Function, localization and regulation of the *Pseudomonas aeruginosa* diguanylate cyclase response regulator WspR

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ABSTRACT

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WspR is a hybrid response regulator-diguanylate cyclase that is phosphorylated by the Wsp signal transduction complex in response to growth of *Pseudomonas aeruginosa* on surfaces. Active WspR produces c-di-GMP, which in turn stimulates biofilm formation. Previous work demonstrated that when phosphorylated in response to growth on surfaces, WspR has a tendency to form oligomers that are visible in cells as subcellular clusters. In this study, I sought to determine the physiological relevance of WspR and its subcellular clustering, as well as the mechanism of how WspR forms subcellular clusters. My results confirm that WspR contributes specifically to production of the Pel polysaccharide. Cluster formation appears to be an intrinsic property of WspR. Analysis of six single amino acid variants of WspR show that the formation of WspR-P subcellular clusters is important for potentiating the diguanylate cyclase activity of WspR, making it more active in c-di-GMP production. Finally, I observed that c-di-GMP inhibition may play a role in the subcellular cluster formation of WspR. Oligomer formation visualized as subcellular clusters is a mechanism by which the activities of response regulator-diguanylate cyclases can be regulated. This work includes a supplementary movie depicting the movement of WspR subcellular clusters in a 5 s time frame.

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CHAPTER I:

INTRODUCTION

c-di-GMP

Bis-(3',5')-cyclic dimeric guanosine monophosphate (cyclic-di-GMP or c-di-GMP) is an intracellular secondary messenger unique to bacteria that controls several cellular functions, including promoting biofilm growth. High levels of c-di-GMP promote exopolysaccharide (EPS) and adhesin production, while low levels allow flagella production and motility (Hengge, 2009; Simm *et al.*, 2004). c-di-GMP is produced by diguanylate cyclases with a GGDEF domain and degraded by phosphodiesterases with either an EAL domain or a HD-GYP domain. Most diguanylate cyclases and phosphodiesterases have various input sensor domains and c-di-GMP effectors have various output domains (Galperin, 2004; Römling et al., 2005).

c-di-GMP regulates cellular functions at several different levels. Some c-di-GMP effectors are transcription factors that bind to promoter DNA to activate or repress transcription (Hickman and Harwood, 2008; Krasteva *et al.*, 2010; Weber *et al.*, 2006). c-di-GMP-binding riboswitches can affect translation (Sudarsan *et al.*, 2008). At the post translational level, c-di-GMP affects protein activity allosterically or influences inter- or intra-protein interactions (Navarro *et al.*, 2011; Weinhouse *et al.*, 1997).

Many bacteria have multiple GGDEF and EAL/HD-GYP proteins, raising the question of how specificity of c-di-GMP signaling is maintained and which environmental conditions control it (Jenal and Malone, 2006). All GGDEF/EAL proteins do not all equally affect c-di-GMP mediated functions (Kulasekara *et al.*, 2006). GGDEF/EAL proteins have varying levels of DGC/PDE activity (Kader *et al.*, 2006; Kulasekara *et al.*, 2006). C-di-GMP receptors respond to different c-di-GMP concentrations (Hengge, 2009; Pultz *et al.*, 2012). Some GGDEF/EAL proteins are

under spatial and temporal regulation (Güvener and Harwood, 2007; Paul *et al.*, 2004; Weber *et al.*, 2006). Specificity of c-di-GMP signaling may either be maintained through the different c-di-GMP ranges that effectors are sensitive to, or perhaps there may be direct delivery or degradation of c-di-GMP.

c-di-GMP in *Pseudomonas aeruginosa*

C-di-GMP signaling in *P. aeruginosa* is studied largely because of its clinical relevance. *P. aeruginosa* is a soil-dwelling Gram negative bacterium, but it is also an important opportunistic pathogen and is a leading cause of mortality in cystic fibrosis patients. Strains with high c-di-GMP and increased biofilm formation ability are isolated from cystic fibrosis patients with poor lung function (Haussler *et al.*, 1999; Smith *et al.*, 2006). These strains are more resistant to antibiotics. In one study, 68% of CF isolates has mutations in *wspF*, one of the genes in the Wsp system (Starkey *et al.*, 2009). Lab-grown biofilms will also produce these mutants (Kirisits *et al.*, 2005).

High levels of c-di-GMP in *P. aeruginosa* result in a loss of motility and an increase in EPS production (D'Argenio *et al.*, 2002). High c-di-GMP increases expression of the biosynthetic machinery for the production of EPS (Hickman *et al.*, 2005; Hickman and Harwood, 2008). *P. aeruginosa* produces three different types of EPS: Psl, Pel and alginate (Ryder *et al.*, 2007). C-di-GMP also activates production of Psl, Pel and alginate at the post-translational level (Lee *et al.*, 2007; Merighi *et al.*, 2007). PAO1, the lab strain of *P. aeruginosa* used in my work, does not produce alginate without a *mucA* mutation so this work focuses on the Pel and Psl EPS (Wozniak *et al.*, 2003).

P. aeruginosa has 40 proteins with GGDEF, EAL or HD-GYP domains. A subset of these proteins has been experimentally verified for in vitro DGC or PDE activity and has characterized cellular functions. SadC and BifA are DGC and PDE respectively that have opposing effects on swarming and Pel EPS production (Merritt *et al.*, 2007). FimX is a PDE that controls twitching motility (Huang *et al.*, 2003; Kazmierczak *et al.*, 2006). SadR/RocR is a PDE that is required for biofilm structure (Kuchma *et al.*, 2005; Rao *et al.*, 2008). Arr regulates biofilm in response to aminoglycosides (Hoffman *et al.*, 2005). MucR is a DGC required for alginate production (Hay *et al.*, 2009). The genes PA4108 and PA4781 encode PDE with HD-GYP domains that control swarming and twitching motility and biofilm architecture (Ryan *et al.*, 2009). TpbB (a.k.a. YfiN or PA1120) is a DGC that contributes to EPS production in response to quorum sensing signals (Malone *et al.*, 2010; Ueda and Wood, 2009).

Ten proteins have been shown to bind c-di-GMP in *P. aeruginosa*, not counting DGC and PDE proteins (Pultz *et al.*, 2012). Seven are proteins of unknown function. One of these seven proteins is encoded by the PA4608 gene. PA4608 expression is influenced by quorum sensing and biofilm development, but not much else is known about its function (Kuchma *et al.*, 2005; Wagner *et al.*, 2003). The three effectors with known functions are Alg44, PelD and FleQ. Alg44 likely facilitates the polymerization and transport of alginate (Merighi *et al.*, 2007; Oglesby *et al.*, 2008). PelD post-translationally promotes the production of Pel EPS, likely by transporting carbohydrate nascent chains across the inner membrane (Lee *et al.*, 2007; Whitney *et al.*, 2012). Finally, FleQ controls the transcription of flagellar genes and *pel* genes (Baraquet *et al.*, 2012; Dasgupta *et al.*, 2003; Hickman and Harwood, 2008).

The Wsp system

The *wsp* operon is conserved in all *Pseudomonas* species and in some *Burkholderia* species (Bantinaki et al., 2007; De et al., 2008). The genes in the *wsp* operon are homologous to chemotaxis signal transduction genes (Bantinaki et al., 2007; D'Argenio et al., 2002). WspA is homologous to methyl-accepting chemotaxis proteins (MCPs) like the *E. coli* Tar and Tsr MCPs. WspA responds to an unknown signal that occurs when cells are grown on an agar surface (Güvener and Harwood, 2007). The Wsp system is predicted to have methyl-group based adaptation using the CheR-like methyl-esterase WspC and the CheB-like methyl-transferase WspF to control the methylation status of WspA. WspB and WspD are predicted to be CheW-like scaffolding proteins connecting WspA to the histidine kinase WspE. WspE is predicted to phosphorylate the response regulator WspR (Fig. 1.1).

Even though the Wsp system and chemotaxis signal transduction systems share homology, their subcellular localizations differ. Chemotaxis receptors form a dense array at the cell poles (Briegel *et al.*, 2009). In contrast, WspA receptor clusters are lateral (Güvener and Harwood, 2007). Judging from our microscopy images WspA clusters contain fewer molecules than the polar clusters formed by other MCPs of PAO1 (Güvener *et al.*, 2006).

WspR

WspR is a response regulator with two domains: an input receiver domain and an output GGDEF domain. Two structure-function studies have been done on the ortholog in *Pseudomonas fluorescens* to determine how mutations and insertions in *wspR* affected the "wrinkly spreader"

colony morphology phenotypes (Goymer *et al.*, 2006; Malone *et al.*, 2007). Mutations and insertions in the stalk region yielded gain of function phenotypes and in the GGDEF domain loss of function phenotypes. More recently, the crystal structure of WspR was solved by the Sondermann group (De *et al.*, 2008). The GGDEF domain of WspR is an active diguanylate cyclase that has been shown to produce c-di-GMP (De *et al.*, 2008; Hickman *et al.*, 2005; Malone *et al.*, 2007). Phosphorylation of WspR has been shown to increase production of c-di-GMP (Hickman *et al.*, 2005). WspR is subject to product inhibition by c-di-GMP, a feature common to GGDEF domains as described below.

In contrast to what we know about the structure, our knowledge of the function of WspR is a bit vague. Screens of transposon libraries of a different *P. aeruginosa* strain, PA14, suggest that *wspR* contributes to biofilm formation (Kuchma *et al.*, 2007; Kulasekara *et al.*, 2006). However, PA14 lacks the Psl EPS, while the lab strain PAO1 produces both Pel and Psl EPS. It was unclear whether WspR in PAO1 contributes to both EPSes or just Pel or Psl. Overexpression and overactivation of WspR leads to overproduction of both EPS and a loss in motility, but the high levels of c-di-GMP involved may not be physiologically relevant. Deletion of *wspR* results in a consistent, perceptible but statistically insignificant loss in biofilm mass in flowcell biofilms (Hickman and Harwood, unpublished).

Response regulators and the receiver domain

Response regulators have a phosphorylatable receiver domain and most often have a separate effector domain. The conserved receiver domain typically is comprised of five alternating alpha helices and five beta strands that form a beta sheet sandwiched by the alpha helices (Volz, 1993).

Three conserved aspartates, one of which is where phosphorylation occurs, cluster together at one side of the domain to bind a divalent cation (Mg^{2+} in the case of WspR) required for phosphoryl chemistry. When the receiver domain is phosphorylated, a conserved lysine and serine/threonine shift over to form a hydrogen bond and a salt bridge with the phosphoryl group (Lee *et al.*, 2001). The shifting rotates one of the alpha helices such that a conserved phenylalanine/tyrosine moves into a hydrophobic cavity. The resulting change to the α4-β5-α5 face of the domain allows protein-protein interaction (Gao and Stock, 2009b). The mechanisms by which the effector domain is activated varies between response regulators, but common themes include dimerization of the receiver domains, relief of inhibition by domain rearrangements, or signal is transduced by interaction with a separate effector protein.

Structural features of diguanylate cyclases

GGDEF domains are named for the conserved GGDEF amino acid motif at the active site, which is required for functional DGC activity. The site only tolerates the GGDEF and GGEEF motifs, as other sequences lack DGC activity (Kirillina *et al.*, 2004; Malone *et al.*, 2007; Paul *et al.*, 2004). Formation of c-di-GMP involves the condensation of two GTP molecules. The active site only holds one GTP substrate molecule, so DGC homodimers are required for c-di-GMP formation.

A majority (>60%) of GGDEF proteins have a conserved c-di-GMP binding pocket, called the inhibition site or I-site (Ryjenkov *et al.*, 2005). Insights about the mechanisms of c-di-GMP negative feedback of DGCs have been derived from crystal structures of WspR and the *Caulobacter crescentus* DGC PleD (De *et al.*, 2008; Wassmann *et al.*, 2007). C-di-GMP binding

involves two sites: the primary I-site which has a conserved RXXD motif and a secondary I-site. An intercalated c-di-GMP dimer binds to the primary I-site on one DGC and the secondary I-site on the other DGC, effectively cross-linking two DGC domains together. Experimental and structural evidence suggest that c-di-GMP does not so much allosterically inhibit by directly changing the active site, but rather sequesters the active sites away from each other.

WspR forms subcellular clusters for unclear reasons

YFP-tagged WspR forms dynamic subcellular cytoplasmic clusters that are visible by fluorescence microscopy when phosphorylated by the Wsp complex (Güvener and Harwood, 2007). In contrast, unphosphorylated WspR-YFP is diffuse throughout the cell. The Wsp complex is activated and phosphorylates WspR when *P. aeruginosa* is grown to a thin film on an agar surface. These and other results have led us to a model in which surface sensing activates the Wsp system to phosphorylate WspR, which in turn, forms subcellular clusters, assumes an active conformation, and produces c-di-GMP.

Understanding how and why WspR forms subcellular clusters will contribute to basic knowledge of response regulators, diguanylate cyclases and the molecular mechanisms that direct biofilm formation. In my thesis, I sought to determine the function of WspR clustering and what structural features of WspR are important for this clustering behavior.

In chapter three I describe some basic information about WspR in the cell. I determined that the deletion of *wspR* did not affect motility, but it did contribute to a small decrease in Pel EPS

mediated biofilm growth. I estimated the number of WspR molecules in a cell and observed that WspR clusters seemed to be intrinsic.

In chapter four I demonstrate that WspR subcellular clustering stimulates its diguanylate cyclase activity. I observed that WspR variants which have increased clustering frequency also have increased diguanylate cyclase activity. I show a correlation between WspR concentration and specific activity in vitro.

In chapter five I discuss the possibility that c-di-GMP may be affecting WspR clustering. I list some circumstantial evidence that c-di-GMP binding not only inhibits WspR cyclase activity but also dampens subcellular cluster formation.

In the appendix I describe the work I have done for papers published by collaborators in the Harwood lab.

