# Sensor histidine kinase is a β-lactam receptor and induces resistance to β-lactam antibiotics

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**β-Lactams disrupt bacterial cell wall synthesis, and these agents are the most widely used antibiotics. One of the principle mechanisms by which bacteria resist the action of β-lactams is by producing β-lactamases, enzymes that degrade β-lactams. In Gram-negative bacteria, production of β-lactamases is often induced in response to the antibiotic-associated damage to the cell wall. Here, we have identified a previously unidentified mechanism that governs β-lactamase production. In the Gram-negative enteric pathogen *Vibrio parahaemolyticus*, we found a histidine kinase/response regulator pair (VbrK/VbrR) that controls expression of a β-lactamase. Mutants lacking either VbrK or VbrR do not produce the β-lactamase and are no longer resistant to β-lactam antibiotics. Notably, VbrK autophosphorylation is activated by β-lactam antibiotics, but not by other lactams. However, single amino acid substitutions in the putative periplasmic binding pocket of VbrK leads its phosphorylation in response to both β-lactam and other lactams, suggesting that this kinase is a β-lactam receptor that can directly detect β-lactam antibiotics instead of detecting the damage to cell wall resulting from β-lactams. In strong support of this idea, we found that purified periplasmic sensor domain of VbrK binds penicillin, and that such binding is critical for VbrK autophosphorylation and β-lactamase production. Direct recognition of β-lactam antibiotics by a histidine kinase receptor may represent an evolutionarily favorable mechanism to defend against β-lactam antibiotics.**

Vibrio | histidine kinase | β-lactam receptor | β-lactamase

The β-lactams are the most widely used and among the most valuable classes of antibiotics (1). These agents contain a β-lactam ring in their structures and inhibit the activity of transpeptidases or penicillin binding proteins (PBPs), the essential enzymes for the biosynthesis of peptidoglycan (PG) (2, 3), thereby damaging the integrity of bacterial cell wall. One of the principal mechanisms by which bacteria develop resistance to β-lactam antibiotics is the production of intrinsic or horizontally acquired β-lactamases that can degrade and inactivate β-lactams (4).

In many bacterial species, β-lactamase expression is inducible in response to β-lactam antibiotic treatment. For example, in Gram-positive bacteria, β-lactamase can be induced directly by β-lactam antibiotics or indirectly by muropeptides that are released from PG after β-lactam treatment (5). In Gram-negative bacteria (e.g., Enterobacteriaceae), muropeptide produced after β-lactam treatment can also induce the expression β-lactamase (6, 7). In addition to β-lactam treatment, β-lactamase expression in Gram-negative bacteria can also be induced by the changes of growth rate (8, 9). The direct role of β-lactam in inducing the expression of β-lactamase in Gram-negative bacteria has not been reported.

Two-component systems (TCSs), which are typically composed of a sensor histidine kinase (HK) and a response regulator (RR), are widely present in many bacterial species (10, 11). Typically, environmental signals are sensed by histidine kinase, leading to its autophosphorylation and subsequent phosphoryl transfer to its cognate response regulator (12, 13). Upon phosphorylation, a response regulator usually controls the expression of genes for adaptation to changing environment. Certain TCSs have been shown to contribute to antibiotic (e.g., glycopeptide) resistance. For example, VanS, the histidine kinase of VanSR TCS, directly binds to vancomycin, leading to the expression of genes that are required for the synthesis of alternate peptidoglycan precursors that have low affinity for vancomycin (14–16). Histidine kinases also contribute to the resistance to the antimicrobial peptides, e.g., LL-37, which activates the histidine kinase PhoQ by directly binding to the acidic surface of the PhoQ periplasmic domain (17). TCSs (e.g., BlrAB in *Aeromonas* or CreBC in *Escherichia coli* and *Pseudomonas*) have also been shown to be involved in the expression of β-lactamase and thus are important for β-lactam resistance (3, 9, 18). The response regulator of BlrAB or CreBC triggers the expression of β-lactamase by recognizing the signature sequences (cre/blr-tag: TTCACnnnnnnTTCAC) located in the promoter of β-lactamase gene (3, 9, 18). Despite the evidence that TCS plays an important role in the induction of β-lactamase expression, the identity of the cues that are recognized and transmitted by TCS to control β-lactamase expression remain completely unknown (19).

Here, we reported a previously unidentified mechanism that governs β-lactamase production in Gram-negative bacterium *Vibrio parahaemolyticus*, the leading cause of seafood-borne diarrheal disease worldwide. Most isolates of *V. parahaemolyticus* from both clinical and environmental settings exhibit resistance to β-lactam antibiotics, thereby limiting treatment options. At the time we initiated these studies, there was minimal knowledge of the mechanisms underlying *V. parahaemolyticus* resistance to β-lactam antibiotics. However, a recent report revealed that essentially all *V. parahaemolyticus* isolates encode a class A chromosomal carbenicillin-hydrolyzing (CARB) β-lactamase ($bla_{V110}$) (20). We

## Significance

**Bacteria can produce β-lactamases, enzymes that destroy β-lactam antibiotics and thereby resist these potent antibiotics that target cell wall synthesis. Production of β-lactamases is often controlled by β-lactam-induced perturbations in the cell wall. Here, we have identified a new mechanism controlling β-lactamase production. We found a signaling system in which a membrane-associated histidine kinase directly binds β-lactams, triggering the expression of a β-lactamase and resistance to β-lactam antibiotics. Direct sensing of β-lactam antibiotics may enable sufficiently rapid induction of β-lactamase to degrade β-lactam antibiotics before the integrity of the cell wall is disturbed.**

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independently identified this chromosome II-encoded enzyme as part of the regulon of a novel TCS (VbrK/VbrR) that controls *V. parahaemolyticus* resistance to β-lactam antibiotics. Notably, we show that the sensor histidine kinase, VbrK, in this system detects β-lactam antibiotics via direct binding and transmits the signal to the response regulator, VbrR, to control the expression of this CARB β-lactamase gene.

