Proteolysis of histidine kinase VgrS inhibits its autophosphorylation and promotes osmostress resistance in *Xanthomonas campestris*

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In bacterial cells, histidine kinases (HKs) are receptors that monitor environmental and intracellular stimuli. HKs and their cognate response regulators constitute two-component signalling systems (TCSs) that modulate cellular homeostasis through reversible protein phosphorylation. Here the authors show that the plant pathogen *Xanthomonas campestris* pv. *campestris* responds to osmostress conditions by regulating the activity of a HK (VgrS) via irreversible, proteolytic modification. This regulation is mediated by a periplasmic, PDZ-domain-containing protease (Prc) that cleaves the N-terminal sensor region of VgrS. Cleavage of VgrS inhibits its autokinase activity and regulates the ability of the cognate response regulator (VgrR) to bind promoters of downstream genes, thus promoting bacterial adaptation to osmostress.

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In both eukaryotic and prokaryotic cells, signalling pathways can be generally divided into two major modes according to the persistence of protein participants: reversible and irreversible. For example, proteolysis is an irreversible, post- translational signalling cascade that modulates cell physiology, whereas protein phosphorylation catalysed by receptor kinases is reversible. Although, the regulatory mechanisms of these two kinds of cellular signalling pathways have been extensively investigated, the cross-regulation between them is not fully understood. Recent studies in eukaryotes revealed that the proteolysis of receptor kinases is critical for regulating development, apoptosis and tumour genesis. However, in prokaryotic cells, how proteolysis modifies receptor kinases and controls their regulatory functions are incompletely understood.

In gram-negative bacteria, regulated proteolysis catalysed by PDZ-domain-containing proteases, such as the HtrA-family proteases (high temperature requirement A) and tail-specific proteases (Tsp), modulates multiple physiological pathways, including virulence, stress response,orum-sensing, protein quality control and antibiotic resistance. Thus, these proteases are potential molecular targets for the development of novel antibacterial agents. These proteases contain multiple domains, including one or more PDZ domains, and they are mostly located in the periplasm of gram-negative bacteria. PDZ-domain-containing proteases regulate physiological processes by binding or cleaving their protein substrates in cells. For example, under stress conditions, the DegS protease of Escherichia coli cleaves anti-σ factor RseA to release σF from the membrane and then activates the transcription of various stress-response genes.

In Vibrio cholerae, a Tsp protease cleaves TcpP to initiate the subsequent proteolysis and inactivation of the membrane-bound transcription factor (TF). In addition to anti-σ factors and TFS, studies revealed that PDZ domain-containing proteases also process misfolded proteins, outer-membrane proteins, phosphatases, haemagglutinin and autotransporters. However, identification of the physiological substrates of proteases remains technically challenging, and thus, the regulatory functions of most proteases are unclear.

The majority of bacterial species belonging to the genus Xanthomonas are plant pathogens. Among them, Xanthomonas oryzae pv. oryzae is the causative agent of rice bacterial blight disease, and Xanthomonas campestris pv. campestris causes the black rot disease of crucifers. Both bacterial species encode at least six PDZ domain-containing proteases. The inactivation of one orthologous gene, prc (also named tsp), results in the attenuation of virulence and hypersensitivities to multiple environmental stresses. Prc of X. oryzae pv. oryzae is a periplasmic protein, and its cleavage substrate remains to be discovered. When the bacterium is grown under osmolarity stress (osmostress), Prc binds to a virulence-associated dipeptidyl peptidase (DppP). Rather than cleaving DppP, Prc stabilizes DppP, suggesting it has a chaperone activity. Therefore, DppP is not a proteolytic substrate of Prc. More direct and effective approaches are necessary to identify the physiological substrates of this protease, which will allow the regulatory role of Prc to be determined.

In the present study, we aim to identify the physiological substrates of Prc and investigate the role of Prc-catalysed proteolysis in controlling the environmental adaptation of X. campestris pv. campestris, which is more genetically amenable than X. oryzae pv. oryzae. Under osmostress, Prc directly binds and cleaves the N-terminal sensor region of VgrS, a canonical histidine kinase (HK) that controls bacterial virulence and stress responses. Cleavage of the VgrS sensor inhibits its autokinase activity, which then modulates the TF activity of the cognate VgrR in binding the promoters of downstream genes. This process significantly promotes bacterial resistance to osmostress. Our study reveal that the proteolytic processing of the sensor region of HK by protease is a molecular mechanism to control the TCS signalling of gram-negative bacteria.

**Results**

**Prc regulates virulence and bacterial stress resistance.** In *X. campestris* pv. *campestris* 8004, *XC_0714* is the orthologue of Prc (PXO_04290) in *X. oryzae* pv. *oryzae PXO99A* (BLASTP search, e-value = 0, identities = 94%). Both proteins encode three domains: an N-terminal PDZ domain, a central peptidase domain and a C-terminal DUF3340 domain with an unknown function (Fig. 1a). The Prc protein of *X. campestris* pv. *campestris* contains a predicted 26-aa signal peptide with a cleavage site between the 26th and 27th positions. According to a MEROPS peptidase database search result, Prc belongs to the S41A serine endopeptidase subfamily (e-value = 2.40 x 10^-99) that contains two conserved catalytic residues (Ser and Lys). This is different from HtrA family proteases that usually contain a catalytic triad (His–Asp–Ser). Western blotting revealed that the Prc of *X. campestris* pv. *campestris* localized to both the periplasm and cytosol (Fig. 1b). In addition, an RT-PCR analysis revealed that prc is located in a four-gene operon that also contains *XC_0711, XC_0712, and XC_0713* because positive RT-PCR products were amplified from a cDNA template derived from the intergenic transcripts of these genes (Supplementary Fig. 1).

We constructed various prc mutants and characterized their phenotypic alterations, including biofilm development, virulence, the production of extracellular polysaccharides and enzymes, and resistance to multiple antibiotics and environmental stresses. In-frame deletions of the prc coding sequence or its PDZ domain, peptidase domain (PEP) or DUF (DUF) domains coding sequences independently caused significant decreases in bacterial virulence and in planta growth against susceptible cabbage (*Brassica oleracea* cv. JingleNG No. 1; Fig. 1c, d, Supplementary Fig. 2a–c) and tolerance to the antibiotics erythromycin and kanamycin (Fig. 1e, f), as well as bacterial resistance to a high Fe^2+ concentration (2.5 mM; Fig. 1g) and osmostress (1.0 M sorbitol; Fig. 1h). Genetic complementation was performed by providing a full-length prc (Δprc) or the heterogeneous prc of *X. oryzae* pv. *oryzae PXO99A* (Δprc-prcC) and fully restored phenotypic deficiencies to levels similar to those of the wild-type (WT) strain (Fig. 1c–h, Supplementary Fig. 2). Thus, prc may regulate bacterial virulence and resistance to multiple environmental stresses, and the biological functions of the prc of *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* appear to be highly conserved. Here, we focused on the role of Prc in regulating bacterial responses to osmostress.

