A cell wall damage response mediated by a sensor kinase/response regulator pair enables beta-lactam tolerance

Tobias Dörr[a], Laura Alvarez[b], Fernanda Delgado[a], Brigid M. Davis[a], Felipe Cava[b], and Matthew K. Waldor[a,1]

[a]Howard Hughes Medical Institute, Brigham and Women's Hospital Division of Infectious Diseases and Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA 02115; and [b]Laboratory for Molecular Infection Medicine Sweden, Department of Molecular Biology, Umeå Centre for Microbial Research, Umeå University, 90187 Umeå, Sweden

Edited by Julian Davies, University of British Columbia, Vancouver, Canada, and approved December 4, 2015 (received for review October 13, 2015)

The bacterial cell wall is critical for maintenance of cell shape and survival. Following exposure to antibiotics that target enzymes required for cell wall synthesis, bacteria typically lyse. Although several cell envelope stress response systems have been well described, there is little knowledge of systems that modulate cell wall synthesis in response to cell wall damage, particularly in Gram-negative bacteria. Here we describe WigK/WigR, a histidine kinase/response regulator pair that enables *Vibrio cholerae*, the cholera pathogen, to survive exposure to antibiotics targeting cell wall synthesis in vitro and during infection. Unlike wild-type *V. cholerae*, mutants lacking *wigR* fail to recover following exposure to cell-wall–acting antibiotics, and they exhibit a drastically increased cell diameter in the absence of such antibiotics. Conversely, overexpression of *wigR* leads to cell slimming. Overexpression of activated WigR also results in increased expression of the full set of cell wall synthesis genes and to elevated cell wall content. WigKR-dependent expression of cell wall synthesis genes is induced by various cell-wall–acting antibiotics as well as by overexpression of an endogenous cell wall hydrolase. Thus, WigKR appears to monitor cell wall integrity and to enhance the capacity for increased cell wall production in response to damage. Taken together, these findings implicate WigKR as a regulator of cell wall synthesis that controls cell wall homeostasis in response to antibiotics and likely during normal growth as well.

peptidoglycan | stress response | antibiotic tolerance | two component system | cell envelope

Nearly all bacteria produce a sturdy cell wall located outside of the cytoplasmic membrane to maintain their shape and structural integrity (1). The cell wall is composed of peptidoglycan (PG), a remarkable macromolecule composed of strands of polymerized disaccharides cross-linked via short peptide sidechains to form a mesh. This complex polymer's crucial role in bacterial cell integrity and consequent survival becomes dramatically apparent when PG synthesis is inhibited by antibiotics, which typically causes rapid cell lysis and death. For this reason, cell wall synthesis inhibitors (such as the penicillins) are among the most potent and widely used antibiotics (2, 3).

PG synthesis proceeds in three principal steps (4), each of which can be inhibited by antibiotics. Starting with the generation of disaccharide–oligopeptide precursors in the cytoplasm [the target of fosfomycin and D-cycloserine (5, 6)] and followed by precursor translocation across the cytoplasmic membrane (CM) [the target of several experimental drugs (7)], the cell wall is assembled outside the CM by membrane-bound enzymes called Penicillin Binding Proteins (PBPs), the targets of beta-lactams such as penicillins. High-molecular-weight PBPs, such as PBP1A and PBP1B, have both polymerizing (transglycosylation) and cross-linking (transpeptidation) activities and are found in large complexes that constitute cell wall assembly machines (8, 9). Cell wall biosynthesis and assembly are required both for bacterial elongation and for division. In addition, bacterial growth requires the activity of PG hydrolases ("autolysins"), enzymes that locally dissolve the meshwork to allow for the insertion of new cell wall material (10, 11). It is thought that an imbalance between synthetic and hydrolytic enzyme activities is ultimately responsible for the lysis phenotype that typically results from inhibition of cell wall synthesis.

Bacteria often use alternative sigma factors or two-component systems (TCS) to adapt gene expression in response to stressors (12). Two-component phosphorelay systems are composed of Histidine Kinase/Response Regulator (HK/RR) pairs. In HK/RR systems, which are ubiquitous in bacteria, the kinase typically relays extracellular information to the response regulator via autophosphorylation and subsequent phosphotransfer. This signal transmission results in changes in expression of stress-responsive transcripts.

Although several stress response systems are known to sense and respond to damaged/misfolded membrane proteins (13–15), there is a paucity of knowledge regarding systems that sense and ameliorate damage to the cell wall. Exceptions are TCS [e.g., WalRK and VraSR (16, 17)] found in several Gram-positive genera that respond to exposure to various cell-wall–acting antibiotics by activating regulons that mediate specialized cell wall synthesis/turnover functions. In addition, some Gram-negative genera encode an AmpR repressor that detects cell wall fragments generated by beta-lactam antibiotics and induces production of the AmpC beta-lactamase that degrades these compounds (18). Notably, TCS or other sensor/response systems

Significance

The cell wall is an essential protective structure for bacteria, and the enzymes required for its biogenesis are the targets for many potent antibiotics. However, there is little knowledge of how bacteria (particularly Gram-negative organisms) respond to damage to the cell wall caused by antibiotics or other agents. Here, we describe a signaling system (the histidine kinase/response regulator pair WigKR) in *Vibrio cholerae*, the cholera pathogen that is triggered by antibiotics that disrupt various steps in cell wall synthesis. Activation of WigR leads to increased expression of genes required for cell wall synthesis and enables the pathogen to recover from the spherical nondividing state induced by antibiotics. Collectively, our findings reveal a new signal transduction pathway controlling cell wall biosynthesis and antibiotic tolerance.