## Results

**Identification of a Histidine Kinase That Is Essential for *V. parahaemolyticus* Resistance to β-Lactams.** *V. parahaemolyticus* isolated from both environmental and clinical settings are routinely resistant to β-lactam antibiotics. As part of an ongoing project to characterize the roles of *V. parahaemolyticus'* TCSs, we attempted to delete all 32 of the predicted histidine kinases encoded in the pathogen's genome. We succeeded in creating 31 mutants, each containing a deletion of an individual ORF encoding a putative histidine kinase. Notably, we found that one of the mutants, harboring a deletion of *vpa920*, could not grow on LB agar plates containing carbenicillin (Fig. S1*A*), ampicillin, or penicillin (Fig. S1*B*). Complementation of the Δ*vpa0920* mutant with a wild-type *vpa0920 in trans* restored resistance to carbenicillin (Fig. S1*C*), indicating that *vpa0920* is important for carbenicillin resistance. These results indicate that the predicted histidine sensor kinase *vpa0920* (now designated as VbrK for *Vibrio* β-lactam resistance sensor kinase) is essential for *V. parahaemolyticus* β-lactam resistance. VbrK is composed of 484 aa, of which amino acids 14–275 contain the domain of DUF3404 with unknown functions. The region encompassing amino acids 274–332 is predicted to be the histidine kinase (HisKA) domain, and the region from amino acids 390–475 is the ATPase domain. VbrK is present in all *Vibrio* species with 60–90% sequence identity across the species. VbrK is predicted to be a membrane protein with two potential transmembrane regions located at amino acids 9–16 and amino acids 240–260, respectively (Fig. S2*A*). Amino acids 25–240 are predicted to be located extracellularly, whereas amino acids 261–484 are predicted to be located intracellularly. We expressed VbrK with a C-terminal 6xHis tag in Δ*vbrK*, and our results showed that VbrK_6xHis was exclusively present in the membrane extracts (Fig. S2*B*), experimentally demonstrating that VbrK is a membrane protein. In the downstream of *vbrK*, there is an ORF (*vpa0919*) encoding predicted response regulator (designated as *vbrR* for *Vibrio* β-lactam resistance response regulator). As expected, VbrR_6xHis is a cytosolic protein (Fig. S2*B*).

**Identification of Genes That Are Regulated by VbrK.** We carried out RNA sequencing (RNA-seq) experiments to identify the VbrK regulon, with the ultimate goal of elucidating the mechanisms by which this histidine kinase regulates β-lactam resistance. In these experiments, we compared the transcriptomes of wild-type and Δ*vbrK V. parahaemolyticus* grown in LB. We found that 230 (4.9%) and 235 (5.0%) of the 4,868 annotated *V. parahaemolyticus* ORFs were significantly up- and down-regulated (more than threefold), respectively, in the Δ*vbrK* strain compared with WT. To gain insight into the pathways that are regulated by VbrK, we grouped the differentially expressed genes based on their GO annotations. This analysis revealed that several GO categories were significantly reduced (more than twofold) in the Δ*vbrK* mutant compared with the WT, including those involving peptidoglycan biosynthesis, vancomycin resistance, pyruvate metabolism, and β-lactam resistance (Fig. 1 and Table S1). Genes in the β-lactam resistance category include *vp0040* encoding TetR transcriptional factor, *vp0039* encoding HlyD membrane fusion protein, *vp0454* encoding penicillin binding protein 3, *vp0545* encoding β-hexosaminidase, *vp2751* encoding penicillin binding protein 1A, and *vpa0477* encoding a class A β-lactamase. The latter gene had the largest fold change (~3.5 folds) between the WT and Δ*vbrK* mutant, and encodes a class A CARB β-lactamase (*blaA*) with 98.36% identity in nucleotide sequence and 98.59% identity in amino acid sequences to the recently described $bla_{V110}$ (20). We performed RT-PCR analyses to verify that *blaA* is regulated by VbrK. Notably, expression of *blaA* was dependent on *VbrK*; we observed *blaA* expression in the WT strain (Fig. 2*A*, lanes 1 and 2), but not in the Δ*vbrK* strain (Fig. 2*A*, lanes 3 and 4). Complementation of Δ*vbrK* with the WT *vbrK* gene *in trans* restored the expression of *blaA* (Fig. 2*A*, lanes 7 and 8), indicating that *VbrK* is essential for the expression of *blaA*.