**Prc is a bona fide serine endopeptidase.** Our previous study failed to detect the in vitro peptidase activity of Prc in *X. oryzae* pv. *oryzae*18. In this work, a mature form of Prc from *X. campestris* pv. *campestris* was successfully obtained by expressing the full-length prc in the Escherichia coli BL21(DE3) strain. N-terminal amino acid sequencing of the purified, soluble Prc showed that the 26-aa signal peptide was removed, indicating that E. coli cells could recognize and process the cleavage site of the signal peptide. As shown in Fig. 2a and Supplementary Fig. 3a, the mature Prc of *X. campestris* pv. *campestris* not only degraded the universal substrate β-casein (Fig. 2a, Lanes 4 and 5) but also had endopeptidase activity that cleaved azocasein, a general chromogenic substrate for endopeptidases (Fig. 2b). When the core catalytic residues Ser475 and Lys500 were substituted, the degradative activities of recombinant Prc^S475A and Prc^K500A, respectively, were completely lost or significantly decreased (Fig. 2a, Lanes 8 and 9, and Lanes 12 and 13, respectively).
respectively; Fig. 2b). Using Michaelis–Menten kinetic curve and Lineweaver–Burk plot analyses, Prc protease azocasein-hydrolysing activities were measured at 37 °C. As shown in Fig. 2c, d, the maximum protease activity and the Michaelis constant (K_m) value were 0.0023 ± 0.001 μmol/min/μg and 2.48 ± 0.32 μM, respectively. This kinetic value is similar to that of the Tsp from E. coli (K_m = 4.4 ± 0.6 μM) and represents a relatively strong peptidase activity relative to other Tsp and HtrA proteases, which usually have K_m values ranging from micromolar to nanomolar levels. Prc protease activity was very stable under various conditions. The optimized temperature range for Prc activity was ~28–37 °C, and changing the pH value from 4.0 to 10.0 did not remarkably affect Prc activity (Supplementary Fig. 3b and 3c). In addition, when the dithiothreitol concentration in the reaction buffer was changed from 0 to 200 mM, the Prc activity level was only slightly affected (Supplementary Fig. 3d). These genetic and biochemical results confirmed the theoretical prediction that Prc is a serine endopeptidase.

**Monomeric Prc directly binds the VgrS sensor.** An affinity proteomic approach using tandem affinity purification (TAP) together with a nanoscale liquid chromatography–mass spectrometry (nanoLC–MS/MS) analysis was employed to screen for Prc-binding proteins. Thus, a recombinant bacterial strain (Δprc-ΔprcS475A-HA-FLAG) was constructed for the TAP analysis. In this strain, HA and FLAG epitope tags were fused in tandem to the C-terminus of an inactive Prc in which the Ser^475 residue has been substituted with Ala. This substitution inactivated Prc protease activity to avoid the degradation of binding proteins during the analysis. The C-terminal HA and FLAG tags did not affect bacterial resistance to osmостress because the corresponding strain (Δprc-prc-HA-FLAG) encoded a functional Prc, showed a growth rate similar to that of the WT strain on NYG–sorbitol plates (Supplementary Fig. 2f). The bacterial cells of the Δprc-prcS475A-HA-FLAG strain were treated with osmостress consisting of 1.0 M sorbitol before being lysed by freeze grinding, which prevented the dissociation of Prc–protein complexes.
Prc is a serine endopeptidase. a Prc degrades β-casein, and its endopeptidase activity is dependent on the Ser475 and Lys500 sites. β-Casein (41.5 μM) was co-incubated with Prc (5 μM) or its recombinant forms (PrcS475A and PrcK500A, 10 μM) at 28 °C for the indicated time. Reactions were stopped and analysed by SDS-PAGE together with Coomassie brilliant blue staining. b Quantification of Prc endopeptidase activity via degradation of the substrate azocasein. Azocasein (424 μM) was mixed with Prc (25 μM), and the reaction was carried out at 28 °C for 30 min. The optical absorbance was measured. Error bars represent standard deviations (n = 3). Asterisks indicate significant differences (Student’s t-test, P < 0.05). c and d The Michaelis-Menten kinetics of Prc activity for azocasein hydrolysis. The Michaelis-Menten curve c and Lineweaver-Burk plot d were obtained from the specific reaction velocity of the hydrolyses of azocasein by Prc. The maximum specific Vmax values of the Prc activity were determined from the graphic representations. The data were derived from three independent experiments, and the goodness of fit values (R²) is indicated. a-d, the experiment was repeated three times.
performed to measure the VgrS autophosphorylation level in the presence of Prc. As shown in Fig. 4a, the addition of mature Prc to the reaction mixture decreased the autophosphorylation level of the full-length VgrS embedded in the inverted membrane vesicle (IMV) in less than 30 s (Lanes 5–8), while the addition of the inactive PrcS475A did not cause a similar effect (Lanes 9–12). This demonstrated that the protease activity of Prc is important in inhibiting the autophosphorylation of VgrS. In addition, neither mature Prc nor inactive PrcS475A affected the autokinase activity of the truncated, soluble VgrS that contained the transmitter domain (MBP-VgrS<sup>ΔS129</sup>; Fig. 4b, Lanes 1–6 and Lanes 10–14, respectively), suggesting that Prc did not interact with the cytoplasmic region of VgrS. Because VgrS transfers the phosphoryl group onto the cognate response regulator (RR) VgrR<sup></sup><sup>23</sup>, we also observed that the addition of Prc did not only decrease the autophosphorylation level of VgrS but also decreased the phosphorylation level of VgrR (Fig. 4c, Lane 6). Inactive Prc<sup>S475A</sup> did not have a similar impact (Fig. 4c, Lane 8). Thus, Prc may inhibit the autophosphorylation of full-length VgrS, and the sensor region of VgrS appears to be indispensable for inhibition.

