The structure–function relationship of WspR, a *Pseudomonas fluorescens* response regulator with a GGDEF output domain

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The GGDEF response regulator WspR couples the chemosensory Wsp pathway to the overproduction of acetylated cellulose and cell attachment in the *Pseudomonas fluorescens* SBW25 wrinkly spreader (WS) genotype. Here, it is shown that WspR is a diguanylate cyclase (DGC), and that DGC activity is elevated in the WS genotype compared to that in the ancestral smooth (SM) genotype. A structure–function analysis of 120 *wspR* mutant alleles was employed to gain insight into the regulation and activity of WspR. Firstly, 44 random and defined pentapeptide insertions were produced in WspR, and the effects determined using assays based on colony morphology, attachment to surfaces and cellulose production. The effects of mutations within WspR were interpreted using a homology model, based on the crystal structure of *Caulobacter crescentus* PleD. Mutational analyses indicated that WspR activation occurs as a result of disruption of the interdomain interface, leading to the release of effector-domain repression by the N-terminal receiver domain. Quantification of attachment and cellulose production raised significant questions concerning the mechanisms of WspR function. The conserved RYGGEEF motif of WspR was also subjected to mutational analysis, and 76 single amino acid residue substitutions were tested for their effects on WspR function. The RYGGEEF motif of WspR is functionally conserved, with almost every mutation abolishing function.

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INTRODUCTION

Investigations into the regulation of surface colonization and aggregative behaviour in prokaryotes have established the central role of the second messenger cyclic-di-GMP (c-di-GMP) (Jenal, 2004; D'Argenio & Miller, 2004; Römling *et al.*, 2005). Although the biological pathways through which c-di-GMP regulates persistence, cell aggregation and the switch to the commensal lifestyle are currently poorly understood, the proteins responsible for c-di-GMP synthesis

and degradation have been determined and, at least partially, characterized. Bacterial diguanylate cyclase (DGC) activity is found in GGDEF domain-containing proteins (Paul *et al.*, 2004; Ryjenkov *et al.*, 2005; Hickman *et al.*, 2005; Kulesekara *et al.*, 2006), whilst EAL domain-containing proteins have been shown to have c-di-GMP-specific phosphodiesterase (PDE) activity (Bobrov *et al.*, 2005; Christen *et al.*, 2005; Schmidt *et al.*, 2005; Kulesekara *et al.*, 2006).

Despite the publication of much biological data concerning GGDEF domains, biochemical demonstration of DGC activity (Paul *et al.*, 2004; Ryjenkov *et al.*, 2005), and the solution of the crystal structure of the GGDEF response regulator PleD (Chan *et al.*, 2004), detailed structure–function analyses of GGDEF proteins are relatively scarce. Christen *et al.* (2006) have analysed the site of c-di-GMP product inhibition (the 'I site') for the *Caulobacter crescentus* enzymes PleD and DgcA. Additionally, site-specific mutagenesis studies have been carried out, focusing on single

Abbreviations: c-di-GMP, cyclic-di-GMP; CR, Congo Red; CV, crystal violet; DGC, diguanylate cyclase; LSWS, large-spreading wrinkly spreader; PSM, pentapeptide scanning mutagenesis; R_f , retardation factor; SM, smooth; SOE-PCR, strand overlap extension PCR; WS, wrinkly spreader.

Lists of mutant *wspR* constructs produced by PSM, mutant *wspR* constructs produced by SOE-PCR, and oligonucleotides used in the study, BLAST alignment data for WspR and PleD, and a figure showing attachment to glass for SM Δ *wspR* versus WS Δ *wspR*, are available as supplementary data with the online version of this paper.

residues of interest (Kirillina *et al.*, 2004; Garcia *et al.*, 2004; Goymer *et al.*, 2006). A comprehensive structure–function analysis stands to contribute insights into various issues, including the role of the GGDEF motif, the mode of activation of individual GGDEF proteins, and potential alternative mechanisms of downstream signal transduction.

The GGDEF domain was first recognized in PleD, a response regulator responsible for mediating the swarmer-to-stalked cell transition in *C. crescentus* (Hecht & Newton, 1995), and has since been identified in large numbers in many bacterial species (Galperin *et al.*, 1999, 2001; Galperin, 2005). GGDEF domains are often associated with receiver, PAS and GAF sensor domains, and are frequently paired with EAL domains (Galperin *et al.*, 2001).

Numerous studies in diverse species have linked GGDEF domain proteins to processes involved in biofilm formation and aggregative behaviour (reviewed by Römling, 2005; Römling *et al.*, 2005). The effects of GGDEF proteins on exopolysaccharide levels have been probed via genetic studies in numerous species, including *Pseudomonas putida* (Gjermansen *et al.*, 2005; Ude *et al.*, 2006), *Pseudomonas fluorescens* SBW25 (Spiers *et al.*, 2002, 2003; Goymer *et al.*, 2006; Ude *et al.*, 2006), *Salmonella typhimurium* (Simm *et al.*, 2004; Garcia *et al.*, 2004; Simm *et al.*, 2005), *Thermotoga maritima* (Johnson *et al.*, 2005) and *Vibrio cholerae* (Bomchil *et al.*, 2003; Rashid *et al.*, 2003; Kovacicova *et al.*, 2005).

In addition, GGDEF-containing proteins have been repeatedly implicated in the regulation of cell motility, attachment and virulence (reviewed by Jenal, 2004; D'Argenio & Miller, 2004; Römling, 2005). For example, PleD controls the onset of motility during the *C. crescentus* cell cycle (Aldridge *et al.*, 2003; Paul *et al.*, 2004). In *Pseudomonas aeruginosa*, autoaggregation is controlled by the GGDEF response regulator WspR (D'Argenio *et al.*, 2002; Hickman *et al.*, 2005), and twitching motility by FimX, which contains GGDEF and EAL domains (Huang *et al.*, 2003; Kazmierczak *et al.*, 2006). Additional examples of the effects of GGDEF domains on cell attachment and motility include ScrC regulation of attachment factors in *Vibrio parahaemolyticus* (Boles & McCarter, 2002), control of curli fimbriae by AdrA in *S. typhimurium* (Simm *et al.*, 2004), and RocS effects on *V. cholerae* motility (Rashid *et al.*, 2003). A recent survey of all GGDEF-containing genes in *P. aeruginosa* PA14 has shown that, in several cases, overexpression or inactivation has profound effects on virulence and cell attachment to surfaces (Kulesekara *et al.*, 2006).

The GGDEF response regulator WspR was initially discovered as a consequence of investigations into the genetic causes of adaptive radiation in experimental populations of *P. fluorescens* SBW25 (Rainey & Travisano, 1998; Spiers *et al.*, 2002). One class of derived genotype colonizes the air–liquid interface of static broth microcosms; these genotypes produce distinctive wrinkled colonies on agar plates and are termed wrinkly spreaders

(WS). Transposon mutagenesis has been used to identify genes that determine WS phenotype, focusing initially on a single WS genotype, the large-spreading wrinkly spreader (LSWS) genotype. Amongst other genes, the wrinkly spreader phenotype (*wsp*) operon has been identified (Spiers *et al.*, 2002; Goymer *et al.*, 2006), which encodes a chemosensory system with homology to the chemotaxis pathway of *Escherichia coli* (Bantinaki *et al.*, 2007).

