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Subcellular location characteristics of the *Pseudomonas aeruginosa* GGDEF protein, WspR, indicate that it produces cyclic-di-GMP in response to growth on surfaces

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Summary

The *Pseudomonas aeruginosa* Wsp signal transduction system produces cyclic-di-GMP (c-di-GMP), an intracellular messenger that stimulates biofilm formation and suppresses motility. The Wsp system is homologous to chemotaxis systems and includes a membrane-bound receptor protein, WspA, and a response regulator GGDEF protein, WspR, that catalyses c-di-GMP synthesis when phosphorylated. We found that the subcellular distributions of fluorescent protein-tagged WspA and WspR differed markedly from their chemotaxis counterparts. WspA–YFP formed patches in cells whereas WspR–YFP was dispersed when unphosphorylated and formed bright cytoplasmic clusters when phosphorylated. WspR formed clusters in cells of a *wspF* mutant, a genetic background that causes constitutive phosphorylation of WspR, but was dispersed in cells of a *wspA* mutant, a genetic background necessary for WspR phosphorylation. In addition, WspR mutated at Asp70, its predicted site of phosphorylation, did not form clusters. C-di-GMP synthesis was not required for cluster formation. WspR–YFP was dispersed in liquid-grown wild-type cells, but formed clusters that sometimes appeared and disappeared over the course of a few minutes in cells grown on an agar surface. Our results suggest that the compartmentalized production of c-di-GMP in response to a stimulus associated with growth on a surface is an important functional characteristic of the Wsp system.

Introduction

We have been studying a chemosensory-type system called Wsp from *Pseudomonas aeruginosa*. The wrinkly spreader phenotype (Wsp) was first recognized as a group of adaptive changes that allowed *Pseudomonas fluorescens* to form thick pellicles in standing liquid cultures and distinctive wrinkled colonies on agar plates (Rainey and Travisano, 1998; Spiers *et al.*, 2002). Subsequent work with *P. aeruginosa* showed that wrinkly colony formation is an indicator of strains that form thick biofilms, communities of cells encased in a self-produced extracellular matrix (Hickman *et al.*, 2005). The mutations that caused the Wsp phenotype mapped to *wspF*, a homologue of the chemotaxis gene *cheB* (D'Argenio *et al.*, 2002). *wspF* is located in a cluster of genes that are predicted to encode a signal transduction system homologous to a bacterial chemotaxis system (Fig. 1A).

The Wsp system is conserved in all *Pseudomonas* species and includes a predicted membrane-bound [methyl-accepting chemotaxis protein (MCP)]-like receptor (WspA), CheW-like scaffolding proteins (WspB and WspD), a CheA/Y hybrid histidine sensor kinase with a receiver domain (WspE), a methyltransferase CheR homologue (WspC), a methylesterase CheB homologue (WspF) and a two domain response regulator GGDEF protein, WspR, which catalyses the synthesis of cyclic-di-GMP (c-di-GMP) (Fig. 1B). By analogy with chemotaxis, the Wsp signal transduction system is predicted to activate WspR by phosphorylation. As expected from this, purified phosphorylated WspR from *P. aeruginosa* catalyses c-di-GMP synthesis at faster rates than the unphosphorylated form of the protein (Hickman *et al.*, 2005).

C-di-GMP is an intracellular signalling molecule that has been proposed to control the transition between biofilm and planktonic modes of growth (Römling *et al.*, 2005; Jenal and Malone, 2006; Römling and Amikam, 2006; Ryan *et al.*, 2006; Cotter and Stibitz, 2007; Tamayo *et al.*, 2007). High intracellular c-di-GMP promotes the production of biofilms, adhesive organelles such as pili or stalks, and reduces motility in number of species (Boles and McCarter, 2002; Aldridge *et al.*, 2003; Garcia *et al.*, 2004; Simm *et al.*, 2004; Tischler and Camilli, 2004; Hickman *et al.*, 2005; Kader *et al.*, 2006; Lim *et al.*, 2006). The synthesis and degradation of c-di-GMP is catalysed by diguanylate cyclases with GGDEF domains and phosphodiesterases with EAL domains respectively. Most species of Gram-negative bacteria encode multiple GGDEF and EAL proteins. *P. aeruginosa* strain PAO1 encodes 17 proteins with GGDEF domains, one of which is WspR, five proteins with an EAL domain and 16 proteins with both domains (Kulasakara *et al.*, 2006).

In chemotaxis, receptors called methyl-accepting chemotaxis proteins bind chemoattractants and then rapidly signal associated CheA proteins to alter the phosphorylation status of the response regulator CheY by phosphotransfer (Fig. 1C). CheY-P interacts with flagella motors to change swimming behaviours and affect chemotactic responses (Stock and Surette, 1996; Falke *et al.*, 1997; Stock *et al.*, 2000). CheA also controls the phosphorylation status of a second response regulator, CheB, with a slower kinetics. CheB-P is active as a methylesterase, which modifies the methylation state of glutamate residues on the receptor proteins in conjunction with a constitutively active CheR methyltransferase. Changes in the methylation state of the receptors resets their signalling activity, thereby allowing them to adapt to the original input stimulus. Receptors are hypermethylated in *cheB* mutants. This causes CheA to become constitutively active, which results in the generation of continuously phosphorylated CheY. By analogy with chemotaxis, the *wspF* mutation likely has the effect of locking the Wsp chemosensory system into a configuration where it is constantly signalling, so that the output protein, WspR, is constantly phosphorylated and constantly producing c-di-GMP. A *P. aeruginosa* *wspF* mutant produces elevated levels of intracellular c-di-GMP (Hickman *et al.*, 2005). Consistent with its role as a signal transduction system, disruption of the Wsp system by mutation of *wspA* or *wspR* causes the *wspF* wrinkly colony phenotype to convert to smooth (Fig. 1D).

The system of rapid response and slower adaptation that is an inherent feature of chemotaxis systems allows motile cells to continuously sense and swim up concentration gradients of chemoattractants. By extension, we hypothesize that the Wsp signal transduction system

allows *P. aeruginosa* to respond and adapt to an as-yet-unknown, but constantly varying, environmental stimulus to fine tune intracellular levels of c-di-GMP. This may provide a mechanism to allow cells to transition in and out of the biofilm state.

Here we analysed the subcellular localization of Wsp proteins as an avenue to gain information about the functional characteristics of this novel chemotaxis-like signal transduction system and to expand our knowledge of the behaviour of a GGDEF protein and accompanying c-di-GMP production in individual cells. We determined that WspA receptor proteins localize in patches all over cells rather than at cell poles, as is the case with *P. aeruginosa* chemoreceptors. We also found that WspR dynamically clusters in the cytoplasm of cells when it is phosphorylated and we present evidence that the formation of cytoplasmic clusters of WspR protein is indicative of Wsp signal transduction. From this we concluded that growth on a solid agar surface stimulates Wsp signal transduction and therefore c-di-GMP production. Our data indicate that the Wsp system produces c-di-GMP at discreet subcellular locations, implying that cells may have specific Wsp-associated targets of c-di-GMP action. Furthermore, although chemotaxis and Wsp systems share many features in common, the details of signal transduction by the two systems differ in important ways.

