Wzb of *Vibrio vulnificus* represents a new group of low-molecular-weight protein tyrosine phosphatases with a unique insertion in the W-loop

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Protein tyrosine phosphorylation regulates the production of capsular polysaccharide, an essential virulence factor of the deadly pathogen *Vibrio vulnificus*. The process requires the protein tyrosine kinase Wzc and its cognate phosphatase Wzb, both of which are largely uncharacterized. Herein, we report the structures of Wzb of *V. vulnificus* (VvWzb) in free and ligand-bound forms. VvWzb belongs to the low-molecular-weight protein tyrosine phosphatase (LMWPTP) family. Interestingly, it contains an extra four-residue insertion in the W-loop, distinct from all known LMWPTPs. The W-loop of VvWzb protrudes from the protein body in the free structure, but undergoes significant conformational changes to fold toward the active site upon ligand binding. Deleting the four-residue insertion from the W-loop severely impaired the enzymatic activity of VvWzb, indicating its importance for optimal catalysis. However, mutating individual residues or even substituting the whole insertion with four alanine residues only modestly decreased the enzymatic activity, suggesting that the contribution of the insertion to catalysis is not determined by the sequence specificity. Furthermore, inserting the four residues into *Escherichia coli* Wzb at the corresponding position enhanced its activity as well, indicating that the four-residue insertion in the W-loop can act as a general activity enhancing element for other LMWPTPs. The novel W-loop type and phylogenetic analysis suggested that VvWzb and its homologs should be classified into a new group of LMWPTPs. Our study sheds new insight into the catalytic mechanism and structural diversity of the LMWPTP family and promotes the understanding of the protein tyrosine phosphorylation system in prokaryotes.

Phosphorylation on protein tyrosine is a posttranslational modification with essential roles in life. In eukaryotes, it has been extensively studied as an essential regulatory mechanism in various cellular processes (1). In contrast, protein tyrosine phosphorylation in prokaryotes was recognized much later than in eukaryotes (2). It regulates a couple of processes in prokaryotes, including polysaccharide synthesis, biofilm formation, and cell growth (3–6). With technique developments in recent years, more proteins with various functions have been identified to be tyrosine-phosphorylated in bacteria and archaea (7, 8). Now it has been recognized that protein tyrosine phosphorylation is a key regulation paradigm in the physiology and pathology of prokaryotes (9–11). Yet, our knowledge on prokaryotic protein tyrosine phosphorylation system is very limited.

*Vibrio vulnificus* is the most deadly foodborne pathogen, causing gastroenteritis, wound infection, and severe septicemia with an over 50% mortality rate (12). It is also an important pathogen for marine animals, causing huge economic loss in aquaculture industry (13). In *V. vulnificus*, protein tyrosine phosphorylation plays a key role in the production of capsular polysaccharide (CPS), one essential virulence factor for the pathogen to escape host immune response (14). The CPS synthesis gene locus of *V. vulnificus* is similar to that of the group 1 or 4 capsules of *Escherichia coli* (15). In *E. coli*, the relationship between tyrosine phosphorylation and CPS production has been basically elucidated. Three conserved proteins Wza–Wzb–Wzc are responsible for polymerization control and translocation of CPS (16). Specifically, Wza is the translocon of CPS, while Wzc and Wzb form a kinase/phosphatase pair required for high-level polymerization of polysaccharide (Fig. 1). Wzb belongs to the family of bacterial tyrosine kinases (BY kinases), a distinct class of kinases that have no homologs in eukaryotes. Wzc acts as an autokinase to phosphorylate its own tyrosine-rich C-terminal tail and other sites, resulting in a high phosphorylated Wzc, while the cognate phosphatase Wzb dephosphorylates Wzc to a low phosphorylation state. The cycling of Wzc between high and low phosphorylation states regulates the production of CPS, with the mechanism remaining elusive (16, 17). The exact situation in *V. vulnificus* is unknown yet but supposed to work in an analogous manner. Nevertheless, there are experiment evidences to directly connect tyrosine phosphorylation to CPS production in *V. vulnificus*. In studies of colony morphologies, Wzb of *V. vulnificus* (VvWzb) was found to be a switch of
morphological transition among opaque, translucent, and intermediate phases through controlling CPS production (18). Deletion of VvWzb led to CPS production defect and attenuated virulence of the bacterium. It was also observed that the expression levels of Wzb and Wzc were correlated to CPS production (19). So far, V. vulnificus Wzb and Wzc have not been biochemically characterized yet, which hinders further understanding of the phosphorylation events in this deadly pathogen. In this study, we would focus on the characterization of VvWzb.

VvWzb belongs to the family of low-molecular-weight protein tyrosine phosphatase (LMWPTP). LMWPTPs are small (ca. 18 kDa), evolutionally conserved and important enzymes involved in various cellular processes of all kingdoms of life (20). They have been extensively studied over decades for the functional importance and druggability potential. So far, a number of structures of LMWPTPs from both eukaryotes and prokaryotes have been determined (21–30). They share a common α/β fold; the active site crevice is composed of three characteristic loops, named the P-, D-, and W-loops, respectively. The P-loop bears the characteristic active-site motif “CXGNNXCRS” (X can be any amino acid) with the first cysteine as the catalytic nucleophile; this loop is at the bottom of the active site crevice, extensively interacting with the phosphate group of substrate, thus named P-loop. The D-loop forms a wall of the active site crevice. It contains a catalytically important aspartate residue, which acts as a general acid/base, and also contains a tyrosine residue to stabilize the phenyl ring of the substrate. The third is a variable loop, forming another wall of the active site crevice and being responsible for substrate recognition. In the structures of eukaryotic and some prokaryotic LMWPTPs, an aromatic residue such as tryptophan is located at the variable loop to interact with the phenyl ring of the tyrosine phosphate. Thus, this loop is conveniently called W-loop. Nevertheless, the study on the structure of E. coli Wzb (EcWzb) uncovered another type of W-loop in prokaryotic LMWPTPs, which lacks this important aromatic residue and thus recognizes substrate in a different way (23). Noticeably, this study also showed that the W-loop type is a good marker to classify LMWPTPs. Indeed, the phylogenetic analysis confirmed that LMWPTPs can be divided into two categories, in agreement with the W-loop forms they have (23, 31). Hereafter, we would name the two categories of LMWPTPs as group I and II, prototyped by mammalian LMWPTPs and EcWzb, respectively. Interestingly, VvWzb is different from any structurally known LMWPTP by harboring a unique short sequence insertion in the W-loop, excluding VvWzb from either group (Fig. 2A). It is unclear if this could challenge the current classification paradigm of LMWPTP family. It also remains to know if the unique insertion would endow VvWzb with some new structural and enzymatic features.

