INTRODUCTION

*Xanthomonas campestris* pv. *campestris* (Xcc), a gram-negative bacterium with a unipolar flagellum, can cause black rot in cruciferous plants (Vicente & Holub, 2013). The polar flagellum of Xcc allows the bacterium to swim in liquid environments and gather on semi-solid surfaces (Bardy et al., 2003). Flagella play multiple roles in the infection of host plants, including in bacterial motility, biofilm formation, biological attachment, and colonization of host tissues (Li & Wang, 2011; Malamud et al., 2011; Ottemann & Miller, 1997; Viducic et al., 2017). In addition, flagella are pathogen-associated molecular patterns that can induce the host innate immune response (Zipfel et al., 2004). Therefore, flagella are considered potential targets of anti-infection drugs. Bacterial flagella are molecular machines driven
by electric motors that facilitate the rotation of long curved filaments (Berg, 2003). In Xcc, flagellum biosynthesis is controlled by a cascade of three-level transcriptional regulation systems (Figure S1) involving the housekeeping sigma factor σ^54 and two alternative sigma factors, σ^54 and σ^28 (Hu et al., 2005; Yang et al., 2009). σ^70 (RpoD) regulates the expression of class I genes such as σ^54 (rpoN2), flgM, and fleQ. Class I proteins are the main regulators of class II genes, such as F-T3SS, rod and hook genes, and σ^28 (fliA). FlgM, an anti-sigma factor, binds FliA (σ^28) and inhibits its activity. Free FliA initiates the expression of class III genes (including flagellin-related genes) (Al Mamun et al., 1996; Liu & Matsumura, 1994; Yang et al., 2009).

In Xanthomonas, there are two rpoN genes encoding σ^54, named rpoN1 and rpoN2. In an rpoN2 deletion mutant, the expression of flgG, fliB, and fliC was significantly down-regulated (Hu et al., 2005). RpoN2 is thus closely related to the transcription of flagellum biosynthesis genes (Li, Wu, et al., 2020). FleQ is the only key activator of all RpoN-dependent flagellar promoters, and the FleQ protein has a central RpoN interaction domain (G140–L357) (pfam00158). It is involved in all RpoN-dependent flagellar promoters, and the FleQ protein has a central RpoN interaction domain (G140–L357) (pfam00158). It is involved in a broad range of processes, such as transcriptional activation and motility (Li et al., 2014). Similar to HpaS/HrpG, hpaS deletion reduces the phosphorylation level of the orphan RR VemR in vivo, and the HpaS/VemR TCS controls the swimming behaviour of Xcc (Li et al., 2020). According to these studies, HpaS phosphorylates at least three RRs (HrpG, VemR, and HpaR2), but how and when HpaS interacts with these RRs needs to be determined.

In addition, Clp is a key regulator of flagellum biosynthesis, EPS production, extracellular enzymes, and the expression of the Hrp system (de Crecy-Lagard et al., 1990; Hsiao et al., 2005; Hsiao & Tseng, 2002; Lee et al., 2003). Clp functions as a c-di-GMP receptor. On binding to c-di-GMP, Clp changes its conformation, which eliminates the interaction between Clp and its target gene promoters (Liu et al., 2013). Clp also up-regulates the expression of the flagellin-encoding gene fliC and positively regulates Xcc motility (Lee et al., 2003). However, how Clp regulates fliC expression remains unclear.

Here, we studied the roles of VemR, RpoN2, and FleQ in Xcc motility and virulence. We also tested the possible interaction between VemR and RpoN2 or FleQ. In addition, we demonstrated that RavA and VemR form a TCS. We also explored the relationship among RavA, RavR, VemR, and Clp and the mechanism by which RavA/VemR regulates bacterial motility. Our results shed light on the complex regulation of Xcc motility and virulence by TCSs.

2 | RESULTS

2.1 | RpoN2 and FleQ function downstream of VemR to negatively regulate Xcc virulence

Our previous studies have shown that VemR, but not RpoN2 or FleQ, was necessary for Xcc 8004 virulence (Tao & He, 2010). However, RpoN2 was recently reported to be vital for the virulence of the Xc17 strain (Li, Wang, et al., 2020). To further determine the roles of rpoN2, vemR, and fleQ in the interaction between Xcc and host plants, we constructed single (ΔrpoN2, ΔvemR, ΔfleQ), double (ΔrpoN2/ΔvemR, ΔvemR/ΔfleQ, ΔfleQ/ΔfliA), and triple (ΔrpoN2/ΔvemR/ΔfleQ) deletion mutants in Xcc strain Δlac8 (Δlac8, a derivative strain of Xcc 8004, has no β-galactosidase activity but has the same other phenotypes as Xcc 8004, including virulence, motility, EPS production, biofilm formation, and growth rate; hereafter, we refer to Δlac8 as the wild-type [WT] strain) (Wang et al., 2018). Then, we compared the virulence of
these strains on *Brassica oleracea* 'Wenxin'. At 14 days after inoculation, the average lesion length of the WT strain was 30.7 mm. The virulence of Δ*vemR* was completely lost but could be restored by complementation with *vemR*. In contrast, the lesion length of the Δ*rpoN2* or Δ*fleQ* strain was not significantly different from that of the WT strain. Furthermore, the average lesion lengths of the Δ*rpoN2/ΔvemR* and Δ*vemR/ΔfleQ* double mutants were approximately 13.7 and 7.7 mm, respectively, demonstrating that their infection abilities were between those of Δ*vemR* and Δ*rpoN2* or Δ*fleQ*. The virulence of the double mutant Δ*rpoN2/ΔfleQ* and the triple mutant Δ*rpoN2/ΔvemR/ΔfleQ* was similar to that of Δ*rpoN2* or Δ*fleQ* (Figure 1a,b). These data indicate that *rpoN2* and *fleQ* are epistatic to *vemR* and act as negative regulators in the VemR-mediated signalling pathway.