To further investigate whether the VgrS sensor is a proteolytic substrate of Prc in vitro, the mature Prc protein was co-incubated with a purified, recombinant VgrS sensor. As shown in Fig. 4d, within 60 min, Prc cleaved the VgrS sensor into two parts and then completely degraded the sensor in vitro. However, when the inactive form of Prc (Prc<sup>S475A</sup>) was added to the reaction mixture, the VgrS sensor remained intact during the co-incubation period (Fig. 4e). Furthermore, to determine whether this proteolytic process took place in vivo, recombinant strains in the ΔvgrS mutant and the ΔprcΔvgrS double-mutant genetic backgrounds were constructed (ΔvgrS-vgrSHA and ΔprcΔvgrS-vgrSHA, respectively; Supplementary Table 1). In the two strains, an HA-epitope tag was fused to the N-terminus of VgrS (between the 6th and 7th aa). Chloramphenicol was added to the bacterial cultures of the two strains to eliminate interference by de novo protein synthesis. Then, the bacterial cells were separately challenged with osmotic stress (1.0 M sorbitol). As revealed by western blotting analysis (Fig. 4f), after 5 min of osmotic stress stimulation, the amount of the N-terminal HA-tag, which was detected by the HA monoclonal antibody, gradually decreased as osmotic stress treatment progressed (Fig. 4f, upper panel, Lanes 1–6). Additionally, western blotting using a polyclonal antibody against VgrS revealed that VgrS was cleaved, and additional bands were detected (Fig. 4f, middle panel, Lanes 6–10). Thus, the N-terminal HA tag was removed from VgrS by Prc cleavage. In the double mutant, in which prc was deleted, the amount of HA-tag remained stable regardless of the presence or absence of osmotic stress stimulation (Fig. 4f, upper panel, Lanes 1–5), and no additional bands were detected by the polyclonal VgrS antibody (Fig. 4f, middle panel, Lanes 1–5). Collectively, in vitro and in vivo experimental evidence demonstrated that the VgrS sensor is a proteolytic substrate of Prc. During the osmotic stress response, Prc directly bound and cleaved the VgrS sensor.
To determine the exact, primary cleavage sites in the VgrS sensor processed by Prc, active Prc and VgrS sensor proteins were co-incubated, and then a matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS/MS) analysis was used to identify the VgrS sensor fragments. A peptide with a mass-to-charge ratio of 2771.5 was obtained (Fig. 5a, upper panel). Subsequent MS/MS analysis revealed that Prc cleaved the VgrS sensor at the site between Ala9 and Gln10 (Fig. 5a, middle panel). To verify this result in vitro, a recombinant VgrS sensor with the Ala9 and Gln10 sites substituted (SensorA9G-Q10A) was obtained and purified. MALDI-TOF-MS/MS analysis revealed that the SensorA9G-Q10A protein was not cleaved by active Prc because no corresponding fragment was identified (Fig. 5a, lower panel). To verify this cleavage site in vivo, recombinant bacterial strains that encoded VgrS, in which the two sites were substituted by the ΔvgrSΔvgrSHA and ΔprcΔavrSΔvgrSHA genetic backgrounds were constructed (ΔvgrSΔvgrSHA-A9G-Q10A and ΔprcΔavrSΔvgrSHA-A9G-Q10A, respectively; Supplementary Table 1). As shown in Fig. 5d, in the two recombinant strains, the N-terminal HA-tag of VgrS remained stable regardless of osmotic stress treatment (Fig. 5d, Lanes 1–5 and Lanes 6–10), indicating that recombinant VgrSΔA9G-Q10A resisted Prc cleavage in vivo. As previously shown in Fig. 4a, b, after Prc cleavage, the difference in the theoretical molecular weights between the full-length and the truncated VgrS was ~1.0 kDa, and the two forms were hard to discriminate by SDS-PAGE analysis. Thus, we employed high-resolution, QTRAP LC–MS/MS analysis to directly detect the N-terminal proteolytic peptide generated by Prc cleavage. An 8-aa peptide (NRNIDFFA, corresponding to 2nd to 9th aa of VgrS) was chemically synthesized and used as a standard. As Fig. 5e and Supplementary Fig. 6 show, after a 5-min treatment of the full-length VgrS IMV by Prc, QTRAP LC–MS/MS analysis detected the NRNIDFFA peptide in the reaction mixture. The amounts of this peptide gradually increased along with the treatment time. These biochemical results demonstrated that Prc primarily cleaved the VgrS sensor between the Ala9 and Gln10 site in vivo and in vitro. Furthermore, to measure the effect of Ala9Gln10 cleavage on the autokinase activity of VgrS, we obtained and purified a truncated, recombinant VgrS protein (VgrSΔ9, embedded in IMV) containing a deletion from the 2nd to the 9th aa at the N-terminus. An in vitro phosphorylation assay then revealed that the autophosphorylation level of the VgrSΔ9 IMV was significantly decreased relative to that of the full-length VgrS IMV (Fig. 5f, Lane 2). As controls, two truncated VgrS IMV proteins (VgrSΔ72 and VgrSΔ84, which were truncated in the sensor region) retained phosphorylation levels that were similar or even
Fig. 5 Identification of the VgrS cleavage site by the Prc protease. a MALDI-TOF-MS/MS analysis revealed that Prc cleaves the VgrS sensor at the Ala9-Gln10 site. Upper panel: proteolysis of the VgrS sensor with active Prc. Middle panel: proteolysis of the VgrS sensor with inactive PrcS475A, which was used as a negative control. Lower panel: proteolysis of the recombinant VgrSΔ9G-Q10A sensor with active Prc. Digested proteins were detected in positive ion reflectron mode over an m/z range of 700–3500. Spectra showed relative intensities in the mass range of m/z 2000–3400. b Schematic view of the secondary structure of VgrS and the cleavage site in the sensor. TM transmembrane helix. c Identification of the Prc cleavage site in the VgrS sensor. A MALDI-TOF/TOF MS/MS spectral analysis of m/z 2771.5 from a Prc + VgrS sensor sample. The magnified MS/MS spectra showed the fragment patterns of peptides. d Substitution of VgrSΔ9G-Q10A resulted in resistance to Prc cleavage. Bacterial strains that encoded recombinant VgrSΔ9G-Q10A were provided to this vgrS deletion mutants in which the coding sequence from the 2nd to the 9th aa of the chromosomal vgrS was deleted (Fig. 5b), was constructed to mimic the truncated protein product cleaved by Prc. Without osmosstress, the mutant vgrSΔ9 exhibited a growth rate similar to that of the WT strain (Fig. 6a). However, when this bacterial mutant was challenged by osmosstress (1.0 M sorbitol), it grew faster than the WT strain (Fig. 6b). Under osmosstress, genetic complementation, in which a plasmid-borne, unrelated vgrS and vgrR were expressed in the IMVs (10 μM). The reaction was performed as described in Fig. 4a. g Substitution of VgrSΔ9G-Q10A did not affect its autokinase activity. Upper panels: autophosphorylation assay. Lower panels: western blotting of the proteins or Coomassie brilliant blue staining was used to check the amount of loaded protein. Two experimental repeats were performed.

Cleavage of VgrS promotes bacterial osmostress resistance. The cleavage of a bacterial HK by a protease has not been previously reported, and the adaptive significance of the signalling process remains unclear. To genetically investigate the consequence of VgrS sensor cleavage on bacterial resistance to osmostress, an in-frame deletion mutant of vgrS (vgrSΔ9), in which the coding sequence from the 2nd to the 9th aa of the chromosomal vgrS was deleted (Fig. 5b), was constructed to mimic the truncated protein

higher than that of the WT VgrS protein (Fig. 5f, Lanes 4 and 5), while the autokinase activity of VgrSΔ58 was also substantially decreased (Fig. 5f, Lane 3). In addition, the recombinant VgrSΔ9G-Q10A protein had autokinase activity similar to that of the WT VgrS (Fig. 5g). Together with the data shown in Fig. 4a, the results of the phosphorylation analyses demonstrate that Ala9-Gln10 of VgrS was the primary cleavage site of the Prc protease. Prc proteolysis resulted in a substantial decrease in the VgrS autophosphorylation level.

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Because Prc-catalysed VgrS cleavage resulted in a substantial decrease in its autokinase activity, we hypothesized that dephosphorylation of the VgrS–VgrR system promotes bacterial resistance to osmostress. As predicted, vgrS deletion mutants and point mutants of vgrS and vgrR, with substitutions in their conserved phosphorylating aa (vgrSΔH186A and vgrRΔS14A, ...

