown function followed by a modified HD-GYP domain, typical of phosphodiesterases implicated in c-di-GMP hydrolysis. However, its implication in c-di-GMP turnover has not been demonstrated. The same phenotypes are apparently regulated by the Wsp system, a chemotaxis-like system whose output (WspR) is a diguanylate-cyclase activity that forms c-di-GMP (Hickman *et al.*, 2005). The relation of SadB and WspR with this important messenger that has not been previously implicated in the regulation of swimming motility in pseudomonads prompted us to select these two mutants for further study.

Phenotypic characterization of sadB *and* wspR *mutants*

To determine that the swimming motility phenotype of both mutants was linked to the transposon insertion, the mutants were reconstructed by insertional mutagenesis using homologous recombination. Reconstructed mutants showed the same hypermotile phenotype (Fig. 1) than the transposon tagged mutants and were used for further characterization. Wild-type F113 produced a 10.25 ± 0.5 mm diameter halo after 18 h. The *gacS*, *sadB* and *wspR* mutants produced haloes of 13.25 ± 0.5, 15.5 ± 1 and 16 ± 0.8 mm. All the mutants produced statistically larger haloes than F113 ($P < 0.05$). No statistical difference was observed between *gacS* and *sadB* or *sadB* and *wspR*. However, *wspR* produced haloes significantly larger than *gacS* ($P < 0.05$). The mutants were also tested for swarming motility and biofilm formation. Conversely to the wild-type strain, both mutants were able to swarm