Herein, we report crystal structures of free VvWzb, VvWzb complexed with benzylphosphonate, and VvWzb(C9A) complexed with phosphate. The structural and enzymatic analyses of VvWzb shed new insight into the classification and catalytic mechanism of the LMWPTP family. Our study also provides critical structural information for antivirulence drug design against V. vulnificus.

Results
Overall structures of VvWzb in free and ligand-bound forms
VvWzb crystallized in the space group P2₁2₁2₁, with two protein molecules in one asymmetric unit. The two molecules formed a dimer in the crystal, with an interface area of about 900 Å². However, this seems to be a crystallographic dimer, since VvWzb existed as a monomer in solution, indicated by gel filtration (Fig. S1). The structural model was built in good quality (Table 1). VvWzb adopts a typical LMWPTP α/β fold (Fig. 2B). In the middle is a twisted parallel β-sheet formed by four β-strands in the sequence of β₁–β₃–β₁–β₂, flanking the central β-sheet are five α-helices, with α₃ at one side and α₁, α₂, α₅ at the other. The P-loop (between β₁ and α₁), D-loop (between β₄ and α₃), and W-loop (between β₂ and α₂) cluster together to form the active site crevice (Fig. 2B). Remarkably, the W-loop of VvWzb is longer than any known LMWPTP structure, due to a “40EKSR43” insertion (Fig. 2A). The W-loop adopts an extended conformation and protrudes from the protein body. The region harboring the unique insertion forms a small β-hairpin including two short β-strands (β₁’ and β₂’).

In the presence of benzylphosphonate, a compound that mimics the phenylphosphate moiety of the substrate phosphotyrosine, VvWzb and VvWzb(C9A) crystallized in space groups P1 and P2₁2₁2₁, respectively. Both crystals contained four protein molecules in one asymmetric unit. The dimeric form observed in the free VvWzb crystal did not show up again, and instead two adjacent molecules were covalently crosslinked via a disulfide bond formed by the Cys61 residues of both molecules. We were able to model one benzylphosphonate molecule (with occupancy of 1 and B-factor of 82.8, 142.8, 109.6, or 89.9 Å²) for each of the four protein chains in the wild-type (WT) structure (Figs. 2C and 3A). However, only phosphate ions were identified in VvWzb(C9A) structure, judged by the unambiguous electron density (Figs. 2D and 3B). The source of the phosphate ion was not defined, presumably from benzylphosphonate degradation or contamination. The overall structures of VvWzb-benzylphosphonate and VvWzb(C9A)-phosphate are similar to that of

![Figure 1. Schematic presentation of the interactions of Wza, Wzb, and Wzc.](Image)
free VvWzb, except that the W-loop underwent significant conformational changes to fold toward the active site in both structures (Fig. 2, C and D).

The active site crevice of VvWzb

Like other LMWPTPs, the active site crevice of free VvWzb is composed of the P-loop (residues Cys9–Ser16), D-loop (residues Lys115–Gln123), and W-loop (residues Ile37–Lys47) (Figs. 2A and 4A). The P-loop, containing the catalytic motif CVGNIICRS, with Cys9 as the catalytic nucleophile, forms the bottom part of the crevice. The residues Asp119, Tyr121, and Arg122 of the D-loop are stacked together above the P-loop to form a wall of the crevice, and the W-loop forms the opposite wall. The W-loop of VvWzb, which is longer than any

Figure 2. Sequence alignment and the overall structures. A, sequence alignment of LMWPTPs. The conserved residues are highlighted in red. The secondary structures as well as the P-, D-, and W-loops are indicated. B–D, structures of free VvWzb, VvWzb-benzylphosphonate complex, and VvWzb-C9A-phosphate complex, respectively. Secondary structures, the N- and C-termini are annotated. The P-, D-, and W-loops are colored in yellow, blue, and magenta, respectively. The ligands benzylphosphonate and phosphate are shown as sticks.
V. vulnificus Wzb structures