Author contributions: T.D. and M.K.W. designed research; T.D., L.A., and F.D. performed research; T.D., L.A., and F.C. contributed new reagents/analytic tools; T.D., L.A., B.M.D., F.C., and M.K.W. analyzed data; and T.D., B.M.D., and M.K.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. Email: mwaldor@research.bwh.harvard.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1520333113/-/DCSupplemental.

that enable Gram-negative bacteria to induce PG synthesis after detection of PG damage (e.g., as a consequence of exposure to cell-wall–acting antibiotics) have not been described. In general, there is little knowledge of pathways that regulate cell wall biogenesis.

We have previously shown that the causative agent of cholera, *Vibrio cholerae*, is intrinsically tolerant to antibiotics targeting cell wall synthesis. Upon exposure to cell wall synthesis inhibitors, *V. cholerae* loses its cell wall and forms viable, nondividing spheres, which readily revert to the normal rod shape upon removal of the antibiotic (19). Here, we describe a two-component system, dubbed WigK and WigR (for wall integrity gauge kinase and regulator), which is critical for *V. cholerae* tolerance to cell-wall– acting antibiotics. Remarkably, the *wigKR* regulon, which includes the entire cell wall synthesis pathway, is induced by exposure to antibiotics that inhibit cell wall synthesis. Moreover, the deletion or overexpression of WigR caused changes in cell width, suggesting that the WigKR two-component system modulates cell wall homeostasis under normal growth conditions in the absence of antibiotics. Collectively, our findings reveal a new signal transduction pathway controlling PG biosynthesis and antibiotic tolerance.

Results

wigKR Locus Is Required for _V. cholerae_'s Tolerance to Inhibitors of Cell Wall Synthesis. A TnSeq-based screen for *V. cholerae* factors required for recovery from penicillin exposure revealed that insertions in *vca0565* and *vca0566* were underrepresented in a transposon insertion library following treatment with penicillin. As noted above we renamed these genes WigKR (wall integrity gauge kinase and regulator) for reasons elaborated below. The two genes are arranged in an operon, in which the *wigR* reading frame overlaps with the 3′ end of the *wigK*-coding sequence. WigK codes for a histidine kinase (HK) of the BaeS family, whereas WigR encodes a response regulator (RR) in the OmpR family (Fig. 1*A*). WigK possesses a well-conserved kinase domain; however, the periplasmic portion of the protein lacks a recognizable, canonical HK signal-sensing domain and instead contains a domain of unknown function. The *wigKR* locus was recently reported to be a regulator of type VI secretion (20), but is otherwise uncharacterized.

To explore the involvement of the *wigKR* locus in *V. cholerae*'s response to cell wall acting antibiotics, we measured the ability of defined mutants lacking *wigK*, *wigR*, or both to survive exposure to the beta-lactam antibiotic penicillin G. As previously observed (19), wild-type *V. cholerae* (El Tor isolate N16961) exhibited no reduction in colony-forming units in response to treatment. In contrast, the colony-forming capacity of the Δ*wigK*, Δ*wigR*, and Δ*wigKR* strains declined by ∼2–3 orders of magnitude in the presence of penicillin G (Fig. 1*B* and Fig. S1). Expression of *wigK* in the Δ*wigK* mutant was sufficient to restore wild-type levels of survival in the presence of penicillin G in vitro, excluding the possibility of polar effects and secondary site mutations (Fig. 1*C*). Likewise, expression of *wigKR* was able to complement the beta-lactam sensitivity of a Δ*wigKR* double mutant (Fig. S1). However, expression of *wigK* alone was insufficient to complement Δ*wigKR* (Fig. 1*C*), suggesting that WigK's effect on survival requires the presence of its putative response regulator WigR.

Exposure of the Δ*wigKR* mutant to penicillin G was not accompanied by a decrease in culture turbidity (Fig. S2*A*), suggesting that the absence of WigKR does not lead to cell lysis. Instead, we hypothesize that WigKR is required for recovery from damage induced by antibiotics that target cell wall synthesis. Consistent with this idea, we found that spherical cells formed by a Δ*wigKR* mutant after penicillin exposure failed to revert to rod shape after the antibiotic was removed (Fig. S2*B*). Additionally, we observed that the *wigKR* locus was critical for *V. cholerae* colony formation after exposure to fosfomycin