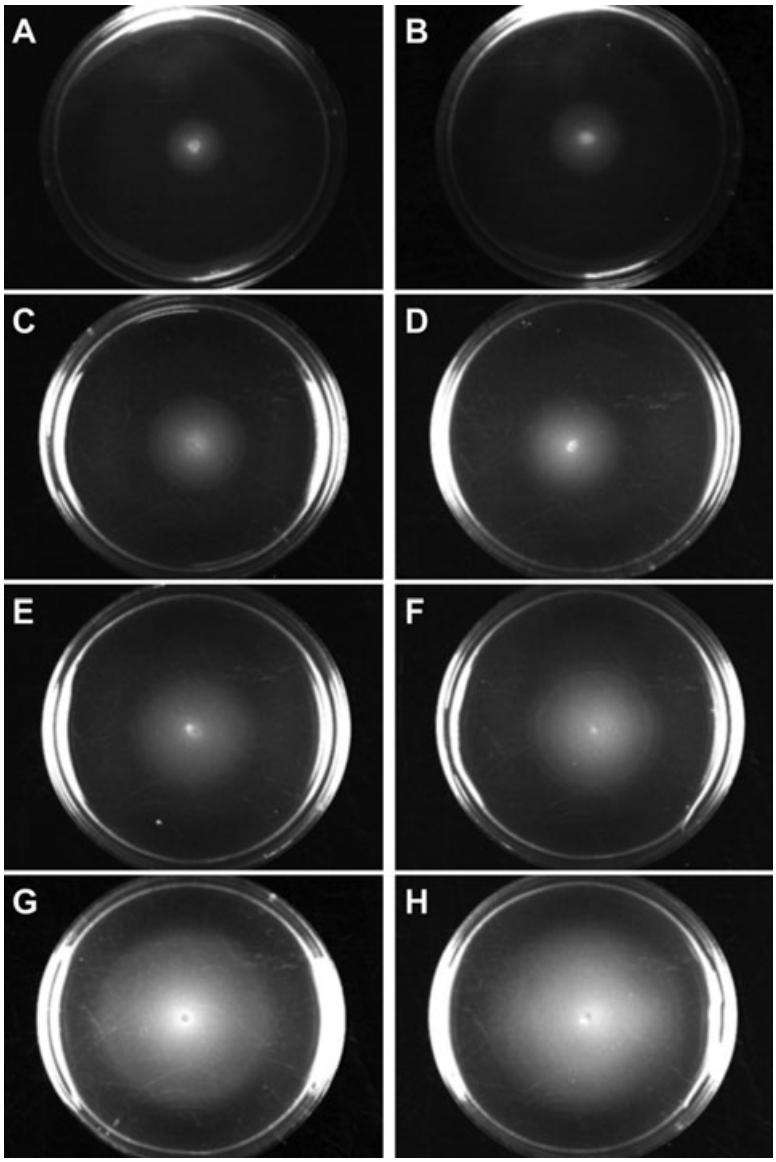

Fig. 1. Swimming motility of *Pseudomonas fluorescens* F113 and hypermotile mutants.
A. F113.
B. *gacS*$^-$.
C. *sadB*$^-$.
D. *wspR*$^-$.
E. *gacS*$^-$*sadB*$^-$.
F. *gacS*$^-$*wspR*$^-$.
G. *sadB*$^-$*wspR*$^-$.
H. *gacS*$^-$*sadB*$^-$*wspR*$^-$.
Exponentially growing cells were inoculated with a toothpick on SA plates containing 0.3% purified agar. Haloes were observed 24 h after inoculation.

(Fig. 2) and were affected in biofilm formation on abiotic surfaces (not shown). Therefore, besides swimming motility, shown here, SadB and the Wsp system appear to be implicated in the regulation of these phenotypes in *P. fluorescens*, as has been described in other pseudomonads.

SadB and WspR define independent signalling pathways

We have previously shown that the two-component GacA/S system repress swimming motility in *P. fluorescens*. To determine whether SadB and/or WspR repress swimming motility through the same signalling pathway than the Gac system, double mutants *gacS-sadB* and *gacS-wspR* were constructed. As shown in Fig. 1, the swimming phenotypes of the double mutants were additive: 22 $\pm$ 0.8 and 24.25 $\pm$ 1.5 mm respectively, indicating that neither SadB nor WspR was acting through the same signalling pathway than the Gac system. A double mutant *sadB-wspR* (26 $\pm$ 0.8 mm) and a triple mutant *gacS-sadB-wspR* (35.75 $\pm$ 1 mm) also showed additive swimming phenotypes (Fig. 1), showing that swimming motility in *P. fluorescens* F113 is repressed by at least three independent signalling pathways.

The double and triple mutants were also tested for swarming motility. As shown in Fig. 2, the wild-type strain was unable to swarm under the experimental conditions used. The swarming phenotype of the *sadB-wspR* mutant was additive (Fig. 2 and Fig. S1) indicating that regulation of swarming by SadB and WspR occur through different pathways. The *gacS* mutant was able to swarm and the

