Subcellular Clustering of the Phosphorylated WspR Response Regulator Protein Stimulates Its Diguanylate Cyclase Activity

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ABSTRACT 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 cyclic di-GMP (c-di-GMP), which in turn stimulates biofilm formation. In previous work, we found that when activated by phosphorylation, yellow fluorescent protein (YFP)-tagged WspR forms clusters that are visible in individual cells by fluorescence microscopy. Unphosphorylated WspR is diffuse in cells and not visible. Thus, cluster formation is an assay for WspR signal transduction. To understand how and why WspR forms subcellular clusters, we analyzed cluster formation and the enzymatic activities of six single amino acid variants of WspR. In general, increased cluster formation correlated with increased in vivo and in vitro diguanylate cyclase activities of the variants. In addition, WspR specific activity was strongly concentration dependent in vitro.

**IMPORTANCE** Bacterial sensor proteins often phosphorylate cognate response regulator proteins when stimulated by an environmental signal. Phosphorylated response regulators then mediate an appropriate adaptive cellular response. About 6% of response regulator proteins have an enzymatic domain that is involved in producing or degrading cyclic di-GMP (c-di-GMP), a molecule that stimulates bacterial biofilm formation. In this work, we examined the in vivo and in vitro behavior of the response regulator-diguanylate cyclase WspR. When phosphorylated in response to a signal associated with surface growth, WspR has a tendency to form oligomers that are visible in cells as subcellular clusters. Our results show that the formation of phosphorylated WspR (WspR-P) subcellular clusters is important for potentiating the diguanylate cyclase activity of WspR-P. Subcellular cluster formation appears to be an additional means by which the activity of a response regulator protein can be regulated.

The intracellular secondary messenger cyclic di-GMP (c-di-GMP) promotes biofilm growth in many gram-negative bacteria by stimulating exopolysaccharide (EPS) and adhesin production. c-di-GMP also inhibits flagellar motility. Multiple diguanylate cyclases with a characteristic GG(D/E)EF domain produce c-di-GMP in response to diverse environmental stimuli (1–4). One of the best-studied diguanylate cyclases is WspR from *Pseudomonas* species (5–8). In the opportunistic pathogen *Pseudomonas aeruginosa*, c-di-GMP production catalyzed by WspR stimulates synthesis of the EPS Pel, which is important for biofilm formation (9, 10).

WspR is the output response regulator of the Wsp signal transduction complex (Fig. 1A). The crystal structure of WspR revealed that it has a CheY-like receiver (response regulator) domain connected to a GGEEF diguanylate cyclase domain via a coiled-coil linker (Fig. 1B) (6). The in vitro diguanylate cyclase activity of WspR is stimulated by phosphorylation (7) and inhibited by c-di-GMP binding at a site of inhibition (I site) on its GGEEF domain (6, 11). In vivo, deletion of the wspF gene in the Wsp operon 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 (7). In a Δ*wspF* strain, yellow fluorescent protein (YFP)-tagged WspR (WspR-YFP) forms dynamic subcellular cytoplasmic clusters that are visible by fluorescence microscopy (12). In wild-type *P. aeruginosa*, YFP-tagged WspR does not form visible subcellular clusters in cells grown in broth but does form clusters in cells grown on an agar surface. Thus, we use WspR cluster formation as an assay for surface-stimulated Wsp signal transduction.

To understand the physiological significance of WspR subcel-
D70A substitution replaces the phosphorylatable aspartate of WspR (we refer to here as the CheY-like receiver domain (Fig. 1B). The WspRV72D), are mutated in the response regulator domain, which were analyzed, the D70A and V72D variants (WspRD70A and WspRV72D) did not have significant levels of clusters when grown in liquid or on an agar surface (Table 1; Fig. 2B). Cells expressing WspRD70A and WspRV72D did not have significant levels of clusters (4%) when grown on agar (Table 1; Fig. 2B). Cells expressing WspRD70A and WspRV72D did not have significant levels of clusters (4%) when grown on agar (Table 1; Fig. 2B). Cells expressing WspRD70A and WspRV72D did not have significant levels of clusters (4%) when grown on agar (Table 1; Fig. 2B).