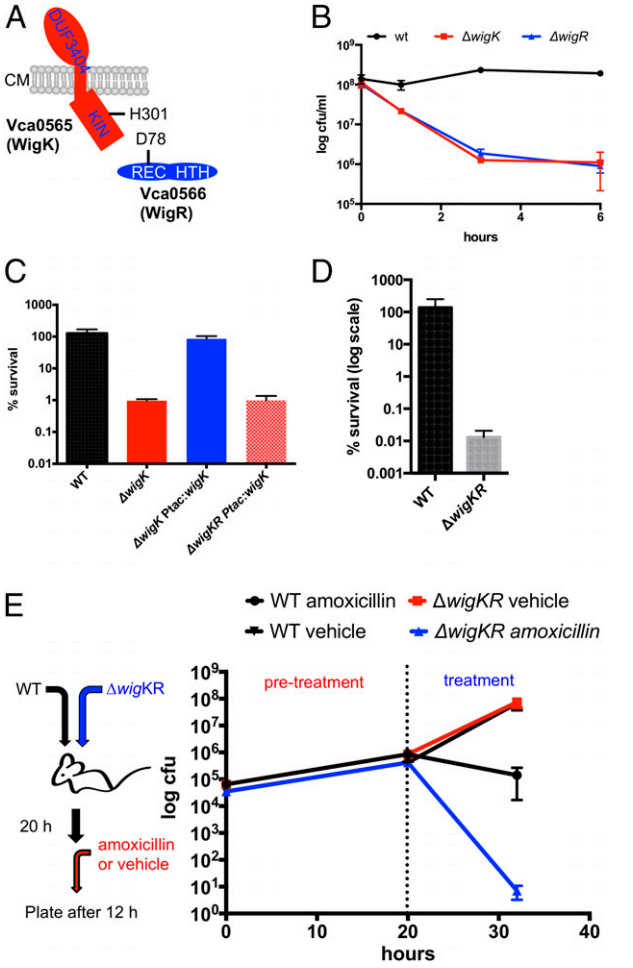

Fig. 1. *V. cholerae* requires the *wigKR* locus to survive inhibition of cell wall synthesis. (*A*) Predicted domain organization of WigK and WigR showing their predicted sites of phosphorylation. Kin: kinase domain; REC: receiver domain; HTH: helix-turn-helix motif; CM: cytoplasmic membrane. (*B*) Survival of WT *V. cholerae* N16961 and its Δ*wigK* and Δ*wigR* derivatives after exposure to penicillin G [100 μg/mL, 20x minimum inhibitory concentration (MIC)] for the indicated amount of time. Data are averages of three biological replicates; error bars represent SE. (*C*) Complementation of *wigKR*-dependent penicillin sensitivity. Strains carrying complementing genes inserted in a single copy in a neutral chromosomal locus under an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter were grown to late exponential phase in LB + inducer and then exposed to 100 μg/mL penicillin G for 3 h; values represent mean of three biological replicates, and error bars represent SDs. (*D*) Survival of a Δ*wigKR* mutant in the presence of fosfomycin (200 μg/mL, ∼4× MIC). (*E*) WigKR are required for *V. cholerae* survival of beta-lactam treatment in vivo. N16961 (lacZ−) parental and Δ*wigKR* (lacZ+) were coinoculated into infant mice. Twenty hours after inoculation, mice were treated orally with either 50 mg/kg amoxicillin or vehicle control ("treatment"), and 12 h later cfu of the parental and Δ*wigKR* strains were determined. Data points are averages of 9 mice (amoxicillin) or 10 mice (vehicle); error bars represent SE.

(Fig. 1*D*), an antibiotic that inhibits MurA, which catalyzes the first step of synthesis of cell wall precursors (6). Thus, WigKR is required for *V. cholerae* to recover from the damage elicited by agents that inhibit distinct steps within PG biosynthesis, including cytoplasmic formation of cell wall precursors as well as cross-linking of PG precursors in the periplasm.

We also tested whether *V. cholerae* requires *wigKR* to survive exposure to a beta-lactam antibiotic during infection in vivo. Following coinoculation of infant mice with wild-type (lacZ−) and Δ*wigKR* (lacZ+) strains, mice were treated at 20 h post infection with amoxicillin (a beta-lactam antibiotic suitable for oral

MICROBIOLOGY

administration) or a vehicle control. Subsequent enumeration of WT and Δ*wigKR* cells revealed that both strains were recovered at equal levels when the antibiotic was not present and thus showed that the Δ*wigKR* strain does not have an intrinsic colonization deficiency. However, there was a dramatic reduction (>10,000-fold) in the recovery of Δ*wigKR* vs. WT colonies after treatment with amoxicillin (Fig. 1*E*). Thus, WigKR contributes to *V. cholerae*'s beta-lactam tolerance in vivo.

To begin to dissect the molecular requirements for WigKR function, we reconstituted a Δ*wigKR* strain with either wild-type *wigKR* or sequences producing point mutations in WigK's and WigR's predicted phosphorylation sites (H301A and D78A, respectively), which are expected to be inactivating. The strains with the H301A and D78A phospho-ablative mutations phenocopied the penicillin sensitivity of the Δ*wigK* and Δ*wigR* mutants (Fig. S3). These results suggest that WigKR's contribution to survival of penicillin exposure relies on its ability to be phosphorylated, strongly suggesting that WigKR are part of a signaling cascade, the output of which ensures cell survival after cell wall damage.

WigR Regulon Includes the Full Set of Genes for Cell Wall Biosynthesis. To begin to identify the genetic circuitry underlying WigKR-mediated beta-lactam tolerance, we used global RNA sequencing analysis to define the output of the WigKR regulon. For these analyses, we overexpressed a putative phosphomimetic version of WigR (D78E) to avoid heterogeneous phosphorylation states that might accompany overexpression of wild-type WigR. Overexpression of D78E caused a slight decrease in growth rate for cells grown in rich medium (LB) (Fig. S4*A*), but a much more pronounced decrease in M9 minimal medium (Fig. S4*B*). Consequently, RNASeq analyses were performed using *V. cholerae* cultured in LB.

These transcriptomic analyses of the consequences of D78E expression vs. empty control plasmid revealed differential expression (defined as greater than twofold differences with a *P* value of < 0.01) of 320 genes (Dataset S1). Of these, 100 genes were down-regulated (Fig. 2*A*), many of which (28%) are associated with either motility (e.g., flagella biosynthesis) or iron acquisition (heme import and siderophore production). Consistent with these data, overexpression of WT WigR or WigR(D78E) resulted in a complete loss of motility in a soft agar swarming assay (Fig. 2*B*). In addition, we explored whether the poor growth of the WigR(D78E) overexpressing strain in M9 medium might reflect the absence of added iron in this media coupled with the down-regulation of iron acquisition genes. We found that addition of Iron(III) partially alleviated the growth defect caused by D78E overexpression (Fig. S4*C*). This result suggests that iron availability can limit growth when the WigR regulon is induced, likely due to down-regulation of siderophore biosynthesis genes.

