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The Wsp system of *Pseudomonas aeruginosa* links surface sensing and cell envelope stress

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Surface sensing is a critical process that promotes the transition to a biofilm lifestyle. Several surface-sensing mechanisms have been described for a range of species, most involving surface appendages, such as flagella and pili. *Pseudomonas aeruginosa* uses the Wsp chemosensory-like signal transduction pathway to sense surfaces and promote biofilm formation. The methyl-accepting chemotaxis protein WspA recognizes an unknown surface-associated signal and initiates a phosphorylation cascade that activates the diguanylate cyclase WspR. We conducted a screen for Wsp-activating compounds and found that chemicals that impact the cell envelope induce Wsp signaling, increase intracellular c-di-GMP levels, and can promote surface attachment. To isolate the Wsp system from other *P. aeruginosa* surface-sensing systems, we heterologously expressed it in *Escherichia coli* and found it sufficient for sensing surfaces and the chemicals identified in our screen. Using well-characterized reporters for different *E. coli* cell envelope stress responses, we then determined that Wsp sensitivity overlapped with multiple *E. coli* cell envelope stress-response systems. Using mutational and CRISPRi analysis, we found that misfolded proteins in the periplasm appear to be a major stimulus of the Wsp system. Finally, we show that surface attachment appears to have an immediate, observable effect on cell envelope integrity. Collectively, our results provide experimental evidence that cell envelope stress represents an important feature of surface sensing in *P. aeruginosa*.

Pseudomonas aeruginosa | surface sensing | cell envelope stress | Wsp | c-di-GMP

Significance

Bacteria must respond quickly to environmental changes to survive. One way bacteria can respond to environmental stress is by undergoing a lifestyle transition from individual, free-swimming cells to a surface-associated community called a biofilm characterized by aggregative growth. The opportunistic pathogen *Pseudomonas aeruginosa* uses the Wsp chemosensory system to sense an unknown surface-associated cue. Here we show that the Wsp system senses cell envelope stress, specifically conditions that promote unfolded or misregulated periplasmic and inner membrane proteins. This work provides direct evidence that cell envelope stress is an important feature of surface sensing in *P. aeruginosa*.

The opportunistic pathogen *Pseudomonas aeruginosa* forms multicellular aggregates called biofilms to promote several different types of chronic infections (1, 2). The first step in biofilm formation involves initial attachment of a free-swimming, planktonic cell to a biotic or abiotic surface (3). This initial attachment event can be sensed by the bacterium through a process loosely termed "surface sensing." Surface sensing induces physiological changes that ultimately lead to the formation of stable biofilm aggregates. In many species, surface sensing involves a cell surface appendage, like the flagellum or pili (4–6). The surface-relevant environmental cues perceived by different surface-sensing mechanisms is currently an area of intense interest.

P. aeruginosa has at least two distinct surface-sensing mechanisms. The first involves a chemosensory system called Pil-Chp, the activation of which is linked to type IV pilus function, while the second is the Wsp chemosensory-like signal transduction system (7–11). Activation of both systems eventually leads to elevated cellular c-di-GMP levels, which stimulates production of biofilm matrix components (7, 8, 12–14). Currently, it's unclear which aspects of a surface are being sensed by these two systems and whether they are independent of one another or represent two branches of the same surface-sensing pathway.

The Wsp signal transduction complex consists of six proteins and is an alternative chemosensory function signal transduction system (15–17). Alternative chemosensory functions are homologous to chemotaxis systems and their proteins are predicted to function similarly, except for the response regulator, which controls cellular functions other than chemotaxis. The response regulator of the Wsp system, WspR, is a diguanylate cyclase that produces c-di-GMP when activated (8, 12) (*SI Appendix*, Fig. S1). The membrane-bound methyl-accepting chemotaxis protein, WspA, senses a surface, leading to the phosphorylation of WspR, causing it to form clusters and synthesize c-di-GMP (8, 9, 18, 19) (*SI Appendix*, Fig. S1*A*).

While much is understood about the Wsp signal transduction cascade and the downstream effects of its activation, little is known about environmental cues that activate the system. This is a particularly interesting question because this system represents one of the few surface-sensing mechanisms that apparently doesn't involve flagella or pili. Previous work has shown that treating *P. aeruginosa* with ethanol (20) increases

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The authors declare no competing interest.

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cellular aggregation and c-di-GMP levels in a Wsp-dependent manner. A role for osmolarity in controlling the Wsp system has been suggested as well, although this is less clear. Güvener and Harwood (12) showed that a minimum level of NaCl in Lennox broth (LB) is required for Wsp activation, while other studies found that high salt (300 mM NaCl) prevented its activation (8, 18). In another study, Blanka et al. (21) showed that addition of 300 mM NaCl to LB activated the Wsp system in a clinical isolate. Interpretation of this result was complicated by the fact that this isolate harbored a point mutation in *wspF* that partially locked the Wsp system into it's active state. This same study suggested a link between inner membrane fluidity and Wsp activity.

We show that the Wsp system is sensitive to chemicals and mutations that perturb the cell envelope. Heterologous expression of the Wsp system in *Escherichia coli* demonstrated that the Wsp system alone is sufficient to promote c-di-GMP production in response to these cell envelope stressors. Additionally, we present evidence that this system is particularly sensitive to stressors that impact the periplasmic space. Mutational and CRISPRi analysis of the periplasmic chaperone *dsbA* suggest that unfolded proteins in the periplasm are a strong inducer of the system. Finally, we provide evidence that the act of surface adherence rapidly exerts stress on the cell envelope. Collectively, our data suggest that surface-induced cell envelope stress represents a key aspect of surface sensing.

Results

The Wsp System Is Activated by Compounds that Perturb the Cell Envelope. Since previous studies found that the Wsp system could respond to osmotic stress, ethanol, and changes in membrane fluidity, we hypothesized that the Wsp system responded to cell envelope perturbations. To test this, we used a plasmid-based c-di-GMP reporter that allowed us to use fluorescent microscopy to screen for c-di-GMP increases (22). We surveyed Wsp activity using compounds that affect the cell envelope: SDS, polymyxin B, lysozyme, carbenicillin, A22, NaCl, glycerol, benzyl alcohol, and ethanol (23–31).

SDS, ethanol, NaCl, lysozyme, carbenicillin, and glycerol were found to activate the reporter in a Wsp-dependent manner (Fig. 1 *A*–*C* and *SI Appendix*, Fig. S2). Ethanol and glycerol treatment induce the reporter in a large portion of WT cells (40 to 75%), while SDS and lysozyme activate the reporter in a smaller fraction of cells (~10%) (Fig. 1 *A* and *C*). Some chemicals induce the reporter in a small proportion of Δ*wspR* cells, although not to the level of WT (Fig. 1 *B* and *C*). Past reports demonstrated that only a subpopulation of cells that attach to a surface exhibit Wsp activation (10). We also observed that reporter induction in the WT strain is heterogenous to all compounds tested (Fig. 1 *A* and *C*). As expected, this heterogeneity is lost in strains with the Wsp system locked on (Δ*wspF*) (*SI Appendix*, Fig. S2*B*). In WT cells, reporter activation in the entire population cannot be achieved by increasing the dose of the chemical treatment, illustrating the inherent heterogeneity of the Wsp system (*SI Appendix*, Fig. S2*C*).