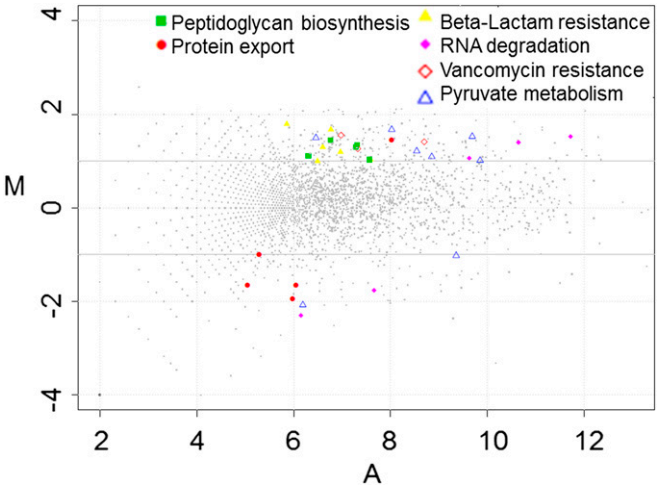


**Fig. 1.** RNA-seq analysis of genes that are regulated by VbrK. MA plots of relative transcript abundance between WT and Δ*vbrK*. The $\log_2$ of the ratio of abundances of each transcript between the two strains (M in *y* axis) is plotted against the average $\log_2$ of abundance of that transcript in both strains (A in *x* axis).

**VbrK Is Essential for *V. parahaemolyticus* β-Lactamase Activity.** Because VbrK is critical for the expression of *blaA* (Fig. 2*A*), and *blaA* is predicted to encode a β-lactamase, we hypothesized that VbrK is also essential for β-lactamase activity. β-Lactamase activity was determined by measuring the ability of bacterial cell lysate to hydrolyze the substrate nitrocefin, which rapidly changes color from yellow to red upon degradation. As seen in Fig. 2*B*, lysates from WT hydrolyzed nitrocefin yielding a red color (Fig. 2*B*) detectable as an increase in $OD_{490}$ (Fig. 2*C*), whereas the lysate from the Δ*vbrK* mutant did not hydrolyze nitrocefin (Fig. 2*B*), yielding a baseline $OD_{490}$ indistinguishable from a Δ*blaA* mutant (Fig. 2*C*). Complementation of the Δ*vbrK* mutant with a WT *vbrK* gene *in trans* restored the strain's ability to hydrolyze nitrocefin (Fig. 2 *B* and *C*) demonstrating that *VbrK* is essential for *V. parahaemolyticus* β-lactamase activity. Furthermore, deletion of *blaA* abolished β-lactamase activity (Fig. 2 *B* and *C*) and β-lactam resistance (Fig. S3). However, expression of *blaA* in the Δ*vbrK* mutant restored the strain's ability to produce β-lactamase and resist β-lactam antibiotics (Fig. 2 *B* and *C*). Thus, these observations strongly suggest that VbrK controls β-lactam resistance via regulating the expression of *blaA* that encodes a functional β-lactamase.

**Transcription of *blaA* and Production of β-Lactamase Require β-Lactam Treatment.** We have shown that *blaA* is expressed in a VbrK-dependent fashion when *V. parahaemolyticus* is grown in LB containing carbenicillin (Fig. 2). We hypothesized that *blaA* transcription is regulated by β-lactams. To eliminate the confounding factors in LB, we grew WT in minimum (M9) medium with or without carbenicillin and determined the expression of *blaA* by RT-PCR. The results showed that *blaA* was transcribed in the presence of carbenicillin, whereas it was not transcribed in the absence of carbenicillin (Fig. 3*A*). We further showed that

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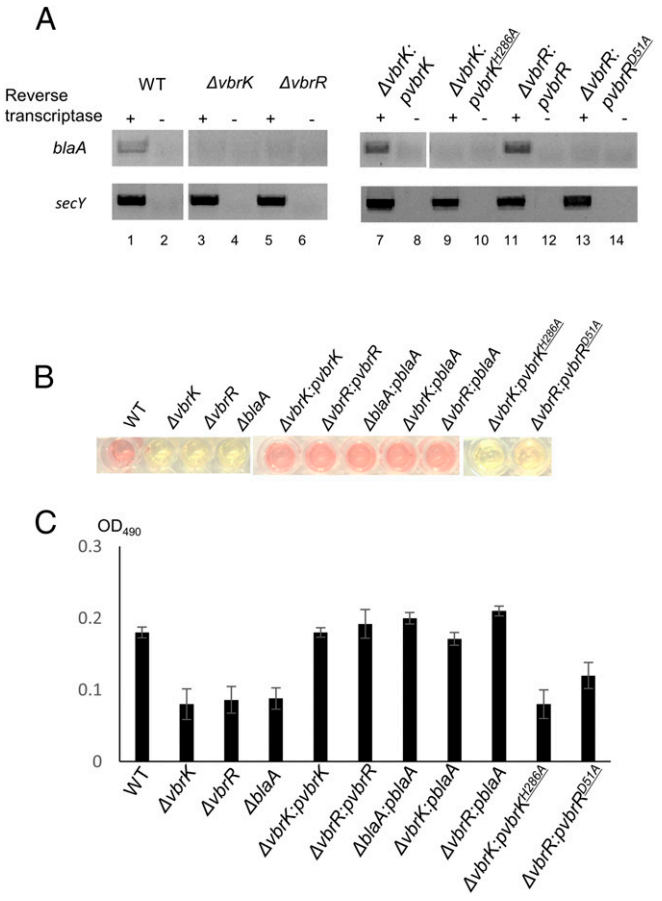