In addition to the genes negatively regulated by WigR, we identified 220 genes that were up-regulated as a consequence of D78E overexpression (Fig. 3*A*). Strikingly, 66 (30%) of the up-regulated genes coded for proteins related to the cell envelope, such as outer membrane proteins, envelope stress responses, and phospholipid biosynthesis. Remarkably, this list also encompassed the entire peptidoglycan biogenesis pathway, including precursor synthesis, precursor translocation, and cell wall assembly, as well as several factors associated with cell wall modification and degradation (Fig. 3*B* and Fig. S5*A*). The increase in the expression of cell wall synthesis genes was accompanied by a significant 1.5-fold increase in cell wall content (Fig. 3*C*) and by a marked (10,000-fold) increase in survival following hypo-osmotic shock (Fig. 3*D*). Thus, induction of the WigR regulon appears to enhance cell envelope strength, probably due to alterations in PG synthesis and/or structure. We did not observe increased expression of most of the type VI secretion genes previously found to be regulated by WigR (20); however, these secretion systems have been found to be expressed by only a small subset of *V. cholerae* strains (21).

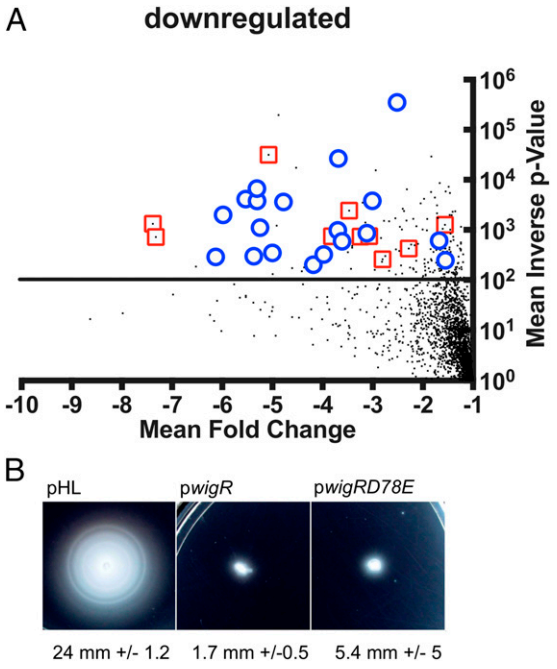

Fig. 2. Overexpression of phosphomimetic WigR leads to the down-regulation of motility and iron acquisition genes. (*A*) Volcano plot representation of down-regulated genes from comparison of transcript abundance in cells overexpressing phosphomimetic WigR(D78E) with empty vector control. Horizontal line indicates $P = 0.01$ (*t* test), the cutoff for significance. Red squares are genes involved in iron acquisition; blue circles indicate genes involved in motility. The full dataset is given in Dataset S1. (*B*) Overexpression of WigR or WigR(D78E) represses motility. Growing cultures were stabbed into soft agar plates and incubated overnight. Average radii of swarming zones were measured from five independent colonies for each strain.

WigKR Regulon Is Activated by Antibiotic-Induced Cell Wall Damage. Given WigR's regulation of cell wall synthesis, coupled with *V. cholerae*'s requirement for *wigKR* to survive beta-lactam exposure, we hypothesized that beta-lactams activate the WigKR-signaling pathway and thereby lead to WigKR-dependent induction of cell wall synthesis genes. To test this possibility, we compared accumulation of epitope-tagged forms of *V. cholerae*'s two major cell wall synthases, PBP1A and PBP1B, following penicillin exposure of cells containing or lacking *wigR*. Strikingly, penicillin induced the expression of both PBPs approximately four- to fivefold (Fig. 4*A*) in a largely *wigR*-dependent fashion.

WigKR-dependent accumulation of PBP1A and PBP1B following inhibition of cell wall synthesis was also observed with untagged membrane proteins that were visualized using the fluorescent penicillin derivative Bocillin-FL (22). Bocillin, like penicillin, covalently binds to the active site of PBPs, and thus one precludes binding of the other; consequently, for this assay we inhibited cell wall synthesis with fosfomycin, which does not directly interfere with bocillin binding. As with penicillin, we found that the abundance of PBP1A and PBP1B increased in a largely *wigKR*-dependent manner when cells were treated with fosfomycin (Fig. 4*B*). Thus, inhibition of distinct steps in cell wall synthesis can induce WigR-dependent production of PBPs. The minor increase in PBP1A levels following antibiotic exposure in the absence of WigKR suggests that PBPs can also be up-regulated by another, unidentified factor.

To gather additional evidence for penicillin induction of the WigR regulon, we measured transcripts from two other cell wall