WspR is the final gene product and primary output component of the Wsp pathway, and is activated by a currently unknown signal processed by the rest of the Wsp complex (Spiers *et al.*, 2002). A *wspR::mini-Tn5* WS mutant displays a smooth colony morphology (Spiers *et al.*, 2002), and does not produce cellulose or attach to surfaces (Spiers *et al.*, 2003). Expression of *wspR* *in trans* stimulates attachment and exopolysaccharide synthesis in various species (D'Argenio *et al.*, 2002; Aldridge *et al.*, 2003; Goymer *et al.*, 2006; Ude *et al.*, 2006). The evolutionary cause of LSWS is a single point mutation in the gene encoding the WspF methylesterase, which results in overactivation of the WspE kinase and constitutive activation of the primary output component of the Wsp pathway, WspR (Bantinaki *et al.*, 2007). Parallel analysis in *P. aeruginosa* PAO1 has linked the emergence of an aggregative, wrinkled colony morphology to disruption of *wspF* (D'Argenio *et al.*, 2002; Hickman *et al.*, 2005). The *wsp* operon is conserved in both SBW25 and PAO1, and the two systems are thought to function in a similar way.

P. fluorescens SBW25 WspR is a 333 residue protein comprising an N-terminal response-regulator receiver domain and a C-terminal GGDEF domain, separated by a linker region (Spiers *et al.*, 2002, 2003). Recently, a combination of random and site-specific mutagenesis has been used to outline basic physical characteristics of WspR (Goymer *et al.*, 2006). Asp 67 has been established as the site of WspR phosphorylation, and WspR activation has been shown to be dependent upon phosphorylation. In addition, constitutively active [e.g. WspR 19 (R129C); D'Argenio *et al.*, 2002; Aldridge *et al.*, 2003] and dominant-negative [e.g. WspR 9 (G296R)] *wspR* alleles have been identified. A model has been proposed to explain the dominant-negative effect seen upon the production of certain WspR variants. In this model, N-terminal receiver domains without functional C termini competitively inhibit the interaction of chromosomally expressed WspR with the Wsp kinase machinery, and hence reduce the downstream signal (Goymer *et al.*, 2006).

The work described here expands on the findings of Goymer *et al.* (2006), employing two distinct mutagenesis strategies to probe in detail the structure–function relationship of WspR. In the first of these, short in-frame stretches of DNA were introduced at positions throughout *wspR*. In the second, the amino acid residues of the RYGGEF motif were substituted via site-specific mutagenesis. Following mutagenesis, phenotypic assays were applied to the libraries of *wspR* mutant alleles. Morphological analysis and assays for

surface attachment and cellulose production were carried out on SBW25 strains expressing the *wspR* insert and substitution libraries *in trans*, allowing for qualitative, quantitative and *in silico* analysis of WspR. These data, combined with biochemical analyses of cell extracts and purified protein, were used to revise and expand upon models for the structure–function relationship of WspR. More broadly, these models were applied to our understanding of both response regulators and GGDEF domain-containing proteins.

METHODS

Strains and growth conditions. Bacterial strains used in this work are listed in Table 1. Bacteria were grown in either King's medium B (KB) or Luria–Bertani (LB) medium (King *et al.*, 1954; Miller, 1972) at 28 °C (*P. fluorescens*) or 37 °C (*E. coli*) with shaking.

KB microcosms contained 6 ml KB medium in 35 ml universal glass vials and were used for attachment and growth assays. Kanamycin was used at a final concentration of 50 µg ml⁻¹, streptomycin at 100 µg ml⁻¹ and tetracycline at 12.5 µg ml⁻¹. Oligonucleotide primers used in this work are listed in Supplementary Table S3.

Molecular biology procedures. Cloning was carried out in accordance with standard molecular biology techniques (Sambrook *et al.*, 1989). pME6010-*wspR* was constructed by ligation of *wspR*, excised as a *Bam*HI/*Eco*RI fragment from pWspR12 (Aldridge *et al.*, 2003) between the *Bgl*II and *Eco*RI sites of pME6010 (Heeb *et al.*, 2000), destroying the *Bgl*II site in the process. pET42b-*wspR* was constructed by ligation of the relevant *wspR* PCR fragment (amplified with primers WsRPurFor and WsRPurRev from pME6010-*wspR*), between the *Xho*I and *Nde*I sites of pET42b (Novagen).

Overexpression and purification of His₆-WspR. Overnight cell cultures of *E. coli* BL21-(DE3) containing pET14b-*wspR* and pET42b-*wspR* were used to inoculate LB medium plus ampicillin, to initial OD₆₀₀ 0.1. Cell cultures were incubated for 150 min at 37 °C

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference*
Strains		
<i>P. fluorescens</i> SBW25		
SM	Environmental isolate, wild-type ancestral smooth genotype	Rainey & Bailey (1996)
WS (also LSWS)	Biofilm former, evolved from SBW25 SM	Spiers <i>et al.</i> (2002)
SM Δ <i>wspA</i> – <i>wspF</i>	SM with <i>wspA</i> – <i>wspF</i> deleted	Bantinaki <i>et al.</i> , (2007)
SM Δ <i>wspA</i> – <i>wspR</i>	SM with <i>wspA</i> – <i>wspR</i> deleted	Bantinaki <i>et al.</i> , (2007)
SM Δ <i>wspR</i>	SM with <i>wspR</i> deleted	S. M. Gehrig (unpublished results)
WS Δ <i>wspR</i>	WS with <i>wspR</i> deleted	Goymer <i>et al.</i> (2006)
WS-4	WS <i>wspR</i> ::mini-Tn5, Km ^R	Spiers <i>et al.</i> (2002)
<i>E. coli</i>		
BL21-(DE3)	Sm ^R , K-12 <i>recF143 lacI^q lacZΔM15 xylA</i>	Novagen
DH5α	<i>endA1 hsdR17(r_K⁺ m_K⁺) supE44 recA1 gyrA (Nal^R) relA1 Δ(lacIZYA–argF)U169 deoR Φ80dlacΔ(lacZ)M15</i>	Woodcock <i>et al.</i> (1989)
DS941	K-12 <i>recF143 lacI^q lacZΔM15 xylA</i> , Sm ^R	Summers & Sherratt (1988)
FH395	DH5α containing pFH395, a Km ^R , conjugative F derivative (pOX38Km) containing transposon Tn4430, inserted <i>in vivo</i>	F. Hayes (unpublished results)
Plasmids		
pET14b- <i>wspR</i>	pET14b with <i>wspR</i> as an <i>Nde</i> I– <i>Bam</i> HI fragment	P. J. Goymer (unpublished results)
pET42b- <i>wspR</i>	pET42b with <i>wspR</i> as an <i>Xho</i> I– <i>Nde</i> I fragment	This study
pME6010	Tet ^R , P _K , 8.3 kb pVS1-derived shuttle vector	Heeb <i>et al.</i> (2000)
pME6010- <i>wspR</i>	pME6010 with <i>wspR</i> <i>Bam</i> HI– <i>Eco</i> RI fragment ligated between <i>Bgl</i> II and <i>Eco</i> RI, <i>Bgl</i> II site destroyed	This study
PSM <i>wspR</i> alleles	pME6010- <i>wspR</i> with 19 separate 15 bp inserts produced by transposon mutagenesis; see Supplementary Table S1	This study
SOE <i>wspR</i> alleles	Twenty-five <i>wspR</i> -SOE alleles produced by SOE-PCR using pME6010- <i>wspR</i> . PCR products ligated between <i>Xho</i> I and <i>Eco</i> RI of pME6010; see Supplementary Table S2	This study
pME6010- <i>wspR</i> - <i>Sac</i> I	<i>wspR</i> - <i>Sac</i> I produced by SOE-PCR using pME6010- <i>wspR</i> . PCR product ligated between <i>Xho</i> I and <i>Eco</i> RI of pME6010	This study
SUB <i>wspR</i> alleles	Seventy-six <i>wspR</i> fragments containing substitutions between codons 246 and 252, produced by PCR with degenerate primers using pME6010- <i>wspR</i> - <i>Sac</i> I, and ligated between <i>Sac</i> I and <i>Eco</i> RI of pME6010- <i>wspR</i> - <i>Sac</i> I; see Table 3	This study