Results

YFP-tagged WspR and WspA proteins are functional

To visualize the subcellular localization of Wsp proteins, we fused a yellow fluorescent protein gene (*yfp*) to the C-termini of the *wspR* and *wspA* genes. We carried out allelic replacements so that *yfp* gene fusions replaced the wild-type copy of the gene and were present at their native sites on the chromosome, expressed from their original promoters. The phenotypes of *wspF* strains expressing *wspR-yfp* or *wspA-yfp* were similar to those of the *wspF* strain carrying wild-type *wspR* and *wspA* alleles in that all strains had wrinkly colonies and were compromised in swimming motility. This indicates that WspR-YFP and WspA-YFP are functional (Fig. 2).

WspA localizes laterally in cells rather than primarily at the poles, as is the case for chemotaxis receptors

WspA-YFP formed multiple lateral patches in wild-type cells (Fig. 3A). As WspA is a predicted membrane-bound protein, it is likely that the patches are located at the peripheries of cells. This pattern of localization differs from that of MCPs involved in chemotaxis, which localize mainly at cell poles as a large patch (Maddock and Shapiro, 1993; Sourjik and Berg, 2000; Güvener *et al.*, 2006), although a few lateral patches of MCPs are also typically present (Maddock and Shapiro, 1993; Sourjik and Berg, 2000; Thiem *et al.*, 2007). The lateral localization of WspA-YFP occurred throughout growth and the pattern of localization that we observed was very similar in broth-grown and surface-grown cells (data not shown). A *wspA-yfp* fusion strain in a wild-type background formed wrinkly colonies (Fig. 3B), suggesting that the addition of YFP to the C-terminus of WspA caused the receptor to adopt a conformation such that it stimulated Wsp signal transduction constitutively in a wild-type genetic background. That a *wspE wspA-yfp* strain formed

smooth colonies (Fig. 3B) confirms the prediction that WspE is necessary for WspA-mediated signalling and is an essential part of the Wsp signal transduction system (see Fig. 1B). The deletion of *wspB*, one of the two *cheW* homologues in the Wsp operon, did not alter wrinkly colony formation by the *wspF* mutant strain (Fig. 3B).

This suggests that WspB does not have an essential role in Wsp signal transduction.

WspR forms cytoplasmic clusters in liquid-grown *wspF* cells, but not in liquid-grown wild-type cells

We only occasionally saw spots of WspR–YFP in liquid-grown wild-type cells observed by fluorescence microscopy. From this we concluded that WspR was likely dispersed in the cytoplasm rather than present at a particular subcellular location (Fig. 4A). WspR has previously been shown to be a cytoplasmic protein (Hickman *et al.*, 2005). In keeping with this, the measured fluorescence intensities were relatively uniform across individual cells (Fig. 4B). By contrast, WspR–YFP localized in clusters visualized as bright spots in liquid-grown *wspF* mutant cells (Fig. 4C). This was reflected as a high ratio of maximum to average fluorescence intensities in individual cells (Fig. 4D). Quantification of fluorescence intensities in single cells indicated that about 20% of *wspF* cell population had WspR–YFP clusters (Table 1). We saw WspR–YFP clustering in 1–2% of the wild-type cell population. Western blot analysis with anti-GFP antibody indicated that the amounts of WspR–YFP were approximately equal in wild-type and *wspF* cells.

Only phosphorylated WspR forms visible clusters in the cytoplasm

Our observation that WspR–YFP formed significant numbers of bright clusters only in *wspF* cells (Fig. 5A), a condition where WspR is predicted to be continuously phosphorylated, suggested that clustering of WspR–YFP is an indication of the presence of phosphorylated WspR. To test this, we mutagenized the predicted phosphor-accepting residue, Asp70, critical for the activity of WspR in *P. aeruginosa* strain PAO1. Mutations in Asp70 disrupt diguanylate cyclase activity, and as a result wrinkly colonies become smooth (J.W. Hickman and C.S. Harwood, unpublished). We created an *yfp* fusion to a mutant *wspR* allele carrying an Asn change at the residue Asp70. *wspF* cells expressing an *yfp*-tagged Asp70Asn allele of *wspR* (WspR[D70N]–YFP) formed smooth colonies as expected (Fig. 5B). WspR[D70N]–YFP was dispersed in *wspF* mutant cells (Fig. 5A) indicating residue Asp70 is also essential for WspR–YFP clustering. Immunoblot assays confirmed that full-length fusion proteins were similarly present in crude cell extracts of strains expressing YFP-tagged wild-type WspR or the YFP-tagged Asp70Asn variant (data not shown). This rules out the possibility that delocalization was due to a cleavage at a junction that would release the Asp70Asn variant and YFP proteins.

WspR–YFP clusters did not form in *wspF wspA* mutant cells (Fig. 5A). This indicates that WspA is essential for WspR–YFP clustering probably through WspA-dependent phosphorylation. The expression of *wspA* *in trans* from an arabinose-inducible promoter on a low-copy plasmid (pWspA) in *wspF wspA* mutant cells restored the lack of WspR–YFP clustering and wrinkly colony formation in this strain (Fig. 5C and D). The percentage of cells with WspR–YFP clusters in a WspA-complemented strain was similar to that of the

wspF strain (Table 1). A cytoplasmic fragment of WspA failed to restore clustering of WspR–YFP or wrinkly colony formation in a *wspF wspA* deletion strain (data not shown). We fused an *yfp* gene to the cytoplasmic fragment of WspA and found that the fusion protein was diffused throughout the cytoplasm (data not shown).

C-di-GMP generation is not important for WspR–YFP clustering. We determined this by examining the subcellular localization of an YFP-tagged Glu253Ala variant of WspR that was unable to catalyse c-di-GMP production due to a mutation in its conserved GGEEF motif (J.W. Hickman and C.S. Harwood, unpublished). This YFP-tagged Glu253Ala variant formed tight clusters similar to the YFP-tagged wild-type WspR in the *wspF* mutant cells (Fig. 5A).

WspR–YFP clustering and wrinkly colony formation occurred in *wspF cheA* cells (data not shown), indicating the Wsp signalling pathway does not depend on the chemotaxis-signalling pathway.

WspR–YFP and WspA–CFP colocalize transiently

That WspA and WspE are required for wrinkly colony formation supports the idea that this receptor and sensor kinase act together to phosphorylate WspR by phosphotransfer between a WspA/WspE complex and WspR as is shown in Fig. 1B. When we examined the behaviour of YFP-tagged WspR and CFP-tagged WspA coexpressed in *wspF* cells, we found that these proteins sometimes appeared to be colocalized, but this was not the general rule (Fig. 6). We reported previously that the *P. aeruginosa* chemotaxis output regulator CheY is always seen colocalized with membrane-bound receptor-CheA complexes at cell poles (Güvener *et al.*, 2006), as is the case in *Escherichia coli* (Sourjik and Berg, 2000). The behaviour of the WspR proteins thus differs from its homologous chemotaxis counterparts.