**Fig. 2.** The role of VbrK/VbrR in the expression of β-lactamase. Indicated strains were cultured in LB. (*A*) RT-PCR analysis of *blaA* expression (*Upper*) with *secY* as an internal control (*Lower*). (*B*) β-lactamase activity was measured by mixing *V. parahaemolyticus* whole-cell lysate with the β-lactamase substrate (nitrocefin). Once hydrolyzed, the degraded nitrocefin compound rapidly changes color from yellow to red. (*C*) $OD_{490}$ was measured to reflect the relative amount of β-lactamase produced by each individual bacterial strain.

β-lactamase activity in WT was controlled by carbenicillin in a dose-dependent manner (Fig. 3*B* and Fig. S4*A*). In addition to carbenicillin, β-lactamase activity can also be induced by other β-lactams, including ampicillin and penicillin (Fig. S4*B*). We used 50 μg/mL carbenicillin in subsequent studies. β-Lactamase activity was gradually increased from 0 to 3 h after β-lactam was supplied (Fig. 3*C* and Fig. S4*C*), indicating that β-lactamase activity was controlled by carbenicillin in a time-dependent manner. Although β-lactamase activity is only slightly increased at 1 h after carbenicillin was added, such an increase was sufficient to trigger β-lactam resistance, because the growth curve of WT in the presence of carbenicillin was similar to that in the absence of carbenicillin (Fig. S4*F*), whereas cfu of Δ*vbrK* was decreased in the presence of carbenicillin (Fig. S4*F*) in the first hour of growth. These results suggest that carbenicillin triggers VbrK-dependent β-lactam resistance quickly (as early as 10 min after carbenicillin was added). β-Lactams contain a four-ring atom structure, whereas other lactams contain five-ring atoms (γ-lactam), six-ring atoms (δ-lactam), seven-ring atoms (ε-lactam), and nine-ring atoms (Fig. S4*E*). We found that β-lactamase activity was only triggered by the β-lactam, but not other lactams (Fig. 3*D* and Fig. S4*D*).

**VbrK Can Be Phosphorylated on Its Conserved Histidine Residue in Vivo.** Histidine kinases usually contain a conserved histidine residue that can be phosphorylated, and such phosphorylation is essential for its signal transducing function. We determined VbrK phosphorylation using Phos-tag, which preferentially binds phosphorylated protein, retarding migration in SDS/PAGE gels (21). In this experiment, we grew bacteria in LB with or without carbenicillin, and whole-cell lysate was obtained for SDS/PAGE and Western blot. In the presence of carbenicillin, a shifted band (VbrK-P) of VbrK_6xHis was detected when proteins were separated in Phos-tag gel (Fig. 4*A*), indicating that VbrK was phosphorylated. When the conserved histidine residue at the 286th amino acid of VbrK was substituted with alanine (H286A), VbrK phosphorylation was abolished (Fig. 4*A*), indicating that VbrK phosphorylation is dependent on its conserved histidine residue. In the absence of carbenicillin, phosphorylation of VbrK was not detected (Fig. 4*A*). Furthermore, the substitution of histidine with alanine in BsrK abolished *blaA* expression (Fig. 2*A*, lanes 9 and 10), β-lactamase activity (Fig. 2 *B* and *C*), and β-lactam resistance (Fig. S1*C*), indicating that VbrK phosphorylation is functionally important for *V. parahaemolyticus* β-lactam resistance.

**VbrK Forms a TCS with Its Response Regulator VbrR.** Deletion of *vbrR*, the putative response regulator located adjacent to *vbrK* on the chromosome, abolished *blaA* gene expression (Fig. 2*A*, lanes 5 and 6), β-lactamase activity (Fig. 2 *B* and *C*), and β-lactam resistance (Fig. S1*C*). Complementation of Δ*vbrR* with a WT *vbrR* gene *in trans* restored the *blaA* gene transcription (Fig. 2*A*, lanes 11 and 12), β-lactamase activity (Fig. 2 *B* and *C*), and β-lactam resistance (Fig. S1*C*), indicating that *VbrR* is essential for β-lactam resistance. Together, these observations strongly suggest that VbrK and VbrR form a TCS that controls cellular production of β-lactamase. To determine if VbrR can be phosphorylated, we expressed VbrR_6xHis in Δ*vbrR*, and phosphorylation was assessed using the Phos-tag assay. A shifted band of VbrR was observed when bacteria were cultured in the presence of carbenicillin (Fig. 4*B*); in contrast, the shifted band was not observed in the absence of carbenicillin (Fig. 4*B*), indicating that VbrR was phosphorylated in response to carbenicillin. More