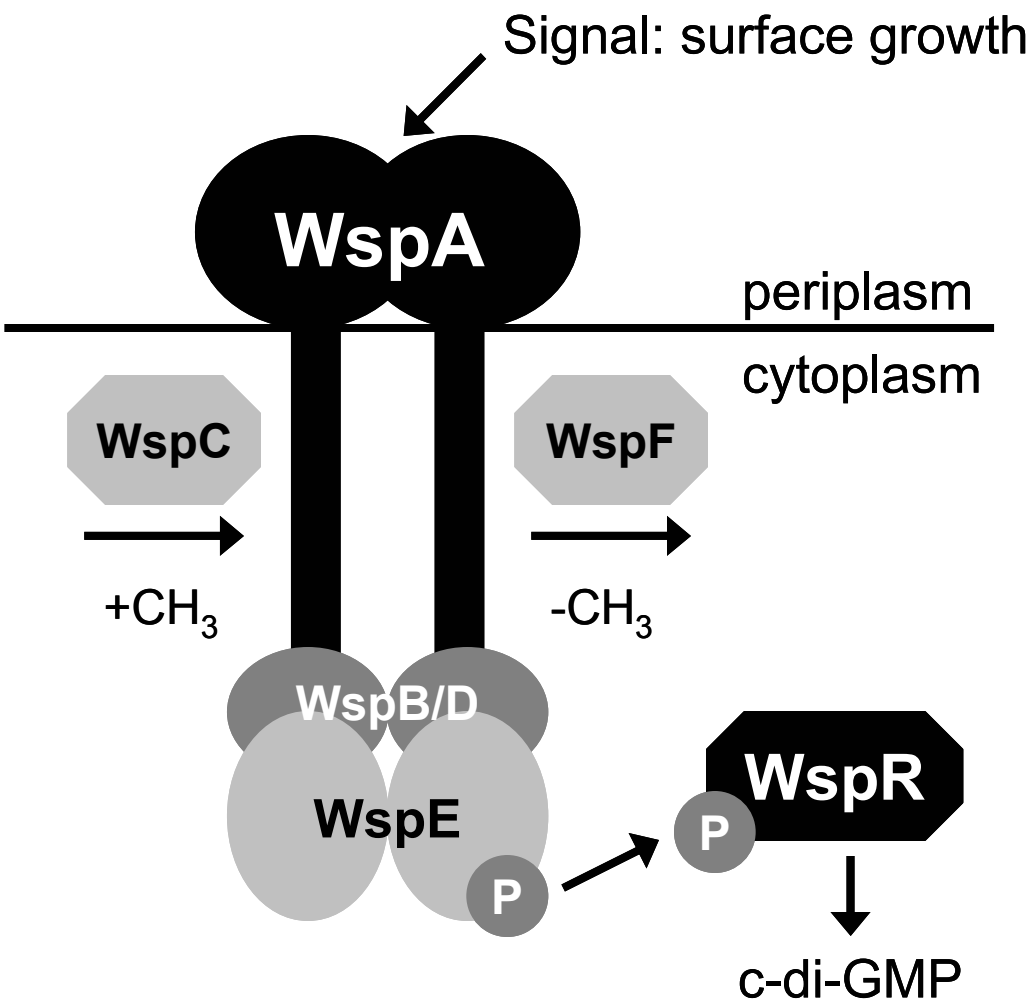

Fig. 1.1 A model for the Wsp signal transduction complex. WspA is a membrane-bound receptor protein, which detects a signal associated with growth on a surface. The signal is transduced to the histidine kinase WspE, which catalyzes phosphotransfer to the response regulator-diguanylate cyclase, WspR. Phosphorylated WspR produces the secondary messenger c-di-GMP. The methyltransferase WspC and the methylesterase WspF likely play a role in adaptation to the surface signal. WspB and WspD are scaffolding proteins important for function and proper localization of the Wsp complex (O'Connor *et al.*, 2012).

CHAPTER II:

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains used in this work are listed in Table 3. Cells were routinely grown in LB medium (10 g L^{-1} tryptone, 5 g L^{-1} NaCl, 5 g L^{-1} yeast extract) at 37°C unless otherwise noted. Media were supplemented with antibiotics at the following concentrations: for *P. aeruginosa*: tetracycline (100 μg ml^{-1}), gentamicin (50 μg ml^{-1}); for *E. coli*: tetracycline (2-20 μg ml^{-1}), gentamicin (10 μg ml^{-1}), ampicillin (50-100 μg ml^{-1}), chloramphenicol (34 μg ml^{-1}) or kanamycin (30-50 μg ml^{-1}). For heterologous expression of WspR in *E. coli*, BW27784 was transformed with a derivative of pTG142 containing $wspR^{D70E}$-yfp (see following section). Cells were grown in LB with tetracycline and 0.01% L-arabinose at 25°C for 8 h before imaging. The plasmid pJNydeH was constructed by ligating the *ydeH* fragment from the plasmid pUT18-ø-ydeH digested with restriction enzymes XbaI and SacI (Baraquet and Harwood, unpublished). Plasmids pJN105, pJN2133, and pJNydeH were transformed into strains by standard electroporated method (Choi *et al.*, 2006; Hickman *et al.*, 2005; Newman and Fuqua, 1999).

WspR immunoblots. PAO1 strains were grown at 37°C in LB to mid-log, diluted to OD_{600} of 0.1 in 10 ml of LB in flasks and incubated with shaking at 22-25°C for 3 h with 1% arabinose. Cells were collected by centrifugation at 4°C for 15 min at 3200 g and stored at -20°C. Frozen cells were resuspended in buffer (20 mM Tris pH 8.0, 250 mM KCl, 0.1 mM EDTA) and sonicated for cell lysis. The sample was centrifuged for 10 and 30 min at 4°C at 16000 g to remove cell debris. Total protein content of the samples was measured using Bio-Rad Protein Assay. SDS-PAGE was performed using NuPAGE Novex Bis-Tris gels and system (Invitrogen). Proteins were transferred to PVDF membranes and blocked overnight with 5% instant nonfat powdered milk in TBS-Tween (20 mM Tris pH 7.8, 150 mM NaCl, 0.05% Tween). The primary

antibody used at 1:1000 concentration was rabbit antisera were raised against purified WspR (Covance Research Products, Denver, PA) and processed with acetone powder of PAO1 Δ*wspR* to reduce nonspecific bands (Harlow and Lane, 1988; Li and Hazelbauer, 2004). The secondary antibody was goat horseradish peroxidase-conjugated anti-rabbit antibodies used at 1:100,000 concentration and was visualized with SuperSignal West Femto substrate (Pierce/ThermoFisher).

Calculation of WspR cellular concentration. Bands in immunoblots were quantified using ImageQuant 5.1 software. A standard curve of pixel intensity vs. ng WspR was generated using pure WspR. The pixel intensity of the WspR blot in cell lysate samples was then converted to ng WspR. The amount of WspR was converted to molecules and divided by the amount (in μg) of cell lysate loaded. PAO1 cells were grown to OD_{600} of 0.5, cells per ml were determined from these samples, cells were harvested and cell lysates prepared. Total protein in cell lysates was determined and calculated as mg total protein per ml of original culture. From this I determined a value of 3.52×10^6 cells per μg cell lysate protein. Replicates (11) across 4 independent experiments give an average of 283 (standard deviation 67) molecules of WspR per cell.

Construction of strains with expression of *wspR* under arabinose-inducible control. *wspR* was translationally fused to *yfp* with a PVPVAT linker and a KpnI site between the two genes as previously described (Güvener and Harwood, 2007). A blunt fragment containing a RBS and *wspR-yfp* was created by cloning *wspR-yfp* into the EcoRI and XbaI sites of pGFP-C (Güvener and Harwood, 2007), excising with NheI and XbaI and treated with T4 DNA polymerase. This fragment was ligated in at the SmaI site of pSW196 (Baynham *et al.*, 2006) which was cured of its KpnI and EcoRI sites. The resulting plasmid, pTG142, contains an arabinose-controllable

promoter upstream of its multiple cloning site and can integrate into the *attB* site on the *P. aeruginosa* chromosome using the mini-CTX system (Hoang *et al.*, 2000). Variant alleles of *wspR* were amplified using PCR from sources noted in Table 2.2 and swapped with the wild-type *wspR* gene between the EcoRI and KpnI restriction sites of pTG142. The resulting plasmids were introduced into the appropriate *P. aeruginosa* strains via conjugation from *E. coli* S17-1 to integrate into the chromosome at the *attB* site (Hoang *et al.*, 2000). Transconjugants were selected for from LB plates containing tetracycline (100 μg ml^{-1}) and chloramphenicol (10 μg ml^{-1}). Integration into the *attB* site on the chromosome was checked using PCR with the primer P_{ser-up} (Hoang *et al.*, 2000) and a primer internal to *wspR* (wspR1 5'-GACTACCTGGTCAAGCTGCCGGACG-3').

Motility assays. Swim plates were composed of 0.3% Noble agar, 1 g/L tryptone, 5 g/L NaCl, 0.5 g/L yeast extract. Cells were inoculated into swim plates by picking overnight colonies with a sterile toothpick and stabbing halfway through the agar. Cells were incubated at 28°C for 48 h. Twitch plates were composed of LB with 1% agar. Cells were inoculated into twitch plates by picking overnight colonies with a sterile toothpick and stabbing all the way to the bottom of the petri dish through the agar. After incubation of upright plates at 28°C for 48 h, the agar was removed and the zone of twitching visualized with 0.1% crystal violet.

Crystal violet attachment assay. Overnight cultures were diluted 1:50 with LB and incubated in Nunc Bacti 96-well plate wells at 22°C for 22 h under static conditions. Non-adherent cells were removed by submerging the plate in deionized water and decanting. Attached biofilm biomass was stained with 0.1% crystal violet for 15 min. Extra stain was washed away with deionized

water and the remaining stain was solubilized by the addition of ethanol to the wells. The OD_{595} reading of the crystal violet stain was normalized to the cell culture OD_{595} reading after the incubation.

Fluorescence microscopy. Sample preparation and microscopy were performed as previously described (Güvener and Harwood, 2007). To analyze liquid-grown cells, cells were grown while shaking to an OD_{600} of 0.3 – 0.5 in LB broth at 37°C, back-diluted to OD_{600} 0.1 in fresh LB with 1% L-arabinose, and incubated with shaking at 25°C for 3 hours for induction of *wspR*. To remove interfering fluorescence from the LB media, cells were washed by centrifuging for 5 min at 10,000g and resuspended in phosphate-buffered saline (pH 7.4). The resuspended cell preparation (3 µl) was spotted onto an 0.8% agarose PBS pad on a microscope slide then covered with a cover slip. To analyze surface-grown cells, liquid-grown cells were diluted to OD_{600} 0.01 and 100 µl were spread on LB 1% arabinose 2.5% agar plates and incubated at 22-25°C for 20 h. Cotton swabs were used to transfer surface-grown cells to PBS agarose pads for imaging. For resuspended surface-grown cells, surface-grown cells were resuspended in 3 ml PBS, transferred to a culture tube and incubated at 22-25°C with shaking. The resuspended cell preparation (3 µl) was spotted onto PBS agarose pads for imaging once every two hours.

Movies showing the movement of WspR clusters were recorded using the Evolve EM CCD Camera (Photometrics) controlled by NIS-Elements Advanced Research software (3.22) (Nikon). Images of liquid-grown PAO1 Δ*wspF wspR-yfp* were continuously acquired with a 123 ms exposure time for 5 s, with the multiplier set at 100, 1x conversion gain, and readout speed 5MHz.

RNA isolation and real-time quantitative PCR. Cell growth, RNA isolation and cDNA synthesis were performed as previously described (Hickman and Harwood, 2008). Cells were incubated at 22-23°C. For analysis of surface-grown cells, cells were grown on plates as described for fluorescence microscopy, then resuspended in a mixture of 6.64 ml RNA Protect Bacteria reagent (Qiagen) and 3.36 ml LB per plate. Transcript levels of *pelA* were normalized to transcript levels of *ampR*. The data represent three biological experiments.

Fluorescence intensity measurements and analysis. The micrographs were analyzed as previously described (Güvener and Harwood, 2007). Briefly, Metamorph software (6.3r2) was used to detect and create regions corresponding to isolated single cells. Average pixel intensity and maximum pixel intensity was acquired for each cell. The ratio of maximum pixel intensity to the average pixel intensity was calculated and used to determine which cells have at least one subcellular cluster. The threshold ratio of 1.74 was determined by eye to lower the incidence of false positives in the *wspA* negative control strain. Analysis of resuspended plate-grown cells (Fig. 3.3) was performed in a similar manner using NIS-Elements Advanced Research software (3.22) (Nikon).

Congo Red colony morphology assay. Broth-grown cells were diluted in LB to an OD_{600} of 0.005 and 2 µl of the dilution were spotted on Tryptone Congo Red plates. The cells were allowed to grow at room temperature for 6 days. Images of surface illuminated colonies were captured on the 6^{th} day, unless otherwise noted, using a digital camera mounted on a dissection microscope (Olympus SZX-ILLK100).

Protein expression and purification. Alleles of *wspR* were amplified by PCR from sources listed in Table 3 and cloned into pET29 or pETDuet vector (Novagen), the latter with the phosphodiesterase PA2133 cloned into the secondary cloning site. The protein was overexpressed in the *E. coli* strain Rosetta 2 (Novagen). Cells were grown to mid-log in LB at 37°C, subcultured at OD_{600} 0.01 in Terrific Broth (12 g L^{-1} tryptone, 24 g L^{-1} yeast extract, 0.4% glycerol, 72 mM K_2HPO_4, 17 mM KH_2PO_4) (Sambrook *et al.*, 1989) and grown at 30°C to OD_{600} of 0.5 – 1.0. Media were supplemented with appropriate antibiotics. The cells were equilibrated to 16-18°C for 30 minutes then induced with 1 mM IPTG for 16 hours. Cells were pelleted at 11,000 x g at 4°C for 15 minutes and frozen at -20°C. Cells were resuspended in wash buffer (25 mM Tris, 0.3 M NaCl, 10 mM imidazole, pH 7.3) and lysed using a French press. Cell debris was removed by spinning at 11,000 x g then 12,000 x g, both for 15 minutes, then filtered through 0.45 μm and 0.22 μm filters. His-tagged WspR proteins were isolated using affinity chromatography with HisPur Cobalt columns (Pierce) and eluted with elution buffer (25 mM Tris, 0.3 M NaCl, 150 mM imidazole, pH 7.3). Arginine (0.1 M) was added to prevent precipitation. Protein was concentrated with Amicon Ultra filter units and stored in 4°C for no longer than 24 h before assaying.

Diguanylate cyclase activity assay. Assays were carried out using the Enzchek Pyrophosphate Assay kit (Invitrogen). The concentration of $MgCl_2$ was increased to 2 mM. Instead of using the pyrophosphatase from the kit, 0.4 U ml^{-1} pyrophosphatase (inorganic from *E. coli*, Sigma) was substituted. Reactions were started by the addition of 0.5 mM GTP (USB). To assay equilibrated WspR, WspR protein was diluted and equilibrated in reaction buffer overnight at 22°C, then

MESG, PNP from the kit and pyrophosphatase was added before starting the reaction with GTP. The accumulation of phosphate was monitored over time by reading OD_{360}.

Beryllium fluoride treatment. Beryllium fluoride was synthesized in situ from beryllium chloride and sodium fluoride (Sigma). Varying amounts of WspR (0.25 μM to 10 μM) were equilibrated in reaction buffer (50 mM Tris-Cl, 10 mM $MgCl_2$, pH 7.5) overnight before activation with 0.1 mM $BeCl_2$ and 10mM NaF for 5 minutes at room temperature. Each reaction was run through a PD-10 desalting column to remove extra BeF_3^-, $BeCl_2$, and NaF from the treated WspR in order to prevent inhibition of the pyrophosphatase in the Enzchek assay. Diguanylate cyclase activity of WspR-BeF_3^- was assayed immediately after BeF_3^- treatment and desalting.

Size exclusion chromatography. Protein (60 μM) was injected into a Superdex 200 10/300 GL column (GE). The buffer used was 25 mM Tris pH 7.5, 100 mM NaCl, 1 mM DTT and 2 mM $MgCl_2$. Chosen fractions were directly assayed for their diguanylate cyclase activity immediately after elution from the column.