Table 1
Crystallographic statistics

| Parameter                          | VvWzb | VvWzb-benzylphosphonate | VvWzbC\(^{13}\)-phosphate |
|------------------------------------|-------|--------------------------|---------------------------|
| Data collection statistics        | BL19H1| BL19H1                   | BL18H1                    |
| X-ray source                       | BL19H1| BL19H1                   | BL18H1                    |
| Wavelength (Å)                     | 0.978 | 0.978                    | 0.9792                    |
| Space group                        | P2\(1\) 2\(1\) | P2\(1\) 2\(1\) | P2\(1\) 2\(1\) |
| Unit-cell dimensions (Å or °)      | a = 47.32, b = 48.74, c = 117.32 | a = 44.11, b = 51.01, c = 68.59 | a = 60.97, b = 68.30, c = 132.96 |
| Resolution (Å)                     | 58.66–1.711 (1.740–1.711) | 48.14–2.785 (2.833–2.785) | 66.48–1.211 (1.232–1.211) |
| Unique reflections                 | 29,957 (1506) | 13,744 (689) | 161,631 (7689) |
| Completeness (%)                   | 99.5 (99.7) | 97.9 (97.9) | 95.7 (92.6) |
| Mean I/σ(I)                        | 19.2 (2.3) | 16.4 (2.3) | 17.9 (2.2) |
| Multiplicity                       | 12.6 (12.9) | 3.6 (3.6) | 13.6 (13.9) |
| Rmerge (%)                         | 8.5 (118.8) | 4.7 (56.8) | 7.8 (107.7) |
| Rmeasure (%)                       | 8.8 (123.8) | 5.5 (66.6) | 8.1 (111.8) |
| Rfree (%)                          | 2.5 (34.3) | 2.9 (34.5) | 2.2 (29.7) |
| Rmeas (%                          | 99.9 (99.7) | 99.9 (99.7) | 99.9 (93.3) |
| Refinement statistics              | 19.9/22.3 | 18.6/22.6 | 14.3/16.9 |
| Resolution range (Å)               | 58.66–1.71 | 48.14–2.79 | 29.72–1.21 |
| Rwork (%)                          | 2.5 (34.3) | 2.9 (34.5) | 2.2 (29.7) |
| Rfree (%)                          | 2.9 (34.3) | 2.9 (34.5) | 2.2 (29.7) |
| Modeled residues                   | A: –1 to 146 | A: 1–146 | A: 1–146 |
|                                   | B: –1 to 146 | B: 0–146 | B: 1–146 |
|                                   | C: 1–146 | C: –2 to 146 | C: –2 to 146 |
|                                   | D: 1–146 | D: 1–146 | D: 1–146 |
| Ligands                           | 2 Cl⁻ | 4 benzylphosphonate | 4 phosphate ions, 1 glycerol |
| Water molecules                    | 270 | None | 679 |
| rmsd bond lengths (Å)              | 0.010 | 0.010 | 0.002 |
| rmsd bond angles (°)               | 1.02 | 1.12 | 0.59 |
| Ramachandran outliers              | 0 | 0 | 0 |
| PDB code                           | 7DHD | 7DHE | 7DHF |

Values in parentheses represent the highest resolution shell.

\(^{a}\) ~5% of the reflections were selected randomly for calculating Rfree.

known LMWPTP by harboring a four-residue insert (\(^{126}\)EKS\(^{129}\)), bulges out, with residues Ser42, Arg43, and Leu44 located at the tip region and residue Glu40 pointing toward the active site. Compared with the D-loop wall, which is relatively orthogonal to the P-loop bottom, the W-loop wall seems to be laid relatively orthogonal to the P-loop bottom, the W-loop wall seems to be laid flat. In such a configuration, the active site crevice of free VvWzb is wide and shallow, being readily accessible for the substrate (Fig. S2). Due to the presence of Glu40, the W-loop wall region of the crevice is negatively charged. In the absence of ligand, the supposed phosphate-binding site is occupied by a chloride ion and a few water molecules; they interact with the P- and D-loop residues via extensive hydrogen bonding (Fig. 4A).

In VvWzb-benzylphosphonate complex, the substrate-mimicking benzylphosphonate is located at the bottom of the active site crevice. Its phosphonate group forms an extensive hydrogen-bonding network with the P-loop, including the main chain amide groups of the residues Val10, Gly11, Asn12, Ile13, Cys14, and Arg15, as well as the sulfhydryl group of Cys9 and the side-chain guanidine group of Arg15. The benzyl group of benzylphosphonate is flanked by residues Val10 of the P-loop and Tyr121 of the D-loop and further stabilized by Leu44 of the W-loop and Ile13 of the P-loop via hydrophobic interactions (Fig. 4B). Compared with the free structure, the W-loop undergoes a dramatic conformational rearrangement from an open to a closed conformation. Consequently, Glu40 is no longer oriented toward the active site cavity but turns outside. Instead, side chains of Ser42 and Leu44 turn to face the active site, forming the W-loop wall. In addition, side chains of Asp119 and Tyr121 of the D-loop also made small local conformational changes to known LMWPTP by harboring a four-residue insert (\(^{126}\)EKS\(^{129}\)), bulges out, with residues Ser42, Arg43, and Leu44 located at the tip region and residue Glu40 pointing toward the active site. Compared with the D-loop wall, which is relatively orthogonal to the P-loop bottom, the W-loop wall seems to be laid flat. In such a configuration, the active site crevice of free VvWzb is wide and shallow, being readily accessible for the substrate (Fig. S2). Due to the presence of Glu40, the W-loop wall region of the crevice is negatively charged. In the absence of ligand, the supposed phosphate-binding site is occupied by a chloride ion and a few water molecules; they interact with the P- and D-loop residues via extensive hydrogen bonding (Fig. 4A).

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Figure 3. Ligands in the complex structures. A, benzylphosphonate in VvWzb-benzylphosphonate structure. B, phosphate in VvWzbC\(^{13}\)-phosphate structure. The representative refined omit Fo-Fc maps contoured at 2.5σ levels were shown as green meshes. The chemical structures of the ligands are displayed as insets. C, ligand-binding differences in VvWzb-benzylphosphonate and VvWzbC\(^{13}\)-phosphate structures. Benzylphosphonate, phosphate, the residues Cys9/Ala9 and Asp119 are shown as sticks and indicated.
interact with the ligand (Fig. 4, A and B). All these conformational changes significantly reconfigure the active site crevice, which is narrower and deeper in shape as well as more positively charged compared with that of free \( \text{Wzb} \) (Fig. S2).

\( \text{Wzb-benzylphosphonate} \) shares a similar active site structure with the \( \text{Wzb-benzylphosphonate} \) complex. Mutating Cys9 to an alanine residue that has a short and uncharged side chain allowed the phosphate ion to bury deeper in the active site than the phosphate moiety of benzylphosphonate (Fig. 3C). As a result, the hydrogen bonds between the phosphate ion and the P-loop residues are generally shorter than those observed in \( \text{Wzb-benzylphosphonate} \), suggesting a stronger binding. Among these interactions, two hydrogen bonds contributed by VvP-loop residues are generally shorter than those observed in \( \text{Wzb-benzylphosphonate} \), supporting the proposal that \( \text{Wzb-benzylphosphonate} \) complex. The P-, D-, and W-loops are colored in green and red spheres, respectively. The hydrogen bonds are shown as red dotted lines.