*SM Δ*wspR*, FH395 and pET14b-*wspR* were the kind gifts of S. M. Gehrig, Oxford University, F. Hayes, University of Manchester, and P. J. Goymer, Oxford University, respectively.

with shaking, before protein expression was induced with IPTG (Sigma) solution at a final concentration of 100 μ M for pET14b-*wspR*, and 1 mM for pET42b-*wspR*. Cell cultures were then incubated for an additional 150 min at 30 °C. Cells were harvested by centrifugation and resuspended in running buffer (25 mM Tris/HCl, pH 8.0, 250 mM NaCl, 1% β -mercaptoethanol), before lysis by sonication and French press. Following lysis, the samples were centrifuged (30 000 g, 15 min, 4 °C) and His₆-WspR was purified from the supernatant via nitrilotriacetic acid (NTA)-nickel chromatography, according to the manufacturer's protocol (Qiagen). Both His₆-WspR samples were eluted in the 200 mM imidazole fraction, and N-terminal His₆-WspR was verified by MS. The protein concentration of samples was determined as described elsewhere (Bradford, 1976) (N-terminal His₆-WspR 1.6 mg ml⁻¹, and C-terminal His₆-WspR 5.0 mg ml⁻¹).

Western blotting. Overnight cell cultures were lysed by incubation (95 °C, 5 min) with 1 volume SDS loading buffer (10 mM Tris/HCl, pH 6.8, 0.005% bromophenol blue, 10% glycerol, v/v, 2% SDS). Proteins from the resulting cell extracts were separated on pre-poured 12% Tris/HCl gels (Bio-Rad) and blotted onto PVDF membranes (Millipore). After overnight incubation in blocking solution (15 mM Tris/HCl, pH 7.0, 10 mM KCl, 10 mM NaCl, 0.01% Tween-20, 10% glycerol, v/v, 5% milk powder), WspR was detected with a 1:2500 dilution of WspR-specific polyclonal antiserum (Harlan Serlabs) and a 1:3000 dilution of anti-rabbit secondary antibody (Invitrogen). Bound antibodies were visualized with ECL chemiluminescent detection reagent (Amersham Biosciences) and photographic film.

Assay for DGC activity. DGC activity was assayed as described previously (Paul *et al.*, 2004). Overnight cultures of strain SBW25 were grown, cells were harvested, resuspended in running buffer, and partially lysed by gentle sonication. Assays were run in 50 μ l (final volume) running buffer containing 10 μ l cell extract or purified WspR, and started by addition of 100 μ M (final concentration) GTP (18.5 kBq [α -³²P]GTP) (Amersham Biosciences). Retardation factor (R_f) values were recorded as the ratio of spot migration distance to the distance migrated by the solvent front.

PCR. A standard reaction contained 5 μ l 2 mM dNTP mix, 2.5 μ l 10 $\times$ polymerase buffer, 1 U *Pfu* turbo polymerase (Stratagene), 0.4 pmol each primer, and 10–20 ng template DNA, made up to 25 μ l with deionized water. Following an initial template denaturation step of 3 min at 94 °C, amplification was performed by 25 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at the appropriate temperature (56–62 °C, depending on the reaction), and strand extension for 1 min 30 s at 72 °C. A final extension step for 5 min at 72 °C was performed before samples were kept at 4 °C.

Pentapeptide scanning mutagenesis (PSM). PSM was used to insert 15 bp sections of DNA into pME6010-*wspR*, as described by Hallet *et al.* (1997), except that a morphological screen was used for transposon insertions in *wspR*. *P. fluorescens* WS-4 (WS *wspR*::mini-Tn5) was transformed with pooled pME6010-*wspR*::Tn4430 DNA, and transformant colonies exhibiting a smooth morphology were selected. These colonies contained constructs unable to complement WS-4; hence they contained transposon insertions in *wspR*. Following mutagenesis, the site of insertion in each case was verified by sequencing.

Strand overlap extension PCR (SOE-PCR). SOE-PCR (Ho *et al.*, 1989) was used during the systematic mutagenesis of *wspR*. DNA on either side of the point of mutation was amplified by a standard PCR with primers SOEFor/Rev 1–25 that complemented at the site for joining (and containing the altered nucleotide sequence). PCR products were purified and 1 μ l of each used as template in the subsequent SOE-PCR. The SOE-PCR was carried out with primers

SOEFOR and SOEREV in a final volume of 25 μ l. Following an initial template denaturation step of 3 min 30 s at 94 °C, strand extension was carried out in two steps: primer annealing for 30 s at 56 °C, followed by extension for 2 min at 72 °C. DNA was then amplified under the conditions described for the standard PCR protocol, with annealing at 56 °C for 30 s. SOE-PCR fragments were confirmed by sequencing, and ligated between the *Xho*I and *Eco*RI sites of pME6010.

Degenerate PCR-based mutagenesis. In order to efficiently mutagenize the GGEEF motif of *wspR*, an upstream restriction site was required. This allowed mutagenesis to proceed via PCR with degenerate primers, and reduced the size of the inserts produced from 1 kb to ~300 bp (the region of *wspR* DNA between the introduced site and the downstream *Eco*RI site). A *Sac*I restriction site (GAGCTC, encoding E-L) was introduced at positions 339/240 of *wspR*, via SOE-PCR with primers *Sac*IFor and *Sac*IRev. PCR was carried out using the degenerate primers DEG1–14 with the primer SOEREV, and pME6010-*wspR*-*Sac*I as a template. PCR products were ligated as a pool between the *Sac*I and *Eco*RI sites of pME6010-*wspR*-*Sac*I. Smooth (SM) cells were transformed with the pooled constructs, and colony morphology on LB agar was determined. Individual colonies were selected on the basis of morphology, and the substituted codon in each case was verified by sequencing.

DNA sequencing. A 50–100 ng aliquot of plasmid DNA was mixed with 1 pmol primer SeqFor or SeqRev and 4 μ l Big-Dye ready reaction mix version 3.0 (Applied Biosystems), in a final volume of 10 μ l. Samples were thermocycled for 25 cycles of 10 s at 96 °C, 5 s at 50 °C, and 4 min at 60 °C, before cooling to 4 °C. Following the reaction, samples were ethanol-precipitated and submitted for sequence analysis on an ABI 3100 sequencer.

Measurement of overnight culture cell density. Assays for attachment and cellulose production described below were inoculated with 24 h cell cultures. Accordingly, the effects of WspR variants on SM culture growth were measured. KB microcosms inoculated with 60 μ l aliquots of overnight cultures, and with the lids loosely taped in place, were incubated at 28 °C with shaking. All cultures displayed similar optical densities (OD₆₀₀) after 24 h growth. A Spectronic-20 spectrometer (Genesys) was used throughout.