### 2.2 | RpoN2 and FleQ are epistatic to VemR in the regulation of Xcc motility

RpoN2 and FleQ positively regulate the expression of flagellum biosynthesis genes in the Xc17 strain (Yang et al., 2009), and VemR is indispensable for the regulation of flagellar motility by the Xcc 8004 strain (Tao & He, 2010). In *Pseudomonas aeruginosa*, FleQ binds to the σ^54^ factor RpoN to regulate flagellum expression (Dasgupta et al., 2002). In *X. citri pv. citri*, VemR regulates the transcription of the flagellar gene *flgG* by interacting with RpoN2 (Wu et al., 2019). Therefore, we wanted to clarify the mechanism by which the *rpoN2-vemR-fleQ* operon regulates Xcc motility.

Two types of motilities, swarming and swimming, are key for bacterial movement. Swarming, which involves pilus-dependent population migration, is an important factor for *Xanthomonas* virulence (Ryan et al., 2007). Compared with the WT strain, Δ*vemR* exhibited significantly reduced swarming (approximately 60%). In contrast, the swarming of the Δ*rpoN2* or Δ*fleQ* strain was not different from that of the WT strain. Compared with the WT strain, the swarming abilities of Δ*rpoN2/ΔvemR* and Δ*fleQ/ΔvemR* decreased by 33% and 16%, respectively, but those of Δ*rpoN2/ΔfleQ* and Δ*rpoN2/ΔvemR/ΔfleQ* increased by 11% and 9%, respectively. These phenotypes could be restored by gene complementation (Figure 1c,d). The above results revealed that VemR positively regulated pilus-dependent population motility, while RpoN2 and FleQ

![Figure 1](image.png)

**Figure 1.** RpoN2/FleQ and VemR antagonistically regulate virulence and motility in *Xanthomonas campestris* pv. *campestris* (Xcc). (a) Virulence phenotypes of the wild type (WT), *rpoN2*, *vemR* and *fleQ* single, double, and triple deletion mutants, and the corresponding complementation strains 14 days postinoculation (dpi) on broccoli cv. Wenxin leaves. (b) Lengths of the lesions on the 14 dpi-infected leaves as shown in (a). (c) Swarming zones of the above Xcc strains inoculated on swarming plates (NY medium containing 2% glucose and 0.6% agar) at 28°C for 3 days. (d) Average diameters of the swarming swimming zones. (e) Swarming zones of the abovementioned strains on swimming plates (0.03% Bacto peptone, 0.03% yeast extract, and 0.28% agar incubated at 28°C for 5 days). (f) Average diameters of the swimming zones. The values given are the means ± SD from triplicate experiments. C indicates the indicated gene(s) complementation strain of a given mutant (including the indicated gene(s) deletion). As our focus was the relationship between *vemR* and *fleQ/rpoN2*, we only analysed the significance of the difference between Δ*vemR* and the double mutants Δ*vemR/ΔfleQ* and Δ*vemR/ΔrpoN2*. **p < 0.01, ***p < 0.001
had antagonistic effects. These data are consistent with the virulence phenotypes, suggesting that Xcc virulence could be affected by this colony movement.

For swimming, the flagellum-dependent motility of \( \Delta vemR \) was 224% higher than that of the WT strain. In contrast, this type of motility was markedly reduced after \( rpoN2 \) or \( fleQ \) deletion. When \( rpoN2/vemR, \ vemR/fleQ, \ rpoN2/fleQ, \) and \( rpoN2/vemR/fleQ \) were deleted, their swimming motility was consistent with the phenotype of the \( rpoN2 \) or \( fleQ \) mutant (i.e., the migration was greatly decreased). All changes in swimming behaviour could be recovered by complementation (Figure 1e,f). These results suggest that VemR is a negative regulator of flagellum-based swimming, but RpoN2 and FleQ act as positive regulators, which is contrary to what was observed in the swimming and virulence results. In summary, RpoN2 and FleQ are epistatic to VemR in regulating Xcc motility and virulence.

