MarR-Family Transcription Factor HpaR Controls Expression of the *vgrR-vgrS* Operon of *Xanthomonas campestris* pv. *campestris*

Yue Pan,^{1,2} Fang Liang,³ Ru-Jiao Li,³ and Wei Qian^{1,†}

¹State Key Laboratory of Plant Genomics, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China;

²School of Life Sciences, University of Chinese Academy of Sciences, Beijing 100049, China; and ³Beijing Institute of Genomics, Chinese Academy of Sciences

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MarR (multiple antibiotic resistance regulator)-family transcription factors (TFs), which regulate the expression of virulence factors and other physiological pathways in pathogenic bacteria, are regarded as ideal molecular targets for the development of novel antimicrobial strategies. In the plant bacterial pathogen *Xanthomonas campestris* pv. *campestris*, HpaR, a typical MarR-family TF, is associated with bacterial virulence, but its mechanism of virulence regulation remains unclear. Here, we dissected the HpaR regulon using high-throughput RNA sequencing and chromatin immunoprecipitation sequencing. HpaR directly or indirectly controls the expression of approximately 448 genes; it acts both as a transcriptional activator and a repressor to control the expression of downstream genes by directly binding to their promoter regions. The consensus HpaR-binding DNA motifs contain imperfect palindromic sequences similar to [G/T]CAACAATT[C/T]TTG. In-depth analysis revealed that HpaR positively modulates transcription level of the *vgrR-vgrS* operon that encodes an important two-component signal transduction system to sense iron depletion and regulate bacterial virulence. Epistasis analysis demonstrated that *vgrR-vgrS* is a core downstream component of HpaR regulation, as overexpression of *vgrR* restored the phenotypic deficiencies caused by a *hpaR* mutation. This dissection of the HpaR regulon should facilitate future studies focused on the activating mechanism of HpaR during bacterial infection.

Xanthomonas campestris pv. *campestris* is a model organism for studying the disease pathogenesis of plant bacterial pathogens. A gram-negative bacterium belonging to class γ -Proteobacteria (Swings and Civetta 1993), this pathovar is the causative agent of black rot disease in cruciferous plants and was selected by plant pathologists as one of the “top 10” bacterial pathogens that are of scientific or economic importance (Mansfield et al. 2012). *X. campestris* pv. *campestris* invades hosts through stomata, wounds, and hydathodes and causes wilting, cankering, and distinctive “V”-shaped necrosis at plant leaf margins (Simpson and Johnson 1990). The genome of *X. campestris* pv. *campestris*, which comprises approximately 5.0 million base pairs (bp), encodes

complex regulatory systems to control the expression of virulence factors and infectious processes (da Silva et al. 2002; Qian et al. 2005; Vorhölter et al. 2008). For example, a number of transcription factors (TFs) among the approximately 190 TFs encoded by the bacterium have been found to control the expression of virulence factors or virulence-associated genes; these include four TFs belonging to the response regulators of two-component signal transduction systems (VgrR, PhoP, HrpG, and XerR/XibR), a CRP-like TF (Clp), an AraC-family TF (HrpX), a GntR-family TF (HpaR1), a LuxR-family TF (XccR), a Rrf2-family TF (IscR), an OxyR-family TF (OxyR), a MerR-family TF (SoxR), two Fur-family TFs (Fur and Zur), and a MarR (multiple antibiotic resistance regulator)-family TF (HpaR). Inactivation of the aforementioned TF-coding genes results in remarkable virulence attenuation (Fuangthong et al. 2015; He et al. 2007; Huang et al. 2009; Ikawa and Tsuge 2016; Koebnik et al. 2006; Mahaviahakanont et al. 2012; Mongkolsuk et al. 2000; Pandey et al. 2016; Peng et al. 2017; Su et al. 2016; Wang et al. 2011, 2016; Wei et al. 2007; Wengelnik et al. 1996). These TFs regulate various physiological processes in *X. campestris* pv. *campestris*, such as the expression of the type III secretion system, production of extracellular polysaccharides or enzymes, metal uptake, envelope stress responses, and cytogenesis, which suggests that bacterial virulence is tightly controlled by a complex network of TFs. To date, however, only the regulons of a limited number of these TFs, such as Clp, VgrR, PhoP, HrpG, and HrpX, have been dissected in *X. campestris* pv. *campestris* by high-throughput transcriptomic methods. Investigation of the regulatory mechanisms of these virulence-associated TFs is needed to facilitate the development of novel antibacterial approaches to artificially manipulate these molecular targets.

MarR-family TFs regulate virulence in a diverse range of pathogenic bacteria (Grove 2013). These TFs control multiple cellular processes, including the production of virulence factors, response to antibiotic and oxidative stresses, and degradation of aromatic compounds (Ellison and Miller 2006). MarR-type TFs contain winged helix-turn-helix (wHTH) structures that directly bind double-stranded DNA (dsDNA) and modulate the transcription levels of genes (Hong et al. 2005). Small molecules, such as salicylates, metals, aromatic compounds, *p*-coumaroyl-CoA, and urate, are bound by the ligand-binding pocket of MarR-family TFs and modulate the DNA-binding activity of these proteins (Perera and Grove 2010). In *X. campestris* pv. *campestris*, HpaR (hypersensitive response and pathogenicity-associated regulator) was the first MarR-type TF found to regulate bacterial virulence. Disruption of this gene by transposon insertion was proved to significantly

[†]Corresponding author: Wei Qian; E-mail: qianw@im.ac.cn

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decrease virulence against its cabbage host (Qian et al. 2005). Subsequent analyses revealed that *hpaR* controls production of extracellular proteases and the capacity to trigger a hypersensitive response (HR) in nonhost pepper (Wei et al. 2007). Inactivation of *hpaR*, however, did not affect production of exopolysaccharides, resistance to oxidative stress, or production of extracellular amylases and cellulases. In addition, the expression of *hpaR* is subject to the control of *hrpG* and *hrpX*, two other TFs that are important in the expression of the bacterial type III secretion system (Wei et al. 2007). cDNA-amplified fragment length polymorphism analysis of a close relative, *X. campestris* pv. *vesicatoria*, revealed that the messenger RNA (mRNA) level of a *hpaR* ortholog was induced in a *hrpG*-dependent manner when the bacterium was grown in a minimal medium (Noël et al. 2001). However, the HpaR regulon in bacterial species belonging to the genus *Xanthomonas* has never been investigated, and how this TF controls virulence factors and virulence-associated factors in *X. campestris* pv. *campestris* remains completely unknown.