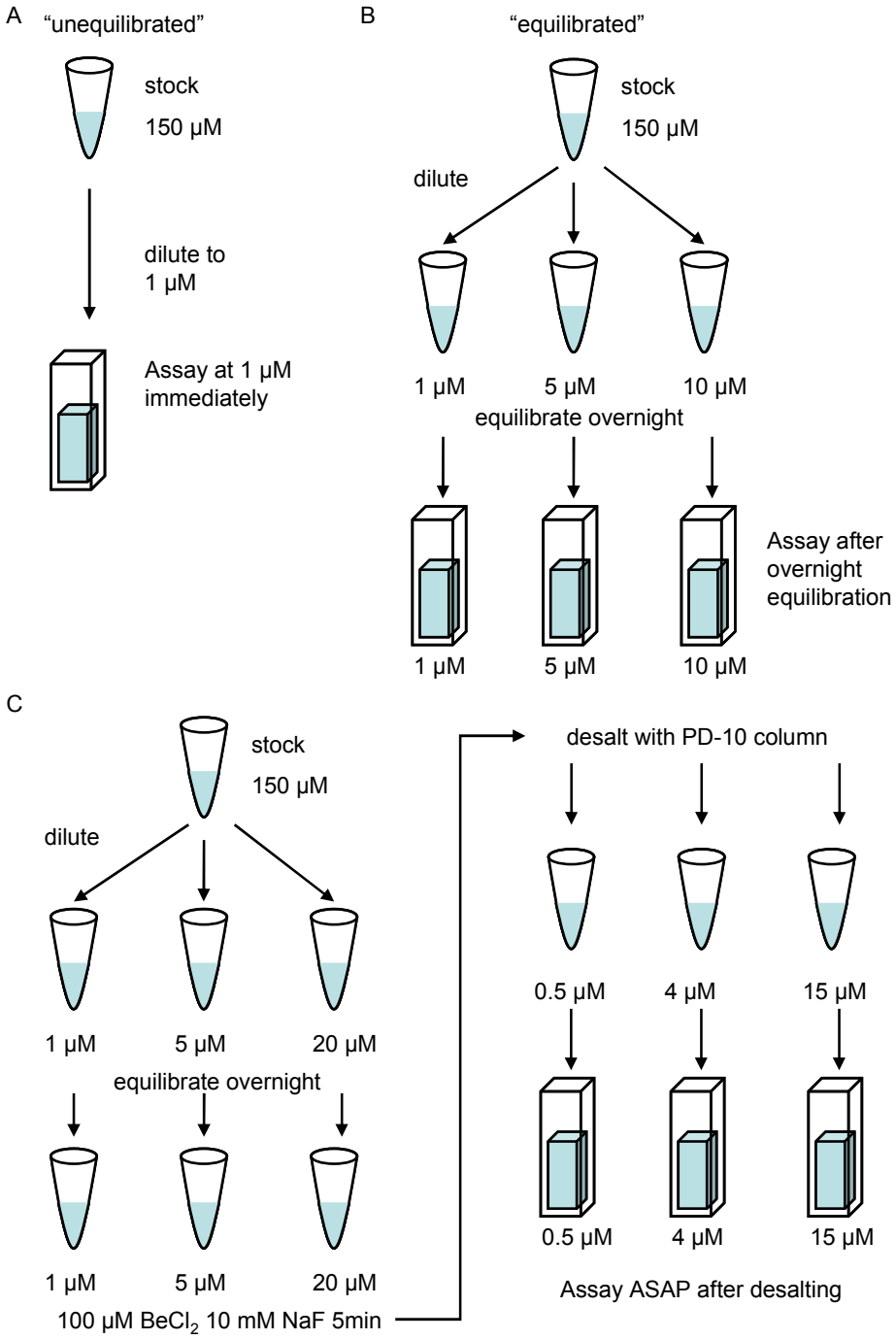

Fig. 2.1 Diagrams of the set-ups of WspR diguanylate cyclase assays. Set-ups for assaying (A) unequilibrated WspR (B) equilibrated WspR (C) equilibrated WspR with BeF_3^- treatment.

Table 2.1 Strains

Strain	Source or reference
Pseudomonas aeruginosa	
PAO1	(Stover *et al.*, 2000)
PAO1 *wspR-yfp*	(Güvener and Harwood, 2007)
PAO1 *wspR*^{E253A}*-yfp*	(Güvener and Harwood, 2007)
PAO1 Δ*wspR*	(Hickman *et al.*, 2005)
PAO1 Δ*pslBCD*	This study, (Kirisits *et al.*, 2005)
PAO1 Δ*pslBCD* Δ*wspR*	This study
PAO1 Δ*pelA* Δ*pslBCD wspR-yfp*	(Güvener and Harwood, 2007)
PAO1 Δ*wspFR*	(Hickman *et al.*, 2005)
PAO1 Δ*wspAR*	(Güvener and Harwood, 2007)
PAO1 Δ*wspR attB::miniCTX-wspR-yfp*	This study
PAO1 Δ*wspR attB::miniCTX-wspR*^{D16N}*-yfp*	This study
PAO1 Δ*wspR attB::miniCTX-wspR*^{D70A}*-yfp*	This study
PAO1 Δ*wspR attB::miniCTX-wspR*^{D70E}*-yfp*	This study
PAO1 Δ*wspR attB::miniCTX-wspR*^{V72D}*-yfp*	This study
PAO1 Δ*wspR attB::miniCTX-wspR*^{L167D}*-yfp*	This study
PAO1 Δ*wspR attB::miniCTX-wspR*^{L170D}*-yfp*	This study
PAO1 Δ*wspR attB::miniCTX-wspR*^{R198A}*-yfp*	This study
PAO1 Δ*wspR attB::miniCTX-wspR*^{E253A}*-yfp*	This study
PAO1 Δ*wspF wspR-yfp*	(Güvener and Harwood, 2007)
PAO1 Δ*wspFR attB::miniCTX-wspR-yfp*	This study
PAO1 Δ*wspFR attB::miniCTX-wspR*^{D16N}*-yfp*	This study
PAO1 Δ*wspFR attB::miniCTX-wspR*^{D70A}*-yfp*	This study
PAO1 Δ*wspFR attB::miniCTX-wspR*^{D70E}*-yfp*	This study
PAO1 Δ*wspFR attB::miniCTX-wspR*^{V72D}*-yfp*	This study
PAO1 Δ*wspFR attB::miniCTX-wspR*^{L167D}*-yfp*	This study
PAO1 Δ*wspFR attB::miniCTX-wspR*^{L170D}*-yfp*	This study
PAO1 Δ*wspFR attB::miniCTX-wspR*^{R198A}*-yfp*	This study
PAO1 Δ*wspFR attB::miniCTX-wspR*^{E253A}*-yfp*	This study
PAO1 Δ*wspA wspR-yfp*	(Güvener and Harwood, 2007)
PAO1 Δ*wspAR attB::miniCTX-wspR-yfp*	This study
PAO1 Δ*wspAR attB::miniCTX-wspR*^{D16N}*-yfp*	This study
PAO1 Δ*wspAR attB::miniCTX-wspR*^{D70A}*-yfp*	This study
PAO1 Δ*wspAR attB::miniCTX-wspR*^{D70E}*-yfp*	This study
PAO1 Δ*wspAR attB::miniCTX-wspR*^{V72D}*-yfp*	This study
PAO1 Δ*wspAR attB::miniCTX-wspR*^{L167D}*-yfp*	This study
PAO1 Δ*wspAR attB::miniCTX-wspR*^{L170D}*-yfp*	This study
PAO1 Δ*wspAR attB::miniCTX-wspR*^{R198A}*-yfp*	This study
PAO1 Δ*wspAR attB::miniCTX-wspR*^{E253A}*-yfp*	This study
Escherichia coli	
DH5α	Gibco-BRL
S17-1	(Simon *et al.*, 1983)
Rosetta 2	Novagen
BW27784 (CGSC#7881)	(Khlebnikov *et al.*, 2001)

Table 2.2 *wspR* alleles

***wspR* alleles**	Source or reference
wspR^{D16N}	(Hickman and Harwood, unpublished)
wspR^{D70A}	(Hickman and Harwood, unpublished)
wspR^{D70E}	This study
wspR^{V72D}	(Hickman and Harwood, unpublished)
wspR^{L167D}	(De *et al.*, 2008)
wspR^{L170D}	(De *et al.*, 2008)
wspR^{R198A}	(De *et al.*, 2008)
wspR^{E253A}	(Güvener and Harwood, 2007)

CHAPTER III:

Basic science of WspR function and WspR subcellular clusters

Introduction

In *P. aeruginosa*, the Wsp system was first described in the context of the *wspF* mutation, which results in high cellular levels of c-di-GMP. This results in increased production of Pel and Psl EPS and a decrease in swimming and swarming motility (D'Argenio *et al.*, 2002; Hickman *et al.*, 2005). What was less clear is the specific role of WspR-produced c-di-GMP. Does WspR contribute to EPS production and to dampening motility? Does it control just one or a few aspects of c-di-GMP mediated functions in PAO1? The *wspR* mutation has been shown to decrease biofilm formation, but that was done in the strain PA14 which only produces Pel but not Psl EPS (Friedman and Kolter, 2004). Other DGC and PDE in *P. aeruginosa* have specific functions and do not target all c-di-GMP mediated functions (Merritt *et al.*, 2010). I was also interested in knowing how many copies of WspR are present in a typical cell and whether clusters were the result of the formation of complexes with c-di-GMP effectors. In this chapter I describe the function of WspR and additional characteristics of WspR subcellular clusters.

Results

WspR contributes to Pel-mediated attachment to surfaces. To probe the function of WspR in more detail I examined c-di-GMP-related phenotypes of a *wspR* deletion mutant. The mutant did not have altered swimming or twitching motility (Fig. 3.1 A and B). The *wspR* mutant did not have a significant defect in *pelA* expression relative to its wild-type parent regardless of whether it was grown in liquid or on an agar surface (Fig 3.1C). However, others in the lab have shown that a *wspRpsl* double deletion mutant had a significant ($p < 0.00005$; one-tailed student t-test) defect in Pel-mediated attachment to a polystyrene surface relative to a *psl* mutant (Fig. 3.1D) (Weaver and Harwood, unpublished). It was necessary to eliminate Psl production, which is the

more dominant EPS in *P. aeruginosa* strain PAO1, to see this phenotype. Elevated c-di-GMP acts at the transcriptional level to stimulate *pel* operon expression through the c-di-GMP responsive transcriptional regulator FleQ (Baraquet *et al.*, 2012; Hickman and Harwood, 2008). It also acts post-translationally to activate Pel biosynthesis by binding to the PelD protein (Lee *et al.*, 2007). Our data suggest that c-di-GMP produced by WpsR acts mainly at the post-translational level to stimulate Pel biosynthesis.

Description of WspR intracellular clusters. I estimated from semi-quantitative immunoblotting experiments that the average *P. aeruginosa* PAO1 cell contains 300 WspR molecules, equivalent to about 1μM (Fig. 3.2). When the Wsp signal transduction system is active and WspR is phosphorylated, individual cells have between one and four visible clusters of WspR-YFP, suggesting that one cluster may comprise 75-300 molecules of WspR. We have previously reported that WspR clusters move or dissolve over observation intervals of 30 s (Güvener and Harwood, 2007). I was able to acquire images of WspR clusters 0.1 s apart over a period of 5 seconds before the YFP fluorophore bleached (Supplemental Movie 1). Some WspR clusters can be observed to move around the cell and across the length of the cell, while others seem to disappear. I was curious as to how long clusters last. In a preliminary experiment, surface-grown cells resuspended in PBS eventually decrease in clustering in about 4 h (Fig. 3.3A). The decrease in clustering may be due to dissociation of clusters or protein degradation. If WspR-YFP is degrading then the fluorescence of the cells should have decreased. However, the mean intensities of the cells did not decrease (Fig. 3.3B), suggesting that the decline in clustering is due to dissociation. Another possibility is that WspR-YFP is cleaved and WspR is degraded while YFP remains stable. Immunoblots performed with anti-WspR on cell lysates derived from

our PAO1 strains expressing WspR-YFP do not give any indication that the fusion protein is being cleaved (data not shown).

Subcellular cluster formation is an intrinsic property of WspR-P. It is possible that WspR-P forms clusters by interacting with or nucleating around another protein. One obvious protein that might play this role is the cytoskeletal protein MreB, which has been shown to play a role in the localization of a number of bacterial proteins (Cowles and Gitai, 2010; Gitai *et al.*, 2004; Shaevitz and Gitai, 2010; White *et al.*, 2010). I tested the possible involvement of MreB in mediating WspA localization. When I treated cells with the drug A22 (Bean *et al.*, 2009), MreB localization was disrupted but intracellular cluster formation by WspR-YFP in a Δ*wspF* strain background was not affected (data not shown). Other candidates for a WspR partner include FleQ, a transcriptional regulator which induces the expression of *pel* genes in response to c-di-GMP (Baraquet *et al.*, 2012; Hickman and Harwood, 2008); SadC and MscL, proteins associated with surface sensing which might have interacted with the surface-responding Wsp complex (Merritt *et al.*, 2010; Moe *et al.*, 1998); and a HD-GYP phosphodiesterase, encoded by the gene PA4108, which could be involved with removing bound c-di-GMP from inhibited WspR (Ryan *et al.*, 2009). The deletion of these proteins did not abolish WspR cluster formation (not shown).

To rule out that WspR-YFP cluster formation requires a *P. aeruginosa*-specific protein or factor I expressed $WspR^{D70E}$-YFP in *E. coli* and observed that it formed clusters in liquid-grown cells (Fig. 3.4). The D70E substitution should activate WspR by mimicking the phosphorylated conformation. $WspR^{wt}$-YFP is diffuse in *E. coli* because there is no Wsp complex to

phosphorylate WspR (data not shown). These results support the interpretation that the clustering behavior of WspR is an intrinsic property of the phosphorylated form of the protein.

Discussion

Subcellular cluster formation by WspR invites speculation that it may be delivering c-di-GMP to a specific site in cells. Such is the case for the response-regulator diguanylate cyclase PleD, which stimulates cell stalk morphogenesis in *Caulobacter crescentus*. PleD-P forms subcellular clusters at the pole of cells and c-di-GMP that it releases at this location activates other polarly localized proteins to initiate a program of stalk morphogenesis (Abel *et al.*, 2011; Lam *et al.*, 2003; Paul *et al.*, 2004). PleD is part of a protein interaction network that exists at the *C. crescentus* cell pole. I cannot say with certainty that WspR is not part of a protein interaction network. However, we have failed to identify proteins that immunoprecipitate with WspR and WspR does not appear to localize to any particular place in cells (Güvener and Harwood, 2007). Thus, our results suggest that c-di-GMP produced from WspR contributes specifically to production of the Pel EPS without stable protein-protein interaction between WspR and the Pel biosynthetic machinery. Our immunoprecipitation efforts did not utilize cross-linking reagents, so future experiments might elucidate whether WspR forms transient interactions with other proteins aside from the Wsp complex.

How then does c-di-GMP produced by WspR specifically affect Pel EPS synthesis but not Psl synthesis or swimming and swarming motility? There are two related possibilities. We know that the depletion in total intracellular c-di-GMP due to a *wspR* mutation is very small. Thus, phenotypes that are very dominant in *P. aeruginosa* may not be much affected by this small

change. Such could be the case for Psl mediated phenotypes. This EPS is primarily responsible for attachment and biofilm formation in *P. aeruginosa* strain PAO1 (Colvin *et al.*, 2012). A related possibility raised by recent work reported with *Salmonella* (Pultz *et al.*, 2012) is that the differential effects of WspR on c-di-GMP sensitive processes is due to differences in the affinities of receptor proteins for c-di-GMP. PelD and FleQ, two proteins that regulate Pel synthesis in response to c-di-GMP, have relatively low affinities for c-di-GMP (2 μM and 15 μM, respectively) (Hickman and Harwood, 2008; Whitney *et al.*, 2012), while other known c-di-GMP receptors have affinities in the nanomolar range (Christen *et al.*, 2010). So a slight change in c-di-GMP caused by WspR DGC activity could affect FleQ and PelD activities, but not the activities of high affinity receptors that remain saturated with c-di-GMP.