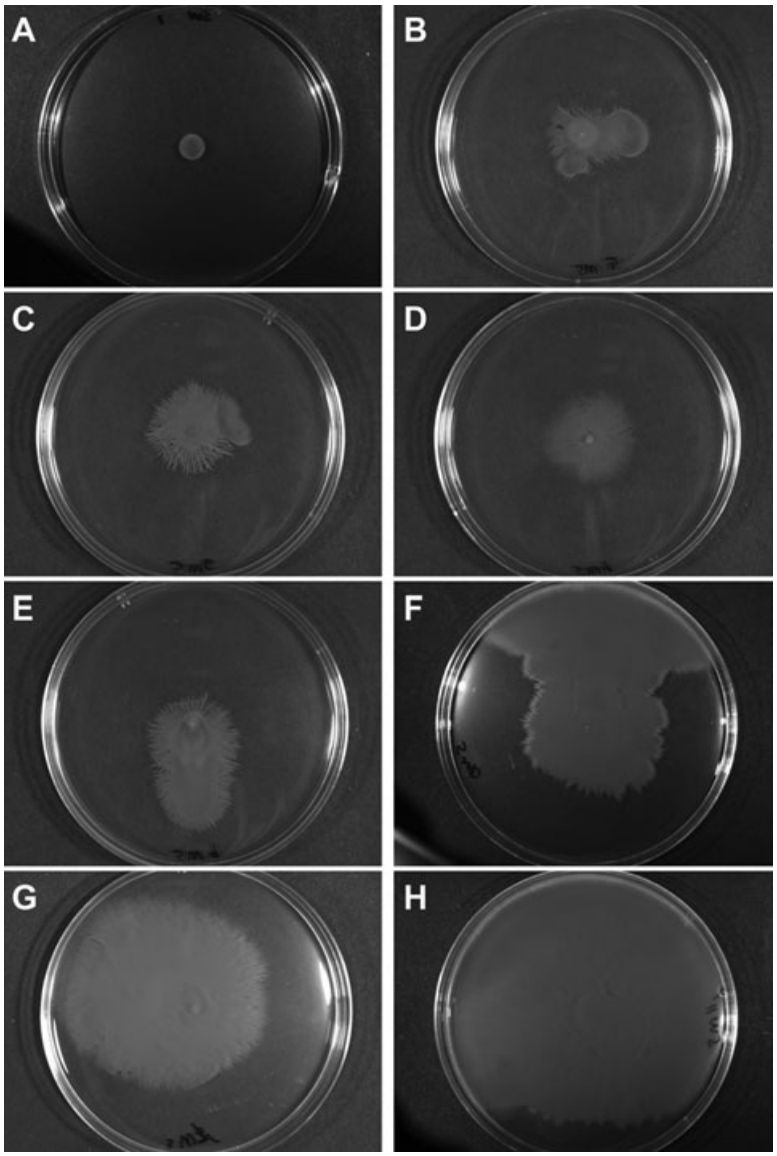

Fig. 2. Swarming motility of *Pseudomonas fluorescens* F113 and hypermotile mutants.
A. F113.
B. *gacS*$^-$.
C. *sadB*$^-$.
D. *wspR*$^-$.
E. *gacS*$^-$*sadB*$^-$.
F. *gacS*$^-$*wspR*$^-$.
G. *sadB*$^-$*wspR*$^-$.
H. *gacS*$^-$*sadB*$^-$*wspR*$^-$.
Exponentially growing cells were inoculated with a toothpick on the surface of swarming plates (0.5% agar). Swarming motility was observed 14 h after inoculation.

swarming phenotype of double and triple mutants were additive (Fig. 2 and Fig. S1), indicating that the same three pathways that repress swimming motility also repress swarming motility independently.

SadB and WspR regulate motility at different levels

A possible target for motility repression is the FleQ protein. This protein acts as a master regulator of most of the genes encoding the synthesis of the flagella in pseudomonads (Arora *et al.*, 1997; Dasgupta *et al.*, 2002). We therefore analysed the expression of the *fleQ* gene in the wild-type and mutant backgrounds by using a transcriptional fusion of the *fleQ* promoter with a promoterless *lacZ* gene (Redondo-Nieto *et al.*, 2008). As shown in Fig. 3A, β-galactosidase assays showed increased *fleQ* expression in the *gacS* and *sadB* mutant backgrounds, but not in the *wspR* mutant.

The *fliC* gene encodes flagellin, the major protein of flagellar filament. By using an antiflagellin antiserum we tested the amount of flagellin in the flagellar filament in the wild-type strain and in the mutants. Figure 3B shows that the amount of flagellin produced by the *sadB* mutant is very high when compared with the wild-type and the other mutants. The amount of flagellin in the *gacS* mutant is higher than in the wild-type strain, but considerably lower than in the *sadB* mutant. No differences in the amount of flagellin were observed between the wild-type strain and the *wspR* mutant. Therefore, good correlation was observed between the expression of the *fleQ* gene and

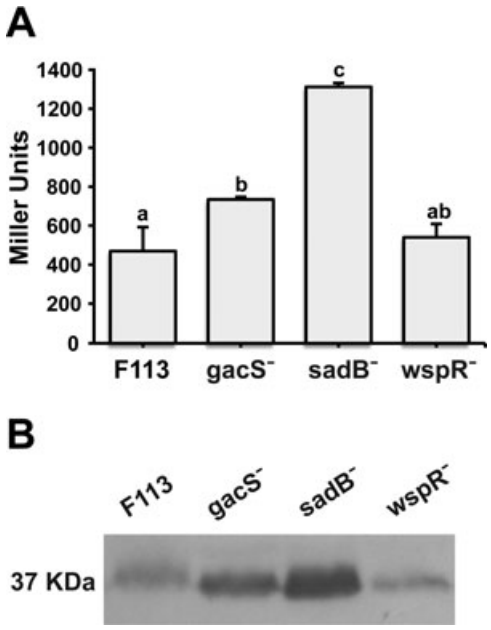

Fig. 3. Flagellin production by *Pseudomonas fluorescens* F113 and hypermotile mutants.
A. Expression of the *fleQ* gene in different backgrounds. A *fleQ::lacZ* fusion was introduced into the different strains and β-galactosidase assays were performed twice in triplicate. Average and standard deviation are shown. Same letters indicate no statistical differences, $P < 0.05$.
B. FliC secretion. Western blot analysis of precipitated extracellular proteins of the different strains. Ten micrograms of proteins was loaded on a 12% polyacrylamide gel, electrophoresed and transferred to a nitrocellulose membrane. An anti-flagellin antiserum was used at a 1:10 000 title. Bands were developed with an ECL system.