Figure 1: (A) 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 communicated to the histidine kinase WspE, which catalyzes phosphotransfer to the response regulator-diguanilate 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 (18). (B) 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) (http://www.pymol.org). WspR is depicted as a dimer, one molecule in grey and the other in black. Red spheres show locations of the mutated residues; cyan indicates the conserved GGEFF motif of the cyclase active site; yellow indicates the conserved aspartate of the phosphorylation site; green balls are Mg^{2+} ions needed for phosphorylation. C-di-GMP dimers bound at the I sites are shown.

We 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. We have previously shown that WspR-YFP is active in cells (12). We estimate from immunoblotting experiments that the average P. aeruginosa PA01 cell contains about 300 WspR molecules, equivalent to about a 1-µmol concentration (see Fig. S1 in the supplemental material). 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 to 300 molecules of WspR. Immunoblot analysis revealed that each of the variants was expressed at approximately the same level as wild-type WspR from its native promoter (data not shown). Immunoblot analysis also confirmed that the YFP tag remained fused to WspR and the WspR variants and was not cleaved off. We observed the clustering behavior of the YFP-tagged proteins using epifluorescence microscopy (Fig. 2) and quantified cluster formation as the percentage of cells in a population with at least one WspR-YFP cluster (Table 1). The variants WspRL167D, WspRL170D, and WspRE253A formed subcellular clusters in broth-grown cells (Table 1; Fig. 2A), a condition in which wild-type WspR (WspRwt) expressed in a wild-type background does not form clusters. However, surface growth further stimulated cluster formation (Table 1). Cells expressing WspRL167D and WspRL170D did not have significant levels of clusters when grown in liquid or on an agar surface (Table 1; Fig. 2B). Cells expressing WspRL167D expressed in a wild-type background had very low levels of clusters (4%) when grown on agar (Table 1; Fig. 2B).

As noted above, phosphorylation of WspR is greatly increased in a ΔwspF strain (7), and WspR forms subcellular clusters not only in cells grown on an agar surface but also in cells grown in shaken liquid culture (12). Consistent with this, WspR variants expressed in a ΔwspF background had increased cluster formation as long as the aspartate at position 70 was intact (Table 1). The one exception was WspRY72D, which did not form subcellular clusters in a ΔwspF background. As we show below, WspRY72D is nevertheless very active in vivo and in vitro. An examination of the in vivo and in vitro behavior of these mutants suggests that subcellular clustering is an additional regulatory mechanism by which WspR activity is controlled.
vitro activity of purified WspR<sup>V72D</sup>, described in the section on WspR concentration-dependent activity below, provides a possible explanation for its anomalous behavior.

None of the WspR variants formed clusters in more than 1 to 2% of cells of a strain deleted for the <i>wspA</i> receptor gene, a background in which phosphorylation is predicted not to occur because the Wsp system cannot form an active signal transduction complex (Table 1). Thus, phosphorylation appears to be essential for WspR subcellular cluster formation.

**Comparison of in vivo clustering frequencies with in vivo diguanylate cyclase activities of WspR variants.** Because of limitations in the sensitivity of assays to quantitate c-di-GMP extracted from cells grown on complex media, we decided to use <i>P. aeruginosa</i> colony morphology as a way to roughly estimate relative levels of c-di-GMP in cells. 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 (7, 13). As shown in Fig. 3, there is generally a correlation between the numbers of subcellular clusters formed by cells harboring various WspR variants and the degree of wrinkliness of their colonies. One exception is the WspR<sup>E253A</sup> variant, which has a smooth colony morphology because the E253A mutation completely abolishes diguanylate cyclase activity. WspR<sup>E253A</sup> is shown here as a negative control, but for these and subsequent studies we do not consider it when drawing a connection between cluster formation and activity. The other exception is the WspR<sup>V72D</sup> variant, which did not form subcellular clusters but did form very wrinkly colonies, indicating that it is highly active in vivo. A WspR<sup>D70A/V72D</sup> double mutant lacked diguanylate cyclase activity and formed smooth colonies (not shown), indicating that an intact aspartate at position 70 is required for the WspR<sup>V72D</sup> variant to be active.