Attachment assay. Bacterial attachment was determined quantitatively using crystal violet (CV), as described by Spiers *et al.* (2003). KB microcosms were inoculated with 60 μ l aliquots of overnight cultures and incubated statically at 28 °C for 48 h. Vials were emptied and washed vigorously with deionized water. One millilitre of 0.05% (w/v) CV (Sigma) was added and mixed for 2 min. The vials were then emptied and washed with deionized water. CV was eluted in 5 ml ethanol with vigorous shaking for 1 h, and A₅₇₀ determined.

Congo Red (CR) binding assay. Cellulose expression was measured using a CR binding assay adapted from that described by Spiers *et al.* (2003). Ten-microlitre drops of overnight cultures were grown on 25 ml KB agar plates for 24 h at 28 °C. Colonies were resuspended in 1 ml 0.005% (w/v) CR (Sigma) and incubated for 2 h at 37 °C. Colony material was then pelleted by centrifugation. The amount of CR remaining in the supernatant was determined by measurement of A₄₉₀ and comparison with appropriate CR standards. CR binding was expressed as a fraction of the CR bound by the control strain.

Homology modelling. BLAST (Altschul *et al.*, 1997) was used to align the primary sequence of WspR and the monomeric structure of *C. crescentus* PleD (Chan *et al.*, 2004; PDB code 1w25B). Twenty-two residues were deleted from the sequence of WspR and gaps were added where necessary to give the optimum alignment (Supplementary Fig. S1), which was then used as a template for SWISS-MODEL analysis (Schwede *et al.*, 2003). The resulting WspR

homology model was visualized and manipulated using DeepView/Swiss-PdbViewer version 3.7 (Guex & Peitsch, 1997). The reliability of the homology model was assessed by comparison of the predicted secondary structures of WspR and PleD with the known secondary structure of PleD (Chan *et al.*, 2004), using PROFsec from the Predict Protein database (Rost *et al.*, 2004).

RESULTS

WspR functions as a DGC *in vitro* and enzymic activity is increased in a WS background

N- and C-terminal His₆-tagged WspR were overexpressed in *E. coli* BL21-(DE3) and purified from cell extracts using NTA-nickel affinity chromatography. For purification of N-terminal His₆-WspR (39.1 kDa), the major eluted band corresponded unambiguously to WspR when tested by MS (data not shown). The smaller minor band (35 kDa) seen in Fig. 1(c) also corresponded to WspR and was likely to be a degradation fragment of WspR.

N-terminal His₆-WspR was supplied to Harlan Seralabs for generation of a WspR-specific polyclonal antiserum. Western blotting with this antiserum was then used to provide a qualitative comparison of relative WspR expression levels in the (wild-type) SM and WS genotypes. A sample of purified N-terminal His₆-tagged WspR was included as a control (Fig. 1c). Approximately equal amounts of WspR were detected in SM and WS cell lysates, supporting the hypothesis that the WS wrinkled phenotype does not arise primarily as the result of WspR overexpression, in agreement with previous observations (Spiers *et al.*, 2002).

Given the sequence homology (74% identity) between *P. fluorescens* SBW25 and *P. aeruginosa* PAO1 WspR (recently shown to function as a DGC by Hickman *et al.*, 2005) and the fact that WspR R129C complements PleD activity in *C. crescentus* (Aldridge *et al.*, 2003), it was reasonable to assume that WspR is a DGC, catalysing the formation of c-di-GMP from two molecules of GTP. Nevertheless, it was important to independently verify DGC activity for SBW25 WspR. N- and C-terminal His₆-WspR were assayed for DGC activity, as described by Paul *et al.* (2004). Radiolabelled GTP was incubated with purified His₆-WspR, and samples were removed at regular intervals. When the nucleotide samples were separated by TLC, a spot corresponding to c-di-GMP was generated over time by C-terminal His₆-WspR, determined by comparison with a c-di-GMP control sample (Christen *et al.*, 2005) (Fig. 1a). No DGC activity was detected for N-terminal His₆-WspR (data not shown), possibly as a result of disruption of the active state by the His₆-tag, similar to that seen for N-terminal His₆-PleD (R. Paul and U. Jenal, personal communication).

To investigate the effects of the WS background on WspR DGC activity, cell extracts from overnight cultures of SM, WS and isogenic strains lacking WspR (SM Δ wspR and WS Δ wspR), and containing pME6010 and pME6010-wspR as indicated, were assayed for DGC activity (Fig. 1b) in the

Fig. 1. Biochemical analysis of WspR. (a) C-terminal His₆-tagged WspR was shown to function as a DGC *in vitro*. c-di-GMP production was observed over time (min) against control (Ctrl) lanes containing [α -³³P]c-di-GMP (22.2×10^{10} kBq mmol⁻¹) (Christen *et al.*, 2005). (b) DGC activity assays were carried out with whole-cell extracts. The furthest-migrating spot, thought to correspond to GMP (R_f 0.54) predominated in the cell extracts. The second-furthest-migrating spot (R_f 0.47) was thought to correspond to GDP. These molecules may have been formed through breakdown of GTP (R_f 0.37) by enzymes in the cell lysate. The slowest-running spot (R_f 0.25) was present in WS cell lysates expressing *wspR* from either a plasmid or the chromosome (lanes 2 and 6). This spot corresponded to c-di-GMP. (c) Protein expression levels were measured for SM and WS cell extracts by Western blotting. WspR expression levels were similar in SM and WS cultures. SBW25 SM Δ wspR cell extract and N-terminal His₆-tagged WspR were included as controls. The small minor band seen in the control N-terminal His₆-tagged WspR lane was shown by MS to correspond to a degradation fragment of WspR.

same manner as above. A slow-running spot, thought to correspond to c-di-GMP, on the basis of comparison with previous studies (Paul *et al.*, 2004), was generated in WS cell lysates expressing *wspR* both from pME6010 and the chromosome. No c-di-GMP generation was seen for the other samples tested. Hence, the WS cell extract activated WspR DGC activity, whereas the SM cell extract did not.

Mutagenesis of WspR

Nineteen *wspR* mutant alleles were produced by PSM (Hallet *et al.*, 1997) (see Supplementary Table S1). The 5 aa

insert resulting from PSM differed among mutants, depending on the specific site of Tn4430 insertion into *wspR*. However, the disruption to protein tertiary structure caused by the insertion of 5 aa into the protein was considered likely to outweigh any effects caused by variation in the amino acid sequence of the insert, and this latter difference was discounted (Hayes & Hallet, 2000).

To complement the PSM variants and fully mutagenize *wspR*, SOE-PCR (Ho *et al.*, 1989) was used to introduce 25 GVPTK insertions at specific positions throughout *wspR* (Supplementary Table S2). The resulting library (44 variants) contained approximately one insert for every 7–8 aa of WspR, ensuring a high probability of disrupting most elements of secondary structure present in the protein.

To determine whether soluble protein was expressed from each pME6010-*wspR* mutant allele, all alleles were expressed separately in SM Δ *wspR*, to allow WspR produced from pME6010 to be detected by Western blotting. Forty *wspR* mutant alleles produced detectable amounts of protein, but no WspR was detected for variants with inserts at residue positions 99, 182, 246 and 264 (Fig. 2), suggesting that either an undetectably small amount of WspR was present in each case, or that these variants produced highly unstable protein that was rapidly degraded during sample preparation. However, when expressed in WS, these four variants ameliorated the wrinkled colony morphology (see below). Given clear phenotypic effects, these alleles were included in subsequent analyses. No correlation was seen between protein levels and the phenotypic effects of expression, suggesting that the amount of protein present was not a major factor in determining the WspR activity state.