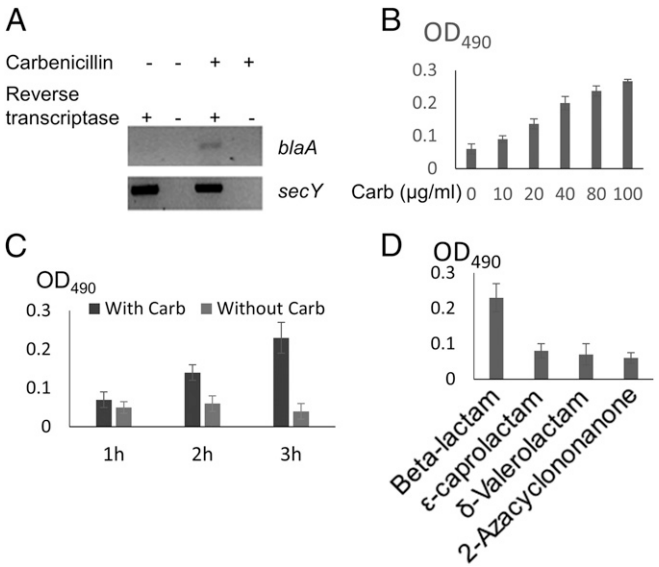


**Fig. 3.** Regulation of *blaA* transcription and β-lactamase activity by β-lactam. (*A*) WT *V. parahaemolyticus* was cultured in M9. RT-PCR analysis of the *blaA* transcription in the presence or absence of carbenicillin. (*B*) The activity of β-lactamase activity in WT *V. parahaemolyticus* treated with increasing dose of carbenicillin. (*C*) β-Lactamase activity was measured at different time points after carbenicillin was added. (*D*) β-Lactamase activity in WT *V. parahaemolyticus* treated with β-lactam and other lactams including ε-caprolactam, δ-valerolactam, and 2-azacyclononanone.

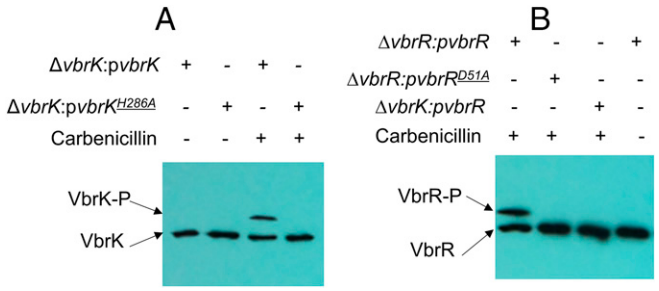


**Fig. 4.** The role of β-lactam in the phosphorylation of VbrK and VbrR when *V. parahaemolyticus* was cultured in LB. (*A*) Phosphorylation analysis of VbrK or VbrK$^{H286A}$ in the indicated strains in the presence or absence of carbenicillin. (*B*) Phosphorylation analysis of VbrR or VbrR$^{D51A}$ in the indicated strains in the presence or absence of carbenicillin.

importantly, when VbrR was expressed in Δ*vbrK*, the shifted band of VbrR was not observed (Fig. 4*B*), indicating that VbrR phosphorylation requires VbrK, and VbrK and VbrR form a functional TCS regulating β-lactam resistance. To determine if phosphorylation of VbrR is important for its function to induce β-lactamase expression, we substituted the conserved aspartate residue at the 51st amino acid of VbrR (a predicted phosphorylation site) with alanine and expressed the VbrR$^{D51A}$ protein in the Δ*vbrR* strain. As expected, D51A substitution completely eliminated the phosphorylation of VbrR (Fig. 4*B*). Furthermore, there was no *blaA* expression (Fig. 2*A*, lanes 13 and 14), β-lactamase activity (Fig. 2 *B* and *C*), and β-lactam resistance (Fig. S1*C*) in the Δ*vbrR:pVbrR*$^{D51A}$ strain, indicating that VbrR phosphorylation by VbrK is essential for β-lactam resistance.

**Mutation in the Extracellular Region of VbrK Alters Its Recognition Specificity for Lactams.** Because β-lactamase activity was triggered only by β-lactams (Fig. 3*D* and Fig. S4*D*), we determined VbrK phosphorylation under these conditions. In these experiments, we obtained whole-cell lysates of bacteria grown in M9 medium supplemented with different lactams. Phosphorylation of VbrK was observed as early as 10 min after carbenicillin was supplied (Fig. 5*A*). In the subsequent studies, we harvested proteins 3 h after carbenicillin was added to get more phosphorylated proteins. VbrK was phosphorylated when *V. parahaemolyticus* was treated with β-lactam, but not with other lactams (Fig. 5*B*), indicating that β-lactam triggers β-lactamase expression via phosphorylation of VbrK. VbrK phosphorylation upon treatment with β-lactam does not necessary indicate that VbrK is directly responding to β-lactam; it is possible, e.g., that β-lactam treatment produces peptidoglycan breakdown products (i.e., muropeptides), which serve as the trigger for VbrK phosphorylation. To test the hypothesis that VbrK is a β-lactam receptor that directly detects β-lactam, we mutated the conserved amino acids in the extracellular region predicted to form a pocket (Fig. S5) to serve as the signal recognition site. Single-mutation L82A in VbrK completely abolished the ability to produce β-lactamase (Fig. 5*F* and Fig. S6). Furthermore, VbrK with L82A mutation cannot be phosphorylated upon treatment with β-lactam or other lactams (Fig. 5*C*), suggesting that L82 is critical for β-lactam recognition. Notably, the mutant harboring VbrK$^{L95A}$ phosphorylated VbrK (Fig. 5*D*) and yielded expression of β-lactamase (Fig. 5*F* and Fig. S6) in response to ε-caprolactam and 2-azacyclononanone in addition to carbenicillin. Similarly, the mutant harboring VbrK$^{P125A}$ phosphorylated VbrK and expression of β-lactamase (Fig. 5 *E* and *F* and Fig. S6) in response to δ-valerolactam as well as carbenicillin. Because ε-caprolactam, 2-azacyclononanone, and δ-valerolactam do not produce peptidoglycan breakdown products, but have the capacity to induce phosphorylation of VbrK containing amino acid substitutions in its periplasmic pocket domain (Fig. 5 *D* and *E*), it is unlikely that peptidoglycan breakdown products activate VbrK phosphorylation. Taken together, these findings strongly suggest that VbrK is a bona fide β-lactam receptor.