In the present study, we used RNA sequencing (RNA-seq) and chromatin immunoprecipitation sequencing (ChIP-seq) to dissect the HpaR regulon. Our analysis revealed that HpaR acts as an activator or a repressor to modulate the expression of approximately 448 genes involved in a diverse set of physiological processes. The consensus HpaR-binding DNA motif was predicted and then experimentally verified by DNase I footprinting and an electrophoresis mobility shift assay (EMSA). Among the HpaR-regulated genes, we found that this TF binds directly to the promoter region of the *vgrR-vgrS* operon (Wang et al. 2016), which encodes a two-component signal transduction system (TCS) that senses iron-depleted environments and regulates bacterial virulence. Overexpression of *vgrR* partially restored the virulence deficiency of the *hpaR* mutant and fully suppressed its protease overexpression and enhanced biofilm formation phenotypes. Our results demonstrate that HpaR is a global regulator of *X. campestris* pv. *campestris*. This TF controls the bacterial virulence through various pathways, including directly modulating the transcriptions of virulence factors and indirectly affecting virulence-associated factors by interacting with other regulators.

RESULTS

Transcriptomic profiling of *hpaR*-regulated genes by RNA-seq.

The *hpaR* gene (XC_2827) encodes a 166-amino acid (aa) TF that contains a wHTH DNA-binding motif (aa positions 52 to 111). Both protein secondary structure prediction by Pfam searching and construction of a three-dimensional structure by Phyre2 modeling revealed that the protein is a well-matched MarR-family TF (confidence >99.9%) (Supplementary Fig. S1). To investigate the HpaR regulon, RNA-seq analysis was used to screen for differentially expressed genes between a *hpaR* mutant and the wild-type (WT) strain of *X. campestris* pv. *campestris* 8004. A nonpolar, in-frame deletion mutant of *hpaR* (Δ hpaR) was constructed by the homologous, double-crossover recombination method (Supplementary Table S1). Bacterial cells were grown in rich nutrient-yeast-glycerol (NYG) medium and *Xanthomonas* virulence mimic 2 (XVM2) minimal medium that induces the expression of the type III secretion system (Schulte and Bonas 1992). Total RNAs were extracted from bacterial cells grown to the logarithmic phase (optical density at 600 nm [OD₆₀₀] = 0.4 ± 0.01). After synthesis of cDNA, adaptor ligation, polymerase chain reaction (PCR) amplification, and high-throughput sequencing, RNA-seq analysis revealed that 139 and 86 genes were upregulated (threshold >1.5-fold) in the *hpaR* mutant grown in NYG and

XVM2 media, respectively, while 94 and 72 genes were respectively downregulated when the bacterium was grown in these media (Fig. 1A to D, Supplementary Tables S3 and S4). A total of 39 genes, including four flagellar assembly genes (*flgG*, *fliH*, *fliQ*, and a hook basal body protein-coding gene), were identified as differentially expressed under both growth conditions. Collectively, the expression levels of 352 nonredundant genes were impacted by the *hpaR* deletion in our test regardless of growth conditions (Fig. 1A and B). These genes were classified into 10 functional categories, such as pathogenicity, metabolism of biomacromolecules, cell structure, and material transportation, which indicates that HpaR directly or indirectly regulates multiple physiological processes in *X. campestris* pv. *campestris*.

When *X. campestris* pv. *campestris* was grown in rich NYG medium, inactivation of *hpaR* noticeably caused upregulation of virulence-associated genes encoding type II and III secretion systems, extracellular cellulase and pectate lyase, and flagellar assembly proteins, suggesting that HpaR directly or indirectly represses the expressions of these virulence-associated genes when the bacterium is grown in a nutrition-rich environment. Under XVM2 growth conditions, the *hpaR* mutation decreased the expression levels of genes responsible for lipopolysaccharide core synthesis, flagellar assembly, chemotaxis, and transportation, indicating HpaR positively controls the transcription of these virulence-associated genes. In addition, the *hpaR* mutation led to increases or decreases in the expressions of many regulatory genes, especially TCS genes and those encoding various TFs belonging to LysR, MerR, DeoR, LacI, and MarR-families, thus suggesting that HpaR plays an important role in complex regulatory networks.

Identification of HpaR-regulated genes by ChIP-seq.

RNA-seq analysis identifies genes regulated directly or indirectly by a TF. Consequently, ChIP-seq analysis is generally superior to RNA-seq for determining directly regulated genes because promoter regions coimmunoprecipitated with the TF are enriched during the analysis (Park 2009). To perform the ChIP assay, we constructed a strain of *X. campestris* pv. *campestris* (*hpaR*-his₆) producing recombinant HpaR fused with a His₆ epitope tag at the C-terminal. Phenotypic profiling revealed that this genetic manipulation had no effect on bacterial virulence, production of extracellular proteases, amylases, and cellulases, or the ability to elicit a hypersensitive reaction in the nonhosts (Supplementary Fig. S2). This result indicates that the His₆ tag had no marked negative impact on HpaR regulation. The recombinant strain was cultured in rich NYG and XVM2 minimal media. During ChIP, the DNA fragments of bacterial cells coimmunoprecipitated with HpaR using monoclonal anti-His₆ antibody were enriched. Subsequent peak calling of the ChIP-seq data showed that HpaR was bound to the 5' regions (<200 bp) of 122 and 71 genes when *X. campestris* pv. *campestris* was grown under NYG and XVM2 conditions, respectively (Fig. 2A and B). There were, thus, 185 redundant genes whose promoter regions potentially bound to HpaR, eight of which were shared between bacterial samples under the two growth conditions. These 185 genes were classified into nine functional categories as shown in Supplementary Tables S5 and S6. Notably, the 5' promoter sequence of *hpaR* was identified among these genes, which suggests that HpaR autoregulates its own transcription by binding to its own promoter.

Comparative analysis of ChIP-seq and RNA-seq data revealed that 89 genes (48.1%) identified by ChIP-seq were also identified by RNA-seq. In the *hpaR* mutant, 34 and 12 ChIP-seq-identified genes were upregulated in NYG and XVM2 media, respectively, while 25 and 18 were correspondingly downregulated. These

results suggest that HpaR acts either as an activator or a repressor depending on the regulated genes.

HpaR regulates transcription of downstream genes by directly binding to their promoter regions.