To verify that increased reporter activity is due to WspR-dependent increases of c-di-GMP pools, we measured c-di-GMP levels directly. Treating WT cells with 1% ethanol or 300 mM NaCl for 30 min increases intracellular c-di-GMP levels, while treatment with benzyl alcohol, which did not activate the reporter, did not increase c-di-GMP levels (Fig. 1*D*).

We also conducted a screen for additional compounds that activate the Wsp system. PAO1 (WT) and PAO1 Δ*wspR* cells harboring a chromosomal c-di-GMP reporter (Tn7::P_{cdrA}:GFP) (22) were grown planktonically and then exposed to a variety of compounds. The OD_{600} and GFP fluorescence were measured over time to assess growth and reporter activity, respectively. Comparing reporter activity in WT and Δ*wspR* backgrounds allowed us to differentiate reporter activating compounds that were Wsp-dependent (Table 1, *SI Appendix*, Table S2, and Dataset S1). Compounds that significantly impacted growth rate were excluded as potential Wsp-activators.

We found that, in general, low levels of osmolytes (≤ 4%) activate the reporter in both PAO1 (WT) and Δ*wspR*, but reporter activity decreases by 4 h. As osmolarity increased (6 to 8% NaCl), reporter activity in WT remains elevated longer, with activity peaking at 3 to 5 h postexposure, and this increased activity is dependent on WspR (Table 1 and Dataset S1, 1–10). However, these increases in osmolarity also impacted growth. Several osmolytes were screened in the presence of NaCl, but only dimethyl glycine in the presence of 6% NaCl increased reporter activity above treatment with 6% NaCl alone. Despite glycerol being a strong Wsp activator (Fig. 1 *A*–*C*), 6% NaCl and glycerol did not increase reporter activity above treatment with 6% NaCl alone.

A few antimicrobials elicited a Wsp-dependent response to low levels. The majority of these Wsp-activating antimicrobials target the cell envelope by inhibiting peptidoglycan synthesis (bleomycin) or compromising membrane integrity (polymyxin B, dodecyltrimethyl ammonium bromide, and benzethonium chloride) (Table 1). Many of these compounds activate the reporter earlier than osmolytes (20 min compared to 2.5 h) (Dataset S1). These antimicrobials do not significantly impact growth at concentrations that activate the Wsp system (Dataset S1).

In a Δ*wspR* background, slight increases in c-di-GMP reporter activity are observed when cells are exposed to penicillin, penimepicycline, sulfathiazole, and sulfamethoxazole (Dataset S1). For these compounds, reporter activity increases 3 to 4 h postexposure. These results indirectly suggest loss of the Wsp system sensitizes *P. aeruginosa* to these antimicrobials, resulting in elevated c-di-GMP, presumably due to the activity of another diguanylate cyclase.

Overall, the screen confirmed that the Wsp system responds to osmolytes and antimicrobials that compromise the membrane. The screen revealed that loss of the Wsp system results in aberrant c-di-GMP signaling when exposed to some antimicrobials.

Wsp Activity Leads to Increased Surface Attachment in Response to Cell Envelope Stress. We next determined if Wsp-activating compounds have a measurable impact on cellular physiology. One of the key consequences of elevated c-di-GMP is an increase in surface attachment. We found that treatment with some Wsp-activating compounds, such as ethanol, increases surface attachment in a *wspR*-dependent manner (Fig. 1*E*), while NaCl increases attachment in both WT and Δ*wspR* cells. These data demonstrate that Wsp activation directly impacts surface attachment. These data also indicate that some environmental signals that stimulate Wsp activity can influence attachment independent of Wsp, perhaps through stimulation of other pathways.

The Wsp System Is Sufficient for Activity in *E. coli*. To avoid potential cross-talk from the Pil-Chp surface-sensing system or the numerous uncharacterized diguanylate cyclases and phosphodiesterases in *P. aeruginosa*, we isolated the Wsp system by expressing it in *E. coli*. We used a construct that takes advantage of elevated Wsp expression and intracellular clustering of WspR-P that occurs upon Wsp activation. This construct