Phenotypic characterization of WspR variants

The WspR variants may be divided into several functional classes: inactive, signal-dependent (i.e. wild-type), signal-independent (i.e. dominant or constitutively active) and dominant-negative. When wild-type *wspR* is expressed *in trans* in the ancestral SM genotype it causes development of the WS phenotype. By extension, the activity states of different WspR variants may be distinguished by their effects

in different SBW25 backgrounds (Goymer *et al.*, 2006). WspR is activated by the WspE kinase (Bantinaki *et al.*, 2007), therefore, development of WS morphology in strains lacking the *wsp* operon (e.g. SM Δ *wspA*–*wspF*) indicates the presence of constitutively active WspR. In cases where a WS morphology develops in the presence of the Wsp machinery (e.g. in SM), but not in a *wsp* deletion background, the protein is signal-dependent and behaves like wild-type WspR. Inactive proteins have no effect in any SBW25 genotype, whilst dominant-negative WspR variants produce a smooth morphology in the WS genotype. To investigate whether the presence of chromosomal *wspR* has any effect on the behaviour of *trans*-expressed *wspR* variants, the morphological analysis was expanded to include several *wspR* deletion strains (e.g. SM Δ *wspA*–*wspR* and SM/WS Δ *wspR*).

To fully distinguish between all possible activity states, the *wspR* insert alleles were expressed in several SBW25 backgrounds: SM, WS, SM Δ *wspA*–*wspF*, SM Δ *wspA*–*wspR*, SM Δ *wspR* and WS Δ *wspR*, and their effects on colony morphology were determined. In general, changes in colony morphology conformed to predictions, and allowed us to assign one of the four activity states described previously to every tested WspR variant (Table 2, Fig. 3). Morphological analysis provided no evidence for interactions between WspR variants and chromosomal WspR; little morphological difference was seen upon WspR production in SM versus SM Δ *wspR*, or SM Δ *wspA*–*wspF* versus SM Δ *wspA*–*wspR*.

The activation states of the WspR variants correlated with the positions of the inserts throughout WspR. N-terminal variants displayed all four states, whilst linker variants were all constitutively active, and C-terminal variants were either dominant-negative or inactive. Several WspR variants (marked with an asterisk in Table 2) produced a wrinkled morphology in WS Δ *wspR*, but had no effect in other tested strains. These variants appeared to have some residual activity; enough to recover the wrinkled morphology in the WS genotype, but not enough to induce a wrinkled morphology in SM. These variants indicate that less WspR activity is required to substitute for deleted chromo-

Fig. 2. Expression levels for WspR insert variants. Protein expression levels were measured by Western blotting for cell extracts of SBW25 SM Δ *wspR* strains containing the WspR insert variants. N-terminal His₆-tagged WspR (1), SM Δ *wspR* pME6010-*wspR* (2) and SM Δ *wspR* pME6010 (3) were included as controls. The smaller band seen in the His₆-WspR control lane corresponded to a degradation fragment of WspR (see Fig. 1c). (a) Insert positions 6–99, (b) insert positions 105–175, (c) insert positions 182–250, (d) insert positions 253–326.

Table 2. Colony morphologies of SBW25 strains expressing the pME6010-*wspR* insert library

Construct*	Colony morphology†						Activity state‡
	SM	WS	SM Δ <i>wspA-F</i>	SM Δ <i>wspA-R</i>	WS Δ <i>wspR</i>	SM Δ <i>wspR</i>	
pME6010	SM	WS	SM	SM	SM	SM	I
pME6010- <i>wspR</i>	WS	WS	SM	SM	WS	WS	D
PSM <i>wspR6</i>	WS	WS	SM	SM	WS	WS	D
PSM <i>wspR10</i>	WS	WS	WS	WS	WS	WS	C
SOE <i>wspR17</i>	WS	WS	WS	WS	WS	WS	C
SOE <i>wspR24</i>	SM	SM	SM	SM	SM	SM	N
SOE <i>wspR31</i>	SM	WS	SM	SM	WS	Int.	I§
PSM <i>wspR38</i>	SM	WS	SM	SM	WS	SM	I§
PSM <i>wspR45</i>	SM	WS	SM	SM	SM	Int.	I
SOE <i>wspR58</i>	WS	WS	WS	WS	WS	WS	C
SOE <i>wspR79</i>	WS	WS	WS	WS	WS	WS	C
PSM <i>wspR89</i>	WS	WS	WS	WS	WS	WS	C
PSM <i>wspR99</i>	SM	SM	SM	SM	SM	SM	N
PSM <i>wspR105</i>	SM	SM	SM	SM	SM	SM	N
SOE <i>wspR113</i>	WS	WS	WS	WS	WS	Int.	C
PSM <i>wspR120</i>	WS	WS	SM	SM	WS	WS	D
PSM <i>wspR124</i>	SM	SM	SM	SM	SM	SM	N
SOE <i>wspR130</i>	WS	WS	WS	WS	WS	WS	C
PSM <i>wspR138</i>	WS	WS	WS	WS	WS	WS	C
SOE <i>wspR144</i>	WS	WS	WS	WS	WS	WS	C
SOE <i>wspR150</i>	WS	WS	WS	WS	WS	WS	C
SOE <i>wspR157</i>	WS	WS	WS	WS	WS	WS	C
SOE <i>wspR169</i>	SM	SM	SM	SM	SM	SM	N
PSM <i>wspR175</i>	SM	SM	SM	SM	SM	SM	N
SOE <i>wspR182</i>	SM	SM	SM	SM	SM	SM	N
PSM <i>wspR189</i>	SM	SM	SM	SM	SM	SM	N
SOE <i>wspR200</i>	SM	SM	SM	SM	SM	SM	N
PSM <i>wspR211</i>	SM	SM	SM	SM	SM	SM	N
PSM <i>wspR213</i>	SM	SM	SM	SM	SM	SM	N
SOE <i>wspR221</i>	SM	SM	SM	SM	SM	SM	N
SOE <i>wspR228</i>	SM	SM	SM	SM	SM	SM	N
SOE <i>wspR239</i>	SM	WS	SM	SM	WS	SM	I§
SOE <i>wspR246</i>	SM	SM	SM	SM	SM	SM	N
PSM <i>wspR249</i>	SM	SM	SM	SM	SM	SM	N
PSM <i>wspR250</i>	SM	SM	SM	SM	SM	SM	N
SOE <i>wspR253</i>	SM	SM	SM	SM	SM	SM	N
PSM <i>wspR258</i>	SM	SM	SM	SM	SM	SM	N
SOE <i>wspR264</i>	SM	SM	SM	SM	SM	SM	N
SOE <i>wspR270</i>	SM	SM	SM	SM	SM	SM	N
SOE <i>wspR276</i>	SM	SM	SM	SM	SM	SM	N
SOE <i>wspR289</i>	SM	WS	SM	SM	WS	SM	I§
PSM <i>wspR296</i>	SM	SM	SM	SM	SM	SM	N
SOE <i>wspR303</i>	SM	Int.	SM	SM	SM	SM	I
SOE <i>wspR310</i>	SM	SM	SM	SM	SM	SM	N
SOE <i>wspR317</i>	SM	SM	SM	SM	SM	SM	N
PSM <i>wspR326</i>	SM	SM	SM	SM	SM	SM	N

*Each construct name contains the amino acid position of the insert.

†Colony morphology on LB agar plates, designated smooth (SM), wrinkled (WS) or intermediate (Int.). Strains containing pME6010 and pME6010-*wspR* (wild-type) were included as controls.