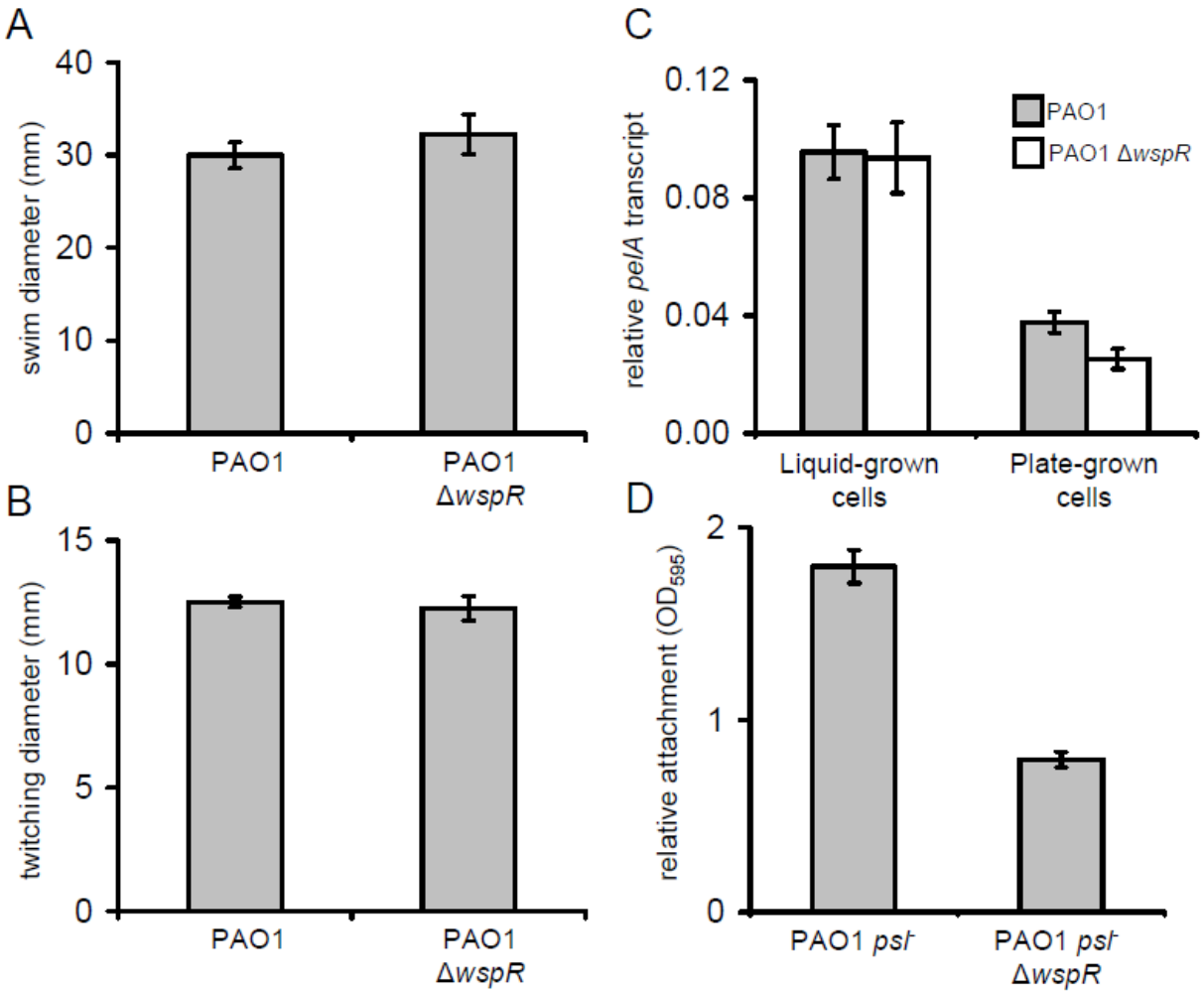

Fig. 3.1 Effects of a *wspR* deletion on c-di-GMP-mediated cellular functions. (A) Swimming motility (n = 4). (B) Twitching motility (n = 4). (C) Relative transcript levels of *pelA* (n = 3). (D) Pel-mediated attachment to 96-well plate wells (n = 30). Bars denote mean value and the error bars represent standard error between replicates.

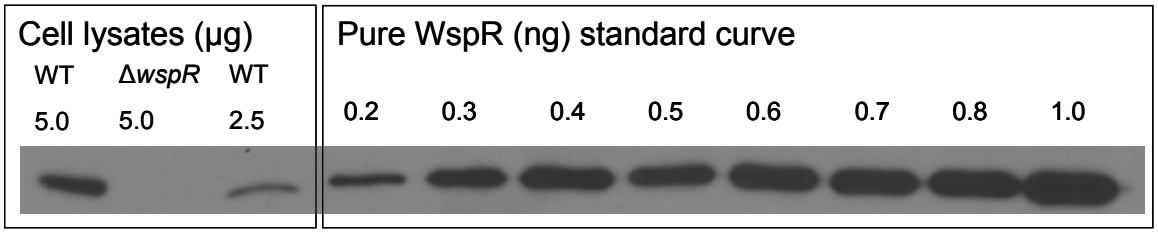

Fig. 3.2 Semi-quantitative immunoblots of WspR to estimate the amount of WspR in a *P. aeruginosa* strain PAO1 cell. Cell lysates of PAO1 and PAO1 Δ*wspR* were run next to a standard curve of purified protein. This immunoblot is representative of four immunoblots used to estimate the cellular concentration of WspR.

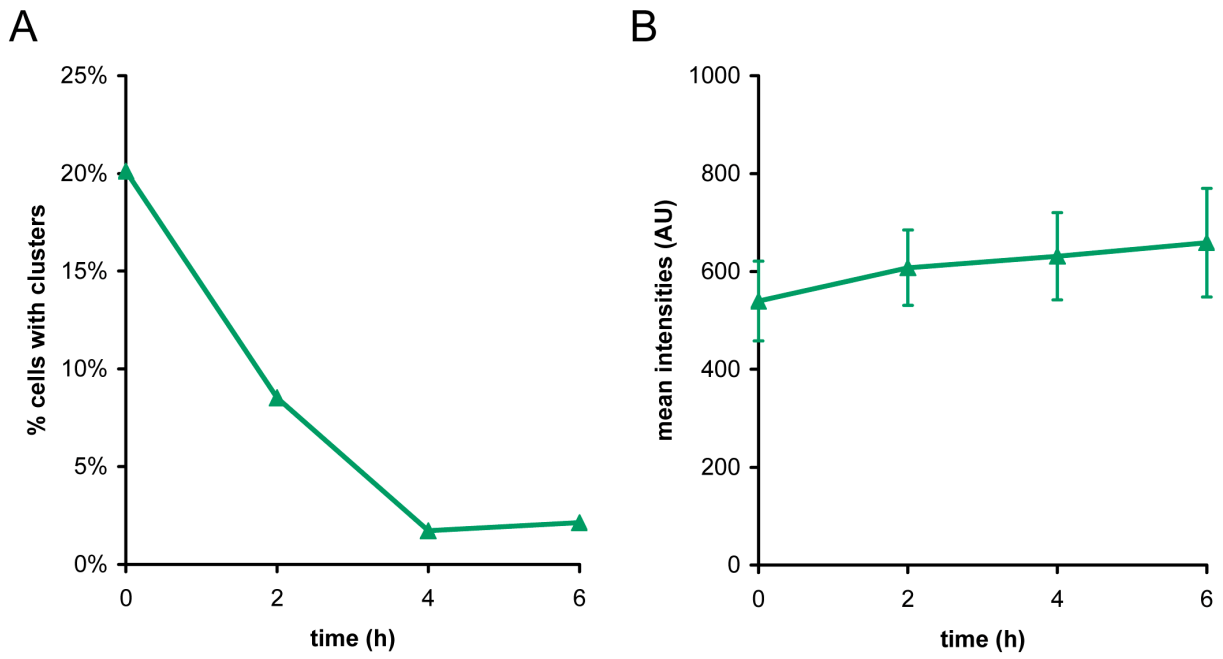

Fig. 3.3 Clustering decreases over time when plate-grown cells are resuspended in PBS. (A) % of cells with clusters over time. (B) Mean intensities of fluorescence in cells over time. Error bars indicate standard deviation.

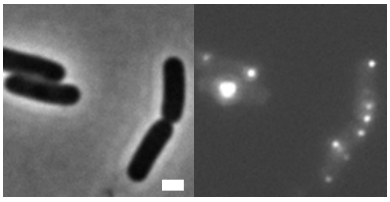

Fig. 3.4 Heterologous expression of the phosphomimic WspR^{D70E}-YFP in *E. coli* strain BW27784. Left: phase contrast image. Right: fluorescent images. The scale bar represents 1 μm. Of 47 cells observed that were expressing WspR^{D70E}-YFP, 44 contained at least one subcellular cluster.

CHAPTER IV:

Subcellular clustering of WspR-P stimulates its diguanylate cyclase activity

Introduction

WspR clustering seems to be intrinsic to the protein itself (chapter III), so I concentrated on the relationship between clustering and DGC activity. I was interested in which domains and features of WspR are important to the localization and activity of WspR. I hypothesized that clustering was needed for WspR cyclase activity.

WspR consists of a CheY-like receiver domain connected to a GGDEF diguanylate cyclase domain via a coiled-coil linker (Fig. 4.1) (De *et al.*, 2008). Similar to other receiver domains, phosphorylation occurs at a conserved aspartate which is position 70 in WspR. The GGDEF domain of WspR contains a site of inhibition (I-site) common to many diguanylate cyclases (Christen *et al.*, 2006). C-di-GMP binding at the I-site has been shown to inhibit WspR cyclase activity (De *et al.*, 2008). In vitro, WspR forms an equilibrium between dimeric and tetrameric species (De et al., 2008). The dimeric form of WspR has low basal levels of activity, while the tetrameric form of WspR has high levels of activity and is considered the active conformation (De et al., 2009).

Results

Description of the WspR variants. To investigate the relationship between WspR clustering and activity, I characterized eight WspR variants, each with a single amino acid residue substitution (Fig. 4.1). Four of these, D16N, D70A, D70E and V72D are in the CheY-like receiver domain. The D16N variant originated from a screen of *wspR* mutants with increased EPS production by *P. aeruginosa* (Hickman and Harwood, unpublished). D16 lies in the N-

terminal region of WspR that is not resolved in the crystal structure and is not conserved across WspR homologues (De *et al.*, 2008). The position of the closest resolved residue at position 18 suggests that D16 is not located near the site of phosphorylation (P-site). Phosphorylation occurs at a conserved aspartate residue (D70) in the receiver domain. The D70A substitution replaces the phosphorylatable aspartate of WspR with an alanine. It is similar to the D70N substitution which renders WspR inactive and insensitive to phosphorylation (De *et al.*, 2009; Güvener and Harwood, 2007). The D70E substitution is predicted to activate WspR by mimicking the phosphorylated conformation of the protein. Converting the aspartate of the conserved phosphorylation site to glutamate activates other response regulators (Aldridge *et al.*, 2003; Klose *et al.*, 1993). The V72D substitution lies close to the D70 residue. Substitutions L167D and L170D are located in the linker stalk region and change WspR oligomerization. $WspR^{L167D}$ tends towards the monomeric species and is inactive, while $WspR^{L170D}$ stabilizes the tetrameric species of the protein and is very active (De *et al.*, 2008). The remaining two variants have substitutions in the GGEEF domain. Like other DGCs, WspR activity is inhibited by binding of c-di-GMP to an inhibition (I-site) on its GGEEF domain (Christen *et al.*, 2006). The R198A substitution is located at the I-site, and $WspR^{R198A}$ does not bind c-di-GMP and is not inhibited by c-di-GMP (De *et al.*, 2008). E253A changes the active site region from GGEEF to GGAEF and abolishes cyclase activity (Malone *et al.*, 2007). The E253A active site substitution does not alter the ability of WspR to form intracellular clusters (Güvener and Harwood, 2007).

Mutations in *wspR* can increase or decrease the frequency of subcellular clustering. I fused the *yfp* gene to the various *wspR* mutant genes and to wild-type *wspR*, integrated the constructs into the *attB* site in the *P. aeruginosa* chromosome and expressed them under the control of the

arabinose promoter in a *wspR* deletion mutant. I observed the clustering behavior of the YFP-tagged proteins using epifluorescence microscopy. I quantified cluster formation as the percentage of cells in a population with at least one WspR-YFP cluster (Tables 4.1 and 4.2). Immunoblot analysis revealed that each of the variants was expressed at approximately the same level as wild-type *wspR* from its native promoter (Table 4.3). Immunoblot analysis also confirmed that the YFP tag remained fused to WspR and the WspR variants and was not cleaved off (data not shown). Variants were considered to be hypercluster formers if they formed subcellular clusters in liquid-grown cells, a condition in which $WspR^{wt}$ does not form clusters (Fig. 4.2A). $WspR^{D16N}$, $WspR^{L170D}$, $WspR^{R198A}$, and $WspR^{E253A}$ belonged to this category. Variants were considered to be hypocluster formers if they did not form visible clusters when expressed in surface-grown cells, a condition in which $WspR^{wt}$ does form clusters (Fig. 4.2B). $WspR^{D70A}$, $WspR^{V72D}$ and $WspR^{L167D}$ were classified as hypocluster formers. The phosphomimic $WspR^{D70E}$ forms clusters when expressed in surface-grown cells but does not form nearly as many clusters as $WspR^{wt}$ (Fig 4.2B, Tables 4.1 and 4.2).

Phosphorylation promotes and is required for cluster formation. We know from our previous studies that the in vivo clustering frequency of $WspR^{wt}$ is correlated with its phosphorylation state. Deleting the methylesterase *wspF* locks the Wsp signal transduction system into an "on" state so that phosphorylation of WspR is greatly increased and levels of intracellular c-di-GMP and biofilm formation increase dramatically (Hickman *et al.*, 2005). Thus, in Δ*wspF* strains WspR forms subcellular clusters not only in cells grown on an agar surface, but also in cells grown in shaken liquid culture (Güvener and Harwood, 2007). Consistent with this, expression of WspR variants with the conserved phosphorylatable aspartate at position 70 in a

wspF background resulted in increased cluster formation (Tables 4.1, 4.2). The one exception was the WspR^{V72D} variant, which did not form subcellular clusters under a condition where it should be phosphorylated. An examination of the in vitro activity of purified WspR^{V72D}, presented below, provides a possible explanation. None of phosphorylatable WspR variants formed clusters in more than 1-2% of cells of strains deleted of *wspA*, a background in which phosphorylation is predicted not to occur because the Wsp system cannot form an active signal transduction complex without the WspA receptor (Tables 4.1 and 4.2.)

The hyperclustering WspR variants and the WspR^{D70E} phosphomimic variant have high *in vivo* diguanylate cyclase activities. I compared the in vivo clustering frequencies of the WspR-YFP variants with their in vivo diguanylate cyclase activities. Levels of c-di-GMP in cells can be roughly determined by examining *P. aeruginosa* colony morphology. High intracellular levels of c-di-GMP stimulate the production of EPS, resulting in "wrinkly" colony morphologies and the uptake of the Congo Red dye (Friedman and Kolter, 2004; Hickman *et al.*, 2005). Cells with relatively lower intracellular c-di-GMP form smooth colonies. The *P. aeruginosa* wild-type strains expressing the hyperclustering variants WspR^{D16N}, WspR^{L170D} or WspR^{R198A} formed colonies that were more wrinkly than WspR^{wt} colonies, indicating that they have elevated in vivo diguanylate cyclase activities relative to WspR^{wt} (Fig. 4.3, middle row). Although WspR^{E253A} is also a hypercluster former, strains expressing WspR^{E253A} have smooth colony morphologies because the E253A mutation completely abolishes diguanylate cyclase activity. WspR^{E253A} is shown here as a negative control, but for these and subsequent studies I do not consider it when drawing a connection between hypercluster formation and activity. The hypocluster variants WspR^{D70A} and WspR^{L167D} had smooth colony morphologies when expressed in a wild type *P.*

aeruginosa, which likely reflects low diguanylate cyclase activity. However the hypocluster variant WspR^{V72D} and the phosphomimic WspR^{D70E} formed wrinkly colonies, reflecting that these enzymes were very active.