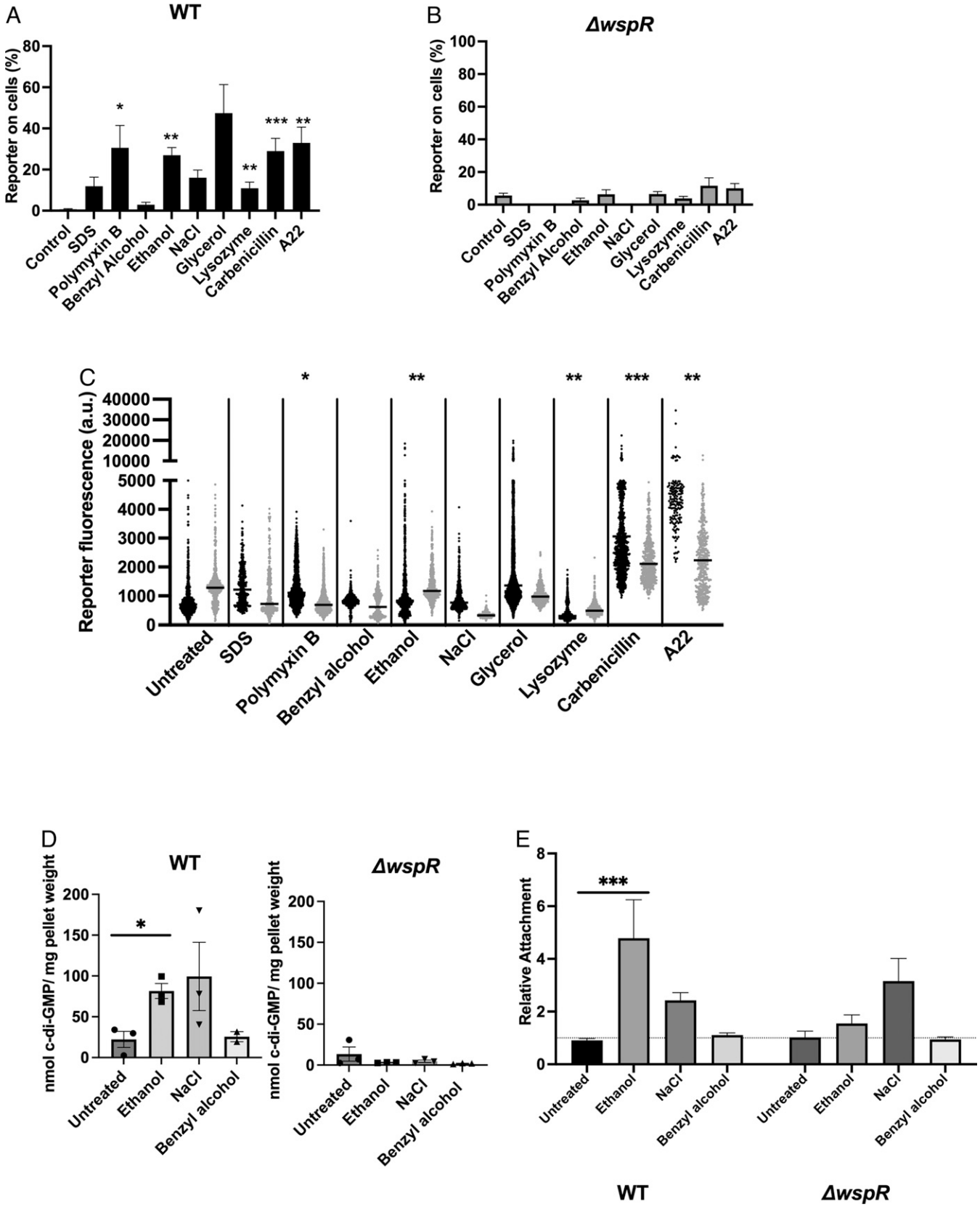

Fig. 1. Wsp can be activated by exogenously added chemicals. The proportion of WT (*A*) and Δ*wspR* (*B*) populations expressing the P_{cdrA}::GFP^{ASV} reporter. The reporter was considered "on" if it was twice the background fluorescence as in Armbruster et al. (10). Shown is the average percentage of reporter on cells in three biological replicates with error. The treatments were conducted at the following concentrations: 0.05% SDS (wt/vol), 1.6 μg/mL polymyxin B, 1 mM benzyl alcohol, 1% ethanol (vol/vol), 300 mM NaCl, 50 μg/ml lysozyme, 40 μg/ml carbenicillin, 5% glycerol (vol/vol), and 40 μg/ml A22. Plots were generated using GraphPad Prism. (*C*) P_{cdrA}:GFP^{ASV} reporter fluorescence in individual cells in WT (black) and Δ*wspR* (gray) backgrounds. Fluorescence intensity was quantified using Volocity software. Plots were generated using GraphPad Prism. (*D*) C-di-GMP levels of WT PAO1 and Δ*wspR* when exposed to Wsp-inducing compounds (ethanol or NaCl), a buffer control (untreated), or a non-Wsp activating chemical (benzyl alcohol). (*E*) Cell attachment to glass coverslip after treatment with Wsp activating compounds. ANOVA was used to verify if treated cells had significantly different fluorescent signal (*A*–*C*), c-di-GMP levels (*D*), or attachment (*E*) from untreated controls. *$P < 0.05$ was considered significant. **$P < 0.01$ and ***$P < 0.001$ are also denoted.

Table 1. Unique P_{cdrA}::GFP activators in PAO1

Target	PAO1 (WT)
Osmolytes	NaCl 6% (3.52 ± 0.17)
	NaCl 6.5% (2.93 ± 0.15)
	NaCl 7% (3.61 ± 0.12)
	NaCl 8% (3.63 ± 0.10)
	NaCl 6% + Dimethyl sulphonyl proprionate (4.15 ± 0.25)
LPS/membrane	Polymyxin B (1.40 ± 0.09)
	Dodecyltrimethyl ammonium bromide (1.21 ± 0.03)
	Benzethonium chloride (1.30 ± 0.02)
Transcription/peptidoglycan	Bleomycin (1.69 ± 0.35)

Biolog screen for chemicals that increase c-di-GMP levels in WT PAO1. PAO1 cells harboring the c-di-GMP reporter P_{cdrA}:GFP at the Tn7 site were exposed to a library of chemicals and fluorescence and OD_{600} was monitored over time. The average fold-increase of P_{cdrA}:GFP fluorescence and SE is included in parentheses.

expressed the entire *wsp* operon, including a YFP-tagged WspR (*SI Appendix*, Fig. S3*A*). Previous work demonstrated that the YFP tag did not impact WspR's cyclase activity (9). As a positive control, we expressed the *wsp* operon containing a *wspF* deletion (that locks the system on) and a YFP-tagged WspR (*SI Appendix*, Fig. S3*A*).

E. coli strains expressing the two constructs displayed phenotypes indicating Wsp is functional. *E. coli* expressing the locked-on Wsp system (pBAD *wspABCDEΔFR-YFP*) produce more curli and cellulose, while having impaired swimming motility (Fig. 2*A* and *SI Appendix*, Fig. S3*B*). These are phenotypes reflective of high levels of c-di-GMP, which are expected in the *wspF* mutation. We found that surface growth stimulates c-di-GMP production in *E. coli* expressing the WT *wsp* operon (Fig. 2*B*). Interestingly, we found that *E. coli* expressing the locked on *wsp* system (pBAD *wspABCDEΔFR-YFP*) still shows some surface responsiveness (Fig. 2*B*). This is consistent with past results in *P. aeruginosa*, where a *wspF* mutant strain retained the ability to respond to surfaces (18).

E. coli expressing the *wsp* operon also exhibited increased WspR-YFP fluorescence when surface grown (Fig. 2*C*) (9). As expected, *E. coli* bearing the locked-on Wsp system display constitutive fluorescence regardless of growth condition (Fig. 2*C*). Western blot analysis shows that both surface and liquid-grown cells make WspR-YFP, with higher levels seen in surface-associated cells (*SI Appendix*, Fig. S3*C*).

Next, we tested whether the *E. coli* expressing the Wsp system can be activated by the same compounds that activate the system in *P. aeruginosa*. We found that NaCl, ethanol, glycerol, or SDS increase WspR-YFP fluorescence in much of the cell population (Fig. 2*D* and *SI Appendix*, Fig. S3*D*). While heterogeneity is still maintained, a higher percentage of the population appears to have active Wsp systems than what is observed in *P. aeruginosa* (compare Fig. 2*D* and *SI Appendix*, Fig. S3*D* to Fig. 1 *A* and *C*). It is unclear if this is due to the differences in the reporters that were used or to intrinsic differences between *P. aeruginosa* and *E. coli*. We also extracted and measured c-di-GMP levels from planktonically grown *E. coli* treated with ethanol. MG1655 (VC) increases c-di-GMP levels upon exposure to 1% ethanol, but c-di-GMP levels are further elevated in *E. coli* cells expressing the *wsp* operon (Fig. 2*E*). Together, these data indicate that the Wsp system is functional and sufficient for surface sensing in *E. coli*.

***E. coli* Cell Envelope Stress-Response Pathways Are Triggered by Wsp-Activating Chemicals.** Many of the compounds that induce WspR activity are known to affect the cell envelope, so we hypothesized that WspA senses some aspect of cell envelope perturbation. *E. coli* is arguably the best-studied species for cell envelope stress-response systems. Three of the key systems include the CpxAR two-component system (32–36), the Rcs signal transduction system (37–40), and the extracytoplasmic function σ factor, σ^E (37, 41–44). Each system regulates its own set of genes, although there is extensive overlap between the regulons. There is also overlap in the environmental signals that induce each system (37, 44). These studies have revealed that cell envelope stress responses are a complex, interconnected web of regulation.

Since *P. aeruginosa* cell envelope stress-response systems are poorly understood relative to *E. coli*, we attempted to gain insight into the Wsp system by assessing the activity of the *E. coli* systems in response to Wsp-activating compounds. Therefore, we monitored induction of the *E. coli* cell envelope stress-response pathways Cpx, Rcs, and σ^E using previously characterized reporters (34, 45). The Cpx system responds to surface association, misfolded proteins in the periplasm and inner membrane, and peptidoglycan damage (32–36). The Cpx system is also activated by exposure to glycerol (Table 2). The σ^E response, which responds to outer membrane protein (OMP) assembly defects (41, 46), is activated by glycerol and SDS (Table 2). The Rcs pathway, which responds to osmotic shock and lipopolysaccharide (LPS) defects (38, 47), is induced when cells are exposed to glycerol, ethanol, and NaCl (Table 2). Since Wsp-activating chemicals also activate *E. coli* cell envelope stress-response pathways, we verified that Wsp activation does not depend upon a functional Cpx, Rcs, or σ^E pathways (*SI Appendix*, Fig. S4). These data suggest that Wsp-activators affect multiple parts of the cell envelope.