‡The deduced activity state of each WspR variant is given as either constitutively active (C), signal-dependent (D), inactive (I) or dominant-negative (N) (see Fig. 2).

§Strains for which some activity was deduced from WS Δ *wspR* morphology (see Results).

Fig. 3. The effects of 5 aa insertions on WspR activity. Morphology data were used to assign activity states to WspR insert variants. The arrows represent the positions of amino acid inserts, and indicate the resultant activity of the insert. Insertions in the linker region and sections of the N terminus produced constitutively active variants, whilst insertions in the C terminus abolished WspR activity, and usually led to suppression of the wrinkled phenotype in WS. The positions of the phosphorylation site (D67) and the GGEEF motif are shown.

somal WspR than is required to induce a wrinkled morphology in an SM genotype. For simplicity, these strains were classed as inactive for the purpose of further analysis.

The emerging model of WspR function was refined through analysis of the effects of WspR variants on different aspects of biofilm formation. WspR is known to be essential for the enhanced levels of attachment seen in WS (Spiers *et al.*, 2003). Therefore, the effect of *wspR* mutant allele expression on attachment in SBW25 strains was measured using a quantitative CV assay (Fig. 4). To distinguish between the effects of constitutively active and signal-dependent variants on attachment, the assay was repeated in SM and SM Δ *wspA*–*wspR* backgrounds.

Variations in bacterial attachment corresponded well with the changes seen in colony morphology. Specifically, constitutively active WspR variants (e.g. WspR138) produced raised levels of cell attachment in both tested backgrounds, relative to the plasmid-only control. Signal-dependent variants produced enhanced levels of attachment in SM only (e.g. WspR6). Insertions in the N terminus (residues 1–130) and in the linker region of WspR (residues 131–163) produced active or constitutively active proteins, with corresponding increases in cell attachment. Insertions in the C terminus (residues 164–333) eliminated WspR function, and these variants did not affect cell attachment. Several variants produced greatly enhanced levels of attachment compared with wild-type WspR.

To determine whether the close correlation between attachment and colony morphology also applied to cellulose production, the effects of WspR variants on cellulose production in SM were determined quantitatively using a CR binding assay, as described by Spiers *et al.* (2003) (Fig. 5). [In addition, the presence or absence of cellulose in each sample was determined qualitatively by staining with calcofluor (CF; Sigma). No major discrepancies emerged between cellulose seen upon CF staining, and the results of the CR assay (data not shown)]. In general, increased cellulose production relative to SM pME6010 was seen for those strains displaying WS colony morphology in SM (Table 2). High levels of cellulose production (relative to the SM pME6010–*wspR* control) were observed for several N-terminal and linker region variants, whilst C-terminal

variants did not affect cellulose production. The CR assay results were then compared with the results for attachment in the SM genotype. Several variants were identified that strongly upregulated cell attachment, but did not effect a corresponding large increase in cellulose production (e.g. WspR6). These results were consistent with several hypotheses, discussed in more detail below.

The solution of the crystal structure of PleD from *C. crescentus* (Chan *et al.*, 2004) made possible the use of homology modelling software to produce a putative 3D structure for WspR (Fig. 6). The PleD structure contains two response-regulator receiver domains and a C-terminal GGDEF domain, which has a fold similar to that of the adenylyl cyclase catalytic domain (Zhang *et al.*, 1997), consistent with the earlier predictions of Pei & Grishin (2001). WspR shares significant sequence homology with PleD across the second response-regulator receiver (R2) and GGDEF (DGC) domains, and the PleD and WspR DGC domains are functionally interchangeable in *C. crescentus* (Aldridge *et al.*, 2003). Therefore, the PleD crystal structure represented the best candidate upon which to base the model. The program SWISS-MODEL (Schwede *et al.*, 2003) was used to produce a homology model for WspR (Fig. 6). This model was then used to refine the structure–function models of WspR. The insertion positions of the N-terminal and linker region WspR variants were mapped onto the WspR homology model (Fig. 6), and the relationship between the position of each insert and its effect on function was analysed.

The GGEEF motif of WspR is functionally conserved

As a final extension of the structure–function analysis of WspR, single amino acid residue substitutions throughout the GGEEF motif, and the preceding two residues RY (aa 246–252), were produced and analysed. To allow for simple, high-throughput mutagenesis, a WspR variant (WspR–*SacI*, containing the substitutions R239E and P240L) was selected for substitution mutagenesis. Analysis of colony morphology and biofilm formation indicated that WspR–*SacI* activity was similar to that of wild-type WspR (Fig. 7). Substitution variants were produced as a random pME6010–*wspR*–*SacI* plasmid pool, in which all residue substitutions

Fig. 4. The effects of *wspR* mutant allele expression on SM and SM Δ *wspA-wspR* attachment to glass microcosms. Data show mean $A_{570} \pm$ SEM for eight biological replicates. The grey bars represent SM Δ *wspA-wspR*, and the white bars SM strains, both containing WspR variants with inserts at the residue positions noted. The bar to the left of the graph represents the domain structure of WspR. The grey section represents the N terminus, the black section the linker region, and the white section the C terminus. A two-way ANOVA showed that the effect of the mutation was dependent on whether it was in the N-terminal domain, the linker region or the C-terminal domain ($F_{2,698} = 118.84$, $P < 0.0001$). The effect of strain background was not significant ($F_{1,698} = 0.32$, $P = 0.571$), but the interaction effect was highly significant ($F_{2,698} = 12.76$, $P < 0.0001$).

Fig. 5. Effects of *wspR* mutant allele expression on SM and WS binding to CR dye. Data show mean $\pm$ SEM CR absorbed relative to WS pME6010 for five biological replicates. The bar to the left of the graph represents the domain structure of WspR. The grey section represents the N terminus, the black section the linker region, and the white section the C terminus. One-way ANOVA revealed a highly significant difference among means ($F_{47,192} = 238.00$, $P < 0.0001$). Dunnet's test was used to identify alleles that gave effects that were significantly greater than the plasmid controls, and are indicated by an asterisk.

(except tryptophan) were possible throughout RYGGEF. This pool was used to transform SM, and WspR substitution variants were recovered and checked. In total, 76 different WspR substitution variants were isolated. Seventy-five of these were from colonies displaying smooth colony morphology (i.e. those in which the WspR variant could not induce the WS phenotype) (Table 3). One additional

Fig. 6. Insert positions in the N terminus and linker region of WspR. The figure shows the WspR homology model visualized from four different angles. The GGDEF domain is coloured grey. The N-terminal receiver domain and the linker region are coloured green, with the sites of 5 aa insertions highlighted according to their resultant activity state. Constitutively active, red; signal-dependent, orange; inactive, light blue; dominant-negative, dark blue. Areas of ribbon coloured grey in the N terminus represent sections with no corresponding WspR sequence.

variant, WspRE250D, produced a wrinkled colony phenotype in SM, suggesting that the protein was active in this case.

Twenty-one WspR substitution variants were selected for further characterization on the basis of the side-chain chemistry of the substituted residues. Firstly, the effects of WspR variant production on WS colony morphology were analysed, and used to predict their activity states, as described above. All but one of the WspR variants, WspRY247F, produced smooth colony morphology in WS, indicating a dominant-negative activity state. WspRY247F was classed as inactive. Given that most of the tested proteins produced a dominant-negative phenotype, quantitative analysis was used to investigate the properties of this phenotype. Therefore, assays for cellulose production and attachment were undertaken in the WS genotype. WspRY247F did not repress WS cellulose production, in contrast with the other substitution variants. Finally, none of the WspR variants significantly reduced WS attachment levels (Fig. 7).