I also determined the colony morphologies of the WspR variants expressed in Δ*wspA* and Δ*wspF* strain backgrounds. A Δ*wspA* strain expressing any of the variants except for WspR^{L170D} WspR^{D70E} or WspR^{V72D}, tended toward a smoother colony morphology relative to the wild type situation, reflecting lower diguanylate cyclase activities and underscoring the importance of WspR phosphorylation for in vivo activity (Compare Fig. 4.3 top row with Fig. 4.3 middle row). This result also draws a rough correlation between cluster formation and in vivo activity (Fig. 4.3 and Table 4.1). The L170D mutation is known to increase the formation of the active tetrameric conformation of WspR and this may overcome the need for phosphorylation. A variant of the *P. fluorescens* WspR with a similar gain-of-function mutation in the linker region was able to retain its activity upon mutation of the P-site (De *et al.*, 2008; Goymer *et al.*, 2006). The V72D mutation seems to result in high levels of activity in the absence of phosphorylation. Colonies of the Δ*wspF* strains expressing phosphorylatable WspR variants tend to be very wrinkly, a reflection of high diguanylate cyclase activities of the phosphorylated forms of the proteins (Fig. 4.3 bottom row). The image for the I-site variant WspR^{R198A} expressed in the Δ*wspF* background had to be taken at an earlier time point (48 h) since smooth suppressor mutants arose and overwhelmed the wrinkly colony.

The *in vivo* clustering frequencies of most WspR-YFP variants correlate with their *in vitro* diguanylate cyclase activities. I next determined the diguanylate cyclase activities of WspR

variant proteins expressed and purified from *E. coli* (Fig. 4.4). It is important to note that I tested the activities of proteins that were not phosphorylated. Thus, as expected, the $WspR^{D70E}$ phosphomimic had very high activity relative to the other WspR variants. Previous work has shown that when expressed and purified from *E. coli*, WspR has c-di-GMP bound at its I-site (De *et al.*, 2008). Thus, to get a full picture of the range of specific activities of purified WspR variants, I also purified the proteins from *E. coli* cells expressing the phosphodiesterase protein PA2133 (Hickman *et al.*, 2005; Kulasekara *et al.*, 2006). Phosphodiesterase treatment dramatically increased the activity of $WspR^{wt}$ and $WspR^{D16N}$. $WspR^{R198A}$ was, as expected, unaffected by c-di-GMP because it does not bind this compound. $WspR^{L170D}$ was also not sensitive to c-di-GMP inhibition. With the exception of $WspR^{V72D}$, variants with relatively high *in vitro* enzyme activity tended to form more intracellular clusters when grown on agar (Fig. 4.4, Table 4.1).

WspR diguanylate cyclase specific activity is concentration-dependent. The data presented so far show that with the exception the $WspR^{V72D}$ variant, the more active WspR variants have a greater propensity to form clusters. But does cluster formation stimulate the activity of a given variant? To test the hypothesis that WspR is most active when it is in a more concentrated form within a subcellular cluster I looked into how WspR concentration affected its *in vitro* activity. When WspR was diluted from a concentrated stock, its specific activity gradually decreased and equilibrated in about 20 hours, after which it stayed constant for at least three days (Fig. 4.5, top). After equilibration, WspR can be reconcentrated to restore its former high specific activity (Fig. 4.5, bottom). Concentration increases the tetrameric species of WspR and others have seen a shift from tetramers to dimers over time when a WspR solution is diluted (De *et al.*, 2008) (H.

Sondermann, personal communication). I assume that the activity and distribution of oligomeric species of WspR which has been kept at a diluted concentration for at least 16 h is representative of WspR at equilibrium at that diluted concentration and I refer to this as "equilibrated WspR". In contrast, the activity and oligomeric species of WspR that exists immediately after dilution and before equilibration, or "unequilibrated" WspR, more closely reflects that of WspR in the concentrated stock solution. The activities shown in Fig. 4.4 were obtained with unequilibrated WspR.

Equilibrated $WspR^{wt}$, when activated with the phospho-analog beryllium fluoride, increased in specific activity as the concentration of WspR protein was increased (Fig. 4.6) (De *et al.*, 2008; Yan *et al.*, 1999). Equilibrated $WspR^{wt}$ that was not treated with BeF_3^- did not demonstrate concentration-dependent activity in the range of WspR concentration tested. There is an upper limit to the concentration of equilibrated WspR that I can test in our assay. At high concentrations of WspR the assay enzymes become rate-limiting and WspR may precipitate from the assay buffer, depending on the variant. $WspR^{D70A}$ had little catalytic activity with or without BeF_3^- addition. The lack of activation of $WspR^{D70A}$ by BeF_3^- demonstrates that BeF_3^- activates $WspR^{wt}$ through mimicking phosphorylation at the P-site and not through another mechanism. $WspR^{L170D}$ is active enough after equilibration to show concentration-dependent activity without BeF_3^-, demonstrating that concentration-dependent activation does occur in the absence of phosphorylation. $WspR^{L170D}$- BeF_3^- is about as active as $WspR^{wt}$- BeF_3^-, suggesting that both have reached the maximum amount of activation by BeF_3^-. However, their activity is still much lower than that of $WspR^{D70E}$ (Fig. 4.6 inset).

Further characterization of WspR^{V72D}. When I examined the concentration-dependent activity of WspR^{V72D}, I found that it had high activity relative to untreated WspR^{wt} and WspR^{L170D} at low protein concentrations. In addition WspR^{V72D} displayed no concentration-dependent activity and was not activated by BeF_3^-. These observations about WspR^{V72D} raised the question of what combination of oligomeric species it contains at equilibrium. Thus, I subjected WspR^{wt} and WspR^{V72D} to size exclusion chromatography (SEC). The SEC profile of both proteins show peak maxima at elution volumes of 12.3 and 13.8 ml which roughly correspond to the tetramer and dimer peak maxima at 11.7 and 13.3 ml, previously reported in the literature (De *et al.*, 2008). I analyzed the diguanylate cyclase activity of the eluted fractions corresponding to each peak. Surprisingly, both dimers and tetramers of WspR^{V72D} show an intermediate amount of activity (Fig. 4.7). This is in contrast to WspR^{wt} where the tetramer fraction demonstrates a high amount of activity while the dimer fraction shows very little activity.

I wanted to determine whether WspR^{V72D} activity is impacted by phosphorylation or c-di-GMP inhibition. I introduced the D70A, D70E and R198A mutations into the V72D background, creating WspR$^{D70A/V72D}$, WspR$^{D70E/V72D}$, and WspR$^{V72D/R198A}$. The colony morphologies of strains expressing the double mutants resemble those expressing WspR^{D70A}, WspR^{D70E}, and WspR^{R198A} (Fig. 4.8). WspR^{V72D} activity appears to be dependent on phosphorylation, as disrupting the phosphorylation site alters the colony morphology greatly. WspR^{V72D} also appears to be susceptible to c-di-GMP inhibition. In strains with the *wspF* deletion, expressing either WspR$^{V72D/R198A}$ or WspR^{R198A} result in similar colony morphologies that are smaller and wrinklier than colony morphologies resulting from expressing WspR^{V72D} or WspR^{wt}, indicating similar and extremely high levels of intracellular c-di-GMP.

Discussion

In this chapter I provide evidence supporting the hypothesis that clustering promotes DGC activity. First, hypercluster formers, those WspR variants that formed clusters with greater frequency than $WspR^{wt}$, also had increased in vivo and in vitro activity. Second, the in vitro specific activities of WspR and WspR variants increased as the protein concentration was increased and third, this effect was magnified by activation of WspR protein by treatment with the phospho-analog beryllium fluoride.

I also confirmed the importance of phosphorylation to cluster formation and in vivo activity of WspR. Phosphorylation is essential for clustering even for hyperclustering WspR variants (Table 4.1). Phosphorylation also seems to overcome problems with oligomerization. For example, the $WspR^{L167D}$ variant is compromised in its ability to polymerize (De *et al.*, 2008). In wild-type surface-grown cells, $WspR^{L167D}$ is not phosphorylated often enough to overcome the polymerization defect, leading us to classify it as a hypocluster former. However, over-phosphorylation of $WspR^{L167D}$ in a Δ*wspF* background restored cluster formation and c-di-GMP production.

$WspR^{V72D}$ is a variant that seems to bypass the need for clustering for activation. $WspR^{V72D}$ is active as a dimer and does not show concentration-dependent activity. This is in contrast to $WspR^{wt}$ which is not active as a dimer and requires tetramerization for activity. In my model, $WspR^{wt}$ needs to cluster to promote tetramerization by increasing its own local concentration. Since $WspR^{V72D}$ requires neither tetramerization nor clustering for activity, $WspR^{V72D}$ is more

active in vivo and in vitro. Some preliminary experiments involving the clustering frequency of the D70E/V72D and V72D/R198A double mutants raise new questions about localization. $WspR^{D70E/V72D}$ is diffuse in liquid-grown cells but clustered like $WspR^{wt}$ in surface-grown cells. $WspR^{V72D/R198A}$ is diffuse in liquid-grown cells, unlike $WspR^{R198A}$ which forms clusters in these conditions. In contrast to the in vivo activity experiments, where the D70E and R198A phenotypes are dominant over the V72D phenotype, here the clustering phenotypes appear to be intermediate. It might be worthwhile to further explore how V72D affects clustering by looking at other double mutations.

I view WspR intrinsic subcellular clustering as a mechanism to activate the protein by increasing the local concentration of protein. There are many examples of oligomeric proteins which show increased activity as their concentration increases, including NtrC, FtsZ, PleD, MinD, and IRE1 (Hu and Lutkenhaus, 2001; Korennykh *et al.*, 2009; Mettke *et al.*, 1995; Paul *et al.*, 2007; Wang and Lutkenhaus, 1993). Although WspR does not have any DNA binding domain, our proposed mechanism most closely resembles proposed mechanisms for DNA-binding response regulators. Binding to enhancer sites on the DNA has been proposed to increase the local concentration of NtrC and to serve as a template for oligomerization of NtrC dimers (Porter et al., 1993). Similarly, OmpR binding to DNA facilitates dimer formation (Maris et al., 2005). WspR clustering may play a similar role in facilitating oligomerization by increasing the local concentration of the protein.

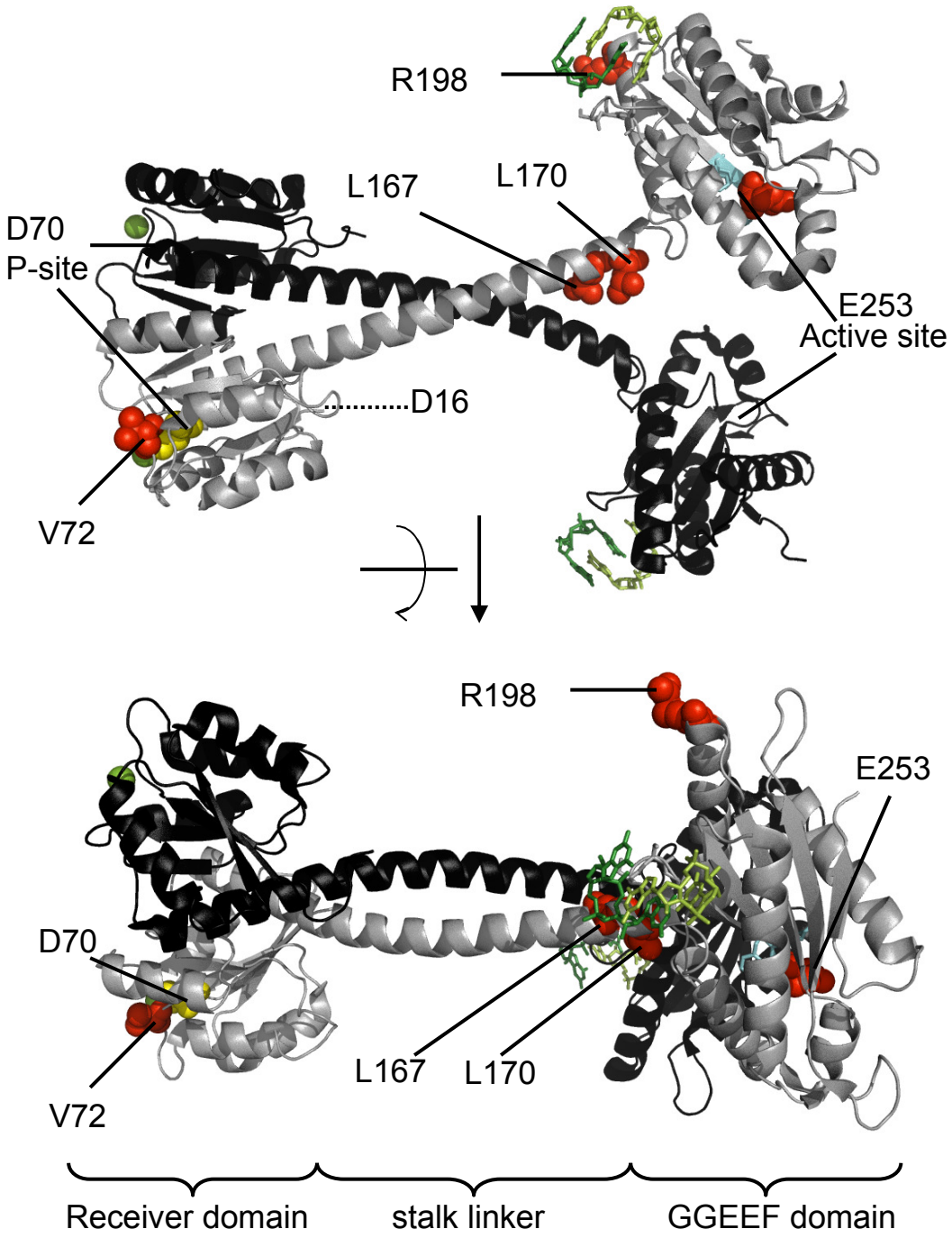

Fig. 4.1 The locations of WspR mutations examined in this study with respect to the published crystal structure of WspR (PDB# 3BRE) rendered by the PyMOL program (v0.99rc6) (www.pymol.org). WspR is depicted as a dimer, one molecule in grey and the other in black. Red spheres: locations of the mutated residues. Dotted line: The position of D16 is not resolved and is approximated by the position of the closest resolved residue, A18. Cyan: conserved GGEEF motif of the cyclase active site. Yellow: conserved aspartate of the phosphorylation site. Green balls: Mg^{2+} ions needed for phosphorylation. Green ball and stick molecules: c-di-GMP dimers bound at the I-sites. The bottom figure is the top figure rotated around the x-axis.