To investigate whether HpaR exerts its TF activity by binding to downstream gene promoter regions, we experimentally verified seven genes identified by RNA-seq and ChIP-seq analyses: *XC_0136* (encoding a RhsD protein), *XC_0141* (α -amylase), *XC_0405* (TonB-dependent receptor), *XC_1057* (fimbrial assembly protein), *XC_1642* (α -glucosidase), *XC_2592* (also a RhsD protein), and *hpaR* (*XC_2827*) itself. The promoter regions of these genes contained sequences obtained by peak calling of the ChIP-seq data (Fig. 3A; Supplementary Fig. S3A). The corresponding DNA promoter sequences were amplified, and the PCR products (153 to 218 bp) were labeled with [γ - 32 P]ATP. Recombinant HpaR fused with a C-terminal His₆ tag was obtained using an *Escherichia coli* BL21(DE3) expression system and was then purified by affinity chromatography. An EMSA revealed that HpaR bound to all seven DNA probes to form protein-DNA complexes (Fig. 3B to F). When unlabeled PCR probes were added to the reaction mixtures as competitors, the isotope signals representing the HpaR-DNA complexes gradually decreased and the amount of free labeled DNA probes aggregating on the bottom of the non-denaturing polyacrylamide gels increased. These results demonstrate that these HpaR-DNA binding events were highly specific.

Based on the above results, quantitative real-time reverse transcription (qRT)-PCR was used to quantify mRNA levels of these seven genes in the *hpaR* mutant. As shown in Figure 3G to K, when the bacterial strains grew in rich NYG medium, the mutation of *hpaR* caused significant decreases in the mRNA levels of *XC_0405* and *XC_1057* to the levels of 47% and 45% compared with the WT strain but increased the expression level

of *XC_0141* to 180% of the WT. It did not markedly affect the expression levels of *XC_0136*, *XC_1642*, *XC_2592*, and *hpaR* itself. These results suggest that HpaR positively controls the expression of *XC_0405* and *XC_1057* under rich nutrition supply. When the bacterial strains were grown in XVM2 medium, however, the amounts of mRNA of *XC_0136*, *XC_1642*, *XC_2592*, and *hpaR* were significantly increased in the *hpaR* mutant (ranging from 213 to 279% of the WT level), while the transcription levels of *XC_0141*, *XC_0405*, and *XC_1057* remains unchanged. Genetic complementation of *hpaR* by in trans provision of a full-length copy of *hpaR* in the mutant significantly restored gene-expression levels relative to those of WT strains (Fig. 3G to K). These results support the idea that HpaR acted as a repressor to control transcription of the four genes (*XC_0136*, *XC_1642*, *XC_2592*, and *hpaR*) when *X. campestris* pv. *campestris* was grown in minimal medium. These observations also confirm that HpaR autoregulated its own transcription through a negative feedback loop when the bacterium was grown in XVM2 medium. Collectively, the above analyses experimentally demonstrate that HpaR has TF activity. The protein directly binds to the promoter region of downstream genes and modulates their transcription. In all the tested genes, including *hpaR* itself, HpaR acts as both activator and repressor to control their expression, which depends on the growth conditions of the bacterium.

Dissection of the consensus HpaR-binding DNA motif.

MEME analysis of potential binding DNA sequences obtained from the ChIP-seq data predicted two HpaR-binding motifs, which corresponded respectively to NYG and XVM2 growth conditions. As shown in Figure 2C, sequences of the two motifs were highly similar with slight variation. Under NYG medium, HpaR bound to a C[A/G][A/G]C[A/G][A/C][T/G][T/G][T/C]TTG-like motif, whereas a [G/T]C[A/G][A/G]C[A/C]A[T/G][T/G]CTT-like motif was observed under the

Fig. 1. Identification of differentially expressed genes associated with a mutation in *hpaR*. **A**, Venn diagram showing the number of differentially expressed genes in the *hpaR* mutant compared with the wild-type (WT) strain grown in nutrient-yeast-glycerol (NYG) and *Xanthomonas* virulence mimic 2 (XVM2) media. **B**, Functional categories of nonredundant genes whose expressions were associated with the mutation in *hpaR*. Bacterial strains were grown in NYG or XVM2 media. **C** and **D**, Differentially expressed genes associated with the *hpaR* mutation. The number of up- and downregulated genes is shown. Functional categories are the same as in B.

XVM2 condition. To experimentally verify the consensus binding motif, we selected the previously mentioned *XC_1642* gene for in-depth analysis. *XC_1642*, which encodes an α -glucosidase, was identified by ChIP-seq analysis in a bacterial sample grown in XVM2 medium. According to RNA-seq analysis, the expression level of this gene was 1.5 to 2.5 times greater in the *hpaR* mutant compared with the WT strain. DNase I footprinting subsequently revealed that the HpaR protein protects two DNA regions (35 and 29 bp in length) at the 5' end of *XC_1642* (Fig. 4A). As shown in Figure 4A, the two DNA regions contained the predicted HpaR-binding motif. We therefore chemically synthesized 40- and 35-bp double-stranded oligonucleotides based on the sequences of the two regions and labeled them with [γ - 32 P]ATP. An EMSA using these two oligonucleotides as probes revealed that the purified HpaR protein directly bound to them (Fig. 4B and C). The binding events were specific, as addition of increasing concentrations of unlabeled DNA probes gradually caused the labeled probes to be out-competed (Fig. 4B and C) and led to a marked decrease in the isotope signals representing HpaR-DNA complexes. To quantify HpaR-oligonucleotide binding affinity, the two oligonucleotides were subsequently end-labeled with 5' FAM (fluorescein) and were subjected to a microscale thermophoresis (MST) assay to determine dissociation constants

between HpaR and probes. In this assay, HpaR bound to the two DNA probes with dissociation constant (K_d) values of 11.23 ± 2.55 and 66.34 ± 2.62 μ M, respectively, corresponding to intermediate to strong binding affinities (Fig. 4D and E). Taken together, these analyses confirmed the consensus DNA binding motifs of HpaR (Fig. 2C).

To theoretically predict the HpaR-binding site in the genome of *X. campestris* pv. *campestris* 8004, we used the consensus motif to screen for matching sequences within the 5' promoter regions of all annotated genes in this genome. The results are listed in Supplementary Table S7. A total of 579 genes were identified as putatively regulated by HpaR; this gene repertoire included 75 (32%) and 55 (30%) of the genes identified by RNA-seq and ChIP-seq, respectively.

HpaR controls the transcription of the *vgrR-vgrS* operon.