DISCUSSION

A systematic biological and biochemical analysis was undertaken of the GGDEF domain-containing protein WspR, in order to address several unanswered questions concerning GGDEF proteins in general, and WspR in particular. Firstly, biochemical analysis was carried out on purified WspR and on SBW25 cell extracts. WspR was shown to function as a DGC (Fig. 1a, b), and the WS phenotype was shown to stimulate WspR DGC activity in cell extracts, in proportion to the level of *wspR* gene

Fig. 7. Effects of WspR substitution variants on WS behaviour. The effects of 21 WspR substitution variants on (a) cellulose production and (b) attachment in WS were measured. Data show (a) mean $A_{490} \pm \text{SEM}$ for five biological replicates and (b) mean $A_{570} \pm \text{SEM}$ for eight replicates. Constructs are named based on the WspR substitution in each case. Wrinkled morphology and cellulose production were repressed in WS upon production of all but one variant protein, WspRY247F. Attachment was not repressed by any WspR variant. A nested ANOVA revealed a significant effect due to substitutions at different positions in the RYGGEF motif ($F_{9,168} = 26.96$, $P < 0.0001$) and a significant effect of the particular residue at each position ($F_{14,168} = 4.50$, $P < 0.0001$). Analysis of a priori contrasts in least-squared-means derived from the nested ANOVA model showed highly significant effects of substitutions at positions 246, 247 and 248 ($P < 0.0001$ in each case), but no significant effect of substitutions at positions 250–252.

Table 3. Amino acid residue substitutions that eliminate activity in WspR-SacI

In every example shown, the ability of the variant protein to produce a wrinkled phenotype in SM cells grown on LB agar plates was abolished.

Altered residue	No. of substitutions*	Substituted residues†			
		Aliphatic	Aromatic	Charged or polar	Aliphatic hydroxyl/sulphur-containing
246R	11	L, I, V, A	F	K, Q	T, S, C, M
247Y	11	L, I, V, P	F, H	D, E	T, S, C
248G	9	L, I, V	F, Y	K, R, Q	T
249G	10	L, I, V, A	F, Y	K, N	S, C
250E	11	G, L, I, A, P	F, Y	Q	T, S, C
251E	13	G, L, I, V, P	F, Y	K, R, N	T, S, C
252F	10	G, L, I, V		R, D, E, N	S, C

*The number of substitutions is out of a possible 18 residue substitutions at each position.
†WspR variants are grouped according to the chemistry of the substituted residue side chain. Standard amino acid definitions are used.

expression. This provided a definite mechanism for WspR function, and suggests that the WS phenotype may arise (at least in part) as the result of increased levels of WspR activation and concomitant c-di-GMP production.

Secondly, to probe the mechanisms of WspR activation and regulation, systematic mutagenesis was used to disrupt WspR at positions throughout its length. The effects of the resulting WspR insert library on SBW25 colony morphology, attachment and cellulose production were analysed, and used to determine the activity state of the various WspR variants. Several distinct observations were made of the relationship between WspR activity and insert position. Firstly, inserts in the C terminus (residues 164–333) of WspR always eliminated enzymic activity. Whilst several insertions in the N terminus also eliminated enzymic function, the same sensitivity to mutation was not seen in the domain as a whole. Secondly, a number of constitutively active WspR variants were found with insertions in the N terminus (residues 1–130), and insertions in the linker region (residues 131–163) produced constitutively active enzymes in every case (Fig. 3). To our knowledge, WspR is the only GGDEF protein for which a comprehensive structure–function analysis of this kind has been undertaken.

Detailed structure–function analysis provides significant insight into the means of protein regulation, and in this case allowed the construction of a model for WspR activation (Fig. 8). In this model, the C-terminal effector domain is inhibited in the inactive state by the N-terminal domain. Regulation through effector-domain inhibition occurs in a number of response regulators, including *E. coli* NarL (Baikalov *et al.*, 1996; Eldridge *et al.*, 2002) and *S. typhimurium* CheB (Djordjevic *et al.*, 1998). Phosphorylation (Fig. 8a), or mutation of the N-terminal domain (Fig. 8b), leads to the release of inhibition. This model expands on the observations of Goymer *et al.* (2006), who have noted that mutations in or near the linker region of

WspR mimic the phosphorylated state of the protein. Biochemical (Ryjenkov *et al.*, 2005) and structural (Chan *et al.*, 2004) evidence suggests that DGCs function as dimers (although no evidence for WspR dimerization was found in this study). If WspR functions as a dimer, disruption of the inactive form of the protein might lead to exposure of the dimer interface, and hence activation (Fig. 8b). These data do not rule out the possibility of an alternative activation mechanism, whereby the phosphorylated N-terminal domain stabilizes the DGC-activated conformation. However, it is unlikely that the precise interactions

Fig. 8. A model for WspR function. For wild-type WspR (a), inhibition of the C-terminal effector domain (oval) by the N-terminal receiver domain (square) is relieved upon phosphorylation, allowing WspR to produce a signal, possibly via dimerization in a similar manner to other GGDEF proteins (Chan *et al.*, 2004; Ryjenkov *et al.*, 2005). Mutation of the N terminus or linker region (b) may remove this inhibition, as exemplified by constitutively active WspR variants. Mutation of the C terminus (c) eliminates function irrespective of the phosphorylation state of WspR, resulting in the inactive or dominant-negative state.

required by such a mechanism would be reproduced by every activating insertion tested, strongly suggesting that the mechanism of WspR activation proceeds via release of effector-domain repression.

A number of insertions produced a dominant-negative phenotype. In these cases, the activity of either the Wsp or the Wss machinery was adversely affected by *trans*-expressed *wspR* mutant alleles. This dominant-negative phenotype could have arisen as a consequence of competitive inhibition of the Wsp signalling machinery by inactive WspR variants, in agreement with the model proposed by Goymer *et al.* (2006) for suppression of the WS wrinkled morphology by the isolated WspR N terminus. Alternatively, inactive heterodimers, formed between inactive WspR variants and chromosomal wild-type WspR may interrupt Wsp signalling at the level of WspR. Neither model of inhibition can be ruled out at this stage.

When the positions of the 5 aa inserts were plotted onto the N terminus and linker region of the homology model (Fig. 6), they clustered according to their effects on WspR activity, suggesting that each group affected WspR function via a common mechanism. N-terminus insertions producing constitutively active proteins were found at the interface between the N- and C-terminal domains, and in adjoining loops and helices. These insertions presumably activated WspR via disruption of the interface between the two domains, a mechanism common to many response-regulator activation mechanisms (Eldridge *et al.*, 2002; Park *et al.*, 2002; Muchova *et al.*, 2004). A comparison of the domain interaction surfaces of four full-length, two-domain response-regulator crystal structures (Robinson *et al.*, 2003) suggests that the activation mechanisms of the different proteins, NarL (Baikalov *et al.*, 1996), CheB (Djordjevic *et al.*, 1998), DrrD (Buckler *et al.*, 2002) and DrrB (Robinson *et al.*, 2003), vary with the extent of their interdomain interfaces. For example, comparison of the DrrB and DrrD crystal structures suggests that different levels of intramolecular communication between the N- and C-terminal domains lead to significant differences in effector-domain regulation in each case (Robinson *et al.*, 2003).