Surface growth stimulates Wsp signal transduction

That WspR forms visible clusters in cells when it is phosphorylated suggested to us that we could use the subcellular localization characteristics of WspR–YFP to identify conditions that stimulate Wsp signalling. We found that although WspR–YFP was dispersed in wild-type cells growing in Luria–Bertani (LB) broth, it formed fluorescent spots in cells grown to confluence on the surface of LB agar for 20 h (Fig. 7A). Quantification of WspR–YFP fluorescence signals in single cells showed that about 30% of the wild-type cells grown on agar surfaces that we observed had WspR–YFP clusters (Fig. 7B and Table 2). Cells did not form WspR–YFP clusters in the absence of WspA, and *wspA* expressed *in trans* (from plasmid pWspA) restored the lack of clustering at levels similar to that of the parent fusion strain (Table 2). The average signal intensity per cell was similar in broth-grown cells (269 ± 14 signal intensity) and surface-grown cells (290 ± 18 signal intensity) expressing WspR–YFP, indicating that the total amount of Wsp protein synthesized was the same under the two conditions. In addition, we found that the amount of *wspR* mRNA transcript synthesized was the same in broth-grown and surface-grown cells. We also observed WspR–YFP cluster formation in *wspF* cells grown on agar surfaces (Fig. 7C and D). Interestingly, the percentage of agar-grown *wspF* cells that had WspR–YFP clusters was higher than the percentage of liquid-grown *wspF* cells that had clusters (Tables 1 and 2). One

interpretation of this is that signal transduction can be further stimulated in the 'locked on' *wspF* mutant.

The amount of agar used to solidify LB medium was not critical for clustering. WspR–YFP clustering occurred in wild-type cells grown on agar surfaces solidified with 1% or 2.5% agar. Changes in the amount of yeast extract or tryptone or elimination of one of the two from LB medium did not have an effect on clustering of WspR–YFP in wild-type cells. Removal of NaCl from LB agar medium abolished clustering in wild-type but not in *wspF* cells. We found that 85 mM NaCl, the amount of salt in LB, was needed for the formation of well-defined fluorescent spots. The substitution of 85 mM KCl or 100 mM KH₂PO₄ for NaCl in LB medium also supported the formation of WspR–YFP clusters. WspR–YFP clusters formed in wild-type cells grown on tryptic soy broth plates. Several defined growth media, including M63, minimal A medium and M9 (Sambrook *et al.*, 1989), solidified with agar, supported the formation of WspR–YFP clustering. Succinate (20 mM), citrate (20 mM) or glucose (17 mM) served as the carbon sources in the defined media tested. WspR–YFP clustering was similarly efficient in cells grown on rich medium or defined minimal medium solidified with Bacto agar, Nobel agar or agarose. However, cells had a slower growth rate on defined medium solidified with agarose. We did not observe WspR–YFP cluster formation in wild-type cells when we made gross changes in viscosity by adding Ficoll to LB broth (not shown). High osmolarity was detrimental to WspR–YFP clustering in wild-type and *wspF* mutant cells. Cells grown on LB plates supplemented with 300 mM NaCl or 700 mM sucrose did not have clusters of WspR–YFP.

Cells growing on a solid surface tend to be touching and making cell-to-cell contact. *P. aeruginosa* has a number of different kinds of surface appendages that bind cells together and that could be important for initiating a Wsp signalling event. We visually inspected WspR–YFP cluster formation in several independently grown cultures of mutants lacking flagella (a *fliC* mutant), type IV pili (a *pilA* mutant), or exopolysaccharide (EPS) (a *pel psl* mutant). We found that the quantity and quality of WspR–YFP clusters formed in the cells of each type of mutant observed after about 20 h of growth on LB agar plates were very similar to those of wild-type cells.

Dynamic behaviour of WspR clusters in cells

WspR–YFP clusters were mobile in cells. We used time-lapse microscopy to obtain fluorescent images of individual wild-type and *wspF* cells grown on agar plates and transferred to an agarose pad. Cells typically had between one and four fluorescent spots. Figure 8 shows representative fluorescence and phase-contrast images of wild-type cells. Images were captured at intervals of 30 s. Several different patterns are evident. Some spots were continuously present but moved inside cells and varied in brightness over the period of observation (Fig. 8A). In some cases spots disappeared and reappeared (Fig. 8B), and we also observed the partitioning of spots from one into two (Fig. 8C). We also observed situations in which spots were continuously present in cells and dynamic without accompanying changes in brightness (not shown). We analysed time lapse images acquired every 30 s over a period of 270 s for 20 *wspF* cells and 49 wild-type cells. The variety of patterns of WspR fluorescent spots, their movement and the numbers of spots that we saw

per cell were approximately the same for *wspF* and wild-type cells. However, a higher percentage of *wspF* cells had spots that were continuously present in cells over the interval of observation (65% of cells) compared with wild-type cells (38%) and the spots were brighter. Wild-type cells tended to have a greater proportion of WspR fluorescent clusters that appeared and disappeared or partitioned during the interval of observation.

Discussion

Our observations indicate that clusters of WspR–YFP protein become visible in the cytoplasm of cells in which the Wsp system is active. Clusters of fluorescent protein-tagged WspR were dynamic and moved around inside cells. Fluorescent spots of WspR increased and decreased in brightness. This could reflect movement of the complexes of protein in and out of the plane of focus, or it may reflect the formation and dissolution of complexes of active WspR. These observations suggest that that Wsp-mediated c-di-GMP formation occurs at discreet subcellular sites. This could allow the Wsp system to deliver localized high concentrations of c-di-GMP to specific c-di-GMP receptor proteins. Known c-di-GMP binding domains include the I site of some diguanylate cyclases (Christen *et al.*, 2006). First defined for the *Caulobacter crescentus* PleD protein, the I site binds c-di-GMP to cause feedback inhibition of diguanylate cyclase activity in a non-competitive manner (Chan *et al.*, 2004). The PelD protein from *P. aeruginosa* binds c-di-GMP at a site that resembles the I site in secondary structure. PelD, which is not a diguanylate cyclase, controls EPS synthesis in response to c-di-GMP by a mechanism that is still to be elucidated (Lee *et al.*, 2007). EAL domain phosphodiesterases that convert c-di-GMP to pGpG also bind c-di-GMP, although the binding site has not been defined. Finally, a c-di-GMP binding domain called PilZ has been described (Amikam and Galperin, 2006; Ryjenkov *et al.*, 2006; Christen *et al.*, 2007; Pratt *et al.*, 2007). *P. aeruginosa* has eight PilZ domain proteins, all of which have been shown to bind c-di-GMP (Merighi *et al.*, 2007; Ramelot *et al.*, 2007). All are conserved hypothetical proteins of unknown function with the exception of PilZ itself (PA2960), which is required for normal pilus assembly (Alm *et al.*, 1996), and Alg44 (PA3542), an alginate bio-synthesis gene (Remminghorst and Rehm, 2006) which responds to c-di-GMP to modulate the production of this EPS (Merighi *et al.*, 2007). Any of one or a combination of the described *P. aeruginosa* c-di-GMP receptor proteins could be targets of Wsp-generated c-di-GMP and it is also possible that Wsp-generated c-di-GMP binds to other protein domains that have yet to be described.