RNA-seq analysis showed that under NYG and XVM2 growth conditions, *vgrR* mRNA levels were decreased in the *hpaR* mutant. In addition, both ChIP-seq analysis and binding site prediction revealed that HpaR potentially binds to the 5' promoter region of the *vgrR-vgrS* operon. These results suggest that HpaR regulates the transcription of *vgrR-vgrS* operon. Our previous study showed that the bicistronic *vgrR-vgrS* operon encodes a TCS (Wang et al. 2016). *VgrS* is a membrane-bound

Fig. 2. Genome-wide analysis of the HpaR regulon by chromatin immunoprecipitation sequencing (ChIP-seq). **A**, Venn diagram showing the number of HpaR-regulated genes and their ChIP-seq-identified promoters. Bacterial strains were grown in nutrient-yeast-glycerol (NYG) (red circle) and *Xanthomonas* virulence mimic 2 (XVM2) (blue circle) media. **B**, Functional categories of downstream genes potentially regulated by HpaR. Black and white columns represent the number of genes identified in bacterial cells grown in NYG and XVM2 media, respectively. **C**, Deduced consensus HpaR-binding DNA motifs based on ChIP-seq data and MEME analysis. WebLogo was used to illustrate the nucleotide composition. The height of each nucleotide is proportional to the level of conservation at that site. Upper and lower panels correspond to motifs predicted under NYG and XVM2 conditions, respectively.

histidine kinase whose function is sensing of iron-depleted environments, while VgrR is a cytosolic response regulator that has TF activity. This TCS plays a critical role in regulating bacterial virulence and stress tolerance in *X. campestris* pv. *campestris*. In this previous study, we found that the promoter region of this operon is located in the 5' region of *vgrR* (*PvgrR*). Using a previously constructed *PvgrR*-GUS fusion reporter (Wang et al. 2016), we showed that β -glucuronidase (GUS) activity of the reporter in the *hpaR* mutant decreased to a level that was 37.7% of the WT strain (Fig. 5A). In addition, qRT-PCR analyses revealed that *vgrR* mRNA amounts significantly decreased, to levels respectively corresponding to 48.5 or 64.2% of the WT strain, when *X. campestris* pv. *campestris* was grown in XVM2 or NYG medium. In the genetic complementary strain (Δ *hpaR*-*hpaR*), the expression of *vgrR* was restored to the level of the WT strain (Fig. 5B and C). These results suggest that HpaR positively controls the transcription of *vgrR-vgrS* operon.

To determine the possible interaction between HpaR and *PvgrR*, a 215-bp DNA fragment of *PvgrR* was amplified by PCR, was labeled with [γ - 32 P]ATP, and was used as a probe in an

EMSA. As shown in Figure 5D, HpaR recombinant protein bound to this DNA probe to form a clear HpaR-*PvgrR* complex. When an unlabeled probe of *PvgrR* was added to the EMSA reaction mixture, the isotopic signal of the HpaR-*PvgrR* complex gradually decreased to an undetectable level. In addition, an MST assay using a 5' FAM-labeled *PvgrR* DNA fragment found that the binding affinity of the HpaR-*PvgrR* interaction was $22.03 \pm 3.21 \mu\text{M}$ (Fig. 5E), which suggests a relatively strong protein-DNA interaction. These results, therefore, demonstrate that HpaR physically binds to the promoter region of the *vgrR-vgrS* operon to positively regulate the transcription of these two important virulence regulatory genes.

vgrR-vgrS is located downstream of the HpaR regulatory cascade.

The above analyses suggest that the *vgrR-vgrS* TCS is an important downstream regulator in the HpaR regulon for controlling physiological processes of *X. campestris* pv. *campestris*. To further investigate this regulatory relationship, we constructed a bacterial strain (Δ *hpaR*-*vgrR*), in which *vgrR* was overexpressed in the genetic background of the *hpaR* mutant,

Fig. 3. Direct binding of HpaR to the promoter regions of downstream genes to control their expression. **A**, Sequences of the promoter regions of five genes. HpaR-binding sequences retrieved from peak calling of chromatin immunoprecipitation sequencing are highlighted by gray backgrounds. Sites of the potential binding motifs relative to the translational initiation sites were labeled. **B** to **F**, Direct binding of HpaR to the promoter regions of downstream genes. An electrophoresis mobility shift assay was used to determine the binding events between HpaR and DNA probes labeled with [γ - 32 P]ATP. Black triangles indicate HpaR-DNA complexes. Polymerase chain reaction (PCR) products of *XC_0136*, *XC_0141*, *XC_0405*, *XC_1057*, and *hpaR* (*XC_2827*) promoter regions were used as probes. Increasing amounts of unlabeled probes (100- to 500-fold) were used as competitors. Each assay was repeated three times. **G** to **K**, Quantification of messenger RNA (mRNA) of downstream genes in bacterial strains. Quantitative reverse transcription-PCR assays were used to quantify gene transcriptional levels. Transfer-messenger RNA cDNA was amplified as an internal control. Each assay was repeated independently three times, and only a representative is shown. Vertical bars indicate standard deviations ($n = 3$). An asterisk (*) indicates a significant difference compared with the wild-type (WT) strain (Student's t test, $P < 0.05$).

by in trans provision of a recombinant vector (pHM1::vgrR). After inoculating the bacterial strains onto the host cabbage (*Brassica oleracea* cv. Jingfeng No. 1), virulence capabilities of the *hpaR* and *vgrR* mutants almost completely disappeared (Fig. 6A) and the amounts of bacterial populations grown in planta significantly decreased (Fig. 6B). Overexpression of *vgrR* in the *hpaR* mutant, however, partially restored bacterial virulence toward the WT strain (Fig. 6A). In addition, the size of the bacterial population grown in planta markedly increased, albeit at a lower level than that of the WT strain (Fig. 6B). We also constructed a strain in which *hpaR* was overexpressed in the *vgrR* mutant. However, virulence and in planta growth assays revealed that overexpression of *hpaR* did not change the phenotypes of the strain (Fig. 6A and B), thus indicating that *hpaR* is not located downstream of *vgrR* regulation.