Periplasmic and Inner Membrane Stress Activate the Wsp System. We next examined if and how Wsp-activators impact the *P. aeruginosa* cell envelope. Initially, we used an NPN (1-*N*-Phenylnaphthylamine) assay to determine if Wsp-activating chemicals alter the integrity of the outer membrane (48). NPN is a large, hydrophobic molecule that cannot penetrate the outer membrane, but if the outer membrane integrity is compromised, NPN integrates into the hydrophobic membrane and fluorescence increases. We exposed WT and a Δ*wspR* mutant strain to Wsp-activating compounds and saw that only SDS (0.05%) significantly increased outer membrane permeability, while other activators of the system do not (Fig. 3 *A* and *B*).

To measure inner membrane integrity, we used propidium iodide staining, which can only enter cells with permeable or compromised inner membranes (49). For WT cells, only benzyl alcohol increases inner membrane permeability (Fig. 3*C*). On the other hand, Δ*wspR* cells show increased inner membrane permeability when exposed to glycerol, polymyxin B, and

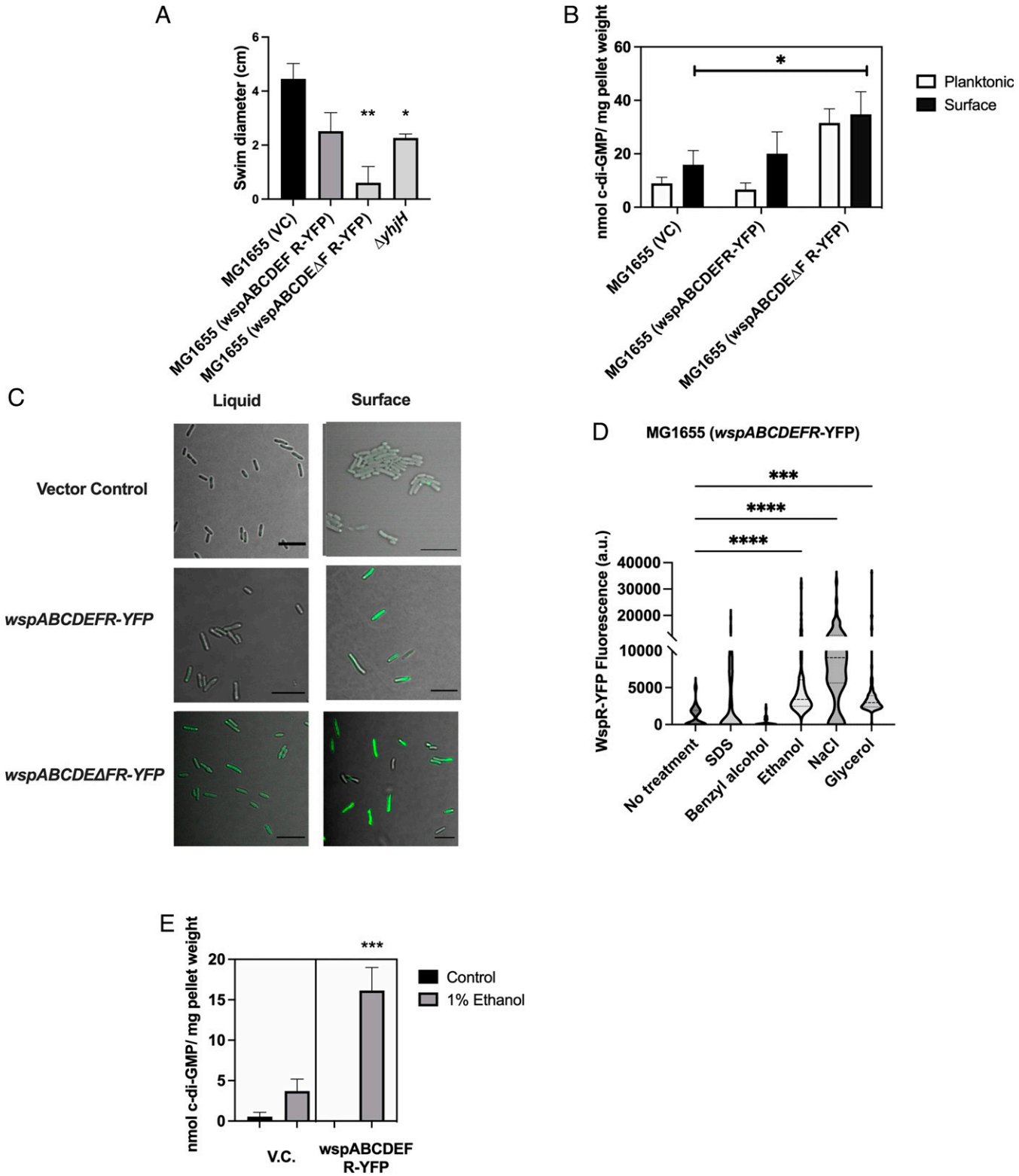

Fig. 2. Wsp is active in *E. coli*. *E. coli* MG1655 cells were transformed with plasmids containing genes from the *wsp* operon under an arabinose-inducible promoter. Assessment of Wsp function using swim plate assay (*A*) or c-di-GMP levels (*B*) for these strains. (*C*) MG1655 cells containing either an empty vector, the surface sensitive (*wspABCDEFR*-YFP), or the locked-on, surface-blind (*wspABCDEΔFR*-YFP) versions of the *wsp* operon were grown planktonically or on a surface, and WspR-YFP fluorescence was used as a proxy for Wsp surface sensing. Shown are brightfield and GFP merged images. Scale bars, 10 μm. (*D*) WspR-YFP fluorescence in MG1655 cells after exposure to listed chemicals. The treatments were conducted at the following concentrations: 0.01% SDS (wt/vol), 1 mM benzyl alcohol, 1% ethanol (vol/vol), 300 mM NaCl, and 5% glycerol (vol/vol). The top and bottom dotted lines in each treatment represents the third quartile and the first quartiles, respectively, while the dashed line represents the median. Plots were generated using GraphPad Prism. (*E*) C-di-GMP levels of ethanol treated and untreated *E. coli* expressing the Wsp operon or carrying a vector control. ANOVA was used to verify if Wsp-expressing *E. coli* had significantly different swim diameters (*A*), c-di-GMP levels (*B* and *E*), or WspR-YFP fluorescence (*D*) from vector controls. **P* < 0.05 was considered significant. ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001 are also denoted.