Although investigators are beginning to identify c-di-GMP binding proteins as having functions that influence motility or biofilm formation, the mechanism by which any protein exerts its effects once it has bound c-di-GMP is not fully understood. In general, c-di-GMP appears to affect the activities of large protein complexes including cellulose synthases, alginate synthase, and flagella motors and pilus assembly (Huang *et al.*, 2003; Kazmierczak *et al.*, 2006; Ryjenkov *et al.*, 2006; Christen *et al.*, 2007; Merighi *et al.*, 2007; Pratt *et al.*, 2007; Ramelot *et al.*, 2007). C-di-GMP also influences the expression of genes for EPS synthesis, virulence and motility (Hickman *et al.*, 2005; Tischler and Camilli, 2005; Mendez-Ortiz *et al.*, 2006; Kim and McCarter, 2007). Our initial characterization of WspR in cells suggests that its function is not to exclusively influence processes that occur at cell poles because it is not exclusively present at poles when in its activated form. In this sense it

differs from the response regulator GGDEF protein PleD from *C. crescentus* and the GGDEF-EAL phosphodiesterase, FimX, from *P. aeruginosa*, both of which localize to a cell pole (Paul *et al.*, 2004; Kazmierczak *et al.*, 2006). PleD resembles WspR in that it has a visible subcellular location only when it is active. The localization characteristics of PleD change when it is activated by phosphorylation and dimerization such that it is sequestered to the cell pole destined for stalk biogenesis (Paul *et al.*, 2004; Paul *et al.*, 2007). A *pleD* mutant is defective in the distinctive flagella ejection and stalk elongation that occur as part of the *C. crescentus* cell cycle (Aldridge and Jenal, 1999) and a *fimX* mutant is defective in type IV pilus assembly (Huang *et al.*, 2003).

Non-polar protein complexes that would be logical targets of activation by WspR-generated c-di-GMP are complexes associated with the synthesis of Pel and Psl EPS in *P. aeruginosa* strain PAO1 (Friedman and Kolter, 2004a,b; Jackson *et al.*, 2004; Matsukawa and Greenberg, 2004; Ma *et al.*, 2007). We know that the *pel* and *psl* operons are responsible for the wrinkly colonies and robust biofilms that are formed when WspR-generated c-di-GMP accumulates in *wspF* strains because we have observed that a *wspF pel psl* strain forms smooth colonies similar in texture to the wild type (J.W. Hickman and C.S. Harwood, unpublished).

Our conclusion that growth on an agar surface stimulates Wsp signal transduction supports the notion that a condition associated with cell-to-cell contact or cell-surface interactions stimulates *P. aeruginosa* to form biofilms. Growth on a solid surface rather than transient contact with a solid surface seems to be required for the stimulation of Wsp signal transduction because WspR–YFP did not form clusters in broth-grown wild-type cells that were transferred to phosphate-buffered saline (PBS) agarose pads and observed by microscopy over a period of an hour. When we observed individual cells inoculated onto M9-citrate medium agarose pads and allowed to grow over a period of about 18 h, we saw that bright spots of WspR–YFP appeared and disappeared from cells photographed at 30 min intervals once cells grew to form a group of about eight or more cells (not shown). These observations suggest that cells need to be touching or very close to each other to signal through the Wsp system. In many ways the Wsp system seems analogous to the Dif chemosensory pathway in *Myxococcus xanthus*, which regulates EPS production, though not necessarily by a c-di-GMP based mechanism (Yang *et al.*, 2000; Bellenger *et al.*, 2002; Black and Yang, 2004). Evidence suggests that type IV pili may stimulate Dif signal transduction (Black *et al.*, 2006). We tested several kinds of *P. aeruginosa* cell surface structures that might bind cells together including flagella, type IV pili and EPS but failed to find any clear evidence for their involvement in Wsp signalling. It is also possible that a physical parameter that is subtly altered during growth on an agar surface stimulates Wsp signal transduction.

A surprising feature of the WspA and WspR proteins is how different their subcellular distributions are from those of their chemotaxis homologues. These differences likely reflect functional differences between the two systems. In chemotaxis, MCPs and associated signal transduction and signal adaptation proteins organize as arrays in large patches at cell poles (Zhang *et al.*, 2007). It has been estimated that on the order of 7500 receptor dimers are packed into *E. coli* polar arrays (Li and Hazelbauer, 2004). The arrays, which include

multiple different receptors, each specific for a different set of chemoeffectors, integrate stimuli for a common output. The structural arrangement of the arrays is also such that input signals are amplified so that environmental stimuli are sensed over a wide dynamic range (Duke and Bray, 1999; Sourjik and Berg, 2004; Studdert and Parkinson, 2004; Wolanin and Stock, 2004). Our observations showing that WspA is distributed at multiple locations around cells and that the WspR protein is transiently associated with WspA suggest that signal integration and signal amplification of the type that occurs in chemotaxis might not be fundamental features of the Wsp system. In the future it will be of interest to determine the structural features of the Wsp proteins that determine their subcellular location and to determine whether the movement of WspR in cells is due to specific associations with the cytoskeleton or other cytoplasmic proteins.

Experimental procedures

Bacterial strains and growth condition

Pseudomonas aeruginosa strains used in this study are listed in Table 3. *E. coli* strains were DH5 α , obtained from Gibco-BRL and S17-1 (Simon *et al.*, 1983), which was the donor strain used to transfer plasmids to *P. aeruginosa* by conjugation. *E. coli* and *P. aeruginosa* strains were grown in LB medium (Sambrook *et al.*, 1989) at 37°C or at room temperature as specified. Strains to be used for microscopy were freshly streaked on LB agar plates from frozen stocks and incubated overnight at 37°C. Cells were inoculated into a 16 mm test tube containing 3 ml LB. Tubes were incubated with shaking until the culture reached an OD₆₀₀ of 0.2–0.3. These broth-grown cells were then diluted to an OD₆₀₀ of 0.005. This served as the inoculum for broth-grown or surface-grown cells. To generate surface-grown cells, a 125 ml sample was spread on an LB agar plate solidified with 2.5% Bacto agar. Plates were incubated at room temperature for about 20 h. Cells scraped from plates at the end of this period of incubation typically yielded an OD₆₀₀ of 0.15–0.3 upon resuspension in 25 ml LB. To generate broth-grown cells, 25 ml diluted cultures were aerated in 125 ml Erlenmeyer flasks at room temperature to mid-log phase in the range of 0.2–0.5 OD₆₀₀. Antibiotic concentrations were 100 mg ml⁻¹ ampicillin and 10 μ g ml⁻¹ gentamicin (Gm) for *E. coli* and 50 μ g ml⁻¹ Gm for *P. aeruginosa*. L-arabinose at a final concentration of 0.1% was included in growth media to induce gene expression from a low-copy plasmid.

Colony morphologies were visualized by spotting 2 μ l of LB broth-grown cells that had been diluted to an OD₆₀₀ of 0.005 onto Congo Red plates (Friedman and Kolter, 2004a) and allowing growth to occur at room temperature for 2 days.

DNA manipulations

Cloning, gel electrophoresis, and related molecular biology work were carried out according to standard protocols (Sambrook *et al.*, 1989). Herculase Enhanced DNA polymerase (Stratagene) was used to amplify DNA. DNA was isolated with Qiagen DNA extraction kits. Oligonucleotides were synthesized by Integrated DNA Technologies. DNA sequencing was performed at the DNA sequencing facility of the University of Washington. *wspR* mRNA transcript levels were determined by real-time PCR as described (Bagge *et al.*, 2004).