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Fig. 6 Deletion of the N-terminus of vgrS suppresses the growth deficiency of the prc mutant under osmostress. a, c, e, and g Bacterial growth in NYG medium. b, d, f, and h Bacterial growth in NYG medium plus 1.0 M sorbitol. The growth curves were measured by an automatic Bioscreen C instrument. Each data point is the average of six samples, and error bars indicate standard deviations.

respectively), also exhibited remarkably increased capabilities to adapt to osmostress (Fig. 6c–f). However, similar to the prc mutant, the vgrR deletion mutant was highly susceptible to sorbitol treatment (Fig. 6e, f), suggesting that intact, dephosphorylated VgrR is indispensable for regulating bacterial responses to stress. To determine the epistatic relationship between prc and vgrS, a set of double mutants was constructed in the Δprc genetic background by mutating vgrS. As shown in Fig. 6g, h, the inactivation of prc almost completely arrested bacterial growth under osmostress, but the growth rates of the double mutants Δprc-vgrSvgrSΔH186A were significantly restored towards the WT level. These epistatic analyses demonstrate that mutations causing the dephosphorylation of VgrS efficiently suppressed the growth deficiency of the prc mutation in resisting osmostress. Thus, vgrS was located downstream of prc regulation during the bacterial osmostress response.

In addition to osmostress resistance, other phenotypic changes in the vgrSΔH186A mutant were measured. The virulence of this mutant against a susceptible host cabbage (B. oleracea cv. Jingfeng No. 1) decreased slightly, while the resistance of the vgrSΔH186A mutant to iron stress and erythromycin was significantly decreased relative to the WT strain (Supplementary Fig. 7). Thus, under osmostress, the phosphorylation of VgrS was detrimental to bacterial growth. Prc-catalysed cleavage of the VgrS sensor to dephosphorylate this TCS system was specifically beneficial to bacterial osmostress adaptation.

Prc controls the VgrR promoter-binding landscape. VgrS autophosphorylates and then transfers the phosphoryl group to the cognate VgrR to control its TF activity in binding promoters.29 Because Prc cleaved the VgrS sensor and decreased its autokinase activity, we hypothesized that Prc will modulate VgrR–DNA-binding affinity.

Chromatin immunoprecipitation sequencing (ChIP-seq) was employed to compare the VgrR-binding promoters in the genome of X. campestris pv. campestris 8004. As shown in Fig. 7a and Supplementary Table 4, after being challenged by 1.0 M sorbitol, ChIP-seq analysis revealed that the VgrR of the WT strain was potentially bound to the promoters of 87 genes (GenBank GEO: GSE120929). In the Δprc mutant, VgrR bound the promoters of 105 genes. Only 41 VgrR-regulated genes were shared by the two strains under osmostress (Fig. 7a), prc may regulate VgrR-promoter binding, and, therefore, a prc deletion may cause changes in genome-scale binding events. The predicted VgrR-binding motif is highly similar to that previously described (Fig. 7b)25. All of the identified VgrR-regulated genes were functionally classified into 16 categories (Fig. 7c, Supplementary Table 4). Among them, the osmostress-related genes, such as those involved in lipopolysaccharide synthesis (XC_3814), lipoic acid synthesis (XC_0713) and transportation (XC_1619, XC_0969 and XC_4223), were absent in the Δprc mutant’s dataset. Notably, The prc promoter was identified by ChIP-seq analysis (Supplementary Table 4). The promoter region of prc also contains a VgrR-binding motif that was then verified by an in vitro electrophoretic mobility shift assay (EMSA) (Fig. 7d). In the WT strain, the prc mRNA level was significantly induced after sorbitol stimulation (Fig. 7e), and in vivo ChIP, together with a quantitative PCR (ChIP-qPCR) assay, revealed that more VgrR was bound to the promoter regions of prc under stress conditions (Fig. 7f). However, in both vgrR and prc mutants containing 669–2,139-bp deletions of prc, prc (or remaining prc sequence, 497–665 bp) mRNA levels were significantly decreased, regardless of the osmostress stimulation (Fig. 7e). In the prc mutant, the level of VgrR occupancy on the prc promoter was significantly decreased, while genetic complementation significantly increased the level of VgrR occupancy (Fig. 7f). Thus, the transcription of prc was directly regulated by VgrR, which may form a feedback regulatory loop between vgrS-vgrR and prc.

We selected six genes from the ChIP-seq data, Xc_0690 (encoding a sugar kinase), Xc_0943 (conserved hypothetical protein), Xc_2164 (YciE orthologue of E. coli that is involved in osmotic stress responses)28, Xc_3300 (outer-membrane protein) Xc_3301 (oxidoeductase), and Xc_3576 (outer-membrane protein), for further functional investigation. As shown in Fig. 8a, independent deletions of Xc_0943, Xc_2164, Xc_3300 and Xc_3301 resulted in decreases in bacterial growth on NYG plus 1.0 M sorbitol plates, while the inactivation of Xc_0690 and Xc_3576 did not have any recognizable impacts, suggesting that Xc_0943, Xc_2164, Xc_3300 and Xc_3301 are involved in the
overexpressing genes were not seen, whereas genetic complementation by mutants, stress-induced increases in the mRNA levels of these (Fig. 8d, e, Supplementary Fig. 8). However, in the P strain.

Prc decreases VgrR phosphorylation level, which unphosphorylated VgrR bound to the promoter regions of downstream genes. To investigate the role of Prc in controlling the transcription of these VgrR-regulated genes, a qRT-PCR analysis revealed that in the WT strain, sorbitol treatment significantly increased the expression levels of the four genes to 158% (\( \text{XC}_{0943} \)), 547% (\( \text{yciE} \)), 412% (\( \text{XC}_{3300} \)) and 174% (\( \text{XC}_{3301} \)) of the untreated sample (Fig. 8d, e, Supplementary Fig. 8). However, in the \( vgrR \) or \( vgrS \) mutants, stress-induced increases in the mRNA levels of these genes were not seen, whereas genetic complementation by overexpressing \( prc \) or \( vgrR \) fully restored deficiencies in the upregulation of the four genes (Fig. 8d, e, Supplementary Fig. 8). This result demonstrated that the inactivation of \( prc \) caused decreases in the mRNA levels of the four genes relative to the WT strain.

\( prc \) control of the transcription levels of VgrR-regulated genes led to the hypothesis that in bacterial cells, proteolysis of VgrS by Prc decreased the phosphorylation level of VgrR, which modulated the TF activity of VgrR. MST analyses then revealed that unphosphorylated VgrR bound to the promoter regions of \( \text{Pprc, PXC}_{0943}, \text{PXC}_{2164}, \text{PXC}_{3300} \) and \( \text{PXC}_{3301} \) with \( K_d \) values from 0.46 to 7.62 \( \mu \)M (Supplementary Fig. 9). However, when VgrR was phosphorylated by VgrS in the presence of ATP, VgrR-P did not bind to the promoter regions of \( \text{Pprc, PXC}_{0943}, \text{PXC}_{2164} \) or \( \text{PXC}_{3301} \) and the binding affinity between VgrR-P and \( \text{PXC}_{3301} \) was decreased (\( K_d = 30.7 \mu \)M). When inactive VgrS was added to the reaction, the binding affinities between VgrR and DNA were similar to those of unphosphorylated VgrR (Supplementary Fig. 9). Thus, unphosphorylated VgrR bound the promoter regions of downstream genes in vitro with higher affinity levels than those of phosphorylated VgrR.