the production of the *fliC*-encoded flagellin. These results show that motility repression by the *sadB* gene and the gac system occurs at the level of flagella synthesis, while repression mediated by the wsp signal transduction pathway occurs at a different level.

Discussion

In order to identify new genes and pathways implicated in swimming motility repression, we have performed a generalized mutagenesis of *P. fluorescens* F113 using transposons. Among 3000 transposon mutants we have found 21 (0.7%) that showed enhanced swimming motility when compared with the wild-type strain and had no alterations in secondary metabolism (pyoverdin and exoprotease production) typical of F113 *gac* mutants (Martínez-Granero *et al*., 2005). Among these mutants, we have found transposon insertions in seven different genes (Table 1). Three of these genes, *wspCE* and *R* form part of the same operon that has been previously described to encode a chemotaxis-like system which in response to an unknown extracellular signal transduces this signal to the WspR protein that in turn synthesizes the messenger molecule cyclic di-GMP (Hickman *et al*., 2005). Structurally, the WspR protein consists of a receiver domain and a GGDEF output domain, responsible of c-di-GMP formation (Hickman *et al*., 2005). The Wsp system has been implicated in acetylated cellulose production (Spiers *et al*., 2003; Ude *et al*., 2006), formation of the wrinkly spreader colony phenotype (Spiers *et al*., 2003; Goymer *et al*., 2006; Bantinaki *et al*., 2007), biofilm formation (Spiers *et al*., 2003; Hickman *et al*., 2005; Ude *et al*., 2006) and repression of swarming motility (Hickman *et al*., 2005).

Other of the genes interrupted by the transposon is the *sadB* gene. This gene was identified in a mutant screening as a gene implicated in surface adhesion in the initial steps of biofilm formation (Caiazza and O'Toole, 2004). It was also implicated in swarming motility repression, through rhamnolipid sensing (Caiazza *et al*., 2005). The SadB protein is located in the cytoplasm (Caiazza and O'Toole, 2004) and contains a modified HD-GYP (phosphodiesterase) domain. Although the SadB protein has been related to the turn-over of cyclic di-GMP, no diguanylate cyclase or phosphodiesterase activity has been demonstrated for this protein. A cytoplasmic membrane protein (SadC) that possesses sensor domains and diguanylate cyclase activity and acts upstream of SadB in repressing swarming motility has been recently described in *Pseudomonas aeruginosa* (Merritt *et al*., 2007). It has been suggested that SadB might sense levels of c-di-GMP produced by SadC.

Mutations on *wspR* and *sadB* in *P. fluorescens* F113 have the same effect as that in other pseudomonads with respect to swarming motility (Fig. 2) and biofilm formation (not shown). However, we have shown a novel role for SadB and the Wsp system in negatively regulating swimming motility in *P. fluorescens* F113. We have previously shown that the Gac system repress swimming motility in this bacterium (Martínez-Granero *et al*., 2005). Here we show that the Gac system is also implicated in the repression of swarming motility (Fig. 2) and biofilm formation. Therefore in *P. fluorescens* F113 these three motility-related phenotypes are co-regulated by the same signalling pathways.

Epistasis analysis has shown that the three signalling pathways are independent. As the GacS and WspA proteins are located in the cytoplasmatic membrane and SadB appears to be a cytoplasm-located protein, it seems likely that repression of motility in this bacterium responds to at least two extracellular signals, sensed by GacS and WspA and an intracellular signal sensed by SadB. The signals recognized by GacS and WspA are yet unknown. It has been proposed that SadB could sense rhamnolipids (Caiazza *et al*., 2005) and cyclic di-GMP (Merritt *et al*., 2007), the product of the reaction catalysed by WspR. Our