WspR diguanylate cyclase specific activity is concentration dependent. The data presented so far show that with the exception of the WspR<sup>V72D</sup> and WspR<sup>E253A</sup> variants, 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, we looked into how the WspR concentration affected its <i>in vitro</i> activity.

When activated with the phosphor analog beryllium fluoride, WspR<sup>wt</sup> increased in specific activity as the concentration of the WspR protein was increased (Fig. 4). WspR<sup>wt</sup> that was not treated with BeF<sub>3</sub>/H<sub>11002</sub> did not demonstrate concentration-dependent activity in the range of protein concentrations tested. We were unable to test higher concentrations of WspR<sup>wt</sup> because it started to pre-

![FIG 2](image-url) 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) Broth-grown cells. (B) Agar-grown cells.

### TABLE 1 Quantitative analysis of subcellular cluster formation in strains expressing different YFP-tagged WspR variants

| Mutation | Location | % cells with clusters (n) for genotype<sup>a</sup> | % cells with clusters (n) for genotype<sup>a</sup> |
|----------|----------|-----------------------------------------------|-----------------------------------------------|
|          |          | Broth grown | Agar grown |
|          |          | Δwsp<sub>A</sub> | WT | Δwsp<sub>F</sub> | Δwsp<sub>A</sub> | WT | Δwsp<sub>F</sub> |
| None     |          | 1 (715) | 1 (295) | 34 (493) | 0 (339) | 43 (411) | 77 (494) |
| D70A     | P site   | 0 (250) | 0 (178) | 0 (138) | 0 (256) | 0 (190) | 0 (139) |
| V72D     | D + 2<sup>b</sup> | 2 (228) | 2 (572) | 0 (256) | 0 (99) | 0 (190) | 1 (310) |
| L167D    | Linker stalk | 0 (158) | 0 (506) | 23 (381) | 0 (288) | 4 (684) | 72 (632) |
| L170D    | Linker stalk | 0 (604) | 29 (632) | 82 (222) | 1 (266) | 75 (468) | 72 (186) |
| R198A    | I site   | 0 (173) | 9 (265) | 36 (140) | 0 (128) | 38 (366) | 48 (261) |
| E253A    | Cyclase active site | 0 (266) | 12 (145) | 75 (232) | 0 (194) | 68 (276) | 75 (130) |

<sup>a</sup> Percentages represent the numbers of cells with at least one well-defined fluorescent spot divided by the total number of visualized cells (n), shown in 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 an empirically determined threshold are considered cells with at least one cluster. WT, wild type.

<sup>b</sup> Two residues downstream of conserved aspartate at the P-site.
concentration-dependent increase in activity without BeF$_3^-$

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- WspR$^{V72D}$ had high activity relative to those of untreated WspR$^{wt}$ and WspR$^{L170D}$ at low protein concentrations (Fig. 4). In addition, WspR$^{V72D}$ displayed no concentration-dependent activity and was not activated by BeF$_3^-$.

- WspR$^{V72D}$ had little catalytic activity with or without BeF$_3^-$ addition. The lack of activation of WspR$^{V72D}$ by BeF$_3^-$ indicates that BeF$_3^-$ activates WspR$^{wt}$ through mimicking phosphorylation at D70 and not through another mechanism.

- When we examined the concentration-dependent activity of WspR$^{V72D}$, we found that it had high activity relative to those of untreated WspR$^{wt}$ and WspR$^{L170D}$ at low protein concentrations (Fig. 4). In addition, WspR$^{V72D}$ displayed no concentration-dependent activity and was not activated by BeF$_3^-$.