Generation of chromosomal fusions of *yfp* or *cfp* to *P. aeruginosa* genes

yfp or *cfp* translational fusions at the C-terminus of *wspA* or *wspR* were generated as previously described by using suicide vectors pEX19EYFP or pEX19ECFP (Güvener *et al.*, 2006). *wspA* was amplified from the *P. aeruginosa* chromosome with a primer pair in which both the 5' and 3' ends had an engineered KpnI sites. The 3' end excluded the stop codon and was followed by two bases prior to the KpnI site. This KpnI fragment was cloned into either pEX19EYFP or pEX19ECFP just upstream of the *yfp* or *cfp* start codon and a clone with the correct orientation of the KpnI fragment was determined. Flanking DNA downstream of *wspA* was amplified from the *P. aeruginosa* chromosome to give a PCR fragment with XbaI and HindIII engineered sites. This XbaI-HindIII fragment was cloned into the previous construct immediately downstream of the *yfp* or *cfp* gene to yield a *wspA*-*yfp* or *wspA*-*cfp* fusion with downstream flanking DNA. PCR amplified fragments containing *wspR* sequences had engineered EcoRI sites at the 5' ends and KpnI sites at the 3' ends. Site-directed mutagenesis to create specific mutations in *wspR* is described below. All final constructs were sequenced to make sure that they were correct and that no PCR mistakes had been introduced during the cloning steps. Gene replacement was used to move the fusion constructs into the *P. aeruginosa* chromosome by homologous recombination (Ferrández *et al.*, 2002). Candidates were screened by PCR. For the allelic replacements in which site-specific mutations were moved to the chromosome, a region of the chromosome was PCR-amplified and sequenced to verify that the correct site-specific mutations were present. Western blot analysis with antiserum to GFP (Clontech) showed that full-length fusion proteins were expressed to the same levels in *P. aeruginosa* cells in all genetic backgrounds tested.

Site-directed mutagenesis to change WspR residue Asp70 to Asn was carried out by overlap extension PCR (Horton *et al.*, 1993). Intergenic primers, which carried mismatched bases to change the codon Asp70 to Asn, were 5'-GTTCGACGCCGGGCATCACCAGGTTCTG GAGG ATC-3' (WspRD70N-R) and 5'-GATCCTCCAGAACCTGGTGATGCCCGGCGTCGAC-3' (WspRD70N-F). Mismatched bases are underlined. Outer primer sequences were 5'-GCCATGGAATTCCCGGGTTCGACTCCGGCCAGGCGCCAG-3' (WspR-F) and 5'-GCTATCGGTACCGGGCCCGGGGCGCGGCACCGGCTGTTCATCAGGCC CACCTGG-3' (WspR-R). EcoRI and KpnI sites are shown in bold letters. A stop codon was omitted in the WspR-R primer but it contained two extra bases and an engineered KpnI site to allow creation of an in-frame fusion to *yfp* in pEX19EYFP. An Ala change in the WspR residue Glu253 was accomplished by a similar strategy. The *wspRE253A* allele was amplified with WspR-F and WspR-R primers to make an in-frame fusion to *yfp* when cloned into pEX19EYFP.

Construction of an in-frame deletion of *wspB*

To make a *wspB* deletion in a *wspA*-*yfp* fusion strain, we created a *wspB* in-frame deletion construct in pEX19Gm (Hoang *et al.*, 1998). The deletion construct was generated by using PCR to amplify two regions of chromosome. One was a 0.757 kb fragment immediately upstream of *wspB*, corresponding to the junction between *wspA*-*yfp* and *wspB* genes. The other was a 1.239 kb fragment immediately downstream of *wspB*. Primers for the

region upstream of *wspB* were 5'-
AGTCCCGGTACCGGTCGCCACCATGGTGAGCAAG-3' (WspB–YFPupF) and 5'-
GCAGGCGTTCGAAGCGATCGTTCATGCGGTCACAGCCTCTA GAG TCGCG-3'
 (WspB–YFPupR), and for the region downstream of *wspB* were 5'-
CGGACTCTAGAGGCTGTGACCGCATGAACGATCGCTTCGAACGCCTGC-3'
 (WspBdownF) and 5'-GC CACAA**AGCTT**CTTTGTTCACTCCACGGGCCCGCGTTG-3'
 (WspBdownR). KpnI and HindIII sites are shown in bold letters and regions complementary
 to downstream of *wspB* (in WspB–YFPupR) or upstream of *wspB* (in WspBdownF) are
 underlined. A mixture of the two PCR products served as templates for a final PCR reaction,
 which generated a fused product of the two templates. This was then crossed into *wspA–yfp*
 strain PAO1275 (Table 3) by conjugation, and homologous recombinants were selected as
 described by Ferrández *et al.* (2002). Mutant candidates were screened by PCR.

Generation of arabinose-inducible gene expression constructs

Plasmid pGFP-C was used to generate an arabinose-inducible *wspA* expression construct. pGFP-C is a derivative of pJN105, a broad host-range vector that carries an α -arabinose-inducible promoter and the *araC* regulator gene (Newman and Fuqua, 1999). We originally constructed pGFP-C to make in-frame translational fusions to green fluorescence protein gene (*gfp*). However, here we used this plasmid because it contained an optimized ribosome-binding site for gene expression. To construct pGFP-C, an NheI-PvuI fragment was obtained from plasmid pDSW208 (Weiss *et al.*, 1999). This fragment contained an optimized ribosome-binding site, a methionine initiation codon (ATG) and multiple restriction enzyme sites followed by the *gfp* gene. The NheI-PvuI fragment was ligated just 3' of the arabinose-inducible promoter of pJN105, to give pGFP-C. The *wspA*-coding region starting with the second codon, and including its stop codon, was amplified from the chromosome of *P. aeruginosa* with a primer set that had engineered XbaI sites. The 3' primer also included an engineered HindIII site prior to the XbaI site. The amplified PCR product was digested with XbaI and cloned into XbaI-digested pGFP-C. A clone carrying the correct orientation of the XbaI fragment was determined. A HindIII digestion released the *gfp* gene from this construct. HindIII-digested and gel-purified vector was self-ligated creating the final construct, pWspA. The cloning procedure introduced 11 codons for amino acids Glu-Phe-Glu-Leu-Gly-Thr-Arg-Gly-Ser-Ser-Arg between the first and second codons of *wspA* in the pWspA construct.

An expression construct of a truncated version of *wspA*, which consisted of only the predicted cytoplasmic domain, was constructed similarly to the pWspA construct. The truncated *wspA* excluded the N-terminal 286 codons predicted to encode its periplasmic sensing and transmembrane domains. A primer pair, of which the 5' primer had an engineered EcoRI site and the 3' primer had a HindIII site, was used to amplify the C-terminus of *wspA*, a region of 256 amino acids with a stop codon. The PCR fragment was digested with EcoRI and HindIII and ligated into EcoRI-HindIII-digested and gel-purified pGFP-C. This procedure also eliminated the *gfp* gene sequence from pGFP-C. The construct encoding the truncated *wspA* had a methionine start codon followed by two non-native codons (Glu and Phe) before the first residue 287Gln of the WspA C-terminus.

Fluorescence microscopy

Samples were prepared for microscopy from broth-grown or surface-grown cells. Broth-grown cultures were appropriately diluted in PBS (pH 7.4) and 3 μ l of cell suspension was spotted on the surface of agarose pads that were prepared with 1.0% agarose made in PBS. An agarose gel had previously been prepared in a standard gel casting system. Blocks of agarose were cut out and transferred on microscope slides and samples were applied and then covered with coverslips. Surface-grown cells were transferred to agarose pads with cotton swabs. Images were acquired with a Nikon 80i microscope equipped with 100 \times PlanApochromat objective (numerical aperture, 1.4), and were recorded with a Cool-Snap HQ camera (Photometrics). The imaging system was operated with Metamorph 6.3r2 software. Filter sets were purchased from Chroma Technology Corp. The filter sets were ET-EYFP (Chroma # 49003) for YFP excitation and ET-CFP (Chroma # 49001) for CFP excitation. All images shown are fluorescence and phase-contrast images. All images that were compared with each other in the text and figures were acquired in the same day with the same microscope settings. Images were minimally processed with Adobe Photoshop 7.0 and ImageJ_{NIH} software. All images that were compared with each other were manipulated (brightness and contrast adjustments and resizing of files) identically. At least three independent experiments were performed, and at least five different fields of view from each experiment were analysed. Figures show representative images.