**VbrK Is Phosphorylated in Vitro.** To further investigate whether VbrK is sufficient to interact with and respond (via autophosphorylation) to β-lactams, we isolated membrane extracts from an *E. coli* strain expressing VbrK. The membrane extracts were treated with both carbenicillin and ATP in vitro and VbrK phosphorylation was assessed with the Phos-tag assay. A shifted band of VbrK was observed when the membrane extract was treated with both carbenicillin and ATP; the intensity of such a shifted band was much lower without carbenicillin treatment (Fig. 6*A*, lanes 4 and 5), indicating that carbenicillin directly triggers the phosphorylation of VbrK. Carbenicillin-dependent phosphorylation requires the presence of ATP (Fig. 6*A*, lane 1). Furthermore, carbenicillin did not trigger phosphorylation of a similar membrane extract from an *E. coli* strain expressing VbrK$^{H286A}$ (Fig. 6*A*, lanes 2 and 3), strongly suggesting that the conserved histidine residue in VbrK is required for carbenicillin to trigger the protein's autophosphorylation. Treatment with other lactams did not induce the phosphorylation of VbrK (Fig. 6*B*). Taken together, these results reveal that VbrK phosphorylation is directly triggered by β-lactam, but not by other lactams.

**Extracellular Sensor Domain of VbrK Directly Binds Penicillin.** To further elucidate the mechanisms by which β-lactam triggers the phosphorylation of VbrK, we determined if penicillin can directly bind to the periplasmic sensor domain of VbrK (amino acids 25–240, VbrK$^{SD}$). We purified GST-VbrK$^{SD}$, GST, and GST$^{SD_L82A}$ to homogeneity as shown by Coomassie blue staining (Fig. S7). A microtiter plate was subsequently coated with these purified proteins, and penicillin binding to the precoated microtiter plate was measured using anti-penicillin antibody and HRP-conjugated secondary antibody. Blue color formation and increases of $OD_{370}$ indicate that penicillin binds to the microtiter plate. Addition of 50 ng penicillin to the wells precoated with GST-VbrK$^{SD}$ results in slight blue color formation and approximately twofold increase of $OD_{370}$ compared

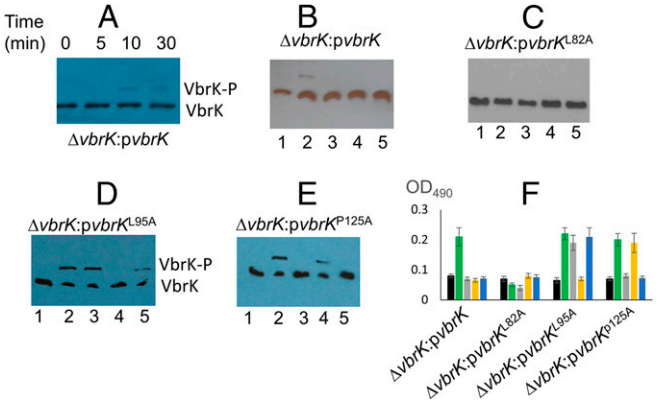


**Fig. 5.** Identification of VbrK amino acid residues essential for specific recognition of different lactams. Indicated strains were cultured in M9. (*A*) VbrK phosphorylation was measured at different time points after carbenicillin was added to the culture. Phosphorylation of VbrK (*B*), VbrK$^{L82A}$ (*C*), VbrK$^{L95A}$ (*D*), and VbrK$^{P125A}$ (*E*) in *V. parahaemolyticus* in the presence of carbenicillin (lane 2), ε-caprolactam (lane 3), δ-valerolactam (lane 4), and 2-azacyclononanone (lane 5) or in the absence of any lactams (lane 1). (*F*) Indicated strains were untreated (black bar) or treated with carbenicillin (green bar), ε-caprolactam (gray bar), δ-valerolactam (yellow bar), and 2-azacyclononanone (blue bar), and $OD_{490}$ was measured to indicate β-lactamase activity.