**Figure 4. Close-up of the active site regions.** A, free \( \text{Wzb} \). B, \( \text{Wzb-benzylphosphonate} \) complex. C, \( \text{Wzb-benzylphosphonate} \)-phosphate complex. The P-, D-, and W-loops are colored in yellow, cyan, and magenta, respectively. Key residues and the ligands are represented in sticks; chloride ion and water molecules are represented by green and red spheres, respectively. The hydrogen bonds are shown as red dotted lines.

Structural comparison with other LMWPTPs

As expected, a number of known LMWPTPs were identified by the Dali server to be structurally similar to \( \text{Wzb} \). Generally, \( \text{Wzb} \) shares more similarity in structure and sequence with the \( \text{EcWzb} \)-prototyped group II LMWPTPs (rmsd of aligned Ca atoms of 0.8–0.9 Å, sequence identity of 52–58%) than the group I LMWPTPs prototyped by mammalian LMWPTPs (rmsd of aligned Ca atoms of 1.4–1.9 Å, sequence identity of 22–37%). In addition, arsenate reductases were also identified to be structurally similar to \( \text{Wzb} \) (rmsd of aligned Ca atoms of about 2.1 Å, sequence identity of about 23%), supporting the proposal that LMWPTP originated from prokaryotic arsenate reductase (20).

We chose two typical LMWPTPs, group II prototype \( \text{EcWzb} \) (PDB ID: 2WJA, sequence identity of 57% to \( \text{Wzb} \)) and group I prototype BPTP (PDB ID: 1DG9, sequence identity of 29% to \( \text{Wzb} \)) for comparison to understand the similarity and difference between \( \text{Wzb} \) and other LMWPTPs (Fig. 5). All structures are in ligand-bound forms. Overall, structures of \( \text{Wzb}, \text{EcWzb}, \) and BPTP could be well aligned for the regulatory secondary structures, with relatively large variations mainly occurring in the loop regions (Fig. 5A). We focused on the comparison of the active site crevices, which directly determine the substrate specificity and catalytic power. The P-loops of \( \text{Wzb}, \text{EcWzb}, \) and BPTP contain the motifs “CVGNICRS,” “CTGNICRS,” and “CLGNICRS,” respectively. Despite the sequence variation at the second position of the catalytic motif, the P-loops of all structures are well superposed in space, in agreement with the conserved phosphate-binding function of this loop. Regarding the D-loop, \( \text{Wzb} \) shares more similarity with \( \text{EcWzb} \) than with BPTP. \( \text{Wzb} \) and \( \text{EcWzb} \) contain one tyrosine residue followed by an arginine residue in the D-loop, while BPTP contains two tyrosine residues (Tyr131 and Tyr132) (Fig. 5B). These two adjacent tyrosine residues are generally found in group I LMWPTPs, endowing their D-loops with a phosphorylation-mediated regulatory function (33). Phosphorylation on the D-loop has also been observed in group II LMWPTP (34) and could occur in \( \text{Wzb} \) as well, due to the similar D-loop compositions. In group I LMWPTPs, the D-loop can undergo conformational changes upon ligand binding (35), but this is not observed in group II LMWPTPs. For \( \text{Wzb} \), there were only certain local conformational changes in the D-loop upon ligand binding. Remarkably, the most
The W-loop of all three enzymes is located at the W-loop (Fig. 5B). The W-loop of EcWzb is the shortest among the three; a leucine (Leu44) is supposed to interact hydrophobically with the substrate. BPTP contains a tryptophan (Trp49) in the W-loop, whose side chain can interact hydrophobically with the phenyl ring of the substrate. In VvWzb, the four-residue insertion is partially overlapped with the side chain of Trp49 of BPTP. These inserted residues do not show direct interactions with the ligand due to a long distance between them. Instead, a leucine residue in VvWzb (Leu44) corresponding to EcWzb Leu44 may provide hydrophobic interaction with the phenyl ring of the ligand. The specific configuration of these loops makes the active site crevice of VvWzb different from those of previously studied LMWPTPs in shape and charge (Fig. S2). In shape, the active site crevices of group II LMWPTPs are relatively small and shallow, while those of group I LMWPTPs appear large and deep, showing a higher W-loop wall, which is attributed to their characteristic aromatic residue in the W-loop. The active site crevice of VvWzb in the ligand-free state is small and shallow but becomes larger and deeper in the ligand-bound state, resembling those of group I LMWPTPs. Regarding charge, the active site crevices of all LMWPTPs share a positively charged bottom, attributed to the conserved Arg residue of the P-loop. Group I LMWPTPs prefer relatively neutral walls for the active site crevices, while group II tends to have more positive ones. In comparison, the active site crevice of VvWzb is the most positively charged, largely due to the extra residue Lys41 in its W-loop wall. Assumingly, the distinct shapes and charge patterns of the active site crevices would exert profound effects on substrate recognition and catalysis for LMWPTPs.

**Enzymatic characterization of VvWzb and its mutants**

VvWzb exhibited clear phosphatase activity in the assay using para-nitrophenyl phosphate (pNPP) as substrate (Table 2, Fig. S3). Compared with EcWzb, VvWzb has a remarkably higher $k_{cat}$ and a comparable $K_m$. Overall, the catalytic efficiency ($k_{cat}/K_m$) of VvWzb is approximately eightfolds of that of EcWzb. To understand the roles of individual residues in catalysis, we made several mutants and measured their activities. Based on structural analysis and previous knowledge of LMWPTPs, residues in the P- and D-loops are supposed to play primary roles in catalysis of VvWzb: Cys9 is a nucleophile; Asp119 serves as the general acid/base in the catalysis; Arg15 is crucial to interact with the phosphate group to stabilize the transition state. Mutating these individual residues of VvWzb to alanine (VvWzbCys9A, VvWzbAsp119A, and VvWzbArg15A) almost abolished the enzymatic activity, conforming their essential roles in catalysis.