To determine the changes in VgrR–DNA-binding affinities in vivo, ChIP-qPCR was used to estimate the levels of VgrR occupancy in the promoter regions of the selected genes, \( \text{XCV}_{0943} \) and \( \text{yciE} \). As shown in Fig. 8f, g, after stimulation by osmotic stress, the amounts of promoter DNA bound by VgrR were significantly increased in the WT strain to 1110% (\( \text{PXC}_{0943} \)) and 362% (\( \text{PyciE} \)) relative to the unstimulated samples. However, in the \( prc \) mutant, the amounts of promoter DNA bound to VgrR after stress stimulation were significantly decreased relative to the levels in the WT strain. In the genetic complementation strain of the \( prc \) mutation, in which \( prc \) was overexpressed, levels of VgrR–DNA binding were restored to, or even higher than, WT levels (Fig. 8f, g). Therefore, these genetic and biochemical analyses revealed that Prc positively regulates the binding between VgrR and the promoters of these osmotic response genes.
Discourse

Bacterial cells, except those of Mycoplasma spp., usually encode several to over a hundred HKs to detect various environmental stimuli.29,30 Because HKs react to stimuli mainly through autophosphorylation and catalyse phosphoryl transfer to their cognate RRs, which control multiple cellular responses, regulation of the tempo and mode of HK phosphorylation is important for bacterial adaptation.31 Chemical ligands, environmental cues, and the protease and the RR. This signalling process significantly and specifically promoted bacterial resistance to osmotic stress (Fig. 9).

Thus, proteolysis of the sensor region of HK appears to be a molecular mechanism for regulating bacterial TCS phosphorylation and has an unambiguous physiological impact on bacterial adaptation.

Although studies investigating the relationship between proteases and HKs are lacking in bacteria, the proteolysis of Thr/Ser/Tyr receptor kinases to modulate their activities has been extensively studied in eukaryotic cells.34,35 In Proteobacteria, Prc/Tsp-family proteases are widely distributed. These proteases not only recognize and process the C-termini of protein activity. Quenching VgrS phosphorylation then modulated the TF activity of VgrR to bind the promoters of downstream genes. In addition, the transcription of prc itself was subject to regulation by VgrR, which then formed a positive feedback loop between the protease and the RR. This signalling process significantly and specifically promoted bacterial resistance to osmotic stress (Fig. 9).

Thus, proteolysis of the sensor region of HK appears to be a molecular mechanism for regulating bacterial TCS phosphorylation and has an unambiguous physiological impact on bacterial adaptation.
substrates but also degrade the substrates into small peptides. For example, the Prc of *E. coli* controls cell wall synthesis and antibiotic resistance by degrading peptidoglycan hydrolases or outer-membrane lipoproteins. The results of our study indicated that the cleavage of HK sensors by proteases localized in periplasmic space, including Prc/Tsp-family (MEROPS S41-family) and HtrA-family proteases (MEROPS S1-family), is important for regulating bacterial adaptation because, in gram-negative bacteria, a majority of the HKs contain periplasmic sensors to detect environmental signals. Compared with reversible dephosphorylation, proteolysis of the HK sensor holds advantages in regulating TCS signalling: cleavage takes place in the periplasm, rather than the cytoplasm, which is more efficient because it shortens the distance travelled by TM signals. In particular, irreversible proteolysis avoids the continuing phosphorylation of existing HKs, which is more effective if reversible phosphorylation is extremely detrimental to bacterial survival in certain circumstances. However, more experimental studies are needed for the following reasons: (1) there is no consensus recognition or processing sequence for the Prc/Tsp-family and HtrA-family proteases, which makes it difficult to predict the physiological substrates of these proteases. (2) as our results revealed, proteolytic processing of the VgrS sensor by the Prc protease is specific and adaptive to the osmotic stress response (Fig. 9), suggesting that protease-HK interactions are highly specific to environmental signals.

VgrS–VgrR is a pleiotropic TCS of *X. campestris pv. campesstris*. It controls virulence, multiple-stress responses, bacterial growth and the capability of eliciting hypersensitivity reactions in non-host plants. The VgrS sensor contains an EPQE motif to directly bind ferric iron. In iron-depleted environments, VgrS maintains a high level of autophosphorylation and then phosphorylates VgrR to regulate the expression of virulence factors. Substitutions of the conserved phosphorylation sites in VgrS (His) or VgrR (Asp) caused decreases in virulence against host cabbage. However, here, in a prc mutant that exhibited a growth deficiency under osmotic stress, various mutations of vgrs, including deletion of the N-terminal sequence that mimics cleavage by Prc, effectively suppressed the growth deficiency caused by the null mutation of prc (Fig. 6h). In addition, substitution of the conserved His of VgrS or the Asp of VgrR (VgrR) increased the bacterial growth rate under stress conditions (Fig. 9). Thus, a low level of VgrS phosphorylation may promote bacterial resistance to osmotic stress, and the inhibition of VgrS autokinase activity by Prc proteolysis is an effective mechanism. In bacteria, a number of RRs are degraded by proteases in the cytosol, such as CtrA of *Caulobacter crescentus* and DegU of *Bacillus subtilis*. Compared with the proteolysis of RRs, the cleavage of HKs by proteases may be more flexible in TCS regulation because of the following: (1) HK is a receptor, rather than an effector, in cell signalling. Thus, degradation of the HK only impacts the phosphorylation level of the cognate RR (effector). This does not cause complete inactivation of the TCS because the dephosphorylated RR can continue to regulate physiological processes. (2) Bacteria usually encode paralogous TCSs that are generated by operon duplication during evolution. These TCSs form regulatory networks, and the proteolysis of an HK enhances the signalling of the existing compensatory pathways of these paralogous TCSs. This is probably important for the VgrS–VgrR system because there are two functionally unknown, paralogous TCSs in *X. campestris pv. campesstris* (XC_3126-XC_3125 and XC_3452-XC_3451). The phosphatase activity of HK is essential for maintaining high levels of specificity between cognate HKs and RRs. The proteolysis of an HK releases the inhibition on the illegitimate phosphorylation of the RR and then provides an opportunity for cross-talk among different TCSs. Future experiments are needed to verify these possibilities.

Our proteolytic experiment showed that Prc cleaved and degraded the purified VgrS sensor in a low-speed in vitro experiment (~60 min, 28 °C, Fig. 4d), which seemed slower than the cleavage of full-length VgrS in vivo (~5–10 min, 28 °C, Fig. 4f). This difference indicated that the purified VgrS sensor may undergo a conformational change that interferes with Prc recognition in vitro. Alternatively, other adaptors or cellular factors are involved in Prc proteolysis within bacterial cells. Adaptors are proteins that bind proteases to help with the recognition of substrates or proteolysis. In *E. coli*, NlpI is an adaptor of Prc that helps facilitate the protease’s degradation of its physiological substrate, MepS, an outer-membrane protein that is associated with peptidoglycan biosynthesis. The presence of NlpI enhances Prc protease activity by 50-fold. Our TAP analysis provided information on Prc-binding proteins, but it is difficult to predict which one is the Prc adaptor used during processing of the VgrS sensor because adaptors are diverse in structure and include lipoproteins, RRs and arginine kinases. In addition, the Prc protease is activated to cleave the VgrS sensor remains unclear. Our preliminary experiment showed that a high concentration of sorbitol (200 mM to 1.0 M) did not affect the enzymatic activity of Prc, indicating that Prc itself is not a sensor that directly detects osmotic stress. As Fig. 3c revealed, Prc exists as a monomer or trimer in vitro, and only monomeric Prc bound and degraded the VgrS sensor (Fig. 3c, e). This implied that alteration of the quaternary structure of Prc might act as a molecular mechanism to activate its protease activity in vivo.