Regarding biofilm formation as quantified by crystal violet staining, inactivation of *hpaR* resulted in a significant increase in biofilm quantity (Fig. 6C). In the Δ *hpaR*-*vgrR* strain, overexpression of *vgrR* effectively suppressed the phenotypic change by decreasing the biofilm formation of the bacterium. As a previous study revealed, *hpaR* negatively regulates the production of extracellular proteases, we also checked this phenotypic alteration in recombinant strains. As shown in Figure 6D, deletion of *hpaR* caused a significant increase in extracellular protease production. In comparison, however, both genetic complementation of *hpaR* as well as overexpression of *vgrR* in the *hpaR* mutant caused extreme decreases in enzyme production. Meanwhile, overexpression of *hpaR* in the *vgrR* mutant (Δ *vgrR*-*hpaR*) did not change the phenocopied the biofilm formation and production of extracellular proteases of the

Fig. 4. Verification of the consensus HpaR-binding motif. **A**, Identification of HpaR-binding sites in *XC_1642* promoter regions based on DNase I footprinting. The exact binding sequences of *XC_1642* are highlighted in red. The control reaction is shown in the electropherogram at the top. The experiment was conducted using an autosequencer. **B** and **C**, Direct binding of HpaR to chemically synthesized oligonucleotides. An electrophoresis mobility shift assay (EMSA) was used to determine the binding events between HpaR and DNA probes. Black triangles indicate HpaR-DNA complexes. Two oligonucleotides were synthesized, were end-labeled with [γ - 32 P]ATP, and were used as probes in the EMSA. Increasing amounts of unlabeled probes (20- to 200-fold) were used as competitors. Each assay was repeated three times. **D** and **E**, Quantification of HpaR-oligonucleotide binding affinity by microscale thermophoresis (MST). The oligonucleotides were labeled with 5'-fluorescein and incubated with the HpaR protein in an NT standard capillary in the MST assay. Titrations of the HpaR protein ranged from 50 nM to 1 mM. The solid curve is the fit of the data points to the standard KD-Fit function. The black bars represent standard deviations ($n = 3$). K_d = dissociation constant.

vgrR mutant ($\Delta vgrR$). Collectively, these genetic results demonstrate that overexpression of *vgrR* suppresses *hpaR*-induced phenotypic alterations in virulence, biofilm formation, and production of extracellular proteases and strongly support the notion that the VgrR-VgrS TCS is an essential regulator downstream of HpaR.

DISCUSSION

MarR-family TFs are widely distributed in bacteria and archaea. Each bacterial genome has been estimated to encode an average of seven such TFs (Perera and Grove 2010). Previous studies have revealed that MarR-family TFs regulate the virulence of many pathogenic bacteria of animals and plants. Examples include SlyA of *Salmonella enterica* (Dolan et al. 2011), Rv1404 of *Mycobacterium tuberculosis* (Healy et al. 2016), PecS of *Dickeya dadantii* (Praillet et al. 1996), SarZ of *Staphylococcus aureus* (Kaito et al. 2006), and RovA of *Yersinia pestis* (Cathelyn et al. 2006). The genome of *X. campestris* pv. *campestris* encodes approximately 10 MarR-family TFs, but only the function of HpaR has been experimentally investigated. In the close relative *X. campestris* pv. *phaseoli*, another MarR-family TF, OhrR, has been found to sense peroxide and modulate oxidative stress responses (Klomsiri et al. 2005; Panmanee et al. 2002). The ortholog of OhrR in *X. campestris* pv. *campestris* 8004 is XC_0273, whose function remains unknown. In the present study, we dissected the regulon of HpaR, using RNA-seq and ChIP-seq analyses. According to our results, HpaR directly or indirectly controls the transcription levels of hundreds of genes, some of which

participate in bacterial virulence and elicitation of HR in host plants (Figs. 1 and 2). Based on these analyses, HpaR-binding consensus motifs containing the imperfect palindromic sequences were predicted and experimentally verified (Figs. 2 and 4). HpaR has TF activity to directly bind the promoter regions of downstream genes and acts both as a repressor and an activator to control gene transcription (Fig. 3). Among these promoter regions, we found that HpaR binds to *cis*-regulatory elements in the 5' upstream region of the *vgrR-vgrS* operon and positively controls its transcription (Fig. 5), thereby playing an important role in bacterial virulence and stress responses (Fig. 6). This dissection of the HpaR regulon should facilitate in-depth study of the molecular mechanism underlying transcriptional networks of *X. campestris* pv. *campestris*.

Our study revealed that HpaR controls the bacterial virulence of *X. campestris* pv. *campestris* through various regulatory pathways. First, HpaR directly binds to the promoter regions of a limited number of genes encoding virulence-associated factors to modulate their transcription; these genes include type IV secretion system genes and genes encoding the type IV pili assembly, RhsD proteins, ABC transporters, and TonB-dependent receptors for iron uptake (Fig. 3). Second, HpaR controls expression levels of regulatory genes that are critical for virulence. ChIP-seq and RNA-seq analyses revealed that the expression of many genes encoding other TFs, two-component signaling proteins, and alternative sigma factors, were down- or up-regulated in the *hpaR* mutant, and we have provided genetic and biochemical evidence to demonstrate that HpaR activates the transcription of the *vgrR-vgrS* operon (Figs. 5 and 6). VgrR-VgrS is one of the TCSs involved in regulating virulence and

Fig. 5. Binding of HpaR to the promoter region of the *vgrR-vgrS* operon to positively control its transcription. **A**, Quantification of the promoter activity of the *vgrR-vgrS* operon by a GUS activity assay. A recombinant vector (pHM2::PvgrR-GUS) was provided in trans to bacterial strains for the GUS activity assay. **B–C**, Messenger RNA (mRNA) levels of *vgrR* in bacterial strains. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays were used to quantify the amount of *vgrR* mRNA in different bacterial strains. Transfer-messenger RNA RNA cDNA was used as an internal control. Each assay was repeated independently three times, and only a representative is shown. In A, B, and C, vertical bars indicate standard deviations ($n = 3$). * indicates a significant difference compared with the wild-type (WT) strain (Student's *t* test, $P < 0.05$). **D**, Direct binding of HpaR to the PvgrR promoter region. The black triangle indicates the HpaR-DNA complex. PCR products of the *vgrR* promoter region were labeled with [γ -³²P]ATP and used as probes. The sequence of the HpaR-binding motif and its location relative to the translational initiation site is shown at the bottom of the figure. Increasing amounts of unlabeled probes were used as competitors. Each assay was repeated three times. **E**, Quantification of HpaR-PvgrR binding affinity by an MST assay. The PCR products of the *vgrR* promoter region were labeled with 5'-fluorescein (45 nM) and were incubated with HpaR in an NT standard capillary for the MST assay. K_d = dissociation constant. The black bars represent standard deviations ($n = 3$).