Compared with other LMWPTPs, the W-loop of VvWzb is special by containing a unique $40^\text{EKSR}^{43}\text{del}$ insertion (Fig. 2A). To probe the function of this insertion, we first deleted the four residues to mimic the W-loop of group II LMWPTPs. In comparison with the WT, the deletion mutant VvWzb played a significant decrease in the catalytic efficiency up to 15 folds (Table 2, Fig. S3). We also replaced these four residues with a tryptophan to mimic group I LMWPTPs. The tryptophan variant VvWzb $40^\text{EKSR}^{43}\text{del}$ exhibited about fourfolds decrease for $k_{cat}$ while the substrate-binding affinity was also significantly lowered judged by the increased $K_m$. Altogether, the deletion mutant showed a significant decrease in the catalytic efficiency up to 15 folds (Table 2, Fig. S3). We also replaced these four residues with a tryptophan to mimic group I LMWPTPs. The tryptophan variant VvWzb $40^\text{EKSR}^{43}\text{del}$ exhibited a similar $k_{cat}$ to that of WT, but the substrate-binding affinity was lower than WT judged by the twofold $K_m$ value. Overall, it showed a half of the catalytic efficiency of WT, which is much higher than the deletion mutant. These results indicate that the four-residue insertion $40^\text{EKSR}^{43}\text{del}$ is required for an optimal activity of VvWzb, and the enhancing effect is even higher than a single tryptophan residue. We were able to find similar four-residue insertion in the W-loops of many VvWzb homologs, showing that Lys41 and Ser42 are conserved in the new type of W-loop (Fig. 2A). These two residues face toward the active site, possibly affecting ligand binding (Fig. 4B). Therefore, we tested the contributions of Lys41 and Ser42 to catalysis by mutagenesis. The mutants VvWzbLys41A, VvWzbLys41E, and VvWzbSer42E maintained about half enzymatic activity of WT, and VvWzbSer42A even showed an activity of 97% of that of WT. Apparently, the activity is not dependent on the side chain types at these two positions. To further test the sequence-independency hypothesis, we replaced $40^\text{EKSR}^{43}\text{del}$ with four alanine residues. Again, the VvWzb $40^\text{EKSR}^{43}\text{del}$AAA mutant
Table 2

| Protein              | \(k_{\text{cat}}\) (s\(^{-1}\)) | \(K_m\) (mM) | \(k_{\text{cat}}/K_m\) (mM\(^{-1}\)s\(^{-1}\)) | Relative activity (%) |
|----------------------|-----------------------|-------------|---------------------------------|----------------------|
| VvWzb                | 25.10 ± 0.24          | 3.94 ± 0.12 | 6.37                            | 100                  |
| VvWzb\(E40\rightarrow\)R43del | 6.00 ± 0.20          | 14.32 ± 1.07 | 0.42                            | 6.58                  |
| VvWzb\(K41A\)        | 25.56 ± 0.23          | 7.90 ± 0.63 | 3.24                            | 50.78                |
| VvWzb\(E11E\)        | 19.68 ± 1.16          | 6.03 ± 1.01 | 3.26                            | 51.08                |
| VvWzb\(S42A\)        | 26.60 ± 0.64          | 7.03 ± 0.49 | 3.78                            | 59.42                |
| VvWzb\(S42E\)        | 25.94 ± 0.20          | 4.21 ± 0.12 | 6.16                            | 96.71                |
| VvWzb\(S42K\)        | 32.08 ± 1.19          | 6.74 ± 0.44 | 4.76                            | 74.78                |
| VvWzb\(S42E\)        | 31.02 ± 0.86          | 8.47 ± 0.27 | 3.66                            | 57.52                |
| EcWzb                | 2.84 ± 0.10           | 3.48 ± 0.24 | 0.82                            | 100 (12.87)\(^{a}\) |
| EcWzb\(A39,L40insEKSR\) | 9.34 ± 0.10          | 2.53 ± 0.03 | 3.69                            | 450 (57.93)\(^{a}\) |
| EcWzb\(A39,L40insAAA\) | 6.09 ± 0.09          | 2.05 ± 0.10 | 3.27                            | 362 (46.63)\(^{a}\) |

The values of \(k_{\text{cat}}\) and \(K_m\) are expressed as mean ± SEM of three independent experiments. Relative activity is defined as the catalytic efficiency \((k_{\text{cat}}/K_m)\) divided by that of WT. The kinetics of VvWzb\(V726A\), VvWzb\(K41A\), and VvWzb\(D116N\) were not determined for their very low activity.

\(^{a}\) The values in the parenthesis indicate the relative activity to that of WT VvWzb.

showed more than half enzymatic activity of WT, with a slightly higher \(k_{\text{cat}}\) and a doubled value of \(K_m\). This result confirmed that the residue type is not critical for the activity enhancing effect of the insertion. Probably, a local structure derived from a simple four-residue skeleton is sufficient for the enhancing effect. Taken together, the four-residue insertion in the W-loop of VvWzb endowed the enzyme with a higher enzymatic activity than null or tryptophan residue at the same position of the W-loop. The enhancing effect is not determined by the sequence specificity of the insertion.

We wondered if this enhancing effect of the four-residue insertion can also be applied to other LMWPTPs. Therefore, we performed a gain-of-function experiment by inserting the four residues in the corresponding position of EcWzb. Both mutants EcWzb\(A39,L40insEKSR\) and EcWzb\(A39,L40insAAA\) showed a significant increase (~fivefolds/fourfolds) in catalytic efficiency (Table 2, Fig. S3). The enhancing effect was mainly reflected by the increase in \(k_{\text{cat}}\), which is about three or two times of that of the WT. For both mutants, the \(K_m\) values are slightly lowered, indicating a better substrate-binding affinity. These results suggest that a four-residue insertion at the W-loop can be a general activity enhancing element for the LMWPTP fold.