Single cell fluorescence intensity measurements

Phase-contrast images were used as a reference point to select well-isolated single cells in the fields of view. The corresponding fluorescence images were then analysed with Metamorph software to quantify greyscale intensity and pixel brightness. Average grey values were extracted that indicated the average of the pixel greyscale values in selected cells. Integrated grey values were extracted that indicated the sum of the greyscale values for all pixels in individual cells. Maximum grey values were extracted that indicated maximum areas of pixel intensity within cells. These values were used to assess WspR clustering. Integrated grey values were plotted against the area of selected individual cells. Data points that deviated from a linear curve were not considered for analysis. Average grey values for the *wspR-yfp* strain were low but were above that seen for a nonfluorescent wild-type strain. By determining ratios of maximum to average grey values, it was possible to combine data obtained from different days. With the microscope settings used, a ratio of 1.64 was determined to be the threshold value for a well-defined fluorescent spot as judged by visual inspection of images of wild-type cells grown on surfaces. Data shown in graphs are the ratios that were plotted against the areas of selected cells. These analyses were done on images acquired from at least three independent experiments from about 10 different fields of view.

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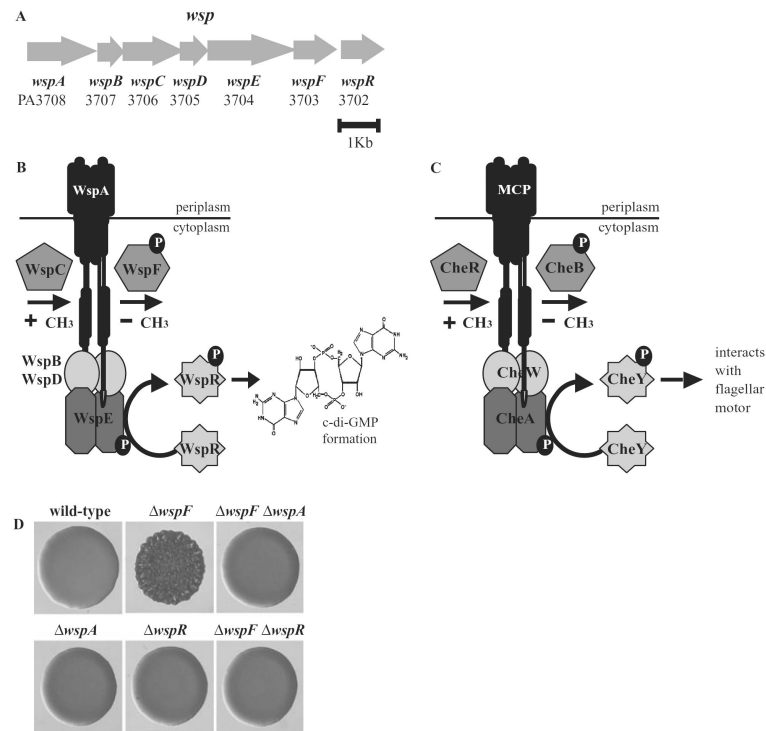
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**Fig. 1.**

Characteristics of the Wsp signal transduction system.

A. Organization of the genes encoding the Wsp system.

B. The Wsp proteins are predicted to be a membrane-bound methyl-accepting chemotaxis protein (WspA), a CheR-like methyltransferase (WspC), a CheB-like methyl-erasing (WspF), two CheW homologues (WspB and WspD) that are predicted to serve as linkers between WspA and a hybrid histidine kinase response regulator (WspE). The response regulator protein, WspR, has a GGDEF domain and catalyses the synthesis of c-di-GMP when phosphorylated. As described in the text, a *wspF* mutation is predicted to lock the Wsp system into a configuration where WspR is constantly phosphorylated and thus constantly producing c-di-GMP.

C. The physical organization of homologous Che proteins (encoded by PA1456–1459, PA1464 and PA3349) and (D) colony morphologies of *P. aeruginosa* PAO1 wild-type and *wsp* deletion strains. *wspF* mutants have high levels of intracellular c-di-GMP relative to wild-type cells (Hickman *et al.*, 2005).

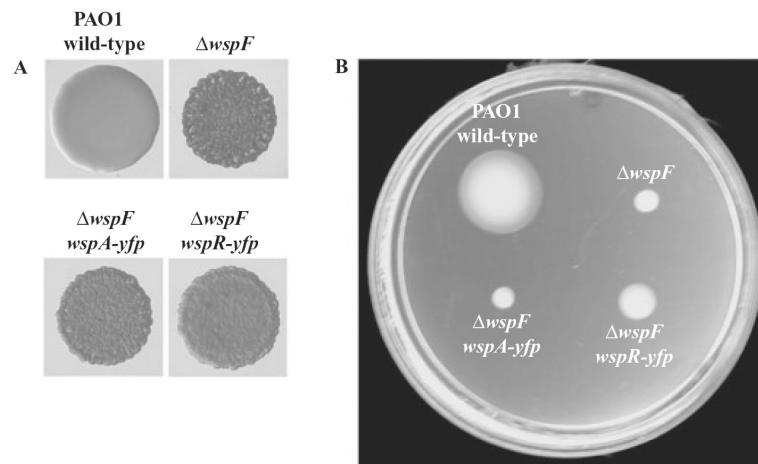


Fig. 2. WspR–YFP and WspA–YFP fusion proteins are functional. The introduction of the fusion constructs into the chromosome of a *wspF* strain by homologous recombination to replace wild-type *wspR* or wild-type *wspA* does not alter the (A) colony morphologies or (B) motility in soft agar swim plates, of the strains. This indicates that the Wsp signal transduction pathway has remained intact.

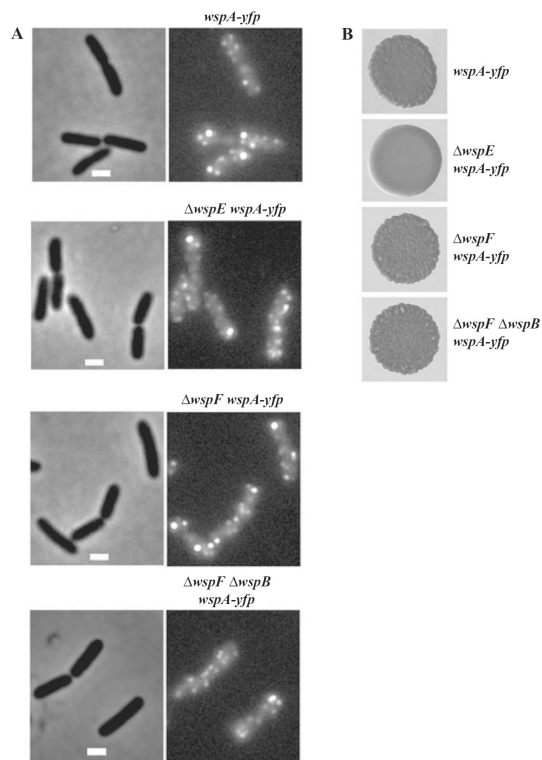


Fig. 3. WspA–YFP localization in cells of wild-type and various mutant strains grown on LB agar. A. Phase-contrast images are shown on left and fluorescent images on the right. The marker bar is 1 μ m. B. Colony morphologies of strains.