stress responses of *X. campestris* pv. *campestris* (Wang et al. 2016). In an iron-depleted environment, such as in host tissues, the autokinase activity of VgrS is maintained at a high level. The phosphorylated VgrS then transfers a phosphoryl group onto VgrR to activate the latter's TF activity. In due course, VgrR modulates the expression of downstream genes associated with virulence and stress responses (Wang et al. 2016). As shown in Figure 6, overexpression of *vgrR* partially but significantly restored the deficiency in virulence caused by the *hpaR* deletion, strongly implying that *vgrR-vgrS* is downstream of the HpaR regulation. Third, HpaR indirectly affects the expressions of many virulence-associated genes. For example, RNA-seq analysis retrieved six promoter sequences of flagellar assembly genes, including *flgF*, *flgI*, *flgG*, and *flgB*. The 5' upstream sequences of these genes contained no HpaR-binding motifs, which suggests they are not directly regulated by HpaR. Flagella contribute to the motility and chemotaxis of bacterial cells. The role of flagella in virulence as well as a pathogen-associated molecular pattern has been reported in various pathogenic bacteria, including *X. campestris* pv. *campestris* (Gómez-Gómez and Boller 2000; Haiko and Westerlund-Wikström 2013; Lee et al. 2003). Furthermore, many virulence-associated genes, including those encoding the type III secretion system, TonB-dependent receptors for iron uptake, and extracellular enzymes such as pectate lyase, were indirectly modulated by HpaR, as these genes identified by RNA-seq had no HpaR-binding motifs as *cis*-regulatory elements.

Collectively, these results support the idea that HpaR is a pleiotropic regulator controlling the level of virulence of *X. campestris* pv. *campestris*.

Although MarR-family TFs usually act as transcriptional repressors in the regulation of downstream genes (Grove 2013), they can also behave as transcriptional activators (Perera and Grove 2010). This dual functionality is applicable to HpaR regulation. As shown in Figure 3, HpaR repressed the transcription levels of five tested genes, including *hpaR* itself, under NYG or XVM2 growth conditions. Inactivation of *hpaR* resulted in upregulation of these genes under certain circumstances. Notably, the binding sites of HpaR in the promoter regions of these genes were located 100 bp upstream of the translational initiation sites. When the bacterium was grown in NYG or XVM2 medium, however, HpaR positively regulated the transcription of the *XC_0405*, *XC_1057*, and *vgrR-vgrS* operon, as the amount of their mRNAs significantly decreased in the *hpaR* mutants. In contrast to the above five genes, the binding site of HpaR to *PXC_0405*, *PXC_1057*, and *PvgrR* are all around the -35 regions of these genes (Fig. 5). As has been shown in previous studies, the general DNA binding mechanism of MarR-family TFs is highly conserved, and the position of their *cis*-regulatory elements determines whether the TFs function as activators or repressors. In regard to activation of gene transcription, for example, the TF OhrR of *Streptomyces coelicolor* binds to the -35 region of the *ohrA* gene and

Fig. 6. Results of epistasis analyses revealing that *vgrR* is a downstream modulator of HpaR regulation. **A**, Overexpression of *vgrR* partially restoring the virulence deficiency caused by the *hpaR* mutation. Bacterial strains were inoculated onto host cabbage (*Brassica oleracea* cv. Jingfeng No. 1), with 10 mM MgCl₂ used as a negative control. Virulence levels were recorded 10 days after inoculation. **B**, Plant growth assay of bacterial strains. Bacterial strains were injected into plant leaves using a 1-ml syringe. Bacterial colonies were isolated from leaf disks and were counted by serial dilution after inoculation. Each value shown is the average number calculated from six leaf disks. **C**, Quantification of biofilm formation of bacterial strains. Biofilms were stained with crystal violet and were measured by optical density (OD) absorbance (OD₅₉₀/OD₆₀₀). **D**, Assay of extracellular protease production. Error bars represent standard deviation ($n = 6$). An asterisk (*) indicates $P < 0.05$, calculated by Student's *t* test.

interacts with RNA polymerase to activate the transcription (Oh et al. 2007), while release of transcriptional repression by interaction with a repressor, such as nucleoid-like proteins (H-NS), has been reported for the TF RovA of *Yersinia pseudotuberculosis* (Heroven et al. 2004). As for transcriptional repression, MarR-family TFs have been found to distort promoter-DNA topology (Deochand et al. 2016), interfere with RNA polymerase binding by promoter occlusion (Stapleton et al. 2002), and hinder mRNA elongation by transcriptional collision (Galán et al. 2003). Consequently, the biochemical basis of how HpaR activates or represses downstream gene expression, especially in the context of stochastic change of environments, is an open question to be investigated.

In addition to TF activity for binding dsDNA, MarR-family TFs can bind small molecules to modulate their DNA-binding behavior (Grove 2013). MarR-family TFs are, therefore, also intracellular sensors for detecting environmental cues, with ligand binding one of the central mechanisms to activate these TFs. Unlike LysR- or LuxR-family TFs that contain ligand-binding domains separate from DNA-binding domains, however, MarR-family TFs have no such domains and, instead, bind small chemicals via their ligand-binding pockets (Perera and Grove 2010). In *E. coli*, for example, Cu²⁺ oxidizes the cysteine residues of MarR to generate disulphide bonds between MarR dimers, which results in the dissociation of MarR from its binding DNA. This process releases the repression of MarR on the transcription of downstream genes responsive to antibiotic treatment (Hao et al. 2014). In *Staphylococcus epidermidis*, sodium salicylate and antibiotics such as β -lactam and aminoglycosides interact with the MarR-family TF TcaR to antagonize the DNA-binding activity of TcaR, which triggers bacterial biofilm formation to resist these antibiotics (Chang et al. 2010). In the present study, our preliminary MST assay excluded the possibility that HpaR binds to salicylates with a mediate affinity (data not shown). In addition, sequence alignment indicated that the primary sequence of HpaR has little similarity to the other MarR-family TFs whose three-dimensional protein structures were dissected. Ligand binding by HpaR, if present, thus remains completely unelucidated. Future study is needed to investigate the activating mechanism of HpaR as a TF during infection, including the identification of small molecules possibly binding to this important virulence regulator of *X. campestris* pv. *campestris*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

Xanthomonas campestris pv. *campestris* strains were routinely cultivated at 28°C in rich NYG medium (per liter, 5 g of tryptone, 3 g of yeast extract, and 20 g of glycerol, pH 7.0) and minimal XVM2 medium (per liter, 5 g of glucose, 1.0 g of sodium citrate, 2 g of (NH₄)₂SO₄, 4 g of K₂HPO₄, 6 g of KH₂PO₄, and 0.2 g of MgSO₄, pH 7.0). *E. coli* strains were grown aerobically at 37°C in Luria-Bertani medium. Antibiotics were added as necessary at the following concentrations: rifampicin (25 µg/ml), spectinomycin (150 µg/ml), and kanamycin (50 µg/ml). *Xanthomonas campestris* pv. *campestris* 8004 electro-competent cells were prepared by washing bacterial cells at the exponential phase three times with 10% ice-cold glycerol.