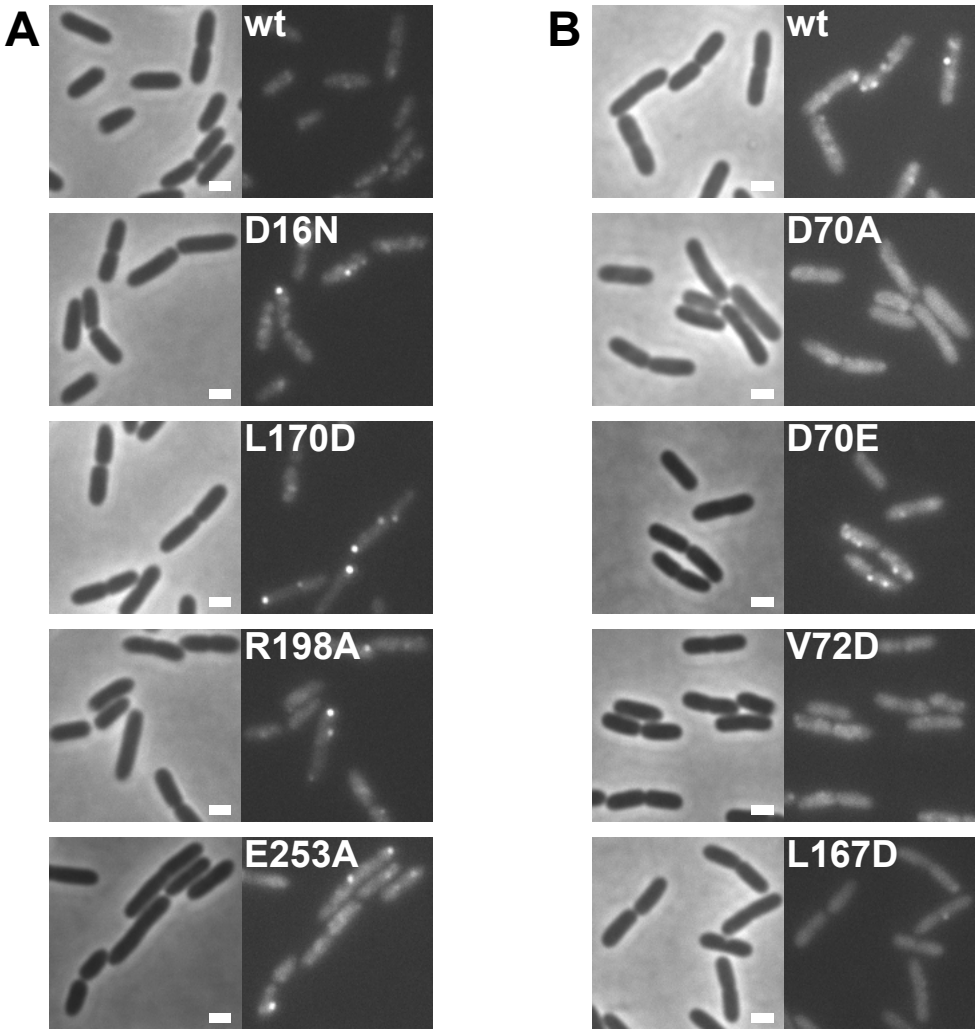

Fig. 4.2 Fluorescence micrographs of strain PAO1 derivatives expressing the denoted *wspR* alleles fused with YFP. Left: phase contrast images. Right: fluorescent images. The scale bar represents 1 μm. (A) Liquid-grown cells. (B) Surface-grown cells.

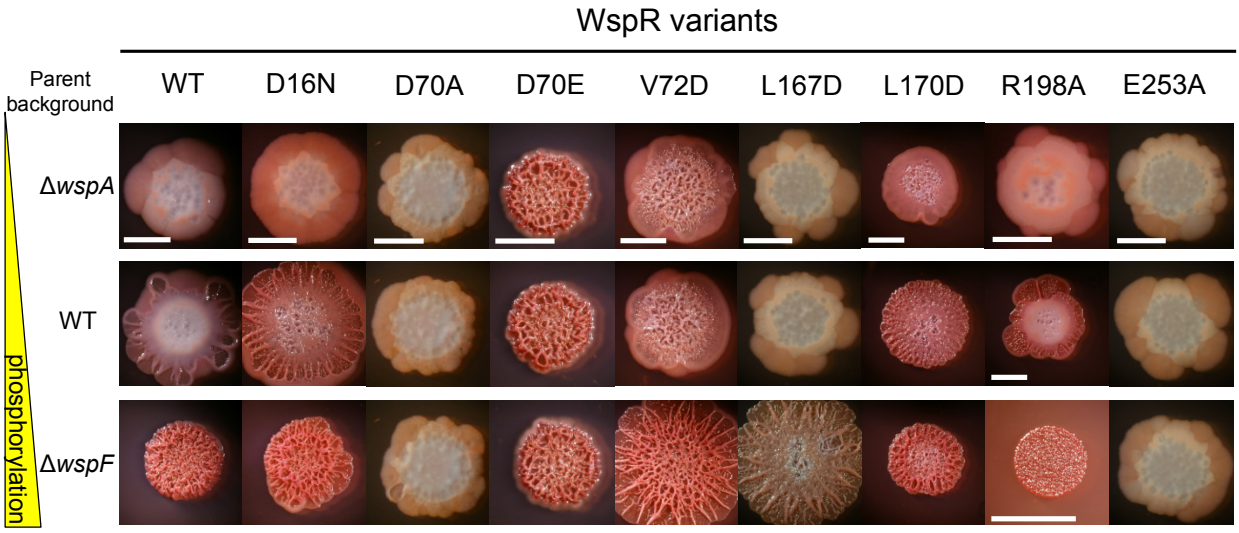

Fig. 4.3 PAO1 derivatives expressing WspR variants spotted onto Tryptone Congo red agar plates with 1% arabinose. Bar = 5mm. Top row: Δ*wspA* background. Middle row: Wild-type background, where the native *wspR* gene is deleted and the selected *wspR* allele is inserted into the neutral *attB* site under an arabinose inducible P_{BAD} promoter. Bottom row: Δ*wspF* background.

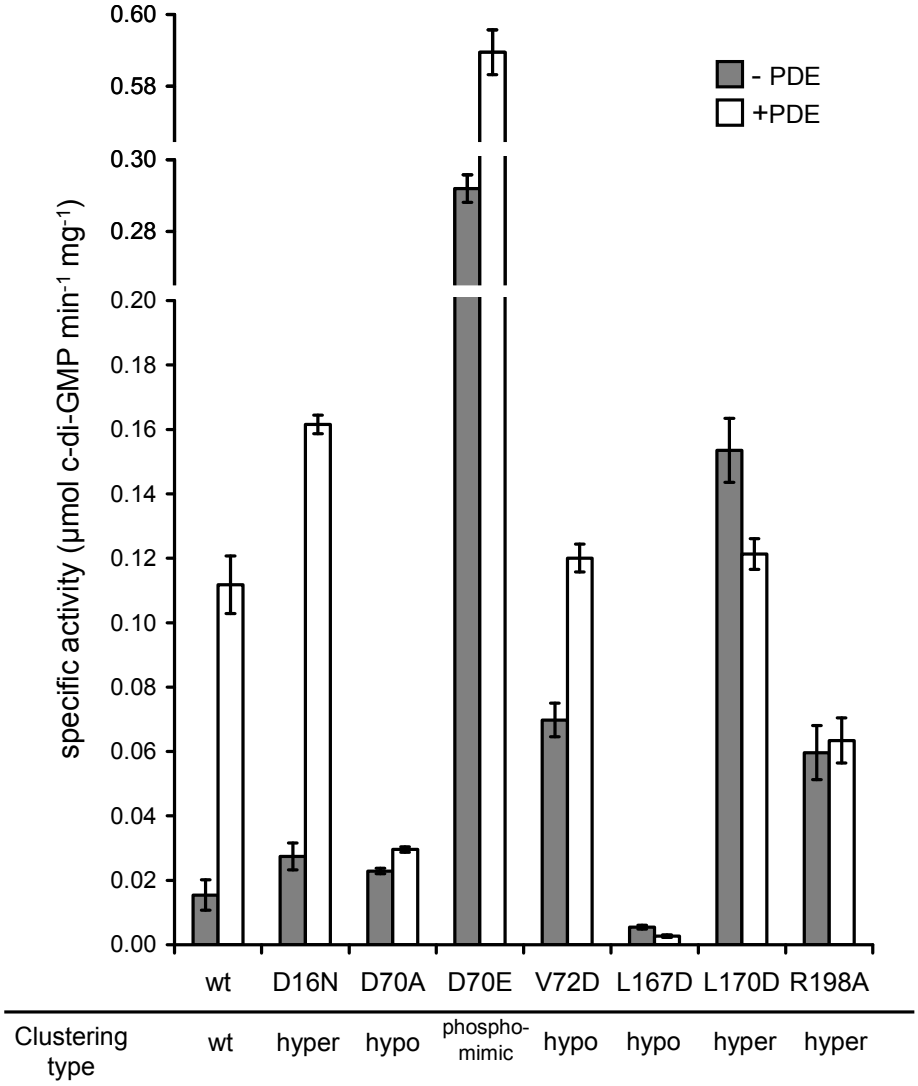

Fig. 4.4 Specific activity of purified WspR protein assayed at a concentration of 1 μM immediately after dilution from a stock solution of 100-1000 μM. To obtain uninhibited proteins, WspR proteins were co-expressed in *E. coli* with the phosphodiesterase encoded by PA2133 and then purified. Data points reflect the mean of at least three replicates and the error bars indicate standard error.

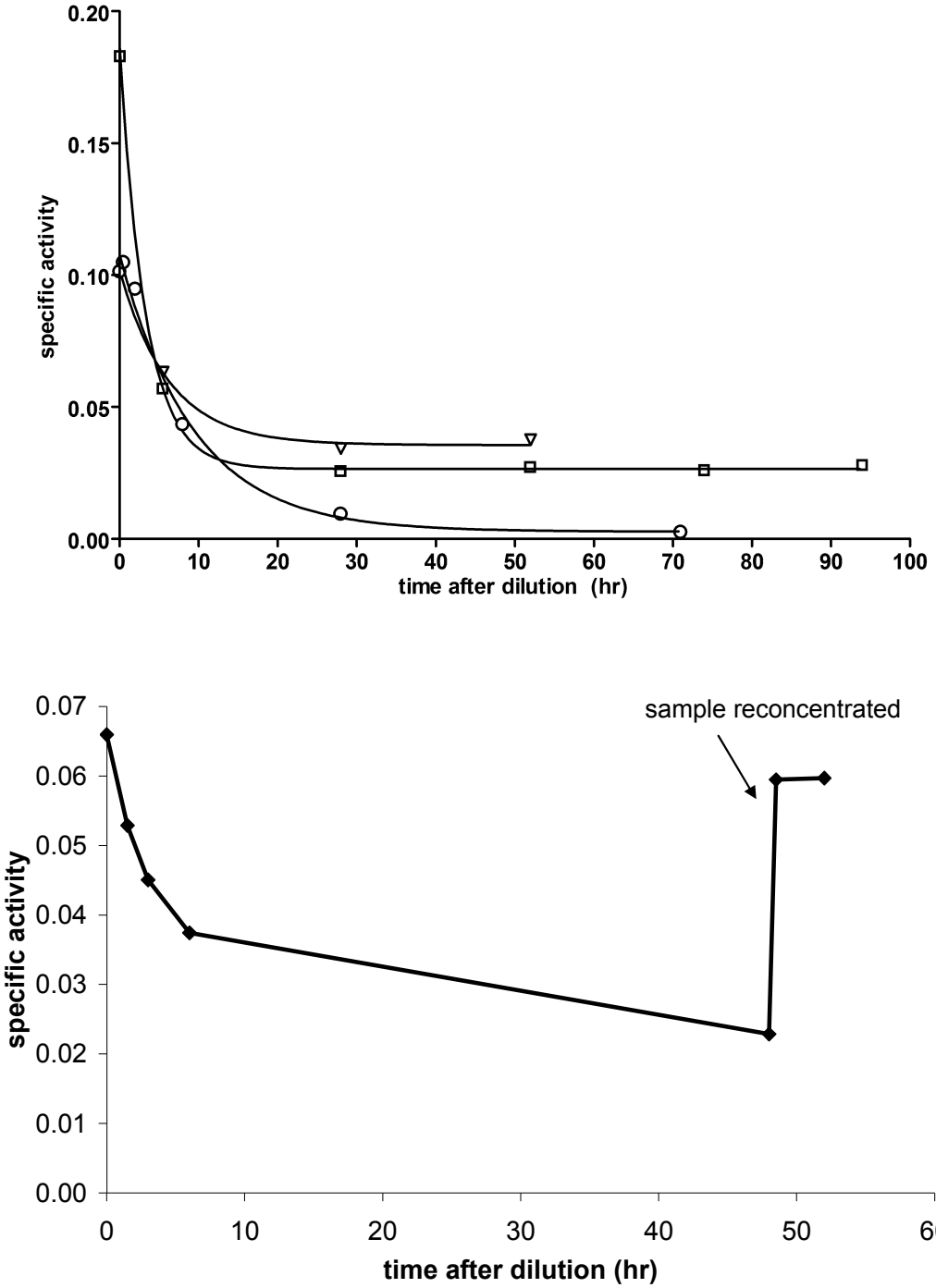

Fig. 4.5 Decrease in WspR specific activity over time after dilution. Top: Circle: $WspR^{wt}$ diluted to 1 μM from a 271 μM stock solution. Square: $WspR^{L170D}$ diluted to 2 μM from a 94 μM stock solution. Triangle: $WspR^{L170D}$ diluted to 4 μM from a 94 μM stock solution. Bottom: $WspR^{L170D}$ diluted to 2 μM from a 94 μM stock solution, then reconcentrated to 33 μM after the 48 hour time point (arrow).

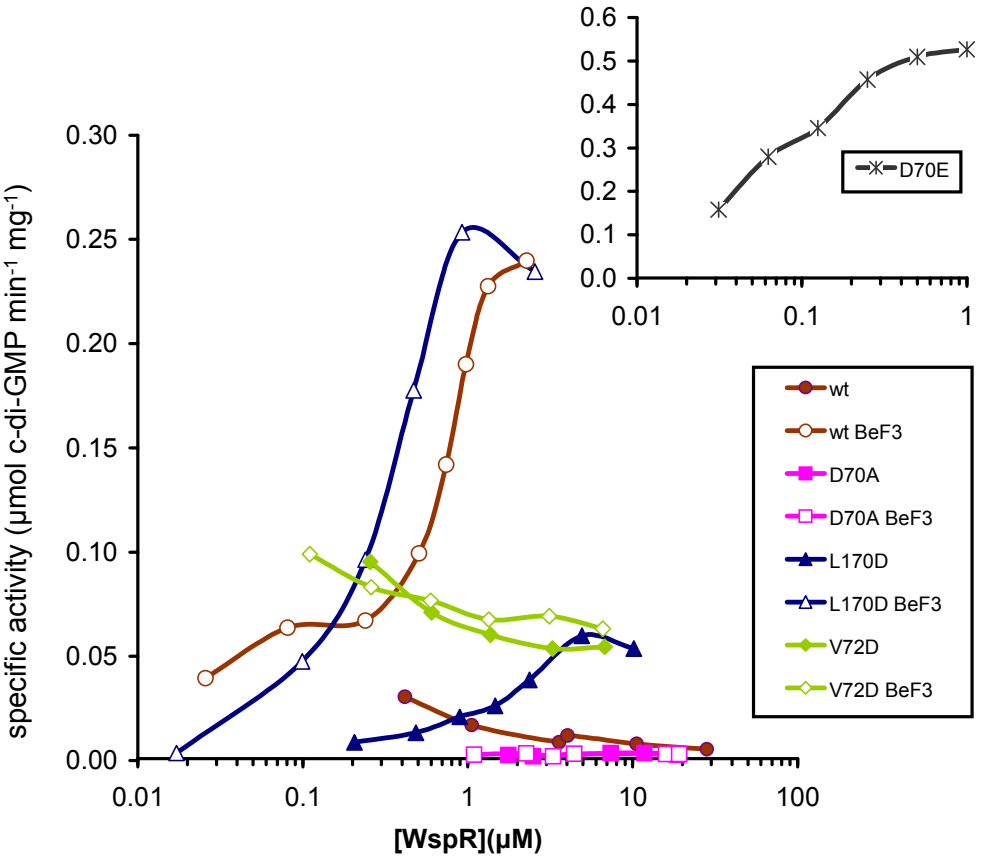

Fig. 4.6 Specific activity of WspR as a function of WspR concentration. The diguanylate cyclase activities of WspR proteins were assayed following their equilibration in assay buffer at concentrations ranging from 17 nM to 28 μM at 22°C for 16-24 hours.