Table 2. Chemical activation of *E. coli* cell envelope stress-response pathways

	Activate Wsp	Cpx	σ^E	Rcs
5% Glycerol	++	6.5 ± 3.3	3.04 ± 1.2	2.5 ± 0.5
1% Ethanol	++	1.02 ± 0.05	1.3 ± 0.13	2.3 ± 0.6
300 mM NaCl	+	1.39 ± 0.06	1.2 ± 0.74	2.04 ± 0.2
0.05% SDS	+	1.23 ± 0.04	2.3 ± 1	1.04 ± 0.2
1 mM Benzyl alcohol	–	0.6 ± 0.02	0.5 ± 0.1	1.3 ± 0.5

MG1655 cells carrying plasmids with lux-based reporters (cpxP-lux, degP-lux, rpoE rseABC-lux, rprA-lux) were exposed to chemicals and luminescence was measured over time. Reporter fold-increase over untreated controls is shown for the cpxP-lux, rpoE rseABC-lux, and rprA-lux. For the Activate Wsp column, a "+" indicates Wsp-dependent c-di-GMP increases in *E. coli* and *P. aeruginosa*; "++" indicates strong Wsp activators with more than 25% of the population responding to that compound by increased P_{cdrA}:GFP^{ASV} reporter activity (*P. aeruginosa*) or increased WspR-YFP fluorescence (*E. coli*).

benzyl alcohol (Fig. 3*D*). Since the assays were performed in liquid culture, these results suggest that the Wsp system plays a role in influencing the cell envelope composition/integrity in planktonic cells. Collectively these results indicate compounds that activate the Wsp system have minimal, if any, effect on membrane permeability in WT cells.

Our *E. coli* experiments demonstrated that Wsp activators induce multiple *E. coli* cell envelope stress responses, suggesting they target various parts of the cell envelope. We used mutagenesis to screen for cell envelope mutations that activated Wsp. Initially, we specifically targeted two nonessential genes that impact features of the cell envelope. Deleting *galU*, which shortens LPS length (50), increases reporter activity independently of WspR (Fig. 4*A*). On the other hand, a *dsbA* mutant strain, which results in periplasmic protein misfolding, increased reporter activity in a Wsp-dependent manner (Fig. 4*A*). Therefore, we assessed periplasmic protein stability after exposing cells to Wsp-activating compounds. We expressed TorAsp-mTurquoise (periplasmic localized) (51) or mTurquoise (cytoplasmic localized) in WT cells and monitored protein fluorescence before and after treatment with Wsp-activators. Since fluorophore function depends on proper folding, a loss of fluorescence indicates improper or complete loss of structure (52). Exposing cells to ethanol (which is known to induce protein unfolding) (53, 54), NaCl, and SDS significantly reduces periplasmic mTurquoise signal (Fig. 4*B* and *SI Appendix*, Fig. S5 *B* and *D*). All other compounds tested marginally reduce periplasmic signal compared to untreated cells. For cytoplasmic-localized mTurquoise, no treatment significantly reduced the fluorescent signal (*SI Appendix*, Fig. S5 *A* and *C*). These results suggest that Wsp activators impact protein folding in the periplasmic space.

A disadvantage of evaluating the mutant strains characterized above is that they give no temporal information regarding the influence of defects in targeted activities in relation to Wsp activity. Toward this end, we utilized a CRISPRi system (55) to repress expression of select cell envelope proteins and evaluate the effects on Wsp-dependent c-di-GMP reporter activity. Additionally, CRISPRi allows targeting of essential functions.

Initially, we focused on an essential gene that is responsible for LPS assembly. LptD performs the essential function of translocating LPS to the outer membrane, and its loss results in LPS build-up in the periplasm and inner membrane (56). Using CRISPRi to deplete *lptD* transcripts causes a slight increase in c-di-GMP reporter activity relative to vector controls (Fig. 4*C*). These data suggest that the Wsp system has at best a minimal role in responding to mislocalized LPS or altered LPS structure (Fig. 4*C* and *SI Appendix*, Fig. S6*A*). Since analysis of the *dsbA* mutant strain suggested unfolded proteins in the cell envelope are a major Wsp cue, we used CRISPRi to target periplasmic protein folding functions. Depleting the chaperone *dsbA*, which promotes folding of periplasmic proteins, increases c-di-GMP in a WspR-dependent manner (Fig. 4*C* and *SI Appendix*, Fig. S6*A*). BamA is an essential OMP that is important for folding and inserting β-barrel proteins into the outer membrane (56–58). Inhibiting BamA should result in unfolded OMPs aggregating in the periplasm (59). Surprisingly, depleting *bamA* in WT cells does not significantly increase reporter activity relative to VC or Δ*wspR* cells. It is possible that AlgU, a homolog of σ^E, is sensing unfolded OMPs and activating another *P. aeruginosa* cell envelope stress-response pathway (60). Another protein that is important for controlling misfolded cell envelope proteins is FtsH. FtsH is an ATP-dependent protease that is important for inner membrane protein maintenance, heat-shock response, and cell morphology and viability during growth arrest in *E. coli* (61–63). We found that depleting *ftsH* induces reporter activity in a WspR-dependent manner. These results suggest misregulation of inner membrane proteins increases Wsp activity (Fig. 4*C*).

Since the Wsp system is also activated by surface contact and attachment, we hypothesized that interaction with a surface induces cell envelope stress. Therefore, we investigated this connection between surface attachment and cell envelope stress using a strain that constitutively expresses periplasmic mTurquoise and cytoplasmic tdTomato in *P. aeruginosa*. To do this, we used a microscopic approach, measuring fluorescence changes in different cell compartments before and after surface adherence. Planktonic cells exhibited periplasmic mTurquoise fluorescence and cytoplasmic tdTomato fluorescence (Fig. 4*D* and *SI Appendix*, Fig. S6*D*). Immediately after surface attachment, the majority of cells lost mTurquoise fluorescence, but retained tdTomato signal. As *P. aeruginosa* spent more time on the surface the mTurquoise signal increased and relocalized to the periphery of the cell (30 to 90 min post surface exposure). This suggests that surface contact either disrupted periplasmic protein stability or compromised membrane integrity, both of which constitute different types of cell envelope stress. We next wondered whether cells that already have active Wsp systems (Δ*dsbA* or WT cells grown on a surface) were still responsive to other Wsp-activating cues. When Δ*dsbA* cells are treated with ethanol or exposed to a surface, a percentage of reporter on cells remained the same. Surface exposure of Δ*dsbA* cells does result in increased reporter fluorescence in some of the "on" cells, suggesting a further increase in c-di-GMP levels (Fig. 4*E* and *SI Appendix*, Fig. S6*E*). Additionally, WT surface-exposed cells experience a small but not statistically significant increase in reporter on cells when exposed to ethanol (Fig. 4*F* and *SI Appendix*, Fig. S6*F*). These results suggest that a small percentage of Wsp-on cells can be hyperactivated by an additional Wsp stimulus, but this does significantly alter the amount of responding cells in a population.