All aforementioned results are based on the enzymatic assays using pNPP as the substrate. We wondered if these results can be verified when using a physiological substrate. We purified VvWzc\(726\) to the Y-cluster of which serves as the natural substrate of VvWzb. The recombinant VvWzc\(726\) showed two bands that were stained by the anti-phosphotyrosine antibody, probably representing two phosphorylation states. After incubation with WT VvWzb and the mutants, we observed dephosphorylation of VvWzc\(726\) to various extents. The WT dephosphorylated VvWzc\(726\) in a short time period, but did not lead to a complete dephosphorylation. The mutants VvWzb\(E40\rightarrow\)R43del, VvWzb\(K41A\), VvWzb\(E11E\), VvWzb\(S42R\), and VvWzb\(E40,R43delinsAAA\) showed 82% to 94% dephosphorylating activities of the WT, while the activity of VvWzb\(E40\rightarrow\)R43del is poor, with 22% activity retained (Fig. 6). In general, the relative activities of these mutants showed the same trend as that observed in the pNPP assays. Interestingly, the mutant VvWzb\(S42E\) showed inconsistent results in the two assays. In the pNPP assay, this mutant showed a high enzymatic activity, similar to that of other point mutations (Table 2, Fig. S3). However, its activity (33% activity of WT) was much lower than other point mutants but comparable with that of the deletion mutant, when using VvWzc\(726\) as substrate (Fig. 6). This phenomenon indicates that an unfavourable interaction between the W-loop of VvWzb\(S42E\) and VvWzc\(726\) occurred, resulting in enzymatic activity loss.

**Discussion**

Herein, we report the crystal structures of VvWzb in free and ligand-bound forms. VvWzb contains an unusual W-loop with a four-residue insertion, which is required for the optimal activity. This is the first report to identify and characterize such a novel W-loop in the LMWPTP family.

Remarkably, the unique insertion in the W-loop endows VvWzb with unprecedented structural and functional features in the LMWPTP family. The P- and D-loop regions of VvWzb resemble those of known LMWPTPs, but its W-loop is longer than any other known LMWPTPs for containing a unique four-residue insertion. The W-loop of VvWzb exhibited an extended conformation in ligand-free state but underwent dramatic conformational changes upon ligand binding, which reconfigured the shape and charges of the active site crevice. Conformational changes in the W-loop were reported in group I LMWPTPs before, but they were only small changes on the orientation of the tryptophan side chain (35). This is the first time to observe such a large conformational change of the W-loop in the LMWPTP family. Importantly, the unique four-residue insertion in the W-loop of VvWzb showed functional importance. The insertion not only was required for an optimal activity of VvWzb but also enhanced the activity of EcWzb once incorporated into the latter at the corresponding position. Roughly, the enhancing effect of the four-residue insertion is comparable with that of a tryptophan residue. The exact mechanism of the enhancement remains to be understood, but it is obviously different from that of an aromatic residue, which stabilizes the phenyl ring of the substrate to gain higher catalytic power (23). Surprisingly, substituting the insertion to a simple four-alanine patch is sufficient to maintain a high enzymatic activity. It appears that the space effect instead of the sequence specificity of the insertion makes the major contribution to activity enhancement. Interestingly, we
also observed that the VvWzb<sup>S42E</sup> mutant showed a largely reduced enzymatic activity when using VvWzc<sub>453−726</sub> as substrate, even though its activity was comparable with that of WT when using pNPP as substrate. In the complex structures, Ser42 points to the active site pocket. When substituting to Glu, the longer side chain may hinder the localization of the Y-cluster of VvWzc<sub>453−726</sub>, whereas this is less hazardous for the smaller substrate pNPP. Alternatively, the W-loop of VvWzb<sup>S42E</sup> interacts with VvWzc<sub>453−726</sub> and thus is not able to take the correct conformation for catalysis. Either explanation inspires a hypothesis of regulation mechanism. It is known that glutamic acid residue is a mimic of phosphoserine. In fact, Ser42 is located at the tip region of the W-loop as shown in the ligand-free VvWzb structure and thus easily accessible by a kinase. It is plausible that Ser42 of VvWzb can be phosphorylated and thus regulate the enzymatic activity. The hypothesis is not unreasonable, considering that phosphorylation-mediated regulation has been reported for LMWPTPs (36). This might explain why the serine residue is conserved in this new type of W-loop (Fig. 2A).

Our study also raises a request to update the current classification of LMWPTP family. In the last decades, a number of LMWPTP structures have been determined and there are only two types of W-loop identified (23). The W-loop containing a characteristic aromatic residue has been identified in the majority of the LMWPTP structures, including BPTP (PDB ID: 1DG9), HCPTPA (PDB ID: 5PNT), HCPTPB (PDB ID: 1XWW), and VcLMWPTP1 (PDB ID: 4LRQ) (25, 26, 32, 37). The other type of W-loop lacks this aromatic residue, found in structures such as EcWzb (PDB ID: 2WJA), EaAmsI (PDB ID: 4D74), and VcLMWPTP2 (PDB ID: 5Z3M) (29, 38, 39). The two types of W-loop employ distinct substrate recognition mechanisms (23). Interestingly, phylogenetic analysis has divided LMWPTPs into two groups, which is parallel to their W-loop types (23, 31). This supports the role of W-loop as an evolutionary marker in classification of the LMWPTP family. Apparently, VvWzb employs a new type of W-loop and is outside of either group. Moreover, this type of W-loop is not limited to V. vulni<sub>ficus</sub>, but spreads widely in the γ-proteobacteria class, including the families of Vibrionaceae, Enterobacteriaceae, and Pasteurellaceae. Further, we carried out a phylogenetic analysis that grouped the LMWPTPs into three clades, including the previously known two groups and the third one containing VvWzb and its homologs (Fig. 7). In agreement with previous observation, the phylogenetic grouping is obviously in parallel to the W-loop types. Taken the W-loop type and phylogenetic relationship together, we suggest to reclassify LMWPTPs into three groups: I to III, prototyped by mammalian LMWPTPs, EcWzb, and VvWzb, respectively. It is known that indels, referring to short insertions and deletions in the protein sequence, are a major force to drive protein evolution to gain new structural folds, new functionalities, and new regulations (40–42). W-loop diversity in LMWPTPs is an excellent example in this context. Based on previous results and our study, it is clear that indel variations in
the W-loop endow LMWPTPs with specific structural, dynamic, and mechanical features. The coincidence between the W-loop types and phylogenetic relationship also demonstrates that indel occurring at the W-loop plays important roles in driving the evolution and diversity of LMWPTP family.