In addition, the regulatory functions of the proteolytic products of VgrS are unknown. In eukaryotes, fragments of receptor kinases generated by proteases are usually stably maintained in cells. These fragments serve as dominant negative factors, decoy receptors or even cellular signals. As Fig. 5 and Supplementary Fig. 6 show, Prc proteolysis produced a short N-terminal peptide and a truncated VgrS. The two fragments were detected by western blotting and MS analysis, and they were relatively stable. In TCS regulation, the majority of HKs constitutively form homodimers that are autophosphorylated in a trans manner. The presence of a truncated, “decoy” VgrS may disturb this process. Furthermore, if VgrS and its paralogous HKs (XC_3125 and XC_3451) constitute regulatory heterodimers,
Vgr5 proteolysis could affect the signaling of other TCSs. The biological roles and fates of the two fragments remain unclear. Future investigations are needed to study the activating mechanism of Pcr under osmostress and the regulatory roles of HK fragments. The results will provide insights into the cross-talk between TCS signaling and regulation through proteolysis.

Methods

Bacterial strains and growth conditions. Strains used in this study are listed in Supplementary Table 1. E. coli strain BL21 (DE3) (Novagen) was used to express recombinant proteins, and E. coli strain DH5α (Lab collection) was used for molecular cloning. The E. coli strain was cultured at 37 °C in Luria–Bertani medium supplemented with the appropriate antibiotics. Xanthomonas campestris pv. campestris 8004 was cultured at 28 °C in NYG medium with the appropriate antibiotics. Xanthomonas oryzae pv. oryzae PXO99 was cultured at 28 °C in PS medium with the appropriate antibiotics. The following concentrations of anti-biotics were used: 100 μg/ml ampicillin, 50 μg/ml kanamycin, 100 μg/ml specti-nomycin, 34 μg/ml chloromycetin and 25 μg/ml rifampicin. The transformation of bacterial competent cells was performed according to previous studies. Bac-terial electro-competent cells were prepared by extensively washing fresh bacterial cells for three times with ice-cold glycerol (10%). Transformation condition of bacterial competent cells were set as 1.6 kV cm⁻¹ to 2.0 kV cm⁻¹ and conducted in a Bio-Rad Pulser XCell (Bio-Rad, USA).

Construction of bacterial recombinant strains. In-frame deletion mutants and recombinant strains of X. campestris pv. campestris were constructed using the homologous, double-cross-over method with the suicide vector pK18mobsacB as described in previous study. Briefly, the upstream and downstream homologous sequences of the region to be deleted were amplified using primers listed in Supplemen-tary Table 2. After restriction enzyme digesting and ligating into pK18mobsacB, the recombinant plasmid was electroporated into competent cells to generate single-cross-over mutants by selection on NYG plates containing kan-a-mycin. The putative single-cross-over mutants were confirmed by PCR, cultured in NYG medium for second-round homologous cross-over, and grown on NYG plates containing 10% sucrose to select in-frame deletion mutants, which were further verified by PCR and subsequent sequencing. Genetic complementation was achieved in trans by providing full-length genes using the broad host vector pHM1.

Phenotypic characterization. Plant inoculations and virulence assays were per-formed as described previously. Briefly, strains of X. campestris pv. campestris were inoculated into susceptible 4-week-old cabbage (Brassica oleracea cv. ‘Jing-Feng 1’) by clamping the leaves with pre-sterilized scissors dipped into the bacterial culture. The lesion length was measured at 10 d after inoculation with at least 12 repetitions. Virulence level was scored by a semi-quantitative standard as follows: 0 (healthy leaves); 1 (lesion length ≤ 0.5 mm); 2 (lesion length ≤ 1 mm); 3 (lesion length ≤ 2 mm); and 4 (lesion length > 2 mm). The bacterial strains were scored as follows: 0 (no symptoms); 1 (mild; symptoms observed only on the cut site); 2 (moderate; chlorosis extending from the cut site); 3 (severe; blackened leaf and severe stunted growth); 4 (extensive; death and drying within the chlorotic area). Virulence level was scored by a semi-quantitative standard as follows: 0 (no symptoms); 1 (mild; symptoms observed only on the cut site); 2 (moderate; chlorosis extending from the cut site); 3 (severe; blackened leaf and severe stunted growth); 4 (extensive; death and drying within the chlorotic area). The virulence level was scored by a semi-quantitative standard as follows: 0 (no symptoms); 1 (mild; symptoms observed only on the cut site); 2 (moderate; chlorosis extending from the cut site); 3 (severe; blackened leaf and severe stunted growth); 4 (extensive; death and drying within the chlorotic area).

Protein expression and purification. The pET System (Novagen, Madison, WI, USA) was used for the cloning and expression of recombinant proteins in E. coli. Recombinant vectors used to express proteins are listed in Supplementary Table 1. Induction, expression, and purification were performed using affinity chromatog-raphy with Ni-NTA agarose beads (Novagen, USA) according to the manu-facturer’s manual. Purified His₅-tagged proteins were concentrated with Amicon Ultra-4 and -15 Centrifugal Filter Units (Merck Millipore, Germany) and dissolved in storage buffer (50 mM Tris-HCL, pH 8.0, 0.5 mM EDTA, 50 mM NaCl and 5% glycerol) before use.

Gel filtration chromatography. The purified proteins were concentrated with Amicon Ultra-4 and -15 Centrifugal Filter Units and subsequently subjected to gel filtration with Fast Protein Liquid Chromatography AKTA Purifier 10 with Frac-tion 900 (GE Healthcare, USA). The AKTA system was pre-equilibrated with 20 mM Tris-HCL, pH 8.0, at a flow rate of 0.5 ml/min and then applied to a Superdex 75 10/300 GL column to separate dimers and oligomers from the monomer. The elution profiles were collected at 280 nm and confirmed by SDS-PAGE gels and western blots.

Analytical ultracentrifugation. Analytical ultracentrifugation was performed at 20 °C in a ProteomeLab XL-1 analytical ultracentrifuge (Beckman, USA) equipped with absorbance optics and an An60-Ti rotor. The sedimentation velocity analysis of the affinity chromatography-purified Prb was conducted at 240,000 g using double sector cells, and data were collected at the concentration of A₄₅₀ = 0.7 in sodium phosphate buffer (pH 8.0) plus 150 mM NaCl for each peak sample. These values were normalized to standard conditions by correcting for buffer density and viscosity. Interference sedimentation coefficient distributions were calculated from the sedimentation velocity data using the SEEDFIT software program (www.analyticalultracentrifugation.com).

Subcellular fraction. To prepare the total cell protein fraction, the bacterial cells were collected and resuspended in 1X SDS-PAGE loading buffer (50 mM Tris-HCL, pH 6.8, 2% SDS, 0.1% bromphenol blue, 10% glycerol and 100 mM DTT) and immediately boiled for 3 min to denature the protein. To prepare the periplasmic fraction, the bacterial cell pellets were thoroughly resuspended in Buffer A (30 mM Tris-HCL, pH 8.0, 20% sucrose and 1 mM EDTA). After rotating at room temperature for 10 min, the cells were centrifuged and the cell pellet was resuspended in ice-cold Buffer B (5 mM MgSO₄). After rotating at 4 °C slowly for 10 min on ice, the periplasmic proteins were released into the buffer. To isolate the periplasmic fraction, the bacterial cells were collected, resuspended and sonicated in a low-salt buffer (100 mM sodium phosphate, pH 7.0, 10% glycerol, 5 mM EDTA, 1 mM PMSF). The periplasmic membrane vesicles were collected by ultracentrifugation (150,000 x g for 60 min at 4 °C) before removing the unbroken cells and debris through two rounds of centrifugation (100,000 x g for 10 min at 4 °C). After washing with high salt buffer (20 mM sodium phosphate, pH 7.0, 2.0 M KCl, 10% glycerol, 5 mM EDTA, 5 mM DTT and 1 mM PMSF), the inverted membrane vesicles were resuspended in storage buffer (20 mM Tris-HCL, pH 7.5 and 10% glycerol).