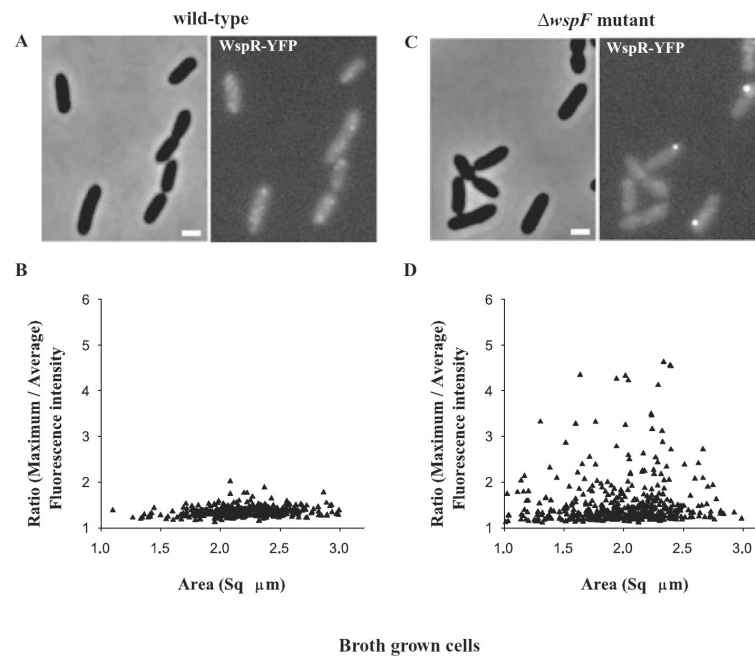


Fig. 4. WspR–YFP localization in (A) wild-type cells grown in liquid cultures. The phase-contrast (left) and fluorescence (right) images of cells expressing *wspR–yfp* are shown. B. Single cell measurements of fluorescence signal intensities expressed as the ratio of maximum to average signal intensities plotted against the area of the respective single cells in wild-type cells expressing WspR–YFP. C. *wspF* mutant cells grown in liquid cultures and expressing WspR–YFP. D. Single cell measurements of fluorescence signal intensities in *wspF* mutant cells expressing WspR–YFP. Single cell fluorescence data were collected from three independent experiments.

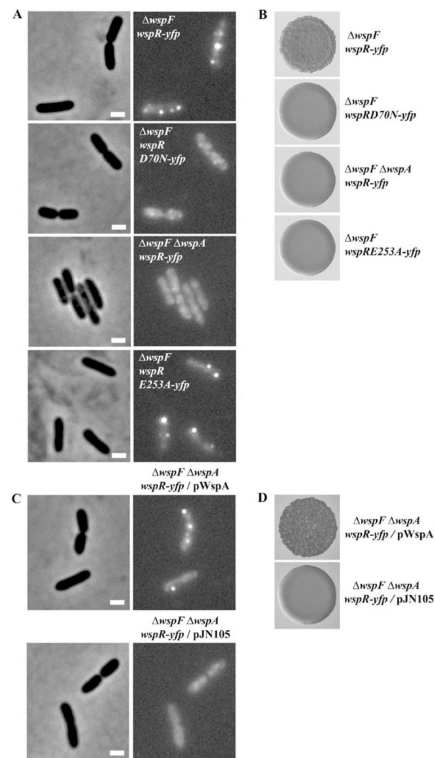


Fig. 5. Phosphorylation is required for WspR–YFP cluster formation.
 A. The localization of WspR–YFP in *wspF* *wspR-yfp* or *wspF* *wspA* *wspR-yfp* mutant cells and the localization of various WspR–YFP mutant proteins in *wspF* cells. Phase-contrast images of cells are shown on the left. The marker bar is 1 μ m.
 B. Colony morphologies of the strains.
 C. WspR–YFP cluster formation is restored in a *wspF* *wspA* *wspR-yfp* mutant strain by providing WspA *in trans*.
 D. The colony morphologies of the *wspF* *wspA* *wspR-yfp* strain complemented with empty vector or *wspA*.

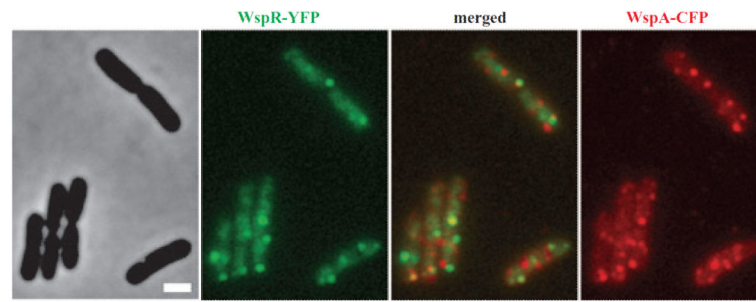
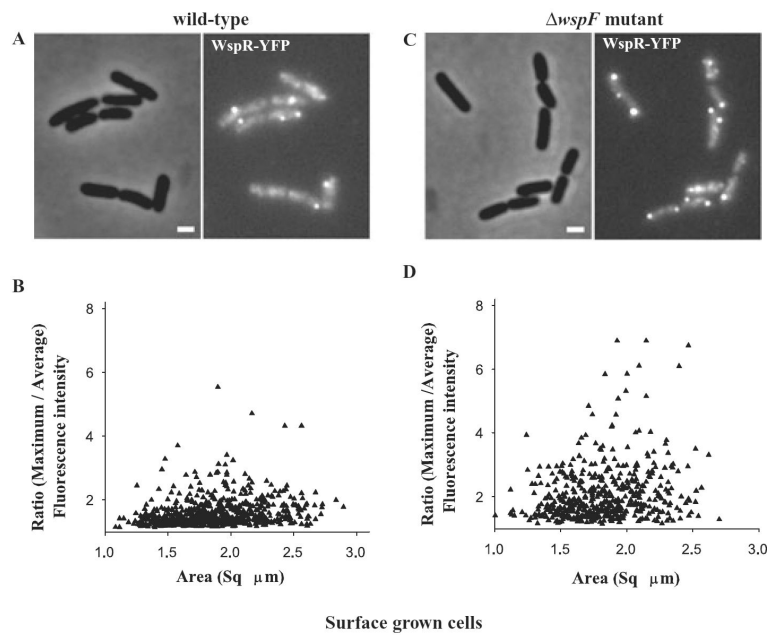


Fig. 6. WspR–YFP and WspA–CFP sometimes colocalize in *wspF* mutant cells. WspR–YFP is coloured green. WspA–CFP is coloured red and the merged image of the two is shown in the middle. A phase-contrast image of the cells is shown on the left. Cells were grown on LB agar. The marker bar is 1 μm .

**Fig. 7.**

Surface growth stimulates WspR–YFP cluster formation in wild-type cells.

WspR–YFP localization in (A) wild-type cells grown on LB agar. The phase-contrast (left) and fluorescence (right) images of cells expressing *wspR-yfp* are shown.

B. Single cell measurements of fluorescence signal intensities expressed as the ratio of maximum to average signal intensities plotted against the area of the respective single cells in wild-type cells expressing WspR–YFP.

C. *wspF* mutant cells grown on agar and expressing WspR–YFP.

D. Single cell measurements of fluorescence signal intensities in *wspF* mutant cells expressing WspR–YFP. Single cell fluorescence data were collected from three independent experiments.