- These observations raised the question of what the configuration of WspR$^{V72D}$ is. To look at this, we subjected WspR$^{wt}$ and WspR$^{V72D}$ to size exclusion chromatography. The elution profile of both proteins showed 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 (6). We analyzed the diguanylate cyclase activities of the eluted fractions corresponding to each peak. Surprisingly, both dimer and tetramer fractions of WspR$^{V72D}$ showed an intermediate amount of activity (see Fig. S2 in the supplemental material). As shown previously (6), the tetramer fraction of WspR$^{wt}$ had high activity while the dimer fraction had very little activity.

**Subcellular cluster formation appears to be an intrinsic property of WspR-P.** One hypothesis is that WspR associates with a c-di-GMP receptor protein and this stimulates WspR cluster formation. Although we cannot exclude this possibility, we have been unable to obtain evidence in its favor. We know that WspR affects Pel EPS biosynthesis. So the Pel EPS machinery, which is known to require c-di-GMP for activity, and the FleQ transcriptional regulator, which controls Pel gene expression in response to c-di-GMP, are two effectors with which WspR might interact. We have previously reported that WspR forms clusters in a Δpel Δpsl double mutant (12), suggesting that it does not nucleate around the Pel EPS biosynthetic machinery. Here we determined that WspR forms clusters in a ΔfleQ mutant, and we found no evidence for an interaction between FleQ and WspR in bacterial two-hybrid assays (not shown).

In considering other proteins around which WspR might nucleate, we know that WspE plays a role in initiating WspR-P clustering behavior because WspE is the protein that transphosphorylates WspR (Fig. 1A). However, WspR-P moves away from WspE, and it stays clustered when it does so. We have observed that WspR-YFP and cyan fluorescent protein (CFP)-tagged WspA (WspA-CFP) (which is in a complex with WspE) are sometimes colocalized in cells, but we do not usually see them in the same place (12). Another obvious protein that might play a role in WspR cluster formation is the cytoskeletal protein MreB, which has been shown to influence the localization of a number of bacterial proteins (14–16). When we treated cells with the drug A22 (17), MreB localization was disrupted but subcellular cluster formation by WspR-YFP in a ΔwspF strain background was not affected (data not shown). In short, while it is not possible to draw firm conclusions from negative data such as these, we were unable to obtain evidence that subcellular cluster formation is anything other than an intrinsic property of WspR-P.

**DISCUSSION**

Our current model is that a surface-associated signal sensed by the WspA receptor protein stimulates the autophosphorylation of WspE, which then transphosphorylates the WspR protein (Fig. 1A). Although WspR-P is active, our results suggest that the formation of WspR-P subcellular clusters potentiates the diguanylate cyclase activity of this protein (Fig. 5). This would have the effect of amplifying the surface signal that is detected by the Wsp system. We do not yet know the identity of the surface stimulus for Wsp. We have speculated that WspA senses changes in the phys-

![FIG 3](https://example.com/image3.png)

*FIG 3* P. aeruginosa strain PAO1 expressing WspR variants spotted onto tryptone-Congo red agar plates with 1% arabinose. Bar = 5 mm. 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.

![FIG 4](https://example.com/image4.png)

*FIG 4* Specific activity of WspR as a function of the WspR concentration. The diguanylate cyclase activities of WspR proteins were assayed at concentrations ranging from 17 nM to 28 μM following their equilibration in assay buffer at 22°C for 16 to 24 h.
Subcellular Clustering Stimulates WspR Activity

FIG 5 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 further stimulates its diguanylate cyclase activity, C-di-GMP bound to c-di-GMP effector phospho-acceptor domain (18). We have tested strains carrying mutations in surface features, including flagella and pili, that have been implicated in surface sensing in other bacteria and did not see a major effect on subcellular clustering of WspR (12), although we have not tested the effects of different surface appendages in combination. Increasing the viscosity of liquid cultures also did not stimulate WspR clustering (12).