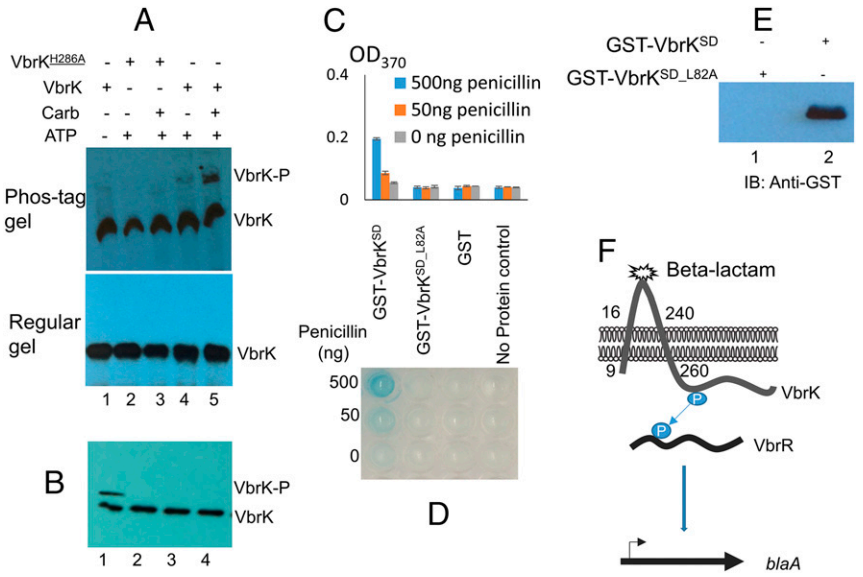


**Fig. 6.** In vitro analysis of VbrK phosphorylation and interaction between VbrK and β-lactam. (*A*) Membrane extracts from *E. coli* expressing 6xHis-tagged VbrK (lanes 4 and 5) or VbrK$^{H286A}$ (lanes 2 and 3) were treated with carbenicillin (lanes 3 and 5) or left without treatment of carbenicillin (lanes 2 and 4). No addition of ATP was used as a negative control (lane 1). Following treatment, each sample was resolved in the Phos-tag gel (*Upper*) or regular gel (*Lower*) and blotted with anti-His antibody. (*B*) In vitro phosphorylation assay for VbrK treated with carbenicillin (lane 1), ε-caprolactam (lane 2), δ-valerolactam (lane 3), and 2-azacyclononanone (lane 4). (*C* and *D*) Analysis of binding between the VbrK sensor domain (VbrK$^{SD}$) and penicillin. Microtiter plate was precoated with GST-VbrK$^{SD}$, GST-VbrK$^{SD_L82A}$, or GST, or without protein coating. Penicillin (50 ng or 500 ng) was added to the precoated plate. Following sufficient washing, anti-penicillin antibody and HRP secondary antibody were added. Blue color formation was observed (*D*) and $OD_{370}$ was measured (*C*) to reflect the relative amount of penicillin that binds to the coated proteins. (*E*) Pulldown assay was performed by addition of equal amount of GST-VbrK$^{SD}$ (lane 2) or GST-VbrK$^{SD_L82A}$ (lane 1) into the protein G Sepharose immobilized with penicillin and anti-penicillin antibody. (*E*) After sufficient washing, protein elution was probed with anti-GST antibody. (*F*) Schematic model for VbrK/VbrR-mediated β-lactamase expression.

with the wells without addition of penicillin (Fig. 6 *C* and *D*). More significant blue color and approximately fourfold increase in $OD_{370}$ was observed when 500 ng penicillin was added (Fig. 6 *C* and *D*), indicating that penicillin directly binds the VbrK sensor domain in a concentration-dependent manner. Such binding was specific because addition of penicillin to the wells precoated with purified GST alone did not result in a significant blue color and increase of $OD_{370}$ (Fig. 6 *C* and *D*). Furthermore, addition of penicillin to the wells precoated with GST-VbrK$^{SD_L82A}$ did not result in an increase in $OD_{370}$ (Fig. 6 *C* and *D*), indicating that L82A mutation in the sensor domain of VbrK abolished its binding with penicillin. To further determine the binding between penicillin and VbrK$^{SD}$, we performed a pulldown assay in which penicillin and anti-penicillin antibody were immobilized into a protein G Sepharose followed by addition of GST-VbrK$^{SD}$ or GST-VbrK$^{SD_L82A}$. The results showed that the elution contained GST-VbrK$^{SD}$ but not GST-VbrK$^{SD_L82A}$, as detected by anti-GST antibody (Fig. 6*E*), further verifying that the sensor domain of VbrK binds penicillin, and L82 is important for such binding. Coupled with the results that L82A mutation abolished VbrK phosphorylation and β-lactamase production in response to β-lactam treatment (Fig. 5 *C* and *F*), we concluded that β-lactam triggers the phosphorylation of VbrK by directly binding to the sensor domain of VbrK.

## Discussion

In this study, we show that histidine kinase VbrK is a β-lactam receptor that triggers the expression of β-lactamase in response to β-lactam antibiotics in a Gram-negative bacterium *V. parahaemolyticus*. This conclusion was supported by the results that (*i*) deletion of VbrK or its response regulator VbrR greatly reduced the expression of β-lactamase and abolished the β-lactam resistance; (*ii*) VbrK activation is specifically triggered by β-lactam antibiotics, but not other lactam; and (*iii*) single amino acid substitution in the predicted sensor domain of VbrK alters its specificity to lactams. Based on these results, we propose a model in which the membrane-associated histidine kinase VbrK directly binds β-lactam antibiotics, leading to the VbrK phosphorylation, phosphoryl transfer to VbrR, and, ultimately, the expression of β-lactamase (Fig. 6*F*).