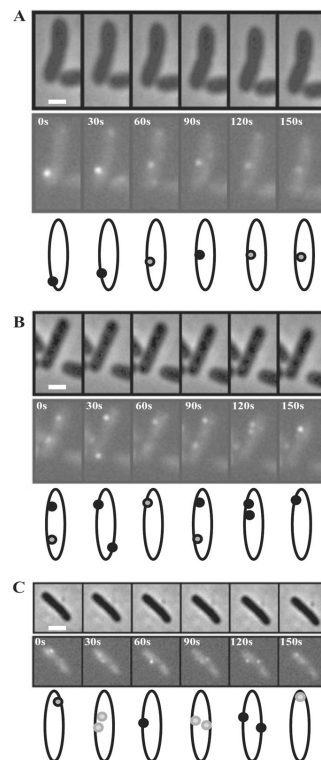


Fig. 8. Time-lapse fluorescence and phase-contrast images of surface-grown wild-type cells expressing the WspR–YFP fusion protein. Images were acquired every 30 s. Individual cells were followed over a 150 s time period in (A), (B) and (C). Cartoons depicting the movement and change in fluorescence intensity of the spots in each cell are shown below each panel. The marker bar is 1 μm .

Table 1

Quantitative analysis of WspR–YFP cluster formation in wild-type and *wspF* mutant cells grown in liquid cultures.

Strain	Percentage of cells with WspR–YFP localized in clusters ^a	Total cells scored
<i>wspR–yfp</i>	1.45 (±1.3)	417
<i>wspF wspR–yfp</i>	19.73 (±4.8)	484
<i>wspF wspA wspR–yfp</i>	2 (±1.67)	426
<i>wspF wspR[D70N]–yfp</i>	1.5 (±0.7)	236
<i>wspF wspR[E253A]–yfp</i>	35.5 (±13.4)	115
<i>wspA wspR–yfp/pJN105</i>	0.45 (±0.6)	262
<i>wspA wspR–yfp/pJNspA</i>	1.58 (±0.9)	571
<i>wspF wspA wspR–yfp/pJN105</i>	1.25 (±0.07)	151
<i>wspF wspA wspR–yfp/pWspA</i>	19.4 (±14.9)	548

^aWspR–YFP clustering was scored based on the presence of a well-defined fluorescent spot or spots in cells. This evaluation was based on the measurement of maximum and average signal intensities in single cells as described in the *Experimental procedures*. After calculating ratios of maximum to average signal intensities, a ratio value corresponding to presence of a well-defined spot was used as a threshold to sort the cells. At least two independent experiments were performed. Average values are given for the percentage of cells that scored above the ratio threshold value. Standard deviations are indicated in parentheses. Cells were grown in LB broth.

Table 2

Quantitative analysis of WspR–YFP cluster formation in various strains grown on agar surfaces.

Strain	Percentage of cells with WspR–YFP localized in clusters ^a	Total cells scored
<i>wspR–yfp</i>	29.7 (±2.9)	692
<i>wspF wspR–yfp</i>	59.8 (±11.8)	493
<i>wspA wspR–yfp</i>	0.22 (±0.3)	253
<i>wspF wspA wspR–yfp</i>	1.32 (±1.2)	270
<i>wspF wspR[E253A]–yfp</i>	74.95 (±1.9)	290
<i>wspA wspR–yfp/pJN105</i>	0.5 (±0.7)	299
<i>wspA wspR–yfp/pWspA</i>	37.6 (±4.3)	280
<i>wspF wspA wspR–yfp/pJN105</i>	0.3 (±0.4)	190
<i>wspF wspA wspR–yfp/pWspA</i>	58.1 (±4.6)	414

^aWspR–YFP clustering was scored based on the presence of a well-defined fluorescent spot in cells. This evaluation was based on the measurement of maximum and average signal intensities of single cells as described in the *Experimental procedures*. After calculating ratios of maximum to average signal intensities, a ratio value corresponding to presence of a well-defined spot was used as a threshold to sort the cells. At least two independent experiments were performed. Average values are given for the percentage of cells that scored above the ratio threshold value. Standard deviations are indicated in parentheses. Cells were grown on LB medium solidified with 2.5% agar as described in *Experimental procedures*.

Table 3

Strains.

Strain	Relative genotype	Source and/or reference
PAO1	Wild-type; twitching motility ⁺	Iglewski (Stover <i>et al.</i> , 2000)
PAO1100	<i>wspF</i>	Hickman <i>et al.</i> (2005)
PAO1101	<i>wspA</i>	Hickman <i>et al.</i> (2005)
PAO1102	<i>wspE</i>	Hickman <i>et al.</i> (2005)
PAO1103	<i>wspR</i>	Hickman <i>et al.</i> (2005)
PAO1104	<i>wspF AwspR</i>	Hickman <i>et al.</i> (2005)
PAO1251	<i>wspR-yfp</i>	PAO1 (this study)
PAO1252	<i>wspA wspR-yfp</i>	PAO1101 (this study)
PAO1254	<i>wspF wspR-yfp</i>	PAO1100 (this study)
PAO1258	<i>wspF wspR-yfp wspA-cfp</i>	PAO1254 (this study)
PAO1260	<i>wspF AwspA wspR-yfp</i>	PAO1254 (this study)
PAO1262	<i>wspF AwspA</i>	PAO1100 (this study)
PAO1265	<i>wspF Ache A wspR-yfp</i>	PAO1254 (Ferrández <i>et al.</i> , 2002; this study)
PAO1268	<i>wspF wspR[D70N]-yfp</i>	PAO1100 (this study)
PAO1275	<i>wspA-yfp</i>	PAO1 (this study)
PAO1276	<i>wspE wspA-yfp</i>	PAO1102 (this study)
PAO1287	<i>wspF wspR[E253A]-yfp</i>	PAO1100 (this study)
PAO1290	<i>wspF Apel Apsl wspR-yfp</i>	PAO1543 (this study)
PAO1501	<i>wspF wspA/pJN105</i>	PAO1262 (this study)
PAO1512	<i>wspF wspA wspR-yfp/pJN105</i>	PAO1260 (this study)
PAO1514	A <i>wspA wspR-yfp/pJN105</i>	PAO1252 (this study)
PAO1516	<i>wspF wspA/pWspA</i> ^(truncated)	PAO1262 (this study)
PAO1526	<i>fliC::Tn5 wspR-yfp</i>	PAO1540 (this study)
PAO1529	<i>pilA wspR-yfp</i>	PAO1541 (this study)
PAO1530	<i>pel psl wspR-yfp</i>	PAO1542 (this study)
PAO1534	<i>wspF wspA/pWspA</i>	PAO1262 (this study)
PAO1535	<i>wspA wspR-yfp/pWspA</i>	PAO1252 (this study)
PAO1536	<i>wspF wspA wspR-yfp/pWspA</i>	PAO1260 (this study)
PAO1537	<i>wspF wspA-yfp</i>	PAO1100 (this study)
PAO1539	<i>wspF wspB wspA-yfp</i>	PAO1537 (this study)
PAO1540	<i>fliC::Tn5</i>	L. L. McCarter
PAO1541	<i>pilA</i>	M. Parsek
PAO1542	<i>pel psl</i>	M. Parsek
PAO1543	<i>wspF pel psl</i>	PAO1542 (this study)