results indicate that SadB is not sensing the cyclic di-GMP produced by WspR because a mutation on *wspR* is not epistatic over a mutation on *sadB* and a double mutant *sadBwspR* shows an additive swimming and swarming phenotype when compared with the individual mutants. It is possible that a different cell localization or differential production of these two proteins prevents the SadB sensing of WspR produced cyclic di-GMP. Supporting this possibility, it has been recently shown that in *P. aeruginosa* non-phosphorilated WspR (inactive form) is dispersed in the cytoplasm while phosphorilated WspR (active form) forms localized cytoplasmatic clusters (Güvener and Harwood, 2007). Furthermore, we have shown here that the signalling pathways defined by SadB and WspR act at different levels: while a mutation in *sadB* has a dramatic effect in the amount of flagellin secreted by the bacterium, this effect is not observed for a *wspR* mutant indicating that the Wsp system is not acting on flagella biosynthesis. It has been shown that in *Caulobacter crescentus* a cyclic di-GMP binding protein, DgrA, regulates motility by controlling a component of the flagellar motor, without affecting flagella assembly (Christen *et al*., 2007). It is interesting to note that conversely to *P. fluorescens* F113, a *sadB* mutation in *P. aeruginosa* had no effect on flagella synthesis and it was hypothesized that the effect of SadB on swarming motility should be at a different level, probably by signalling to members of the CheIV chemotaxis gene cluster (Caiazza *et al*., 2007). Therefore, control of motility through cyclic di-GMP seems to be different in *P. aeruginosa* (reviewed in Verstraeten *et al*., 2008) and *P. fluorescens*. However, differences in the regulation of the synthesis of the flagellar filament between *P. aeruginosa* and *P. fluorescens* and even between *P. aeruginosa* strains possessing type a and type b flagella have been shown (Redondo-Nieto *et al*., 2008).

Regulation of *fleQ* through the gac system is likely to be indirect. The gac system transcriptionally increases the level of small RNAs able to tritrate the CsrA homologues RsmA and RsmE (Kay *et al*., 2005). These proteins are able to bind specific mRNAs and repress their translation by blocking the ribosome binding site. This binding depends on conserved secondary structure of the mRNA around the ribosome binding site (Schubert *et al*., 2007). The *fleQ* mRNA, although possesses a conserved stem-loop structure around the ribosome binding site, lacks all other structural determinants that have been described as important for RsmAE binding. Furthermore, a translational blockage of the *fleQ* messenger by the Rsm proteins would result in the inverse phenotype for a *gac* mutant, which should exhibit decreased FleQ production and therefore reduced motility. It is therefore likely that the gac system acts through the Rsm proteins in blocking the translation of a messenger RNA encoding a yet unidentified *fleQ* repressor. On the other hand, it is also possible that the Rsm proteins are able to stabilize the *fleQ* messenger as has been shown for the CsrA protein in stabilizing the messenger encoding the flagellar master regulator in enterobacteria (Wei *et al*., 2001).

Figure 4 shows a model for the environmental repression of motility in *P. fluorescens* F113. According to this model, environmental signals are perceived by the GacS, WspA and SadB proteins. Signals perceived by GacS (unknown) and SadB (cyclic di-GMP?) are independently transduced to repress flagellar synthesis by repressing the transcription of *fleQ* resulting in reduced swimming and swarming motility. It is interesting to note that the FleQ protein of *P. aeruginosa* has been shown to bind and respond to c-di-GMP, affecting the negative regulation of other genes not implicated in flagella synthesis (Hickman and Harwood, 2008). Also independently, the unknown signal perceived by WspA is transduced through a chemotaxis-like system to the WspR protein resulting in increased and probably localized levels of cyclic di-GMP, leading to repression of swimming and swarming motility.