Characterization of the protein tyrosine phosphorylation system in prokaryotes is still at infancy in comparison with that in eukaryotes (43). LMWPTPs are important components in both prokaryotic and eukaryotic tyrosine phosphorylation systems. Here, we notice a difference in their LMWPTP diversities. Based on the phylogenetic tree, eukaryotes use only group I LMWPTPs (Fig. 7). This sole type of LMWPTPs can dephosphorylate an array of protein substrates in various cellular processes (1). In contrast, prokaryotes employ all three groups of LMWPTPs. It is not unusual that one species contains two groups of LMWPTPs, being a combination of any two groups. For example, *V. vulnificus* contains group I (protein ID: WP_130193014.1) and III (protein ID: WP_017420711.1) LMWPTPs. Even, some species contain all three groups, exemplified by *E. coli* (with protein IDs: WP_153671227.1, WP_052935621, and AYN88831.1) and *Vibrio cholerae* (with protein IDs: WP_001909008.1, WP_001880745.1, and WP_114808776.1). It is also interesting to find that a similar biochemical process in different species can employ different groups of LMWPTPs. For instance, the above-mentioned EcWzb (group II) and VvWzb (group III) are all related to polysaccharide synthesis (18, 44). Since W-loop plays an important role in substrate recognition and ligand regulation (25), using diverse LMWPTPs with all types of W-loops may endow prokaryotes with certain advantages in substrate spectrum and regulation to adapt to their large variety of cellular processes. It is known that most prokaryotes use BY-kinases, with no homologs in eukaryotes (45). Now, from both the aspects of kinase and phosphatase, it is clearer that prokaryotic tyrosine phosphorylation system is not analogous to that of eukaryotes, but has its own unique characteristics. The evolutional relationship between prokaryotic and eukaryotic tyrosine phosphorylation systems is an interesting question for future study.

Finally, our study also provides an opportunity to develop antivirulence agents against *V. vulnificus*. LMWPTP family members have been proposed in recent years as promising drug targets for human diseases, including cancer, diabetes, and infectious diseases, with many potential inhibitors developed (46–48). Obviously, inhibiting VvWzb would damage the CPS production of *V. vulnificus* and thus render the bacterium to the biocidal attack of the host immune system, making VvWzb an excellent antivirulence target. Our structural analysis of VvWzb in free and ligand-bound forms has described a clear profile of its ligand-binding pocket, facilitating inhibitor development through a structure-based approach. The chemical compounds developed for other LMWPTPs can be a good start to design VvWzb inhibitors. More importantly, the unique insertion in the W-loop endows VvWzb with an active
V. vulnificus Wzb structures

site pocket distinct from that of the LMWPTPs of the eukaryotic hosts and most microbiota bacteria, giving a good opportunity to develop pathogen-specific inhibitors. To note, conformational changes of the W-loop of VvWzb may be induced by inhibitor binding, which should be taken into account during inhibitor developing process.

Experimental procedures

Gene cloning and protein production

The DNA sequence encoding VvWzb was amplified from the genomic DNA of V. vulnificus ATCC 27562 by standard PCR method using primers (forward, 5’-AGTCCATGGGCTTAAACAAATCCTTAGTCGTTTG-3’; reverse, 5’-CCGCCTGAGCTACAATTTTTTTGCCCAG-3’). The PCR product was inserted into the vector pETM11 (EMBL) using Ncol and XhoI sites. The product from this construct would be composed of an N-terminal 6×His tag, a tobacco etch virus (TEV) cleavage site, and the protein. The induced cells were further cultured at 16°C for 20 h with vigorous shaking. Subsequently, the cells were harvested by centrifugation at 6000 g for 1 min. The cell pellets were resuspended in the binding buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM DTT. The puriﬁed protein was eluted with the buffer containing 300 mM benzylphosphonate and 150 mM NaCl, 1 mM DTT was titrated by NaOH to result in a 300 mM benzylphosphonate solution of pH 7.5. Then, VvWzb or VvWzbCOA solution was mixed with the benzylphosphonate solution and incubated at 4°C for 1 h. Complex crystals were grown in drops containing 1.5 μl of protein solution (14 mg/ml protein in the buffer 10 mM HEPES, 30 mM benzylphophonate, 150 mM NaCl, 1 mM DTT, pH 7.5) and 1.5 μl of reservoir solution (100 mM HEPES pH 7.0, 200 mM NaCl, 31% (w/v) PEG3350). The reservoir solution supplemented with 4% (w/v) PEG3350 was used as cryoprotectant solution. We also crystallized VvWzb and VvWzbCOA in the presence of benzylphosphonate. First, benzylphosphonic acid dissolved in 10 mM HEPES pH 7.5, 150 mM NaCl, 1 mM DTT was prepared by Phenix.refine (58). Ligands were modeled at late stage of refinement, and the geometry restraints of ligands were generated by the GRADE server (http://grade.globalphasing.org). All the crystallographic data are summarized in Table 1.

Crystallization and structure determination

Crystallization was conducted with the sitting-drop vapor-diffusion method at 20°C. Free VvWzb crystals were grown in drops containing 1.5 μl of protein solution (14 mg/ml in the buffer 10 mM HEPES pH 7.5, 150 mM NaCl, 1 mM DTT) and 1.5 μl of reservoir solution (100 mM HEPES pH7.5, 200 mM NaCl, 31% (w/v) PEG3350). The reservoir solution supplemented with 4% (w/v) PEG3350 was used as cryoprotectant solution. We also crystallized VvWzb and VvWzbCOA in the presence of benzylphosphonate. First, benzylphosphonic acid dissolved in 10 mM HEPES pH 7.5, 150 mM NaCl, 1 mM DTT was titrated by NaOH to result in a 300 mM benzylphosphonate solution of pH 7.5. Then, VvWzb or VvWzbCOA solution was mixed with the benzylphosphonate solution and incubated at 4°C for 1 h. Complex crystals were grown in drops containing 1.5 μl of protein solution (14 mg/ml protein in the buffer 10 mM HEPES, 30 mM benzylphophonate, 150 mM NaCl, 1 mM DTT, pH 7.5) and 1.5 μl of reservoir solution (100 mM HEPES pH 7.0, 25% (w/v) PEG3350). The reservoir solution supplemented with 10% glycerol and 30 mM benzylphosphonate pH 7.5 was used as cryoprotectant solution. The crystals were shortly soaked in their respective cryoprotectant solutions, prior to flash-freezing in liquid nitrogen for data collection.