In our model, WspR in its active conformation, WspR-P tetramers, tends to oligomerize (cluster), and the oligomerized form of WspR-P is even more active. We found that a concentrated and phosphorylated form of the WspR protein is the form that has the highest diguanylate cyclase specific activity in vitro. Phosphorylation seems to stimulate oligomerization. When WspR variants were not phosphorylated by WspA due to being expressed in a ΔwspA mutant background, they did not form subcellular clusters. The same was true of the unphosphorylatable variant WspRΔ170A. De and coworkers found that the WspR167D variant is compromised in its ability to tetramerize and tends to form monomers and be inactive (6). Consistent with this, this variant formed very few subcellular clusters when expressed in wild-type cells grown on agar (Table 1). However, overphosphorylation of WspR167D in a ΔwspF background restored cluster formation and c-di-GMP production as assessed by wrinkly colony formation.

The WspRV72D variant is highly active in vivo and in vitro but does not form visible subcellular clusters in P. aeruginosa. We found that WspRV72D differed from WspRWT and WspR170D in that its specific activity did not increase as its concentration was increased and its activity also was not potentiated by treatment with beryllium fluoride (Fig. 4). In addition, we determined that the dimer form of WspR170D is active, in contrast to WspRWT, which is active only as tetramers. Thus, WspR170D behaves in vitro as we would predict from our observation that it does not form clusters in vivo.

It seems likely that WspR-P clusters can dissociate as well as form, and one hypothesis is that c-di-GMP stimulates cluster dissolution. Indeed, the active tetramer form of WspR is also the species that is subject to product inhibition by c-di-GMP, and there is evidence that the c-di-GMP-inhibited tetrameric form of WspR dissociates further into inactive elongated dimers (5). From this, one would predict that WspR variants that are resistant to c-di-GMP inhibition would be better cluster formers. The data in Table 1 support this. WspR variants WspRE253A, WspR170D, and WspR198A are all not subject to c-di-GMP inhibition in vivo, and all form clusters in wild-type cells grown in liquid. In contrast, WspRWT forms almost no clusters under these conditions. If binding of c-di-GMP to WspR stimulates cluster dissolution, then one would predict that cells that are overexpressing a phosphodiesterase in trans would have greater numbers of WspR clusters. We carried out this experiment with the phosphodiesterase PA2133 and found that its expression had no effect on WspR-YFP clustering in P. aeruginosa. Also, WspR produces high levels of c-di-GMP when expressed in a ΔwspF background, as evidenced by the formation of wrinkly colonies, yet a high percentage of such cells have WspR-YFP clusters. It is possible that highly phosphorylated WspR is more resistant to c-di-GMP inhibition, and we also cannot rule out that WspR has a specific interaction with a phosphodiesterase. Thus, although we are unable to conclude at this point that c-di-GMP binding to WspR-P alters its ability to stay oligomerized and maintain clusters in cells, this is an intriguing possibility that warrants follow-up.

The concept that increasing the local concentration of a protein is a mechanism to stimulate its activity is not new. There are many examples of oligomeric proteins that increase in specific activity as their concentration increases, including NtrC, FtsZ, PleD, MinD, and IRE1 (19–23). The functional consequences of oligomerization differ depending on the protein. For example, phosphorylation of the response regulator domain of the AAA+ ATPase protein NtrC promotes its assembly into a hexameric ring structure that has ATPase activity necessary for remodeling a σ54-RNA polymerase closed complex into an open complex able to transcribe genes (24). PleD is a diguanylate cyclase from Caulobacter crescentus with two response regulator domains and resembles WspR in that it also forms subcellular clusters. PleD-P forms 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 (25–27). PleD is part of a protein interaction network that exists at the cell pole, and its location activates other polarly localized proteins to initiate a program of stalk morphogenesis (25–27).

We cannot say with certainty that WspR is not functioning this way or that it is not part of a protein interaction network. However, we have
failed to identify proteins that interact with WspR, and WspR does not appear to localize to any particular place in cells (Fig. 2) (12).