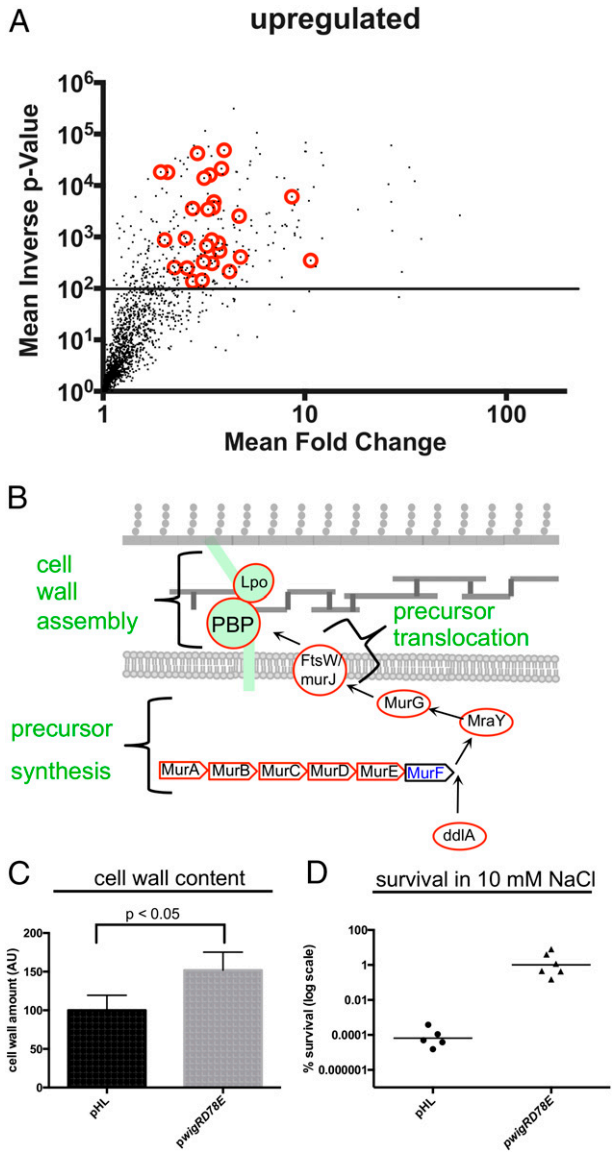

Fig. 3. Overexpression of phosphomimetic WigR leads to the up-regulation of cell wall synthesis genes. (*A*) Up-regulated genes from transcriptomic analysis described in Fig. 2*A*; note that, unlike Fig. 2*A*, the *x* axis is plotted on a logarithmic scale. Red circles indicate cell wall synthesis genes. (*B*) Schematic showing WigR-up-regulated cell wall synthesis genes. Black font indicates genes at least twofold up-regulated after WigR(D78E) overexpression. (*C*) Comparison of cell wall content in a strain overexpressing WigR(D78E) or empty vector control. Cell wall content was measured by ultra performance liquid chromatography (UPLC)-mediated quantification of digested cell wall material derived from cultures normalized to the same cfu/mL. (*D*) Osmoresistance caused by overexpression of WigR(D78E). Cells were grown to exponential phase in LB (200 mM NaCl), pelleted, and resuspended in 10 mM NaCl for 5 min, followed by adjustment to 200 mM NaCl. Individual data points of six biological replicates are shown.

synthesis genes, *mraY* (lipid2 biosynthesis) and *murJ* (lipid2 flippase), using qRT-PCR. Expression of both genes was strongly (17- and 88-fold) induced by penicillin in a *wigKR*-dependent fashion (Fig. 4*C*). Thus, taken together, our findings reveal that *wigKR* activates multiple steps of the cell wall biogenesis process in response to cell wall synthesis inhibitors.

We then asked whether WigKR-dependent induction of cell wall synthesis was associated only with cell wall damage or with assaults on the cell envelope in general. To test this, we exposed our PBP1A-His reporter strain to two unrelated cell envelope stressors, cerulenin (an inhibitor of fatty acid biosynthesis) and crude bile, which acts as a general membrane-damaging agent. Neither agent induced a significant up-regulation of PBP1A expression (Fig. S5*B*), suggesting that the WigKR regulon is not induced by cell envelope damage in general.

***wigKR* Controls Its Own Expression in Response to beta-Lactams.** Many HK/RR systems are known to autoregulate their expression; i.e., these systems are expressed constitutively at low levels but increase their own expression upon signal recognition (23). Indeed, *wigK* was up-regulated in response to expression of WigR(D78E) in our RNASeq dataset (Dataset S1). We tested whether beta-lactams induce WigKR in conjunction with its regulon by measuring protein levels of an mCherry-WigK translational fusion and of C-terminally His-tagged WigR-His (both encoded at their native chromosomal loci) after exposure to antibiotics. Baseline WigK and WigR levels were very low, but increased dramatically upon addition of penicillin (Fig. 5). The baseline level and penicillin-induced increase in WigK abundance were markedly reduced in a *wigR* mutant, confirming that the *wigKR* operon autoregulates. Increased WigR levels were likewise observed after exposure to fosfomycin and to D-cycloserine (Fig. S6), an inhibitor of D-Ala-D-Ala ligase (5), which

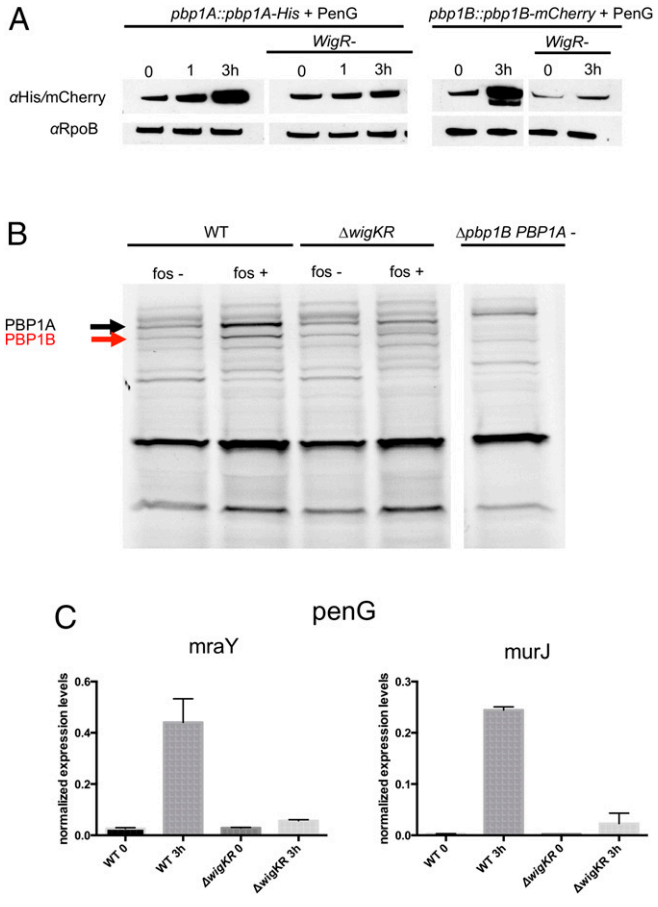

Fig. 4. Cell-wall–acting antibiotics induce expression of cell wall synthesis genes in a WigKR-dependent fashion. (*A*) Western blot analysis of His- or mCherry-tagged PBP1A or PBP1B, respectively, expressed from their native chromosomal loci. Cells were grown to late exponential phase and exposed to penicillin G (100 μg/mL). (*B*) Bocillin-stained membrane proteins from the indicated strains, grown in the absence or presence of fosfomycin. (*C*) qRT-PCR analysis of cell wall precursor synthesis/translocation genes. Values are means of biological triplicates; error bars represent SD. Expression levels were normalized to an internal control transcript, *rpoC*.