Several activating insertions were clustered in the α -helical section of the linker region (Fig. 6). Mutational analysis of the linker region between the receiver and effector domains of OmpR by Mattison *et al.* (2002) has shown that the composition of the linker region affects the interplay of the two domains, and hence plays a role in transducing the receiver signal to the effector. In addition, the importance of interdomain linkers in the determination of the response-regulator activation mechanism has been shown using OmpR–PhoB hybrid proteins (Walthers *et al.*, 2003). The linker region appears to play an important role in regulation of WspR activation. However, studies with NarL have assigned little functional significance to the linker region (Eldridge *et al.*, 2002), suggesting that linker-mediated regulation is not a universal feature of response regulators.

Insertions producing inactive and dominant-negative WspR variants were spread over the surface of the model, furthest from the domain interface, surrounding the predicted phosphorylation site (Fig. 6). N-terminal dominant-negative insertions may have disrupted protein folding without affecting the domain interface or promoting dimerization, in the same manner as was seen for the activating insertions. Alternatively, these insertions could have affected WspR interaction with the Wsp chemosensory kinase.

For the purposes of this study, two morphological states (SM and WS), and four activity states for WspR were described. These distinctions are a necessary simplification of the situation *in vivo* that allows us to propose models for WspR function. Both in this study and elsewhere (Römling *et al.*, 2005), it is evident that the phenotypic consequences of changes in c-di-GMP levels are highly complex and rely on a number of interrelated factors. For example, the wrinkled phenotype caused by overexpression of wild-type *wspR* in the SM background was accompanied by little increase in cellulose production or attachment compared to the LSWS phenotype, yet both were classified here as WS. (In the first case, most WspR in the cell was thought to be in the inactive form. In the second, less WspR was present but a far larger proportion of this was activated). The physiological relevance of overexpression phenotypes should not be overstated. Nonetheless, within the context of isolated, controlled experiments, overexpression is a valid tool for the analysis of GGDEF domain proteins.

Quantitative analysis of the effects of WspR variants showed that generation of the wrinkled phenotype in SM strains was associated with increases in both cell attachment and cellulose production (Figs 3, 4 and 5). An important exception, however, was the discovery of several WspR variants whose expression produced little effect on cellulose production, but greatly stimulated cell attachment, relative to the other WspR variants tested (e.g. WspR6 and WspR120). In addition, greatly decreased levels of cellulose production in WS were observed upon expression of the 20 dominant-negative *wspR* substitution mutant alleles. However, cell attachment to glass in WS strains expressing these *wspR* mutant alleles was not repressed, relative to that seen with the wild-type control (Fig. 7).

There are several possible explanations for these data. The first is that WspR regulates attachment and cellulose production via distinct and separable mechanisms, possibly regulating one function through DGC activity and another via protein–protein interactions. Andrade *et al.* (2006) have suggested that some GGDEF proteins may function in this way. Secondly, if *wspR* functions solely via DGC activity, and if upregulation of attachment requires lower levels of c-di-GMP than does cellulose production, then WspR variants that upregulate attachment only (e.g. WspR6) presumably do not produce enough c-di-GMP to trigger cellulose production. Likewise, inhibition of DGC activity by dominant-negative WspR substitution variants (e.g. WspRR246L) would reduce c-di-GMP levels to the point

at which cellulose production, but not attachment, is repressed. Finally, the Wsp system may modulate different processes through two or more response regulators, including WspR. Repression of DGC activity (and hence cellulose production) in WS by dominant-negative WspR variants would have no effect on attachment in this scenario. If a second Wsp-controlled response regulator existed, we would expect to see high levels of attachment for WS Δ wspR relative to that for SM Δ wspR. In fact, attachment levels in this strain are comparable to those of SM Δ wspR (Supplementary Fig. S2), strongly arguing against this model. Biochemical analysis of DGC levels for the different classes of WspR variants is required to confirm which explanation of the data is correct. Alternatively, the discovery of a hypothetical WspR variant that upregulates cellulose production whilst having no effect on attachment would provide strong support for the first model.

For several *wspR* insert variants (e.g. SOE*wspR130*), higher levels of attachment were observed upon expression in SM Δ wspA–*wspR* than in SM (Fig. 4). This may be related to the loss of pellicle structure in SM Δ wspA–*wspR* compared to SM, allowing more cells to attach at the meniscus than would be possible for strains producing a structured biofilm. Similarly, repression of cellulose production, and hence loss of pellicle structure, may explain why several dominant-negative WspR substitution variants (e.g. SUB*wspRR246F*) produced increased WS attachment levels compared to those of wild-type WspR (Fig. 7). It is probable that biofilm formation in SBW25 consists of numerous interconnected processes (Spiers *et al.*, 2003), and care must be taken when interpreting the phenotypic data.

Finally, the RYGGEF motif of WspR was investigated using site-specific mutagenesis, and was shown to be highly sensitive to mutation. Substitution of residues 246–252 (RYGGEF) led to a loss of protein function in almost every case tested. Of the 76 *wspR* substitution alleles produced, only one produced an active protein (wrinkled morphology in SM). As WspR functions as a DGC, it is reasonable to suggest that the level of functional conservation seen for the WspR RYGGEF motif may also apply to other DGCs; that this motif (GGD/EEF) is absolutely required for DGC activity. [The GGD/EEF motif is not always required for domain function, however, as functional proteins containing GGDEF domains with degenerate active site motifs have been identified (Christen *et al.*, 2005; Kazmierczak *et al.*, 2006)]. Mutagenesis of single residues in RYGGD/EEF has eliminated protein function in numerous species (Paul *et al.*, 2004; Simm *et al.*, 2004; Kirillina *et al.*, 2004; Garcia *et al.*, 2004; Goymer *et al.*, 2006), and the crystal structure of PleD places the RYGGEF motif close to the ribose group and alpha-phosphate of bound c-di-GMP, suggesting a critical role in nucleotide binding or catalysis (Chan *et al.*, 2004).

The single active WspR variant, WspRE250D, contained the motif RYGGDEF, rather than RYGGEF. An analysis of sequence conservation among GGDEF domains (Galperin *et al.*, 2001) has indicated that aspartate and glutamate are

found with similar frequency at position 250. It is possible that the conformational constraints on the first glutamate in the RYGGEF motif are not so pronounced as for the second, allowing aspartate or glutamate to be present. Interestingly, Tyr 247 was absolutely conserved in this study, despite the residue at this position showing substantial variation across different GGDEF domains (Galperin *et al.*, 2001). When three WspR substitution variants were chosen to represent each position of the RYGGEF motif and analysed in more depth, almost every variant tested produced a dominant-negative effect on WS morphology (Table 3). The only exception was WspRY247F, which produced an inactive protein. The structural similarity between tyrosine and phenylalanine suggests that an undefined protein misfolding event, avoided in the case of a structurally conserved substitution such as that in WspRY247F, was responsible for the observed dominant-negative effect of the tested residue substitutions on WspR activity.

This work sheds light on several previously unanswered aspects of the WspR structure–function relationship. WspR functions as a DGC, whose enzyme activity is stimulated in the WS genotype compared with the (wild-type) SM genotype. Upon phosphorylation, or disruption of the linker region or the interdomain interface, activation proceeds via release of effector-domain repression by the receiver domain. Quantitative analysis of biofilm formation has raised questions concerning the downstream regulatory mechanisms of WspR. Finally, the RYGGEF motif of WspR is highly conserved functionally, with almost every substitution tested abolishing function in a dominant-negative fashion. As the time and effort required to produce high-quality structural models of proteins continues to decrease, this study underlines the continued utility of systematic mutagenesis to structure–function analyses.

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