How then does c-di-GMP produced by WspR specifically affect Pel EPS synthesis but not Psl synthesis or swimming and swarming motility (9, 10; V. Huangyutitham and C. S. Harwood, unpublished)? There are two related possibilities. We know that the depletion in total intracellular c-di-GMP due to a

**Table 2** Strains used in this study

| Strain | Source or reference |
|--------|---------------------|
| *Pseudomonas aeruginosa* | |
| PAO1 | This study |
| PAO1 wspR-yfp | 35 |
| PAO1 ΔwspR | 12 |
| PAO1 ΔwspFR | 7 |
| PAO1 ΔwspAR | 7 |
| PAO1 ΔwspAR attB::miniCTX-wspR-yfp<sup>a</sup> | This study |
| PAO1 ΔwspR attB::miniCTX-wspR<sup>704D</sup>-yfp<sup>a</sup> | This study |
| PAO1 ΔwspR attB::miniCTX-wspR<sup>272D</sup>-yfp<sup>a</sup> | This study |
| PAO1 ΔwspR attB::miniCTX-wspR<sup>167D</sup>-yfp | This study |
| PAO1 ΔwspR attB::miniCTX-wspR<sup>170D</sup>-yfp<sup>a</sup> | This study |
| PAO1 ΔwspR attB::miniCTX-wspR<sup>196A</sup>-yfp<sup>a</sup> | This study |
| PAO1 ΔwspR attB::miniCTX-wspR<sup>2254A</sup>-yfp<sup>a</sup> | This study |
| PAO1 ΔwspR wspR-yfp<sup>a</sup> | 12 |
| PAO1 ΔwspFR attB::miniCTX-wspR-yfp<sup>a</sup> | This study |
| PAO1 ΔwspFR attB::miniCTX-wspR<sup>270D</sup>-yfp<sup>a</sup> | This study |
| PAO1 ΔwspFR attB::miniCTX-wspR<sup>167D</sup>-yfp<sup>a</sup> | This study |
| PAO1 ΔwspFR attB::miniCTX-wspR<sup>170D</sup>-yfp<sup>a</sup> | This study |
| PAO1 ΔwspFR attB::miniCTX-wspR<sup>196A</sup>-yfp<sup>a</sup> | This study |
| PAO1 ΔwspFR attB::miniCTX-wspR<sup>2254A</sup>-yfp<sup>a</sup> | This study |
| PAO1 ΔwspR wspR-yfp<sup>a</sup> | 12 |
| PAO1 ΔwspAR attB::miniCTX-wspR-yfp<sup>a</sup> | This study |
| PAO1 ΔwspAR attB::miniCTX-wspR<sup>270D</sup>-yfp<sup>a</sup> | This study |
| PAO1 ΔwspAR attB::miniCTX-wspR<sup>167D</sup>-yfp<sup>a</sup> | This study |
| PAO1 ΔwspAR attB::miniCTX-wspR<sup>170D</sup>-yfp<sup>a</sup> | This study |
| PAO1 ΔwspAR attB::miniCTX-wspR<sup>196A</sup>-yfp<sup>a</sup> | This study |
| PAO1 ΔwspAR attB::miniCTX-wspR<sup>2254A</sup>-yfp<sup>a</sup> | This study |
| *Escherichia coli* | |
| DH5α | Gibco-BRL |
| S17-1 | American Type Culture Collection |
| Rosetta 2 | Novagen |

<sup>a</sup> The miniCTX backbone of pTG142 was integrated in the chromosome alongside wspR. See Materials and Methods and reference 34.

**Materials and Methods**

**Bacterial strains and media.** Bacterial strains used in this work are listed in Table 2. Cells were routinely grown in LB medium (10 g liter<sup>−1</sup> tryptone, 5 g liter<sup>−1</sup> NaCl, and 5 g liter<sup>−1</sup> yeast extract) at 37°C unless otherwise noted. Media were supplemented with the following antibiotics: for *P. aeruginosa*, tetracycline (100 μg ml<sup>−1</sup>), and gentamicin (50 μg ml<sup>−1</sup>); for *E. coli*, tetracycline (2 to 20 μg ml<sup>−1</sup>), gentamicin (10 μg ml<sup>−1</sup>), ampicillin (50 to 100 μg ml<sup>−1</sup>), chloramphenicol (34 μg ml<sup>−1</sup>), or kanamycin (30 to 50 μg ml<sup>−1</sup>).
tryptone-Congo red plates (7). The cells were allowed to grow at room temperature for 6 days. Images of surface-illuminated colonies were captured on the 6th day using a digital camera mounted on a dissection microscope (SZX-ILLK100; Olympus).