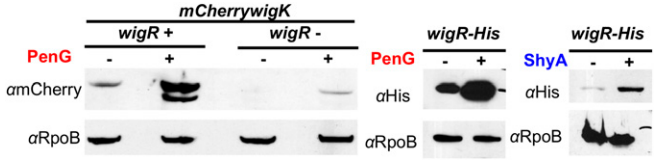

Fig. 5. *wigKR* expression is autoregulated and increased in response to antibiotics targeting cell wall synthesis. Cells harboring either mCherry-WigK or a WigR-His fusion were grown to late exponential phase and exposed to penicillin G for 3 h or overexpression of ShyA for 1 h, followed by Western blotting using antibodies specific to mCherry (for WigK detection) or His (for WigR detection).

catalyzes a later cytoplasmic step in cell wall synthesis. Notably, WigR levels were also increased upon overexpression of the PG endopeptidase ShyA (Fig. 5), a housekeeping enzyme required for PG degradation in the context of cell elongation (24). Thus, therapeutics that target various steps in cell wall synthesis, as well as endogenous agents of cell wall damage, can all induce WigR.

***wigKR* Locus Plays a Role in Cell Wall Homeostasis During Normal Growth.** Given the large set of genes in the WigR regulon that mediate cell wall biogenesis, we speculated that *wigKR* might modulate cell wall synthesis/degradation during normal growth, as well as in response to antibiotics that target cell wall synthesis. Although mutants lacking *wigK* and/or *wigR* exhibited only minor growth defects, which interestingly were restricted to the late exponential phase (Fig. S7*A*), we observed a marked increase in cell diameter (Fig. 6) and cell volume (Fig. S7*B*) in mutants lacking *wigR*. Conversely, overexpression of WigR caused a reduction in cell width from 1 μm on average to ∼0.8 μm (Fig. 6). Because cell width is determined by the cell wall and is typically tightly controlled (25, 26), these striking aberrations suggest a fundamental role for *wigR* in cell wall homeostasis, the molecular details of which warrant further investigation.

Discussion

WigKR is an unusual two-component system that responds to damage caused by antibiotics that target cell wall biosynthesis and to endogenously generated stimuli, such as those elicited by overexpression of the ShyA endopeptidase. Similar cell wall damage response systems have not been described in Gram-negative bacteria. The VraSR and WalRK two-component systems in *Staphylococcus aureus* and *Bacillus subtilis* are thought to respond to cell wall stress induced by antibiotics, but, in marked contrast to the WigKR system, the regulons controlled by these Gram-positive two-component systems primarily include specialized, single PBPs and modulators of autolysin activity, and not the entire pathway for PG biogenesis. WigKR's control of the complete PG synthesis pathway explains why this system is critical for *V. cholerae* tolerance to antibiotics that target the cell wall. Treatment of *V. cholerae* with cell-wall–acting antibiotics results in formation of PG-deficient spheres; WigR and its regulon, which are induced by these agents, are required for *V. cholerae* recovery presumably because they enable *V. cholerae* to regenerate its cell wall and restore its rod shape.

Although WigK is conserved only in the *Vibrionaceae*, it is possible that other bacteria have evolved analogous stress response systems to fulfill similar functions. For example, the *Escherichia coli* Rcs phosphorelay system (homologs of which are absent from *V. cholerae*) has been shown to be required for recovery from a spherical state induced by lysozyme treatment in an osmotically stabilized medium (27) (a state that resembles *V. cholerae* after treatment with beta-lactam antibiotics). Moreover, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, like *V. cholerae*, fail to lyse in the presence of cell wall synthesis inhibitors under some conditions (19, 28). These pathogens lack Rcs systems as well, but may harbor specialized stress response systems functionally equivalent to WigKR that aide in recovery from cell wall damage. Inhibition of such cell wall stress response systems may be a promising avenue for discovery of adjuvants that enhance the efficacy of antibiotics that target cell wall synthesis.

In addition to mediating antibiotic tolerance, *wigKR* likely contributes to cell wall homeostasis in the absence of antibiotics. The Δ*wigR* strain exhibited increased cell width, whereas *wigR* overexpression led to cell slimming. WigR(D78E)-induced upregulation of the major PBPs and their activators (PBP1A/B and LpoA/B, respectively) is of particular interest. Cell wall assembly in the periplasm was thought to be a constitutive housekeeping process until the discovery of outer membrane-localized activators of PBP1A and PBP1B activity (8, 9). Our data suggest that, in addition to posttranslational regulation, at least some bacteria are also capable of modulating transcription of cell wall synthesis pathways. Very few transcriptional regulators of PBPs have been described to date, especially in Gram-negative bacteria. *E. coli* regulates the expression of low-molecular-weight PBPs (which are thought to play roles in PG modification rather than synthesis) via the transcriptional regulator BolA in response to an unknown signal upon entry into stationary phase (29). *Neisseria gonorrheae*'s principal PBP, encoded by the *ponA* gene, is under control of a repressor also controlling expression of type IV pili and a multidrug efflux pump (30). The WigKR system is unusual as it regulates the entire cell wall synthesis pathway, including precursor synthesis, precursor translocation, and cell wall assembly (PBPs and PBP activators) as well as PG hydrolases, which are also required for growth. By governing the complete pathway for PG synthesis, WigKR presumably allows for control of total cell wall content. Consistent with this idea, we found that cells overexpressing phosphomimetic *wigR* had increased PG content, which likely accounts for their markedly increased resistance to osmotic stress. Thus, the WigKR system is a bona fide regulator of total cell wall content.