The diffraction data were collected at 100 K on the beamlines BL18U1 and BL19U1 of Shanghai Synchrotron Radiation Facility (SSRF) (49, 50). The diffraction data were processed by autoPROC (Global Phasing) calling the programs XDS and Aimless (51–53). The structure of free VvWzb was solved by molecular replacement using Phaser (54), with the structure of EcWzb (PDB ID: 2WMY) as search model. Refinement of the atomic coordinates, B-factors, and TLS parameters using autoBUSTER (55) and model building using Coot (56) were carried out alternately. The chloride ions in free VvWzb structure were identified on the basis of electron density, anomalous signal, and stereochemistry (57). The ligand-bound structures were solved by molecular replacement using the ligand-free structure as search model. The refinement protocol of the ligand-bound WT VvWzb is similar to that of the free form. For ligand-bound VvWzbCOA, which diffracted to atomic resolution, the atomic coordinates and anisotropic B-factors were refined using Phenix.refine (58). Ligands were modeled at late stage of refinement, and the geometry restraints of ligands were generated by the GRADE server (http://grade.globalphasing.org). All the crystallographic data are summarized in Table 1.

Structure similarity searching was performed on the Dali webservice (59). Sequence alignment was performed on the
Phosphatase activity assays

The phosphatase activities of VvWzb and mutants were measured by an assay using pNPP as substrate. VvWzb can dephosphorylate pNPP into p-nitrophenol, whose absorbance can be recorded at 405 nm. The determination of kinetic parameters was carried out in a 300 μl reaction system containing 10 mM HEPES pH 7.5, 150 mM NaCl supplemented with 0.45 to 57.6 mM pNPP and different amount of enzyme (100 nM for VvWzb and its mutants except 400 nM for VvWzb C453–726; 200 nM for EcWzb and its mutants). Absorbance at 405 nm was recorded continuously on a Cary 60 UV-Vis spectrophotometer (Agilent Technologies) with 10-mm path length cuvettes at 20 °C to obtain the progress curve, which was used to calculate the initial velocity. A standard curve of the product p-nitrophenol was drawn to convert absorbance values to p-nitrophenol concentrations. Kinetics parameters were estimated by nonlinear fitting of the initial velocity versus substrate concentration data to the Michaelis–Menten equation \( v = \frac{V_{\text{max}}[S]}{K_m+[S]} \) using the Origin program.

The phosphatase activities of VvWzb and the mutants were also tested using recombinant VvWzc453–726 as substrate. In detail, 10 μM VvWzc453–726 with 3 μM VvWzb or its mutants was incubated in 10 μl reaction buffer (50 mM HEPES pH 7.5, 150 mM NaCl) at 4 °C for 0.5 h. Then the SDS-PAGE sample buffer was added to terminate the reactions. Samples were heated at 95 °C for 5 min, separated on 12% SDS-PAGE gels, and transferred onto PVDF membranes using a wet transferring protocol with constant current 200 mA. Membranes were firstly incubated with monoclonal anti-phosphotyrosine antibody (1:2000 diluted ab10321; Abcam), followed by incubation with goat anti-mouse IgG-HRP (1:5000 diluted gc0125s; cwbio). Immunodetection was performed using the DAB kit (cw0102s; cwbio). The intensity of the bands was quantified using the Image J software. The activity was evaluated by the intensity difference between samples and the control.

Phylogenetic analysis

The rootless protein phylogenetic tree was constructed by MEGA6 software using the neighbor-joining method. In total, 26 representative LMWPTP sequences including eukaryotic and prokaryotic ones were used for analysis (with NCBI numbers in the parentheses): BPTP Bos taurus (NP_776403.1), HCPPTA human (NP_004291.1), Stpt1 S. pombe (NP_001342920.1), LMWPTP Mus (NP_001103709.1), LMWPTP1 V. cholerae (WP_001889901.1), PTP A. fumigatus (WP_000482223.1), Wzb K. pneumoniae (AAD30006.1), Wzb S. enterica (WP_122056837.1), Wzb V. vulnificus (WP_017420711.1), LMWPTP V. cholerae (WP_114808776.1), Wzb Vibrio parahaemolyticus (WP_005458338.1), Wzb Vibrio harveyi (WP_021018901.1), Wzb Vibrio crassostreae (WP_055320062.1), PTP A. sfae (WP_061013219.1), PTP P. damselae (WP_065171812.1), LMWPTP E. coli (WP_122056837.1), and LMWPTP G. parasuis (WP_010785901.1). One thousand bootstrap replicates were used to test the inferred phylogeny relationship. The final clustering result was generated and displayed with radiation.

Data availability

The coordinates and diffraction data of the free VvWzb, VvWzb-benzylphosphonate, and VvWzbC453A-phosphate have been deposited in the PDB (www.rcsb.org) with accession numbers of 7DHD, 7DHE, and 7DHF, respectively. All remaining data are contained within the article and the supporting information file.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

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Author contributions—X. W. and Q. M. designed, performed and analyzed the experiments, and wrote the paper; Q. M. conceived and supervised the study; X. W. and Q. M. reviewed the results and approved the final version of the manuscript.

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Abbreviations—The abbreviations used are: CPS, capsular polysaccharide; IPTG, isopropyl-β-D-thiogalactopyranoside; LMWPTP, low-molecular-weight protein tyrosine phosphatase; PEG, polyethylene glycol; pNPP, para-nitrophenyl phosphate; rmsd, root-mean-square deviation; TEV, tobacco etch virus.

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