Enzymatic and endo-protease activity assays. The qualitative enzymatic activity assay was performed in the enzymatic reaction buffer (50 mM Tris-HCL, pH 7.5, 2 mM DTT, 25 mM NaCl, 25 mM KCl and 5 mM MgCl₂) with the proper amounts of protease and substrate co-incubated at 28 °C for the indicated time, or at a different temperature, DTT concentration, or pH value for 5 min. The reactions were stopped by adding SDS-PAGE loading buffer before loading the samples into a 12% PAGE gel and performing the electrophoresis. The gel was stained with Coomassie brilliant blue to determine the remaining substrate.

The quantitative endo-protease activity assay was performed for azocasein (Megazyme) as the substrate. The pre-equilibrated protease (5 μM) was co-incubated with pre-equilibrated azocasein (400 μM) in Buffer A (100 mM sodium phosphate). The solution was fully mixed and incubated for 10 min at 37 °C. The enzymatic reaction was terminated by the addition of 2.5 times the volume of 5% trichloroacetic acid to precipitate the non-hydrolyzed azocasein. The reaction was vigorously stirred for 5 s and stood for 5 min at room temperature. Then, it was centrifuged at 3000 x g for 10 min, and the absorbance of the supernatant solutions at 440 nm was determined. The samples, minus the protease, which was substituted with Buffer A, served as blanks.

To measure the enzyme kinetics of the protease, the same amount of protease was co-incubated with different amounts of azocasein in Buffer A (100 mM sodium phosphate). The subsequent procedure was performed in the same manner as the qualitative endo-protease activity assay. The Km, which is the Michaelis–Menten constant, and Vmax, which is the maximum reaction velocity, values were determined using the Lineweaver–Burk plot method.

TAP and MS identification. TAP was carried out following the protocol of the FLAG HA Tandem Affinity Purification Kit (Sigma-Aldrich). Briefly, the bacterial cells were collected and ground before being resuspended in lysis buffer [50 mM Tris-HCL, pH 8.0, 0.13 M NaCl, 1 mM EDTA, pH 8.0, 1 mM PMSF and 1 tablet/20 ml protease inhibitor cocktail tablets (Roche)]. They were centrifuged at 13,000 x g for 20 min at 4 °C, and the supernatant represented the whole cell lysate. Pre-washed ANTI-FLAG M2 resin was added to the sample lysate and incubated for 2 h to overnight at 4 °C with slow shaking. For each wash, the resin was gently agitated in the lysis buffer, centrifuged at 3000 x g for 1 min, and then the remaining final wash volume was decanted without losing any resin. The resin–protein complex was washed with lysis buffer using three rounds of low-speed centrifugation. The remaining resin was transferred into a spin column, and 2.5 volume of 3X FLAG peptide (150 ng/μl) was added and co-incubated for at least 2 h at room temperature. The resin was washed with lysis buffer using three rounds of low-speed centrifugation and then eluted with 3X FLAG peptide solution. The elution profiles were collected and confirmed by SDS-PAGE gels and western blots.
10 min at 4 °C. The column was spun before removing the tip to a clean micro-centrifuge tube and keeping the eluate, which contained the eluted FLAG–protein complex. The elution process was repeated twice. Then, 40 µl pre-washed ANTI-HA resin in lysis buffer was added and incubated 30 min to 2 h at 4 °C with slowly shaking. The supernatant was removed after the incubation, and the ANTI-HA resin–protein complex was washed with lysis buffer through three rounds of low-speed centrifugation. Then, 50 µl of 8 M urea was added and co-incubated for a minimum of 10 min at room temperature. The column was spun for 1 min, and the eluate was loaded and separated on a 12% SDS-PAGE gel. After silver staining, the differential gel bands between sample and control were manually excised, and each band was then enzymatic digestion.

The gel plugs were further cut into small plugs, washed in 100 µl freshly made 30 mM K2HPO4 and 100 mM Na2SO4 (1:1, v:v) buffer for seconds, rinsed with 25 mM NH4HCO3, dehydrated in 100% acetone for 10 min, and dried in a SpeedVac (Labconco) for 15 min. The reduction procedure was performed in solution A (10 mM DTT and 25 mM NH4HCO3) for 45 min at 56 °C, and then, the alkylation procedure was performed in solution B (40 mM iodoacetamide and 25 mM NH4HCO3) for 45 min at room temperature in the dark. The gel plugs were washed with 50% acetone in 25 mM ammonium bicarbonate twice. The gel plugs were then dried in the SpeedVac for 15 min and digested with sequence-grade modified trypsin (50 ng per band) in 25 mM NH4HCO3 overnight at 37 °C. Formic acid was added to a 1% final concentration to stop the enzymatic reaction before transferring the solution to a sample vial for subsequent LC–MS/MS analysis.

The nanoLC–MS/MS identification of proteins was performed on a Thermo Finnigan LTQ linear ion trap mass spectrometer in line with a Thermo Finnigan Surveyor MS Pump Plus HPLC system. Tryptic peptides were loaded onto a trap column (500 µm inner diameter, 5-µm particle, iAsp Technologies, CA, USA). The peptides were eluted over gradient solution C (80% acetonitrile and 0.1% formic acid) at a flow rate of 300 nL/min and introduced into the online linear ion trap mass spectrometer (Thermo Fisher Corporation, San Jose, CA, USA) using nano electrospray ionization. The five most abundant ions (one microscan per spectrum) were selected, and their fragmentation was monitored on the scan mass spectrum by collision-inducement dissociation for data-dependent scanning.

MS data were analysed with SEQUEST against NCBI’s X. campestris pv. campestris 8004 protein database and displayed with Bioworks 3.2. Peptides with +1, +2, or +3 charge states and with cross correlations of ≥1.90, ≥2.5, and ≥3.0, respectively, were accepted. Carbamidomethylation on cysteine and oxidation on methionine were selected as residue modifications. SEQUEST was searched with a peptide tolerance of 3.0 Amu and a fragment of 1.0 Amu.

### Microscale thermoresistance (MST) measurement

The MST measurement was used for detecting protein–protein interactions. Briefly, 10 µM purified VgrS sensor was labelled on a Monolith NT Protein Labeling Kit Red-NS (Nano Temper Technologies GmbH, München, Germany) using red fluorescent dye NT-647 N-hydroxysuccinimide (amine-reactive) according to the manufacturer’s instructions. The reagents were removed by buffer-exchange at amitogen chromatography, the labelled VgrS sensor was eluted in NTA buffer (300 mM NaCl and 50 mM sodium phosphate buffer, pH 7.0). The binding assays were performed on a Monolith NT.115 Microscale Thermoresistance instrument (Nano Temper Technologies GmbH) using standardly treated capillaries. Equal amounts of labelled sensor were mixed in solution A with unlabeled protein and purified PrcS475A, and PEP + DUF-A2047573A, which were exchanged into the IX NTA buffer with 0.05% Tween, using a 1:1-series dilution method. The curves were fitted by Nano Temper Analysis Version 1.5.41 from three replicates, and the value of dissociation constant (Kd) was calculated.