A key challenge for future investigation is identification of the signal that activates the WigKR regulon. It is formally possible that WigK directly senses antibiotics or depletion of PG synthesis precursors, which has recently been described as a lethal consequence of exposure to beta-lactam antibiotics (31). However, direct sensing of antibiotics is highly unlikely because structurally diverse agents (beta-lactams, D-cycloserine, fosfomycin) induce WigR and WigR governs cell width even in the absence of antibiotics. Sensing of precursor depletion is unlikely because WigR is also induced by overexpression of ShyA, an endopeptidase that cuts cross-links in the PG meshwork, which in theory

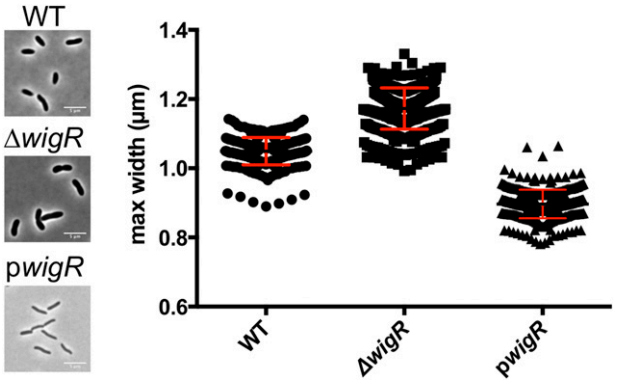

Fig. 6. WigKR regulates *V. cholerae* cell size during exponential phase growth. Phase contrast images and comparison of cell widths in WT and Δ*wigR V. cholerae* and in *V. cholerae* overproducing WigR during exponential phase growth in LB.

should not affect precursor availability. ShyA preferentially releases short PG chains from the PG network and is primarily responsible for cell wall degradation after inhibition of cell wall synthesis (19, 24); consequently, WigK may be activated by endogenous cell wall fragments. Such fragments could be the product of normal growth processes; result from the action of antibiotics; or potentially be induced by phage or type VI effector attack, both of which can damage the cell wall.

Materials and Methods

Strains, Plasmids, and Oligos. Strains, plasmids, and oligos are summarized in Dataset S2. All *V. cholerae* strains are derivatives of N16961, an El Tor clinical isolate (32). For details on strain construction, media, and growth conditions, see *SI Materials and Methods*.

Killing and Osmoresistance Assays. For time-dependent antibiotic survival experiments, cells were grown with shaking at 37 °C to midexponential phase (OD_{600} ~0.3) in LB (~200 mM NaCl), pelleted, and resuspended in 10 mM NaCl for 5 min, followed by addition of 1/10 volume of 10× PBS (1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4) and then serial-diluted and spot-plated onto LB agar plates.

RNAseq Analysis. RNA was extracted using the TRIzol method (33) (for details see *SI Materials and Methods*). RNAseq and differential gene expression analysis was conducted by Genewiz.

Bocillin-FL Staining. Membrane fractions were obtained by differential centrifugation (see *SI Materials and Methods* for details). PBPs in the membrane fractions were stained using 50 μM Bocillin-FL (Invitrogen) for 20 min at 37 °C.

PG Quantification. For PG quantification and analysis, cultures were normalized to the same cfu/mL, and muropeptides were isolated following previously described methods (34, 35).

Microscopy and Image Analysis. Cells were imaged on 0.8% agarose pads using a Zeiss Axiovision light microscope and 100× objective. Cell width/volume in cells grown in LB to OD_{600} = 0.5 was determined using MicrobeTracker software (36).

For additional methods, see *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Stavroula Hatzios for assistance with illustrations. Research in the M.K.W. laboratory is supported by Howard Hughes Medical Institute and NIH Grant R37 AI–042347. Research in the F.C. laboratory is supported by the Molecular Infection Medicine Sweden (MIMS), the Knut and Alice Wallenberg Foundation, and the Swedish Research Council. L.A. is supported by Grant JCK-1326 from the Kempe Foundation (Kempestiftelserna).