In Gram-positive bacteria (e.g., *Staphylococcus aureus* and *Bacillus lichenformis*), β-lactam resistance is mediated by the expression of β-lactamase (*blaZ*) or the production penicillin binding protein 2a (PBP2a or MecA), which has low affinity for β-lactam antibiotics (22–25). Expression of *blaZ* is initiated upon the direct interaction of β-lactam with the membrane-associated β-lactam receptor (BlaR1). Here, we show that Gram-negative bacterium, *V. parahaemolyticus*, uses a novel β-lactam receptor VbrK to induce the expression of β-lactamase. Although both BlaR1 and VbrK are membrane associated and recognize β-lactam via direct binding, the subsequent signaling pathway for β-lactamase expression is different. Upon binding to the β-lactam, BlaR1 becomes irreversibly acylated, which results in the autoproteolytic cleavage within the cytoplasmic domain of BlaR1. The cleaved form of BlaR1 is an active metalloprotease and can inactivate the repressor BlaI, leading to the dissociation of BlaI from the promoter of *blaZ* and consequent expression of *blaZ* (26, 27). In contrast, VbrK becomes autophosphorylated upon binding to β-lactams. The phosphoryl group is subsequently transferred to the response regulator VbrR to control the expression of β-lactamase. When we screened for VbrK mutants that can detect other lactams, we identified P125A and L95A. These two substitutions may modify the β-lactam binding pocket to allow for the recognition of other lactams, leading to the subsequent VbrK phosphorylation, phosphoryl transfer to the VbrR, and production of β-lactamase. We also identified L82A mutation that abolished the ability of VbrK to bind to β-lactam and induce the expression of β-lactamase, suggesting that L82 is critical for VbrK binding to β-lactam. Alternatively, L82A mutation may alter appropriate protein folding, leading to an inactive VbrK. β-Lactam binding to the pocket of VbrK may result in a conformational change that is transmitted to the HisKA domain, leading to a closer association with the ATPase domain and consequent phosphorylation of the histidine residue.

β-Lactamase in Gram-negative bacteria is often induced by the β-lactam-associated perturbation of cell wall integrity (6). In Enterobacteriaceae, this induction mechanism is complex and involves multiple proteins and steps (6, 7). β-Lactam antibiotics disrupt the murein biosynthesis, leading to the accumulation of muropeptides in the periplasm. These muropeptides can be transported through the inner membrane channel created by a membrane protein AmpG. Within the cytoplasm, muropeptides bind the transcriptional factor AmpR, and such binding activates AmpR's DNA binding activity, leading to the transcription and expression of AmpC β-lactamase (28). It is conceivable that such multistep

defensive mechanisms occur at the cost of damages in the cell wall integrity. Here, we provide evidence that Gram-negative bacterium *V. parahaemolyticus* can use a histidine kinase VbrK to directly sense β-lactam antibiotics, leading to the production of β-lactamase. Direct sensing of β-lactam antibiotic to induce the expression of β-lactamase could occur potentially in a rapid fashion and at no cost of damages in the cell wall integrity, which may represent a novel mechanism to defend against β-lactam antibiotics.

Although TCSs are present in a wide range of bacterial species, and their function in the recognition and transduction of environmental signals has been well documented, very few physiological signals for the histidine kinases are known. Particularly, there is only one antibiotic, vancomycin, that has been reported to be the signal molecule for the histidine kinase VanS (15, 16). Because of the lack of the defined signals that histidine kinase can recognize, structural analyses of ligand–histidine kinase interactions are very limited (11, 29, 30). Defining the ligand, β-lactam, as the signal molecule for the histidine kinase VbrK provides molecular tools to study the structure–activity relationship of VbrK and the mechanisms by which histidine kinase is phosphorylated and activated. Importantly, VbrK is present in almost all *Vibrio* species, and the residues (L82, L95, and P125) that are responsible for specific recognition of lactams are conserved in different *Vibrio* species (Fig. S8). The gene encoding β-lactamase was also found in many *Vibrio* species, including non-O1/non-O139 *Vibrio cholerae*, *Vibrio harveyi*, and *Vibrio alginolyticus* (31, 32). These results suggest that direct recognition of β-lactam antibiotic by VbrK is a well-conserved mechanism to induce β-lactamase gene expression in *Vibrio* species. Application of β-lactamase inhibitors could potentially reduce the prevalence of β-lactam resistance (33, 34). However, the inhibitors exhibit variable affinity to different β-lactamases, and the efficacy may be compromised by the overwhelming quantity of β-lactamases they produced. Defining the VbrK/VbrR regulatory pathway that controls the expression of β-lactamase would provide a promising target to inhibit β-lactamase production and β-lactam resistance. Lead compounds that can inhibit the activity of histidine kinase have been identified for a number of TCSs (35–38). There is future promise in rationally designing VbrK inhibitors to enhance the efficacy of β-lactam antibiotics in treating infections caused by *Vibrio* species.

## Materials and Methods

The strains and primers used in this study are shown in Tables S2 and S3. The detailed protocols for construction of mutants, complemented strains, RNA-seq, RT-PCR, β-lactamase assay, in vitro and in vivo phosphorylation assay, and penicillin binding assay are provided in *SI Materials and Methods*.

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