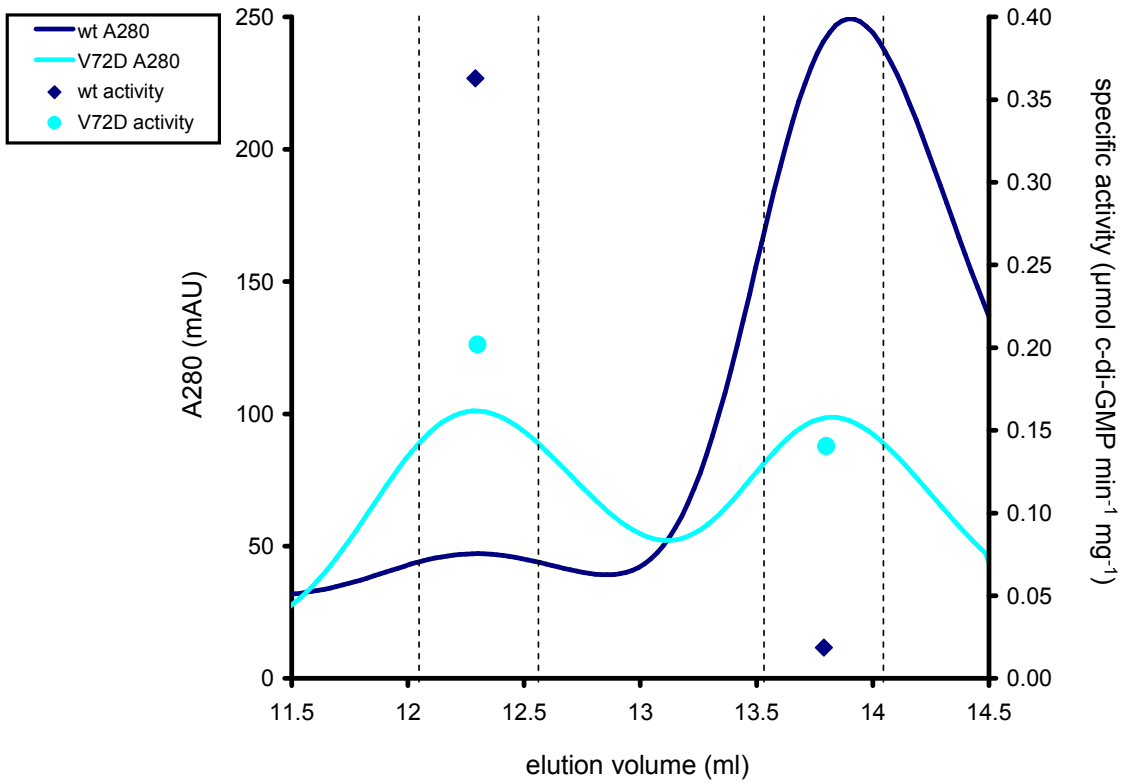

Fig. 4.7 Size exclusion chromatography of $WspR^{wt}$ and $WspR^{V72D}$ and the specific activities of fractions. The dashed lines denote the elution volumes corresponding to the fractions analyzed.

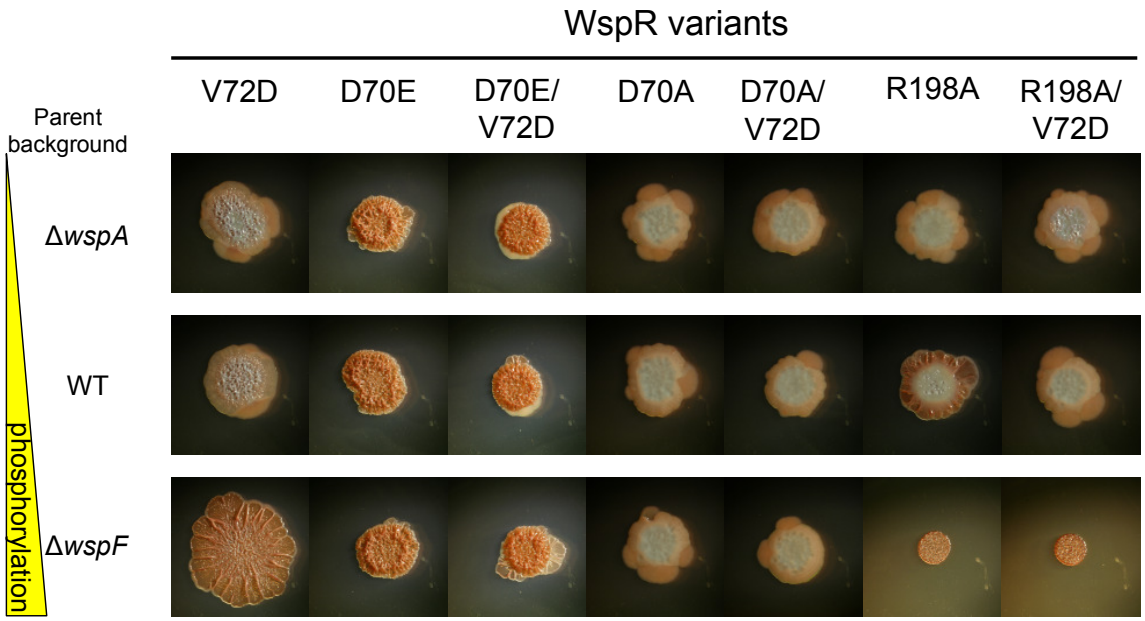

Fig. 4.8 Colony morphologies of PAO1 derivatives expressing $WspR^{V72D}$ double mutation variants. Top row: Δ*wspA* background. Middle row: Wild-type background, where the native *wspR* gene is deleted and the selected *wspR* allele is inserted into the neutral *attB* site under an arabinose inducible P_{BAD} promoter. Bottom row: Δ*wspF* background.

Table 4.1 Quantitative analysis of WspR-YFP cluster formation in strains expressing different variants of WspR grown on an agar surface.

Location	Mutation	% surface-grown cells with clusters[a]		
		Δ*wspA*	(wt)	Δ*wspF*
	wild-type	0% (339)	43% (411)	77% (494)
unsolved N-term	D16N	0% (130)	68% (644)	76% (386)
P-site	D70A	0% (256)	0% (190)	0% (139)
P-site	D70E	7% (204)	7% (229)	19% (200)
D + 2	V72D	0% (99)	0% (599)	1% (310)
linker stalk	L167D	0% (228)	4% (684)	72% (632)
linker stalk	L170D	1% (266)	75% (468)	72% (186)
I-site	R198A	0% (128)	38% (366)	48% (261)
cyclase active site	E253A	0% (194)	68% (276)	75% (130)

[a]Percentages represent the number of cells with at least one well-defined fluorescent spot divided by the total number of visualized cells, shown in the parentheses. A well-defined fluorescent spot is defined by dividing the maximum pixel intensity by the average pixel intensity in the cell. All cells with a resultant number above a certain threshold, determined visually, are considered cells with at least one cluster.

Table 4.2 Quantitative analysis of WspR-YFP cluster formation in strains expressing different variants of WspR grown in liquid cultures. The percentage of cells with at least one cluster is determined as described in Table 4.1

Location	Mutation	% liquid-grown cells with clusters		
		Δ*wspA*	(wt)	Δ*wspF*
	wild-type	1% (715)	1% (295)	34% (493)
unsolved N-term	D16N	2% (270)	16% (397)	57% (522)
P-site	D70A	0% (250)	0% (178)	0% (138)
P-site	D70E	0% (301)	0% (116)	0% (218)
D + 2	V72D	2% (228)	2% (572)	0% (256)
linker stalk	L167D	0% (158)	0% (506)	23% (381)
linker stalk	L170D	0% (604)	29% (632)	82% (222)
I-site	R198A	0% (173)	9% (265)	36% (140)
cyclase active site	E253A	0% (266)	12% (145)	75% (232)

Table 4.3 Quantitative analysis of immunoblots of WspR variants. WspR bands were subjected to densitometry using ImageQuant software (v5.1). The data are mean band intensities derived from two experiments and normalized to the $WspR^{wt}$ band intensity. Standard deviation is shown within the parentheses.

Location	Mutation	Band intensity
	wild-type	1.0 (0.0)
unsolved N-term	D16N	1.1 (0.2)
P-site	D70A	0.7 (0.6)
P-site	D70E	0.9 (0.1)
D + 2	V72D	3.2 (1.7)
linker stalk	L167D	2.3 (1.4)
linker stalk	L170D	2.5 (1.3)
I-site	R198A	1.4 (0.5)
cyclase active site	E253A	1.4 (0.7)

CHAPTER V:

C-di-GMP inhibition may dampen WspR subcellular clustering

Introduction

While studying the clustering behavior of the WspR variants, I saw a unexpected correlation between c-di-GMP inhibition and clustering. WspR variants that are insensitive to c-di-GMP inhibition ($WspR^{L170D}$ and $WspR^{R198A}$) or do not produce c-di-GMP ($WspR^{E253A}$) are hyper-clusterers (Fig. 4.2, Table 4.2). That led me to hypothesize that c-di-GMP may dampen WspR subcellular clustering.

Results

Addition or removal of c-di-GMP in trans did not change clustering frequency. If c-di-GMP dampens WspR subcellular clustering then the addition of c-di-GMP should reduce clustering and the removal of c-di-GMP should increase clustering. I expressed the *ydeH* diguanylate cyclase from *E. coli* or the phosphodiesterase PA2133 from *P. aeruginosa in trans* and looked for any effect on WspR clustering. I also expressed *ydeH* in a strain lacking the Pel and Psl EPS in order to side-step any consequences of overproduction of EPS, such as underrepresentation from cells in aggregates or on biofilms stuck to the side of the culture tube. In addition, I expressed *ydeH* in a strain expressing the WspR variant with the active site mutation, $WspR^{E253A}$, to see if influx of c-di-GMP can reduce the cluster formation in liquid-grown cells. However, the addition or degradation of c-di-GMP *in trans* had no apparent effect on WspR clustering in *P. aeruginosa* (Table 5.1). c-di-GMP production by YdeH was confirmed by colony morphology (data not shown).

c-di-GMP inhibition dampens concentration-dependent activity. Although I did not detect changes in WspR clustering when modulating cellular c-di-GMP levels *in vivo*, there are some *in*

vitro data that suggests c-di-GMP does dampen clustering, assuming that subcellular clustering is a mechanism to activate WspR via concentration-dependent activity. In figure 4.6, I did not show the specific activity of $WspR^{wt}$ equilibrated above 30 μM for two reasons. First, the assay enzymes pyrophosphatase and purine ribonucleoside phosphorylase became rate-limiting and therefore WspR cyclase activity would be underestimated. Cyclase activity above 0.3 μmole c-di-GMP/min is underestimated by the assay. Second, at high concentrations WspR tends to precipitate in the assay buffer. Therefore, I compared the activities of equilibrated vs. unequilibrated protein to approximate comparing WspR activity at high and low concentrations. Unequilibrated $WspR^{wt}$ has a much higher specific activity compared to equilibrated $WspR^{wt}$, demonstrating the concentration dependent activity of $WspR^{wt}$ (Fig. 5.1). When inhibited by c-di-GMP, $WspR^{wt}$ does not show this concentration-dependent increase in activity. In contrast, the I-site variant $WspR^{R198A}$, which is unable to bind c-di-GMP and should not be affected by c-di-GMP inhibition, increases its activity by the same amount regardless of whether it is co-expressed with a phosphodiesterase. A similar result has been demonstrated by the Sondermann lab (De *et al.*, 2008).

c-di-GMP increases solubility of purified WspR protein. $WspR^{D16N}$, when purified after co-expression with a phosphodiesterase and therefore free of bound c-di-GMP, tends to form a loose jelly-like precipitate when at concentrations above 5 μM in the cyclase assay buffer. When GTP is added the precipitate is solubilized and the solution becomes clear (Fig. 5.2). The accumulation of phosphate is detected by the assay to confirm that cyclase activity is occurring after the addition of GTP. The evidence suggests that the c-di-GMP binding depolymerizes

WspR from macroscopically visible oligomeric structures, which is consistent with the formation of "elongated dimers" when WspR is inhibited by c-di-GMP (De *et al.*, 2008).

Discussion

Expressing a DGC or PDE in trans did not cause clustering to decrease or increase, so c-di-GMP may not affect clustering and the c-di-GMP insensitive variants cluster more frequently for some other unknown reason. Alternatively, the c-di-GMP inhibition dampening effect on clustering may require c-di-GMP locally produced by WspR itself. The *in vitro* data suggests that c-di-GMP does affect some sort of higher order oligomerization with WspR protein in vitro. c-di-GMP inhibition prevents concentration-dependent increase in specific activity and solubilizes the precipitate of at least one WspR variant. I assume that c-di-GMP is formed from the added GTP since the assay for DGC activity detects the phosphate by-product from the formation of c-di-GMP. The c-di-GMP then binds to the apo-$WspR^{D16N}$, and forces the oligomers of $WspR^{D16N}$ that are visible as a precipitate to dissociate. I have only recorded this phenomenon in $WspR^{D16N}$ because the other WspR variants do not reliably precipitate as much. The next step would be to systematically find at what concentrations other WspR variants precipitate and whether those precipitates also dissolve upon addition of c-di-GMP, to determine whether this can be generalized to a mechanism utilized by $WspR^{wt}$ or if it is a quirk specific to the $WspR^{D16N}$ variant.

Evidence is mixed regarding whether the effects of phosphorylation on WspR activity and oligomerization are dominant over the effects of c-di-GMP inhibition. Strains that have high levels of c-di-GMP, such as Δ*wspF* strains, still have high frequencies of WspR clustering (Table

4.1, Table 4.2). If c-di-GMP inhibition dampens clustering, then strains with high levels of c-di-GMP should show less WspR clustering. Perhaps constitutive phosphorylation can overcome c-di-GMP inhibition, or there may be a phosphodiesterase that is involved in removing c-di-GMP from WspR *in vivo*. *In vitro*, BeF_3^- will only activate uninhibited WspR and does not activate c-di-GMP-bound WspR (De et al., 2009). Perhaps *in vitro* BeF_3^- activation does not fully recapitulate *in vivo* phosphorylation by the Wsp system.