1. Höltje JV (1998) Growth of the stress-bearing and shape-maintaining murein sacculus of Escherichia coli. *Microbiol Mol Biol Rev* 62(1):181–203.
2. Lewis K (2013) Platforms for antibiotic discovery. *Nat Rev Drug Discov* 12(5):371–387.
3. Schneider T, Sahl HG (2010) An oldie but a goodie: Cell wall biosynthesis as antibiotic target pathway. *Int J Med Microbiol* 300(2-3):161–169.
4. Typas A, Banzhaf M, Gross CA, Vollmer W (2012) From the regulation of peptidoglycan synthesis to bacterial growth and morphology. *Nat Rev Microbiol* 10(2):123–136.
5. Lambert MP, Neuhaus FC (1972) Mechanism of D-cycloserine action: Alanine racemase from Escherichia coli W. *J Bacteriol* 110(3):978–987.
6. Kahan FM, Kahan JS, Cassidy PJ, Kropp H (1974) The mechanism of action of fosfomycin (phosphonomycin). *Ann N Y Acad Sci* 235(0):364–386.
7. Huber J, et al. (2009) Chemical genetic identification of peptidoglycan inhibitors potentiating carbapenem activity against methicillin-resistant Staphylococcus aureus. *Chem Biol* 16(8):837–848.
8. Paradis-Bleau C, et al. (2010) Lipoprotein cofactors located in the outer membrane activate bacterial cell wall polymerases. *Cell* 143(7):1110–1120.
9. Typas A, et al. (2010) Regulation of peptidoglycan synthesis by outer-membrane proteins. *Cell* 143(7):1097–1109.
10. Vollmer W (2012) Bacterial growth does require peptidoglycan hydrolases. *Mol Microbiol* 86(5):1031–1035.
11. Uehara T, Bernhardt TG (2011) More than just lysins: Peptidoglycan hydrolases tailor the cell wall. *Curr Opin Microbiol* 14(6):698–703.
12. Capra EJ, Laub MT (2012) Evolution of two-component signal transduction systems. *Annu Rev Microbiol* 66:325–347.
13. Bury-Moné S, et al. (2009) Global analysis of extracytoplasmic stress signaling in Escherichia coli. *PLoS Genet* 5(9):e1000651.
14. Raivio TL (2014) Everything old is new again: An update on current research on the Cpx envelope stress response. *Biochim Biophys Acta* 1843(8):1529–1541.
15. Majdalani N, Gottesman S (2005) The Rcs phosphorelay: A complex signal transduction system. *Annu Rev Microbiol* 59:379–405.
16. Dubrac S, Bisicchia P, Devine KM, Msadek T (2008) A matter of life and death: Cell wall homeostasis and the WalKR (YycGF) essential signal transduction pathway. *Mol Microbiol* 70(6):1307–1322.
17. Kuroda M, et al. (2003) Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in Staphylococcus aureus. *Mol Microbiol* 49(3):807–821.
18. Jacobs C, Frère JM, Normark S (1997) Cytosolic intermediates for cell wall biosynthesis and degradation control inducible beta-lactam resistance in gram-negative bacteria. *Cell* 88(6):823–832.
19. Dörr T, Davis BM, Waldor MK (2015) Endopeptidase-mediated beta lactam tolerance. *PLoS Pathog* 11(4):e1004850.
20. Cheng AT, Ottemann KM, Yildiz FH (2015) Vibrio cholerae response regulator VxrB controls colonization and regulates the type VI secretion system. *PLoS Pathog* 11(5):e1004933.
21. Ho BT, Dong TG, Mekalanos JJ (2014) A view to a kill: The bacterial type VI secretion system. *Cell Host Microbe* 15(1):9–21.
22. Zhao G, Meier TI, Kahl SD, Gee KR, Blaszczak LC (1999) BOCILLIN FL, a sensitive and commercially available reagent for detection of penicillin-binding proteins. *Antimicrob Agents Chemother* 43(5):1124–1128.
23. Mitrophanov AY, Groisman EA (2008) Signal integration in bacterial two-component regulatory systems. *Genes Dev* 22(19):2601–2611.
24. Dörr T, Cava F, Lam H, Davis BM, Waldor MK (2013) Substrate specificity of an elongation-specific peptidoglycan endopeptidase and its implications for cell wall architecture and growth of Vibrio cholerae. *Mol Microbiol* 89(5):949–962.
25. Taheri-Araghi S, et al. (2015) Cell-size control and homeostasis in bacteria. *Curr Biol* 25(3):385–391.
26. Huang KC, Mukhopadhyay R, Wen B, Gitai Z, Wingreen NS (2008) Cell shape and cell-wall organization in Gram-negative bacteria. *Proc Natl Acad Sci USA* 105(49):19282–19287.
27. Ranjit DK, Young KD (2013) The Rcs stress response and accessory envelope proteins are required for de novo generation of cell shape in Escherichia coli. *J Bacteriol* 195(11):2452–2462.
28. Monahan LG, et al. (2014) Rapid conversion of Pseudomonas aeruginosa to a spherical cell morphotype facilitates tolerance to carbapenems and penicillins but increases susceptibility to antimicrobial peptides. *Antimicrob Agents Chemother* 58(4):1956–1962.
29. Santos JM, Lobo M, Matos AP, De Pedro MA, Arraiano CM (2002) The gene bolA regulates dacA (PBP5), dacC (PBP6) and ampC (AmpC), promoting normal morphology in Escherichia coli. *Mol Microbiol* 45(6):1729–1740.
30. Folster JP, Dhulipala V, Nicholas RA, Shafer WM (2007) Differential regulation of ponA and pilMNOPQ expression by the MtrR transcriptional regulatory protein in Neisseria gonorrhoeae. *J Bacteriol* 189(13):4569–4577.
31. Cho H, Uehara T, Bernhardt TG (2014) Beta-lactam antibiotics induce a lethal malfunctioning of the bacterial cell wall synthesis machinery. *Cell* 159(6):1300–1311.
32. Heidelberg JF, et al. (2000) DNA sequence of both chromosomes of the cholera pathogen Vibrio cholerae. *Nature* 406(6795):477–483.
33. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162(1):156–159.
34. Desmarais SM, De Pedro MA, Cava F, Huang KC (2013) Peptidoglycan at its peaks: How chromatographic analyses can reveal bacterial cell wall structure and assembly. *Mol Microbiol* 89(1):1–13.
35. Dörr T, et al. (2014) A novel peptidoglycan binding protein crucial for PBP1A-mediated cell wall biogenesis in Vibrio cholerae. *PLoS Genet* 10(6):e1004433.
36. Sliusarenko O, Heinritz J, Emonet T, Jacobs-Wagner C (2011) High-throughput, subpixel precision analysis of bacterial morphogenesis and intracellular spatio-temporal dynamics. *Mol Microbiol* 80(3):612–627.
37. Donnenberg MS, Kaper JB (1991) Construction of an eae deletion mutant of enteropathogenic Escherichia coli by using a positive-selection suicide vector. *Infect Immun* 59(12):4310–4317.
38. Gibson DG, et al. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* 6(5):343–345.