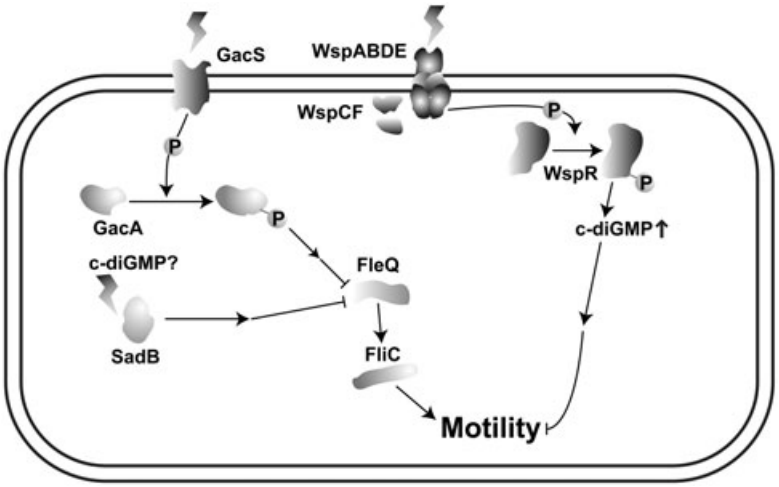

Fig. 4. Hypothetical model showing signalling in environmental repression of motility in *Pseudomonas fluorescens* F113. Extracellular signals perceived by GacS and the Wsp system are independently transduced to the GacA and WspR proteins through phosphorylation. The GacA protein negatively regulates the expression of the flagellar master regulator gene *fleQ* lowering the amount of flagellar proteins (FliC) production. The WspR protein, through its diguanylate cyclase activity, increases the concentration of c-di-GMP that represses motility independently of FleQ. The SadB protein senses a cytoplasmic signal (probably local c-di-GMP concentration) and negatively regulates the expression of *fleQ*. Regulation through SadB is independent from the Gac and Wsp signalling pathways.

Cyclic di-GMP levels have been shown to inversely regulate swarming motility and biofilm formation in pseudomonads and other bacteria (Simm *et al.*, 2004). We have shown here that in *P. fluorescens* F113, this messenger appears to regulate also swimming motility. The importance of motility for rhizosphere competitive colonization and the differences observed with other pseudomonads may reflect different strategies for substrate colonization.

Experimental procedures

Strains, plasmids and growth conditions

Strains and plasmids used are listed in Table 2. Strains of *Pseudomonas* were grown on SA medium (Scher and Baker, 1982) at 28°C, 1.5% purified agar (Pronadisa) was used for solid medium. When necessary, antibiotics were used at the following concentrations (μg ml^{-1}): rifampicin 100; kanamycin 50; gentamicin 5; spectomycin 100. *Escherichia coli* strains were grown on Luria–Bertani (LB) medium at 37°C. Antibiotics were used at the following concentrations (μg ml^{-1}): kanamycin 25; gentamicin 10; spectomycin 100; ampicillin 100; chloramphenicol 30.

Physiological and enzymatic assays

Swimming was tested on SA medium with 0.3% purified agar. Plates were inoculated with a sterile toothpick and incubated at 28°C. Swimming haloes were observed and measured after 18 h. Swarming assays were performed in media containing meat extract 5 g l^{-1}, proteose peptone 3 g l^{-1}, glucose 5 g l^{-1} and 0.5 % American Bacteriological Agar. Plates were inoculated on the media surface with a sterile toothpick imbibed with cultures in a state of exponential growth and incubated at 28°C. Swarming dendrites were observed after 14 h.

Exoprotease production was tested on skimmed milk medium (Martínez-Granero *et al.*, 2005) by the appearance of a degradation halo after 24–48 h. Pyoverdine production under iron-sufficient conditions was observed by exposing cultures grown on LB plates to UV light as previously described (Martínez-Granero *et al.*, 2005). β-Galactosidase activity was determined by the method of Miller (1972), using a transcriptional fusion (out of frame) of the *fleQ* promoter with a promoterless *lacZ* gene present in the pMP220 plasmid. Triplicate overnight cultures on LB medium with an OD_{600} 0.75–0.95 were used. Strains harbouring the empty vector pMP220 and growth under the same conditions were used as negative controls.

Generation and analysis of mutants

Generalized transposon mutagenesis was performed by introducing suicide vectors pRL1063a and pCAM140, containing respectively transposons Tn5*luxAB* and miniTn5*gusA* into *P. fluorescens* F113 by triparental mating. Both transposons contain a replication origin functional in *E. coli.* Transposants were screened for swimming motility in duplicate and those producing haloes larger than the wild-type strain were isolated. Isolates were tested for exoprotease and pyoverdine production, and those showing a phenotype consistent with a *gac* mutation (exoprotease negative and pyoverdine production on LB medium) were discarded. The remaining isolates were tested for single transposon insertion by Southern blot. To determine the point of insertion of the transposon, genomic DNA from the putative mutants was digested with EcoRI (Tn5*luxAB*) or PstI (miniTn5*gusA*), religated and trans-

Table 2. Strains and plasmids used.