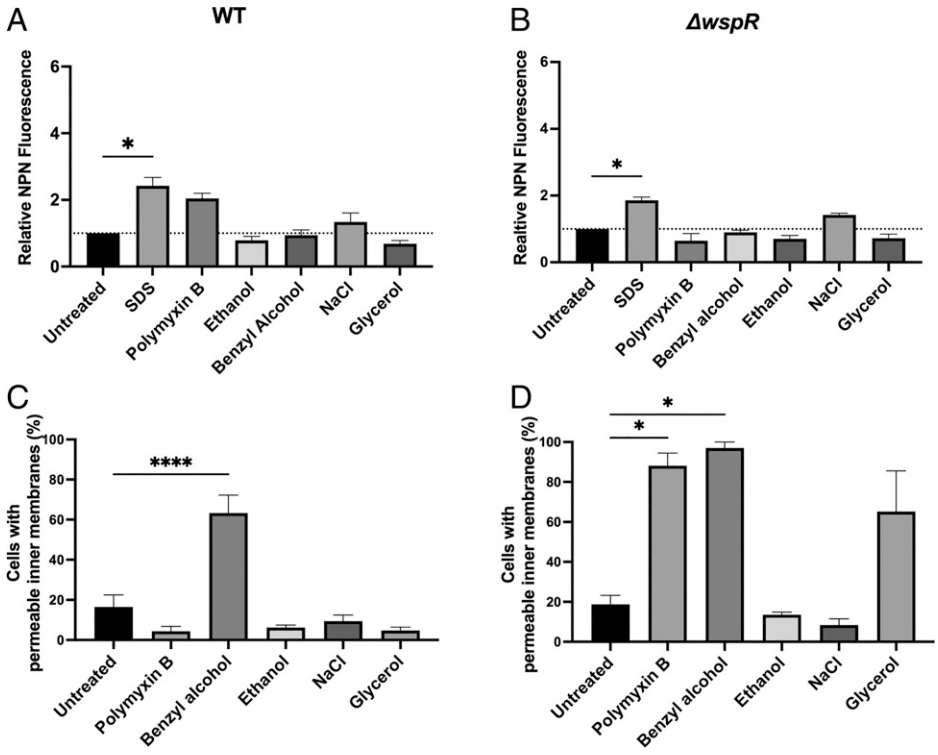

Fig. 3. The impact of Wsp-activating chemicals on *P. aeruginosa* membrane integrity. Wsp-activating compounds impact on the outer membrane (*A* and *B*) and inner membrane (*C* and *D*). Outer membrane permeability was assessed using NPN fluorescence. Inner membrane permeability was measured using propidium iodide and Syto 9 staining. The treatments were conducted at the following concentrations: 0.05% SDS (wt/vol), 1.6 μg/mL polymyxin B, 1 mM benzyl alcohol, 1% ethanol (vol/vol), 300 mM NaCl, and 5% glycerol (vol/vol). ANOVA was used to verify if treated cells had significantly different fluorescent signal from untreated controls. *$P < 0.05$ was considered significant. *$P < 0.05$ and ****$P < 0.0001$ are denoted.

Discussion

In this study, we present evidence that the Wsp system is stimulated by a wide range of compounds with diverse structures capable of perturbing the cell envelope. Our data suggest that unfolded proteins in the periplasm and inner membrane serve as a major stimulus of Wsp activity (Figs. 3 and 4). Furthermore, reconstitution of the Wsp system in *E. coli* suggests that it is sufficient for sensing environmental stimuli, such as surface attachment (Fig. 2). Finally, our data also suggest that cell envelope stress is an important feature of surface sensing in *P. aeruginosa*.

One lingering question is how the Wsp system can sense such diverse cues. Previous work demonstrated that the periplasmic ligand binding domain of WspA is dispensable for surface sensing (18). Therefore, it is unlikely that the ligand binding domain of WspA is directly binding to a ligand in cells treated with these compounds. One possible explanation is the environmental changes that destabilize periplasmic and inner membrane proteins also induce structural changes in WspA which, in turn, activates the system. This phenomenon has been suggested for other methyl-accepting chemotaxis proteins. For example, pH, phenol, and osmotic stress all alter the structure of *E. coli* chemotaxis receptors and impact the chemotactic output (swimming direction) (64–66). It is possible that a similar mechanism is inducing Wsp signaling. The absence of DsbA activates Wsp. Since WspA has no cysteine residues, it is unlikely that DsbA activity is directly responsible for Wsp function and folding. Therefore, WspA structure and signaling could be influenced by periplasmic redox changes and increases in periplasmic hydrophobicity due to high levels of unfolded proteins and exposed cysteines in a *dsbA* mutant. Understanding exactly how WspA senses cell envelope stress is an ongoing area of research.

The role of the Wsp system in the cell envelope stress-response network in *P. aeruginosa* remains to be defined. *P. aeruginosa* has at least two putative cell envelope stress-response systems, AmgRS and AlgU, which are predicted to be akin to Cpx and σ^E, respectively (67–69). In *E. coli*, the Cpx and σ^E responses are capable of sensing unfolded, aggregated proteins in the periplasm (41, 42, 70). Despite the similarities between *P. aeruginosa* and *E. coli* systems, it is currently unknown if/how AmgRS and AlgU respond to cell envelope stress. Our data revealed that Wsp does not perfectly mimic any *E. coli* stress-response system (Fig. 4*A* and Table 2). Wsp activators have broad effects on the cell envelope (Table 2), and Wsp strongly responds to unfolded periplasmic proteins. The *P. aeruginosa* cell envelope stress response is likely an interconnected network, much like *E. coli*, but the degree of overlap between the Wsp, AmgRS, and AlgU pathways, the signals they respond to, and the functions they regulate are currently unclear.

Here we show that the Wsp system is activated when unfolded periplasmic proteins are present. We also demonstrate that upon surface attachment, periplasmic proteins lose functionality (Fig. 4*D*). While this indicates that surface contact and attachment generate cell envelope stress, it is unclear if the Wsp system solely responds to cell envelope stress or can respond to additional surface-induced cues. It is possible the surface sensing and cell envelope stress are two separate pathways that are sensed via the Wsp system and cause increased biofilm formation. Cell envelope stress has been postulated to be a feature of surface sensing in other species. Most of the evidence is indirect. In *E. coli*, the Cpx system is activated when *E. coli* contacts a surface and Cpx activation induces *dsbA* expression (33) and increases c-di-GMP levels via DgcZ (36, 71), hinting that surface attachment might initially result in unfolded proteins. Loss of DsbA also induces biofilm formation