### SPR

The binding kinetics of protein–protein interactions were estimated by the SPR assay, which was performed using a Biacore 3000 (GE Healthcare) at 25 °C. The running buffer was PRS (0.27 g/L KH2PO4, 1.42 g/L NaH2PO4, 8 g/L NaCl, 0.2 g/L KCl, pH 7.4 and 0.005% Tween-20). As the manufacturer described, one flow cell of a CMS sensor chip was activated with a mixture of 0.2 M N-(2-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride and 0.05 M N-hydroxy-succinimide in ddH2O. The ligand protein, in 10 mM sodium acetate (pH 4.5), was injected for 10 min at a flow rate of 0.1 µL/min. The remaining binding sites were blocked by 1 M ethanolamine (pH 8.5). In total, 3000 response units of the ligand protein were immobilized. Protein at different concentrations (double dilution) was injected over the immobilized protein and blank flow cells for 5 min at a flow rate of 30 µl/min. The bound protein was removed with 0.01% SDS for 30 s after the 5-min dissociation phase. The curves were then fitted by the KD Fit function of NanoTemper Analysis software version 1.5.41 was used for curve fitting and calculation of the value of dissociation constant (Kd).

### EMSA

**PCR products of the promoter region were labelled with [32P]-ATP using T4 polynucleotide kinase (Fermentas) and purified with a ProbeQuant G-50 column (GE, New York, NY, USA) that removed free [32P]-ATP.** The unfractionated DNA probe (0–10 µmol) and purified protein (0–10 µg) were incubated together for 30 min at 28 °C in reaction buffer (10 mM Tris-HCl pH 7.5, 50 mM KCl, 1 mM DTT, 50 mM poly(dIdC) and 10 mM EDTA, pH 8.0). Then, 10 fmol labelled DNA probes were subsequently added to each reaction for binding and competition for another 30 min at 28 °C. To stop the EMSA reaction, 4 µL DNA loading buffer (0.25% bromophenol, 40% sucrose) was added. The samples were loaded onto a 5% native PAGE gel and performing the electrophoresis under 120 V for 40 min with 0.5X TBE buffer (5× g/L Tris, 2.75 g/L boric acid and 2 mM 0.5 M EDTA, pH 8.0) before autoradiography.

**ChIP-seq and ChIP-qPCR.** ChIP-seq was performed as previously reported. Briefly, the bacterial strains grown in NYG medium until OD600 = 0.8 ± 0.05 were treated with or without 800 mM sorbitol for 5 min. After collection by
centrifugation, the samples were crossing-linked with 1% (V/V) formaldehyde for 20 min and subsequently quenched with 20% (V/V) 0.5 M glycine for 10 min. Then, the bacterial cells were recollected, washed with cold PBS (0.27 g/l K2HPO4, 1.42 g/l Na2HPO4, 8 g/l NaCl, and 0.2 g/l KCl, pH 7.4) twice, and resuspended in ChIP lysis buffer (10 mM Tris, pH 8.0, 20% sucrose, 50 mM NaCl, 10 mM EDTA, pH 8.0, 10 mg/ml lysozyme, and 1 mM PMSE). Quadruple the lysis buffer volume of immunoprecipitation buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8.0, 1% Triton X-100, 0.1% sodium deoxycholate and 0.1% SDS) was added to the bacterial cell suspension, and the suspension was sonicated with a Diagenode Bioruptor (Diagenode, Liège, Belgium), generating 100–300 bp DNA fragments. The cell lysis was pre-cleared with 20 µl Protein A sepharose (GE) for 10 min at 4 °C on a rotator, and 300–500 µl aliquots were retained as the inputs. For the ChIP assay, 50 µl Protein A sepharose and 2 µl anti-His, monoclonal antibody (Abmart, China, M20001L) was added to a 1.5-ml aliquot of the cell lysis. The mixture was incubated at 4 °C overnight or at least 8 h on a rotator. The protein A sepharose beads were collected and washed with immunoprecipitation buffer twice, and subsequently with wash buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, pH 8.0, 0.5% Nonidet P40 and 0.5% sodium deoxycholate), high salt wash buffer (50 mM HEPES, pH 7.9, 500 mM NaCl, 1 mM EDTA, pH 8.0, 0.1% SDS, 1% Triton X-100 and 0.1% deoxycholate) and TE buffer (pH 8.0) once. After adding 100 µl of elution buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, pH 8.0 and 1% SDS) at 65 °C for 10 min, the immuno-precipitated chromatin DNA was removed from the beads. Then, the eluted DNA was treated with RNase A to remove contaminating RNA at 42 °C for 90 min and subsequently with Proteinase K to remove contaminating protein at 65 °C overnight. Further, the DNA was extracted with 24:1 (v:v) chloroform:isoamyl alcohol, subsequently with Proteinase K to remove contaminating protein at 65 °C overnight and finally with phenol:chloroform:isoamyl alcohol. The DNA was precipitated with 1 mM EDTA, pH 8.0) once. After adding 100 µl of elution buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, pH 8.0 and 1% SDS) at 65 °C for 10 min, the immuno-precipitated chromatin DNA was removed from the beads. Then, the eluted DNA was treated with RNase A to remove contaminating RNA at 42 °C for 90 min and subsequently with Proteinase K to remove contaminating protein at 65 °C overnight. Further, the DNA was extracted with 24:1 (v:v) chloroform:isoamyl alcohol, and precipitated with ethanol. Finally, DNA fragments of 100–500 bp were collected and purified using a 2% agarose gel and PCR purification kit (Qiagen, Duesseldorf, Germany), respectively. An Illumina HiSeq 2000 system (Illumina, San Diego, CA USA) was used for high-throughput sequencing, which was conducted by the Beijing Institute of Genomics genome science. The high-throughput sequencing reads were aligned to the genome sequence database of X. campestris pv. campestris 8004. Peak calling was conducted by MACS2. The consensus binding motif analysis was completed with MEME and FIMO tools in the MEME software suite.

ChIP-qPCR was performed to quantify the amount of promoter DNA bound by the His-tagged protein or VgrR in vivo. Input DNA (10 pg) was quantified with qPCR using Maxima SYBR Green (Fermentas). The percentage of immunoprecipitated promoter DNA was calculated in comparison to the amount of input.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All relevant data in this work has been included in the manuscript. The original, uncropped images were shown in Supplementary Fig. 10. Data and experimental materials are available from the corresponding author upon request. The accession number for the ChIP-seq dataset reported in this study is GenBank GEO: GSE120292.

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Author contributions
W.Q. conceived the study. C.-Y.D. and W.Q. designed the experiments. C.-Y.D., H.Z., L.-L.D., Y.J.L., Y.P., S.-T.S., Y.W., L.W. and W.Q. performed the experiments and analysed the data. C.-Y.D. and W.Q. drafted the manuscript, and all authors contributed to the revisions. All authors read and approved the manuscript.

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