	Characteristics	Reference
Strains		
DH5α	*E. coli* cloning strain	Gibco-BRL
F113rif	*P. fluorescens* wild-type Rif^R	Shanahan *et al.* (1992)
F113*gacS*-1	F113rif *gacS*⁻ Rif^R Km^R	Martínez-Granero *et al.* (2006)
F113*gacS*-2	F113rif *gacS*⁻ Rif^R Gn^R	This work
F113*sadB*-1	F113rif *sadB*⁻ Rif^R Km^R	This work
F113*sadB*-2	F113rif *sadB*⁻ Rif^R Gn^R	This work
F113*wspR*-1	F113rif *wspR*⁻ Rif^R Spc^R	This work
F113*wspR*-2	F113rif *wspR*⁻ Rif^R Km^R	This work
F113*gacS-sadB*	F113rif *sadB*⁻ *gacS*⁻ Rif^R Km^R Gn^R	This work
F113*gacS-wspR*	F113rif *wspR*⁻ y *gacS*⁻ Rif^R Spc^R Km^R	This work
F113*sadB-wspR*-1	F113rif *sadB*⁻ y *wspR*⁻ Rif^R Km^R Spc^R	This work
F113*sadB-wspR*-2	F113rif *sadB*⁻ y *wspR*⁻ Rif^R Gn^R Spc^R	This work
F113*gacS-sadB-wspR*	F113rif *sadB*⁻ *wspR*⁻ *gacS*⁻ Rif^R Km^R Spc^R Gn^R	This work
F113v35	Hypermotile phenotypic variant	Martínez-Granero *et al.* (2005)
Plasmids		
pGEM®T-easy vector	Cloning vector Amp^R	Promega
pK18*mobsacB*	Suicide vector *sacB* Km^R	Schäfer *et al.* (1994)
pG18*mob*2	Suicide vector *sacB* Gn^R	Kirchner and Tauch (2003)
pRK2013	Helper plasmid Km^R	Figurski and Helinski (1979)
pRK600	Helper plasmid Cm^R	Finan *et al.* (1986)
pBG1410	Transcriptional fusion p*fleQ*::*lacZ*	Redondo-Nieto *et al.* (2008)

Rif, rifampicin; Km, kanamycin; Gn, gentamicin; Spc, spectomycin; Amp, ampicillin; Cm, chloramphenicol.

formed into *E. coli*-competent cells. The resulting plasmids were isolated and custom sequenced (Parque Cientifico de Madrid) using primers from the transposon ends. Sequence analysis was performed with BLAST programs.

Directed mutagenesis was performed by cloning an internal fragment of the target gene into the suicide vectors pK18*mobsac* (Schäfer *et al*., 1994) or pG18*mob*2 (Kalogeraki and Winans, 1997; Kirchner and Tauch, 2003). The constructs were introduced into F113 or F113-derived strains by triparental mating and selected for homologous recombination. The same methods were used in the construction of double and triple mutants. All mutants were checked by Southern blot and PCR.

Protein electrophoresis and Western blot

In order to detach the flagella, cultures at 0.9–1.2 OD_{600} 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 and extracellular proteins were extracted from the supernatant by precipitation for 16 h at 4°C with 10% (w/v) trichloroacetic acid, followed by two washes with chilled 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. Acrylamide gels were transferred to nitrocellulose membranes for 1 h under standard conditions. The membranes were incubated with a 1:10 000 dilution of an anti-flagellin antiserum (de Weger *et al*., 1987) and then with a peroxidase-tagged secondary antibody (anti-rabbit immunoglobulin) for 1 h. The enhanced chemiluminescence (ECL) method and Hyperfilm ECL (Amersham Biosciences) were used for development.

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Supporting information

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

Fig. S1. Swarming motility of *Pseudomonas fluorescens* F113 and mutants. Bars represent the area of the agar (mm^2) covered by the swarm after 14 hours incubation. Different letters indicate that differences are statistically significant ($p < 0.05$).

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