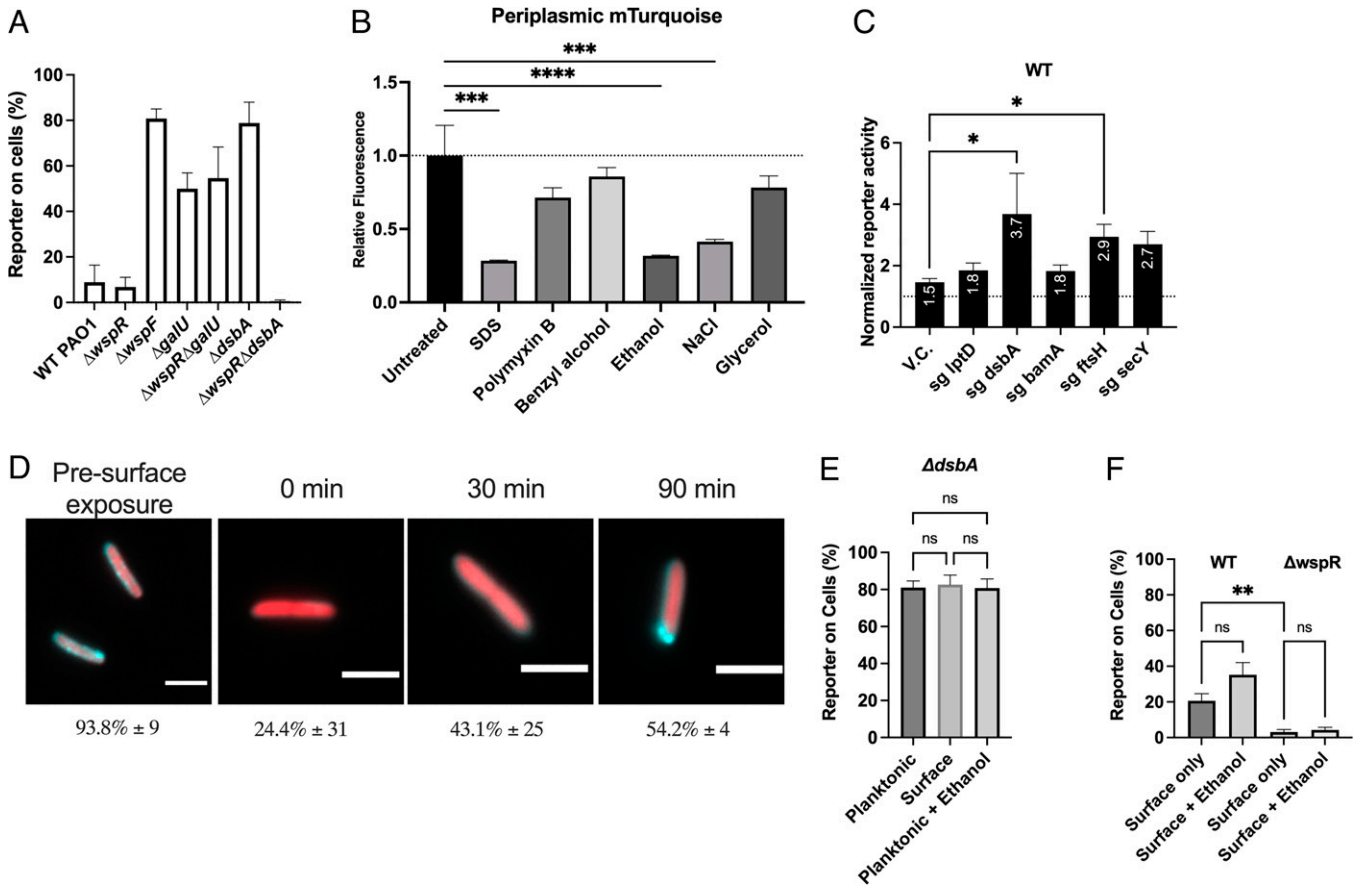

Fig. 4. Functions that impact folding of cell envelope proteins activate the Wsp system in *P. aeruginosa*. P_{cdrA}:GFP^{ASV} activity in cell envelope mutants. (*A*) Fluorescence in individual cells was measured using confocal microscopy and Volocity software was used to determine reporter activity in the population. (*B*) Periplasmic protein fluorescence in WT cells after treatment with Wsp-activating chemicals. Fluorescence was measured using a plate reader 30 to 60 min after treatment. The treatments were conducted at the following concentrations: 0.05% SDS (wt/vol), 1.6 μg/mL polymyxin B, 1 mM benzyl alcohol, 1% ethanol (vol/vol), 300 mM NaCl, and 5% glycerol (vol/vol). (*C*) P_{cdrA}:GFP activity after CRISPRi knockdown of transcripts involved in cell envelope maintenance. Reporter activity is normalized to uninduced controls. sg is the single-guide RNA used to knock down expression. VC had no guide RNA but did constitutively express Cas9. (*D*) Periplasmic mTurquoise and cytoplasmic tdTomato signal stability before and after surface contact. Percentages below the micrographs represent percentage of cells with mTurquoise signal localized to the periphery of the cell. Presurface exposure cells were fixed with 2.5% paraformaldehyde prior to imaging. (Scale bar, 2.5 μm.) (*E*) Percent of cells with PcdrA:GFP reporter activity in Δ*dsbA* mutants grown on a surface, planktonically, or planktonically with 1% ethanol. (*F*) WT and Δ*wspR* strains PcdrA:GFP reporter activity after surface exposure or after surface + ethanol exposure. ANOVA was used to verify if treated cells had significantly different periplasmic fluorescent signal (*A*) and if CRISPRi knockdown of cell envelope protein transcripts had significantly higher reporter activity that vector controls (*C*). *$P < 0.05$ was considered significant; ns, not significant. **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ are also denoted, while ns indicates no signficant difference.

in *Pseudomonas putida* and *Salmonella* Typhimurium (72, 73). Additionally, the *E. coli* Rcs cell envelope stress response regulates the production of exopolysaccharides, and an *rcsC* mutant is defective in biofilm development (74). This suggests that the cell envelope stress and periplasmic protein integrity is linked to the biofilm mode of growth in multiple species.

When considering the other surface-sensing mechanism in *P. aeruginosa*, the Pil-Chp chemosensory system, it's clear that the cell can respond to different types of surface-associated signals. The nature of the Pil-Chp signal has been a focus of recent research and is linked to the type IV pilus. An output of both Wsp and Pil-Chp is cyclic-di-GMP production. It should be noted that cAMP is an output of the Pil-Chp response as well, so the two systems are not completely redundant in their output. The presence of these two systems begs the question: Will a surface always stimulate both systems? If not, what are the different situations where one system provides the predominant surface sensing response?

In summary, our findings emphasize that *P. aeruginosa* senses surface association through diverse cues. Many of the previously described surface-sensing systems, like Pil-Chp, depend upon an extracellular appendage (3, 4, 6, 75, 76). Here, we show that cell envelope stress is one consequence of surface association and that *P. aeruginosa* can use the Wsp system to sense surface-induced cell envelope stress. Accounting for the different mechanisms by which bacteria sense surface and the heterogeneity in the responses will be critical for understanding the initial steps of biofilm formation and, ultimately, our ability to prevent them from forming.

Materials and Methods

Detailed materials and methods can be found in the *SI Appendix*.

Bacterial Strains and Growth Conditions. The strains and plasmids used in this study are listed in *SI Appendix*, Table S1. *E. coli* and *P. aeruginosa* were maintained on LB agar and grown at 37 °C. For all assays, *P. aeruginosa* was planktonically grown in LB media, unless otherwise indicated.

Biolog Plate Screen. Biolog plates PM9 (Cat. No.: NC0198006), PM 11 (NC1369169), and PM 12B (NC1369172) were obtained from Fisher Scientific. PAO1, Δ*wspR*, and Δ*wspF* strains carrying the c-di-GMP reporter inserted at the Tn7 site (pTn7 P_{cdrA}::gfp^{C}) were grown overnight in M9 medium with glucose as a carbon source and then exposed to compounds in the Biolog plates. OD_{600} and GFP fluorescence (excitation: 488 nm, emission: 515 nm) were measured using a BioTek Synergy H1 plate reader.

Measuring P_{cdrA}::GFP Reporter Induction. Planktonic *P. aeruginosa* strains PAO1, Δ*wspR*, and Δ*wspF* carrying the plasmid based short half-life c-di-GMP reporter (pP_{cdrA}::GFP^{ASV}) were exposed to potential Wsp activators for 2 h, and then GFP fluorescence was measured using Zeiss LSM 800 confocal laser scanning microscope.