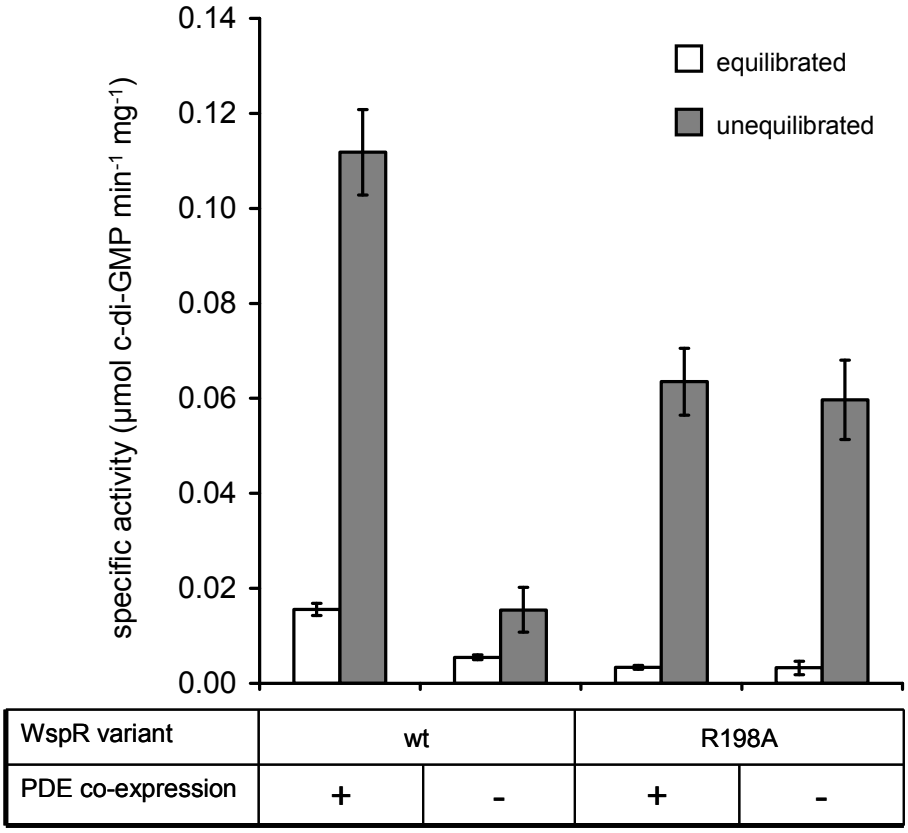

Fig. 5.1 c-di-GMP inhibition dampens concentration-dependent activity. White bars indicate specific activity of WspR assayed at 1 µM from a solution that has been diluted to and equilibrated at 1µM overnight. Grey bars indicate specific activity of WspR assayed at 1µM immediately after dilution from concentrated stock solutions (180-1000 µM). When overexpressing the protein for purification, WspR was either co-expressed with the PA2133 phosphodiesterase to remove c-di-GMP or expressed by itself, in which case c-di-GMP would be produced and bind to $WspR^{wt}$.

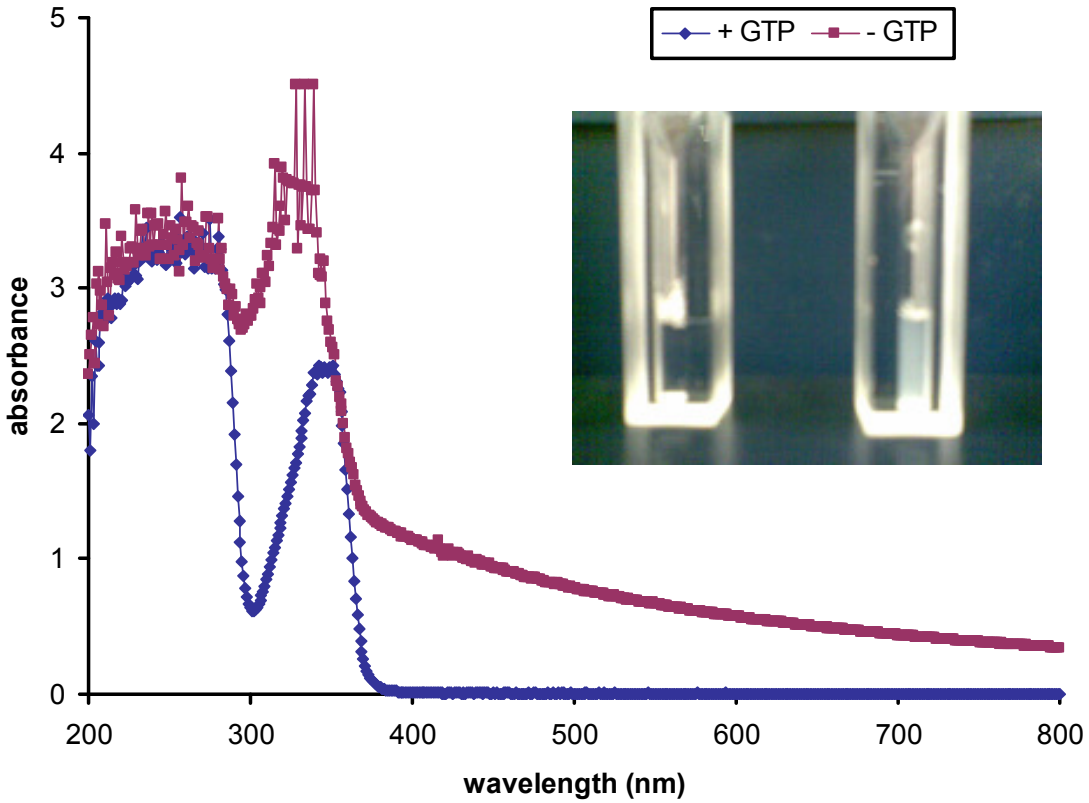

Fig. 5.2 Absorbance wavelength scans of $WspR^{D16N}$ solution (30 μM) before and after adding GTP (0.5 mM). The peak at 360 nm in the GTP+ line represents the reaction of MESG to the formation of pyrophosphate. Inset: Photo of the same $WspR^{D16N}$ solutions in quartz cuvettes before and after addition of GTP (right and left, respectively).

Table 5.1 Quantitative analysis of WspR-YFP cluster formation in strains expressing the diguanylate cyclase YdeH or the phosphodiesterase PA2133 in trans. pJN105 is the empty vector. The percentage of cells with at least one cluster is determined as described in Table 4.1

Strain	% liquid-grown cells with clusters[a]	% surface-grown cells with clusters[a]
PAO1 wspR-yfp /pJN105	0% (250)	11% (202)
PAO1 wspR-yfp /pJN2133	2% (218)	12% (235)
PAO1 wspR-yfp /pJNydeH	1% (125)	12% (178)
PAO1 ΔpelA ΔpslBCD wspR-yfp /pJN105	0% (381)	12% (298)
PAO1 ΔpelA ΔpslBCD wspR-yfp /pJNydeH	0% (231)	7% (284)
PAO1 $wspR^{E253A}$-yfp /pJN105	12% (244)	32% (180)
PAO1 $wspR^{E253A}$-yfp /pJNydeH	8% (313)	44% (92)

CHAPTVER VI:

Conclusions and future perspectives

From previous work, we understood the Wsp complex to respond to unknown signals that are present when cells are growing on a surface (Güvener and Harwood, 2007). WspR is phosphorylated and forms subcellular clusters in surface-grown cells. WspR~P is also the active form that produces c-di-GMP which promotes exopolysaccharide production, ultimately promoting biofilm growth. My thesis work has both confirmed and updated our model of Wsp function and WspR activation (Fig. 6.1). I have narrowed down the effects of WspR-produced c-di-GMP specifically on Pel in the PAO1 strain of *P. aeruginosa*. I have found that WspR cluster formation promotes its cyclase activity. I have confirmed the important of phosphorylation to cluster formation. I have some preliminary evidence suggesting that c-di-GMP may be dampening subcellular cluster formation and oligomerization. Overall, WspR subcellular cluster formation appears to be a necessary intermediate in the signal transduction pathway in the Wsp system.

The importance of clustering and localization may be generalized to other two component systems. A systematic screen for localized proteins in *Caulobacter crescentus* identified 7.7% of all predicted proteins to form subcellular clusters (Werner *et al.*, 2009). Genes involved in signal transduction were overrepresented in the screen hits. In addition, 6% of response regulators are involved in c-di-GMP metabolism and it would be interesting to see if their activities are also regulated by higher-order oligomerization (Gao and Stock, 2009a).

It was known that binding of c-di-GMP to the I-site of WspR inhibits WspR cyclase activity, but it was less clear what role this plays in vivo. The clustering frequencies of the WspR variants suggest that c-di-GMP inhibition causes the dissociation of WspR clusters, so that the formation

of active species of WspR is decreased. In vitro, inhibited tetramers have been shown to dissociate into inhibited dimers (De *et al.*, 2008). I have also observed the dissolution of WspR protein precipitate when c-di-GMP is formed (Fig. 5.2). Taken together these data support a model where c-di-GMP binding inhibits WspR activity not only by active site sequestration but also through the dissolution of higher order oligomers, possibly preventing concentration-dependent activation and the disruption of localization. However, expressing a DGC or PDE in trans did not seem to have an effect on WspR cluster formation. Further experiments will be needed to determine why c-di-GMP insensitive WspR variants cluster more often or whether only c-di-GMP formed by WspR itself is able to affect its cluster formation.

Chemotaxis receptors in *P. aeruginosa* and *E. coli* localize at the cell poles where they form a dense array (Briegel *et al.*, 2009; Güvener *et al.*, 2006). Participation in an array increases the sensitivity and dynamic range of signal detection by a receptor (Duke and Bray, 1999; Li and Hazelbauer, 2005; Sourjik and Berg, 2004). The Wsp system is homologous to chemotaxis signal transduction proteins, but the receptor WspA localizes at lateral clusters (Güvener and Harwood, 2007). Wsp signal transduction is thus without the advantages of sensitivity from participation in a polar lattice. The clustering activation of WspR could serve as a positive feedback loop to amplify response to contact with surface or cell-cell contact.

The dephosphorylation rate of WspR is unknown. Unlike other chemotaxis signal transduction systems there is no gene coding for a phosphatase in the *wsp* operon. Different dephosphorylation rates in WspR variants may explain the surprising phenotypes of the

phosphomimic D70E and the D+2 V72D mutations (data not shown). In CheY, similar mutations have been shown to affect dephosphorylation rates (Silversmith *et al.*, 2001).

It would be interesting to understand the polymeric structure WspR forms when it is in a subcellular cluster. Starting from our estimates of 300 copies of WspR and 3-4 clusters per cells, we estimate that there are about 20 tetramers per cluster. Insights might be gathered by higher resolution microscopy techniques such as PALM to detect the actual locations and structure of clusters.

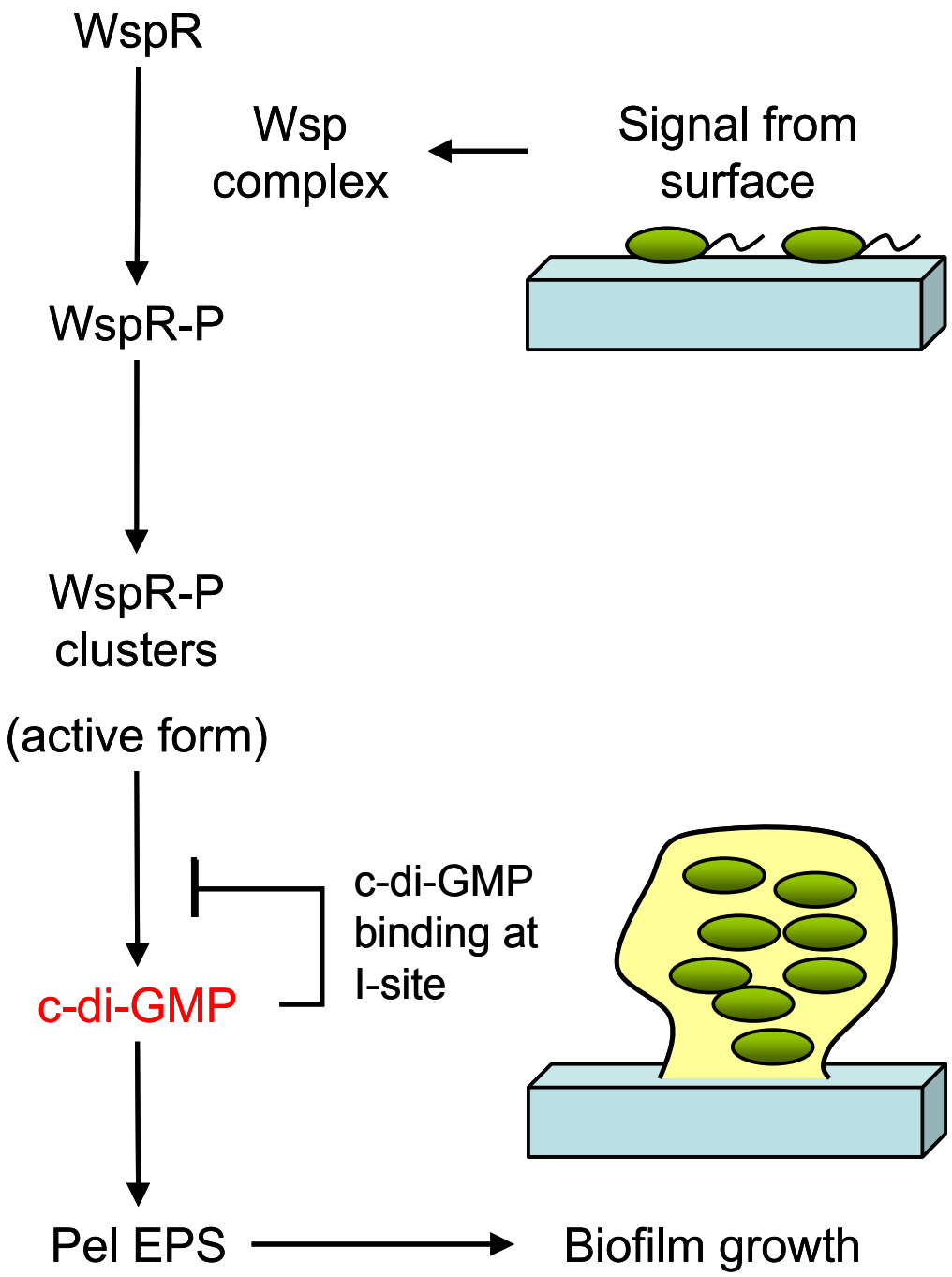

Fig. 6.1 Model for WspR-P activation in the context of Wsp signal transduction and *P. aeruginosa* biofilm growth. A surface-associated signal activates the Wsp complex to phosphorylate WspR. WspR-P assumes a conformation that allows subcellular cluster formation. WspR-P subcellular clustering stimulates its diguanylate cyclase activity. C-di-GMP bound to c-di-GMP effectors in the EPS biosynthetic machinery increases production of Pel EPS, resulting in biofilm growth of the *P. aeruginosa* cells. c-di-GMP can bind to WspR to inhibit its activity, thereby decreasing c-di-GMP production.

CHAPTER VII

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APPENDIX

Contributions to other works during doctoral training.

Harwood and Hickman , unpublished: I constructed plasmids used to express WspR variants in the Pseudomonas aeruginosa strain PAO1. I performed immublots to verify their presence and lack of degradation.

O'Connor et al. 2012: I constructed the *pctB-yfp* gene fusion allele replacement vector.