Bacterial genetic manipulation and epistasis analysis.

Construction of in-frame deletion mutants was based on the homologous double-crossover method, using the suicide vector pK18mobSacB according to previous studies (Bürckstümmer et al. 2006; Wang et al. 2016). Genetic complementation or over-expression of genes was performed by constructing recombinant

pHM1 vectors, which were inserted in trans into corresponding bacterial strains. All primer sequences used in this study are listed in Supplementary Table S2.

To determine the epistatic relationship between *hpaR* and *vgrR*, a full-length *vgrR* gene was overexpressed in the *hpaR* in-frame deletion mutant or vice versa, by providing a recombinant pHM1 vector (pHM1::*vgrR* or pHM1::*hpaR*). In this vector, *vgrR* or *hpaR* is subject to the control of a *PlacZ* promoter. The recombinant strains were employed for phenotypic characterization.

Phenotypic characterization of bacterial strains.

Extracellular enzyme assays and plant inoculation was carried out according to a previous report (Qian et al. 2008). In brief, strains of *X. campestris* pv. *campestris* were cultivated to nearly 10⁶ CFU per milliliter and were then inoculated onto susceptible, 8-week-old cabbage plants (*Brassica oleracea* cv. Jingfeng No. 1) by clipping the leaf tips with presterilized scissors dipped into the bacterial cultures. Lesions were measured and photographed at 10 days after inoculation. For in-planta growth assays, bacterial populations of each strain were determined on six inoculated leaves every 48 h. After grinding each harvested leaf in 10 mM MgCl₂, the leaf homogenate was serially diluted and was plated onto NYG plates. Bacterial numbers were counted after incubation at 28°C for 48 h. Each assay was independently repeated three times, and bacterial populations of each strain were calculated as average values from eight inoculated leaves at each time point.

qRT-PCR analyses.

Total bacterial RNA was extracted from bacterial strains at the exponential growth phase (OD₆₀₀ = 0.4 ± 0.05), using Trizol reagent (Invitrogen, Waltham, MA U.S.A.) according to the manufacturer's instructions. After measuring RNA concentrations with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, U.S.A.), DNA contamination was removed by digestion with RNase-free DNase I (Ambion, Austin, TX, U.S.A.). cDNA was synthesized using random primers (Promega, Madison, WI, U.S.A.) and Superscript III reverse transcription (Invitrogen). mRNA levels of target genes were quantified by qRT-PCR using Maxima SYBR green (Fermentas, Waltham, MA, U.S.A.) in a DNA Engine Opticon 2 system (Bio-Rad, Hercules, CA, U.S.A.), according to the manufacturer's instructions. cDNA of transfer-messenger RNA was amplified as an internal control. Each qRT-PCR experiment was typically repeated independently three times, with three technical repeats per sample. One representative of each set of biological repeats was selected and reported.

High-throughput RNA-seq.

Bacterial strains were cultured in minimal NYG or XVM2 medium overnight to OD₆₀₀ nm = 0.4, and then, cells were collected by centrifugation before total RNA extraction. The mRNA was enriched by removing 16S and 23S rRNAs with a MICROBEnrich kit (Ambion). The RNA libraries for sequencing were prepared using TruSeq RNA kits (Illumina, San Diego, CA, U.S.A.), and RNA-seq was performed by Illumina HiSeqTM 2000. All clean reads were aligned to the complete genomic sequence of *X. campestris* pv. *campestris* 8004 with SOAPaligner/SOAP2. The number of clean reads aligning to each genomic position on each DNA strand was calculated. The total numbers of reads aligning to each functional category and each putative gene were calculated. Analysis of differentially expressed genes was performed using normalized RPKMO values (reads per kilobasepair of gene per million reads aligning to annotated open reading frames) by CLC Genomic Workbench for RNA-seq (CLC Bio, Aarhus, Denmark). the

differential expression analyses were performed in *R* version 3.1.1 using the DESeq2 package. *P* values were adjusted by the default method in DESeq2 and genes were considered differentially expressed when adjusted *P* values below 0.05 were obtained. The 1.5-fold threshold was set as a core data set.

GUS reporter activity assays.

To construct a promoter-GUS transcriptional fusion, DNA sequences corresponding to the promoter of *vgrR-vgrS* were amplified and ligated to the *gusA* gene, with the Shine-Dalgarno sequence of *gusA* acting as the ribosome-binding site. The inserts were ligated into vector pHM2 and were transformed into bacterial strains. For GUS activity assays, bacterial cells were collected by centrifugation and were stored immediately in liquid nitrogen. Cells were resuspended in 1× GUS extraction buffer (50 mM sodium phosphate, pH 7.0, 5 mM dithiothreitol [DTT], and 1 mM EDTA, pH 8.0), were lysed by sonication, and were centrifuged, with the supernatants used for GUS activity assays. GUS activity was measured by the fluorometric method using 4-methylumbelliferyl β-D-glucuronide (4-MUG) (Goldbio, St. Louis) as the substrate. A standard curve was established by diluting 4-MUG (Sigma) stock solution. The fluorescence of samples and standard curve solutions was measured using an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

Protein expression, purification, and extraction.