C-di-GMP Extraction and Liquid Chromatography-MS Measurements. C-di-GMP was extracted from solid grown or liquid grown *E. coli* and *P. aeruginosa*, as previously described (8), using 2-chloro AMP as an internal standard. C-di-GMP was extracted from pelleted cells by incubating with 70% perchloric acid on ice for 1 h. The supernatant was retained and neutralized using potassium bicarbonate. Liquid chromatography MS/MS measurements were performed using an Acuity UPLC with a Synergi 4μ Hydro RP 80A column and a C18 Guard Cartridge (Phenomenex) on a Premier XL triple-quadrupole electrospray mass spectrometer (Waters). The *m/z* 691 > 152 transition was used for c-di-GMP and 382 > 170 for 2-chloro-AMP. The cone voltages and collision energies were 40 V/30 eV and 35 V/20 eV, respectively.

Attachment Assay. Cultures were grown overnight and the next morning reinoculated and allowed to grow until midlog phase. At $OD_{600} = 0.5$, cultures were treated with Wsp activating compounds or controls for 1 h. After treatment, cells were diluted 1:10 and then 100 μL of cells were pipetted onto a coverslip and allowed to attach for 10 min. After 10 min, coverslips were washed three times with sterile water and then imaged. Cells were counted using FIJI's cell counter and were normalized to total area imaged.

Generation of *E. coli* Heterologously Expressing *wsp* Operon. The *wsp* operon was amplified from PAO1 *wspR*-YFP or PAO1 Δ*wspF wspR*-YFP and ligated into pBAD18 to generate *wspABCDEFR-yfp* and *wspABCDE*Δ*FR-yfp*, respectively. The plasmids were transformed and maintained in DH5α. The plasmids were maintained with carbenicillin, and wsp genes were induced with 1% arabinose in liquid culture and on plates. Plasmid sequences were confirmed with sequencing (Plasmidsaurus) and maps were generated using Benchling.

***E. coli* Wsp Activity and Microscopy.** For Wsp activity under surface growth conditions, MG1655 cells carrying an empty vector or a vector containing *wsp* operon variants were grown overnight on LB medium (1.5% agar) supplemented with 1% arabinose and carbenicillin (100 μg/mL). Cells were scraped the next morning, resuspended in 1× PBS, and 10 μL was placed on a prepared 1% low melting point agarose slide and imaged using confocal microscopy. For planktonic growth, cells carrying an empty vector or a vector containing *wsp* operon variants were grown overnight in LB liquid supplemented with carbenicillin (100 μg/mL). The next morning, cultures were diluted to $OD_{600} = 0.1$ in LB liquid containing carbenicillin and 1% arabinose and allowed to grow to midlog phase. Ten microliters of culture were placed on a prepared 1% low melting point agarose pad and imaged using confocal microscopy.

For chemical treatment, MG1655 cells carrying an empty vector or a vector containing *wsp* operon variants were grown overnight in M9 medium supplemented with glucose and carbenicillin (100 μg/mL). Wsp protein production was induced with 1% arabinose. The next morning, cells were diluted 100× and allowed to grow to early log phase ($OD_{600} = 0.2$ to 0.4) at 37 °C. Cells were then treated with various Wsp activating chemicals (i.e., 1% ethanol, 5% glycerol, 0.01% SDS) and allowed to continue to grow for 2 h. After 2 h, cells were either imaged by aliquoting on a 1% agarose pad using confocal microscopy or c-di-GMP was extracted from the entire culture for quantification by MS analysis (as described above). For fluorescent imaging, cells were imaged immediately using Zeiss LSM 800 confocal laser scanning microscope and five fields-of-view from each agarose pad were captured. Three biological replicates per strain and treatment were imaged. For each replicate, at least 100 cells were counted. Images were analyzed using FIJI software and WspR-YFP fluorescence intensity per cell was quantified using the MicrobeJ plugin. For each tested chemical, growth curves were conducted to find the MIC, and all chemicals were used at sub-MIC levels.

***E. coli* Cell Envelope Reporter Assay.** We used a series of previously verified plasmid-based transcriptional reporters (24, 34, 70) to determine how Wsp activators impact the *E. coli* cell envelope. All experiments were carried out as in Price and Raivio (34). All reporters were verified in mutant backgrounds that eliminated or promoted reporter activity (24, 70). Briefly, MG1655 cells carrying the reporter plasmid were grown overnight and the next day diluted to an $OD_{600} = 0.004$ in either LB or LB containing the putative Wsp activator (1% ethanol [vol/vol], 5% glycerol [vol/vol], 0.01% SDS [wt/vol], 50 μg/mL lysozyme, 0.1 mM benzyl alcohol). One milliliter cultures were grown for 5 h at 37 °C shaking in a 96-well plate. Luminescence and OD_{600} were determined from 200-μL aliquots using a BioTek H1 Synergy plate reader equipped with GenTech software. Luminescence was normalized to OD_{600} and then to untreated samples.

Generation of Cell Envelope Mutants. To construct chromosomal deletion of genes important in cell envelope maintenance, DNA flanking the genes was amplified and spliced together using Gibson assembly and cloned into pEX18GmGW. The resulting plasmid was transformed into S17.1 and verified by sequencing. The plasmid was then mobilized into PAO1 or PAO1 Δ*wspR*. Transconjugants were selected for by plating on VBMM (Vogel-Bonner minimal medium agar) media containing gentamicin (60 μg/mL), followed by screening for recombination on NSLB (No Salt Lennox Broth agar) containing 10% sucrose and PIA (*Pseudomonas* isolation agar). Colonies were screened for mutants using colony PCR and verified with sequencing.

CRISPRi. CRISPRi experiments were performed as in Stolle et al. (55). Reporter fluorescence of arabinose induced cells was normalized to uninduced controls. Graphs were generated using fluorescence at $T = 60$ min and statistical significance was determined using a one-way ANOVA.

Induction of *cas9* and knockdown of *dsbA* were confirmed using qRT-PCR and were normalized to the housekeeping gene *rpsL*, as previously described (55).

NPN Uptake Assay. To measure outer membrane permeability, we used the NPN assay previously described (23, 48). Additional details can be found in the *SI Appendix, Supplemental Materials and Methods*.

Periplasmic Protein Unfolding. PAO1 cells carrying pSW002 Pc TorA-mTurquoise or pSW002 mTurquoise (55) were grown to $OD_{600} = 0.5$ and then treated with Wsp activating compounds for 1 h. Cells were washed and resuspending in 5 mM Hepes buffer and mTurquoise (excitation: 430 nm, emission: 475 nm) emission spectrum fluorescence was acquired using a plate reader. Aliquots of treated and untreated cells were imaged using a Nikon Ti2.

Periplasmic and Cytoplasmic Fluorophore Stability. mPAO1 Tn7:PA1/04/03 td Tomato cells carrying pSW002 Pc TorA-mTurquoise (51) were grown in LB media supplemented with tetracycline (100 μg/mL) overnight. The next morning, cells were diluted 1:1,000 and allowed to grow to $OD_{600} = 0.4$. A 1-mL aliquot of the culture was fixed with 2.5% paraformaldehyde and fluorescence was imaged on 1% low melting point agarose pads using a Nikon Ti2. Unfixed cells were placed on an agarose pad and imaged immediately and every 30 min for up to 2 h after surface exposure. Fluorescence was quantified using FIJI.

Statistical Analysis. All statistical analyses were conducted using GraphPad Prism 8. Unless indicated differently above, all experiments were tested using a one-way ANOVA and differences were considered statistically significant when the $P < 0.05$.

Data Availability. All study data are included in the main text and supporting information.

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