**Protein expression and purification.** Alleles of wspR were amplified by PCR from sources listed in Table 2 and cloned into the PET29 or pETDuet vector (Novagen), the latter with the phosphodiesterase PA2132 cloned into the secondary cloning site. The protein was overexpressed in the E. coli strain Rosetta 2 (Novagen). Cells were grown to mid-log phase in LB at 37°C, subcultured at an OD600 of 0.01 in terrific broth (12 g liter−1 tryptone, 24 g liter−1 yeast extract, 0.4% glycerol, 72 mM K3PO4, and 17 mM KH2PO4), and grown at 30°C to an OD600 of 0.5 to 1.0. Media were supplemented with appropriate antibiotics. The cells were centrifuged to 16 to 18°C for 30 min and then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 16 h. Cells were pelleted at 11,000 g for 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 g for 15 min (each) and 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 6.3). Arginine (0.1 M) was added to prevent precipitation. Protein was concentrated using Amicon Ultra filter units and stored at 4°C for no longer than 24 h before assaying. When WspR was diluted from a concentrated stock, its specific activity remained relatively high for a period of a few hours. Activity then decreased gradually to a constant level after about 20 h. We used these “equilibrated” preparations of WspR for the experiments shown in Fig. 4.

**Calculation of WspR cellular concentration.** Rabbit antisera was raised against purified WspR (Covance Research Products, Denver, PA). Bands in immunoblots were quantified using ImageQuant 5.1 software. A standard curve of pixel intensity versus ng of WspR was generated using pure WspR. The pixel intensity of the WspR blot in cell lysate samples was then converted to ng of WspR. The amount of WspR was converted to mg of total protein per ml of culture. From this, we determined a value of 3.52 × 106 cells per μg cell lysate protein. Replicates (11) across 4 independent experiments gave an average of 283 g (standard deviation, 67) of cell lysate protein per ml.

**Diguanylate cyclase activity assay.** Assays were carried out using the Enzchek pyrophosphate assay kit (Invitrogen). The concentration of MgCl2 was increased to 2 mM, GTP (USB) was at 0.5 mM, and the pyrophosphatase from the kit was replaced with 0.4 U ml−1 pyrophosphatase (inorganic from E. coli; Sigma). To assay WspR activity, WspR was equilibrated in reaction buffer overnight, and then enzymes from the kit and pyrophosphatase were added to reaction mixtures before starting the reaction with GTP. The accumulation of phosphate was monitored over time at OD600.

**Beryllium fluoride treatment.** Beryllium fluoride was synthesized in situ from beryllium chloride and sodium fluoride (Sigma). Various amounts of WspR (0.25 μM to 10 μM) were equilibrated in reaction buffer (50 mM Tris·Cl, 10 mM MgCl2, pH 7.5) overnight before activation with 0.1 mM BeCl2 and 10 mM NaF for 5 min at room temperature. Each reaction was run through a PD-10 desalting column to remove extra BeF3−, BeCl2, and NaF from the treated WspR protein in order to prevent inhibition of the pyrophosphatase in the Enzchek assay. Diguanylate cyclase activity of WspR−BeF3− was assayed immediately after BeF3− treatment and desalting.

**Size exclusion chromatography.** Protein (60 μM) was injected into a Superdex 200 10/300 GL column (GE). The buffer used consisted of 25 mM Tris (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol (DTT), and 2 mM MgCl2. Chosen fractions were directly assayed for their diguanylate cyclase activity immediately after elution from the column.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.orglookup/suppl/doi:10.1128/mBio.00242-13/-/DCSupplemental.

Figure S1, DOCX file, 0.1 MB.

Figure S2, DOCX file, 0.1 MB.

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