A prokaryotic expression system with vector pET30a and *E. coli* BL21 (DE3) (Novagen, Darmstadt, Germany) was used to express recombinant HpaR fused with a C-terminal His₆ epitope. Protein was expressed and purified by affinity chromatography using Ni-NTA agarose beads (Novagen), according to the manufacturer's manual. In brief, *E. coli* strains were grown to an OD₆₀₀ of 0.6 and were then induced with 0.5 mM isopropyl-β-D-thiogalactoside for 18 h at 16°C. The bacteria were collected by centrifugation and were treated with lysis buffer (50 mM Tris-HCl, pH 7.5; 0.5 M NaCl; 10% glycerol; 0.1% Triton X-100; and 1 mM phenylmethylsulfonyl fluoride [PMSF]). Buffers used for protein purification with Ni-NTA beads (Novagen) were as follows: Ni-NTA binding buffer (300 mM NaCl; 50 mM sodium phosphate buffer, pH 7.0; and 10 mM imidazole, pH 8.0), Ni-NTA washing buffer (300 mM NaCl, 50 mM sodium phosphate buffer, pH 7.0; and 30 mM imidazole, pH 8.0) and Ni-NTA elution buffer (300 mM NaCl, 50 mM sodium phosphate buffer, pH 7.0; and 10 mM imidazole, pH 8.0). Purified His₆-tagged proteins were dissolved in storage buffer (50 mM Tris-HCl, pH 8.0; 0.5 mM EDTA; 50 mM NaCl; and 5% glycerol).

EMSAs.

To detect HpaR-DNA binding, PCR products of the corresponding promoter region or chemically synthesized dsDNA probe were labeled with [γ-³²P]ATP using T4 polynucleotide kinase (NEB) and were purified using a ProbeQuant G-50 column (GE Healthcare). Binding reactions were carried out in 20-μl volumes of reaction buffer (10 mM Tris-HCl, pH 7.5; 50 mM KCl; 1 mM DTT; and 1 μl of 50 ng/μl poly[dI:dC]). Labeled DNA probe (2 to 4 fmol) and 2 to 6 μM HpaR were used in each EMSA. For the competition reaction, a certain amount of unlabeled DNA probe was co-incubated for 20 min at room temperature before electrophoresis. Prior to electrophoresis, 4 μl of DNA loading buffer (0.25% bromophenol and 80% glycerol) was added to stop the EMSA reaction. The samples were then loaded onto a 5% native polyacrylamide gel electrophoresis gel. Prior to autoradiography, electrophoresis was performed at 120 V for about 40 min in 0.5× Tris-borate-

EDTA buffer. Cold probes (not isotope-labeled) were also used in the competitive experiments.

ChIP-seq.

ChIP was performed according to a previous study (Wang et al. 2014). Briefly, bacterial strains were grown in NYG and XVM2 media until the OD₆₀₀ reached 0.4 ± 0.05. Bacterial cells were collected by centrifugation, were cross-linked with 1% formaldehyde, and were subsequently quenched with 0.5 M glycine for 10 min. The cells were re-collected by centrifugation, were washed in 10 ml of cold Tris-buffered saline (150 mM NaCl and 20 mM Tris-HCl, pH 7.5), and were resuspended in 1 ml of lysis buffer (10 mM Tris, pH 8.0; 20% sucrose; 50 mM NaCl; 10 mM EDTA; and 10 mg of lysozyme per milliliter). IP buffer (50 mM HEPES-KOH, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1% Triton-X 100; 0.1% sodium deoxycholate; 0.1% sodium dodecyl sulfate [SDS]; and 1 mM PMSF) was added to the bacterial cell suspension, and the cells were sonicated, using a Diagenode Bioruptor (Diagenode, Liege, Belgium), to generate DNA fragments of about 100 to 500 bp. After centrifugation, the solution was precleared with 20 μl of protein A sepharose, at 4°C for 10 min on a slow rotator, and a 100-μl aliquot was retained as the loading control DNA (input sample). For the ChIP assays, 50 μl of protein A sepharose (50% slurry) and 2 μl of an anti-His₆ antibody were added to an 800-μl aliquot of the DNA sample and the mixture was incubated at 4°C overnight, with slow rotation. The beads were collected by centrifugation and were washed with IP buffer and wash buffer (10 mM Tris-HCl, pH 8.0; 250 mM LiCl; 1 mM EDTA; 0.5% NP40 [equivalent to Triton X-100]; and 0.5% sodium deoxycholate). The immunoprecipitated chromatin was removed from the beads by adding 100 μl of elution buffer (50 mM Tris, pH 7.5; 10 mM EDTA; and 1% SDS), and the solution was incubated for 10 min at 65°C. RNase A and proteinase K were used to remove RNA and protein, respectively. The DNA was purified by PCR purification sequencing on an Illumina Highseq-2000 system by BGI (Beijing). The resulting high-throughput sequencing reads were analyzed by the Burrows-Wheeler aligner method. The cleaned reads were aligned to the genomic sequence of *X. campestris* pv. *campestris* 8004. Peak calling was conducted with MACS2 (Zhang et al. 2008). The consensus HpaR-binding motif analysis was completed using the MEME and FIMO tools in the MEME software suite (Bailey et al. 2015).

DNase I footprinting assay.

A DNase I footprinting assay was performed to determine the HpaR-binding sites on the promoter region of *vgrR*. For preparation of fluorescent FAM-labeled probes, the promoter region of *XC_1642* was PCR-amplified with Dpx DNA polymerase T plasmid, using primers M13F-47 (FAM) and M13R-48. The FAM-labeled probes were purified using a Wizard SV gel and PCR clean-up system (Promega, Madison, WI, U.S.A.) and were quantified on a NanoDrop 2000C instrument (Thermo Scientific). DNase I footprinting assays were performed similarly to those reported by Stock et al. (2000). For each assay, 400-ng probes were incubated with different amounts of HpaR protein in a total volume of 40 μl. After incubation for 30 min at 25°C, 10 μl of a solution containing approximately 0.015 units of DNase I (Promega) and 100 nmol of freshly prepared CaCl₂ was added, with incubation then continued for 1 min at 25°C. The reaction was stopped by adding 140 μl of DNase I stop solution (200 mM unbuffered sodium acetate, 30 mM EDTA, and 0.15% SDS). Samples were extracted with phenol/chloroform, were precipitated with ethanol, and the pellets were dissolved in 30 μl miniQ water. Preparation of the DNA

ladder, electrophoresis, and data analysis were as described previously (Stock et al. 2000), except that a GeneScan-LIZ500 size standard (Applied Biosystems, Foster City, CA, U.S.A.) was used.

MST measurement.

MST was used to quantify HpaR-DNA binding affinities. Oligonucleotide primers (50 bp) for target genes, labeled with 5'-FAM, were synthesized by Invitrogen and were annealed to an unlabeled complementary primer to form dsDNA. The labeled dsDNA was added to serially diluted protein reaction volumes (at an initial concentration of 40 μ M) containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM MgCl₂, and 0.05% (vol/vol) Tween-20. The KD fit function of NanoTemper analysis software version 1.5.41 was used for curve fitting and calculation of the K_d value.

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