2.3 VemR interacts with FleQ and regulates its own expression

Our data above show that \( rpoN2 \) and \( fleQ \) are epistatic to \( vemR \) in regulating bacterial virulence and motility, indicating that the expression of VemR could be regulated by RpoN2 and FleQ or that VemR could interact with FleQ and/or RpoN2 to affect their functions. Therefore, we first analysed \( rpoN2-vemR-fleQ \) operon expression levels in the WT, \( \Delta rpoN2, \Delta vemR, \) and \( \Delta fleQ \) strains. The \( rpoN2 \) promoter was fused to the \( \beta\)-glucuronidase (\( gusA \)) gene to construct the \( rpoN2p-gusA \) plasmid. After transforming this construct into the WT, \( \Delta rpoN2, \Delta vemR, \) and \( \Delta fleQ \) strains, the GUS activity of the transformants was analysed. The intensity of the blue GUS staining of \( \Delta vemR \) was stronger than that of WT (Figure S2), and the quantification of GUS activity showed that the \( rpoN2 \) promoter activity increased by 799% in \( \Delta vemR \) versus the WT strain (Figure 2a). When \( rpoN2 \) and \( \Delta vemR \) were both deleted, the GUS activity of the transformants was reduced by 36.9%-44.9% compared with that in the WT strain (Figure 2a). These results show that VemR down-regulates \( rpoN2-vemR-fleQ \) expression, while FleQ and RpoN2 activate the expression of this operon.

Then, we analysed whether VemR could interact with FleQ and/or RpoN2. To this end, we first used bacterial two-hybrid (B2H) assays to detect the interaction between RpoN2/FleQ and VemR. Only the reporter strains expressing both VemR and FleQ grew on the selection plates and produced blue colonies. The strains expressing either VemR or FleQ did not grow on the selection plates and did not produce blue colonies (Figure 2b,c). These data suggest that VemR and FleQ interact in Escherichia coli cells. However, no interaction was found between RpoN2 and VemR in the B2H experiments (Figure S3).

Previous studies have predicted that aspartic acid residues at positions 11 (D11) and 56 (D56) in VemR are key for its phosphorylation. D56 might be the phosphorylation site, and D11 coordinates the \( \text{Mg}^{2+} \) cation, allowing proper D56 phosphorylation (Appleby & Bourret, 1999; Delgado et al., 1993). After substituting these aspartic acids with lysine and alanine, the reporter strains expressing the VemR mutants (D11K, D56A, and D11KD56A) and FleQ still grew and turned blue on the selection plates (Figure 2b,c), illustrating that these residue substitutions, which may affect the phosphorylation of VemR, do not influence the interaction between FleQ and VemR.

To further verify the interaction between VemR and FleQ, pull-down assays were carried out. His-FleQ, a c.55 kDa protein, could be pulled down by maltose-binding protein (MBP)-VemR (58 kDa) but not by MBP (44 kDa) (Figure 2d). Consistent with the results of B2H experiments, protein pull-down analysis also showed that there is a physical interaction between VemR and FleQ.

2.4 RpoN2-VemR-FleQ is epistatic to RavA in the regulation of Xcc virulence and motility

Our previous study showed that RavA was involved in Xcc virulence, EPS production, biofilm formation, and motility (Tao et al., 2014). The phenotypes of the \( ravA \) mutant were highly similar to those of the \( vemR \) mutant (Tao & He, 2010; Tao et al., 2014). RavA acts as an HK, and the cognate HK of VemR has not been found, suggesting that RavA may interact directly or indirectly with VemR. To determine whether RavA plays a role in the regulation of bacterial motility via the RpoN2-VemR-FleQ signalling pathway, we constructed double mutants (\( \Delta rpoN2/\Delta ravA, \Delta vemR/\Delta ravA, \) and \( \Delta fleQ/\Delta ravA \)), triple mutants (\( \Delta rpoN2/\Delta vemR/\Delta ravA, \Delta vemR/\Delta fleQ/\Delta ravA, \) and \( \Delta rpoN2/\Delta fleQ/\Delta ravA \)), and quadruple mutants (\( \Delta rpoN2/\Delta vemR/\Delta fleQ/\Delta ravA \)).

For bacterial virulence, the average lesion length of \( \Delta ravA \) was 10 mm, while those of \( \Delta rpoN2/\Delta ravA \) and \( \Delta fleQ/\Delta ravA \) double mutants increased to 20 and 20.7 mm, respectively, 100% and 107% higher than that of \( \Delta ravA \). When \( vemR \) and \( ravA \) were both deleted, the virulence was completely lost, similar to the virulence phenotype of \( \Delta vemR \) (Figure 3a,b). The lesions of the triple mutants \( \Delta rpoN2/\Delta vemR/\Delta ravA, \Delta vemR/\Delta fleQ/\Delta ravA, \) and \( \Delta rpoN2/\Delta fleQ/\Delta ravA \) were similar to those of the double mutants \( \Delta rpoN2/\Delta vemR, \Delta vemR/\Delta fleQ, \) and \( \Delta rpoN2/\Delta fleQ \), respectively. Similarly, the virulence of the quadruple mutant \( \Delta rpoN2/\Delta vemR/\Delta fleQ/\Delta ravA \) was consistent with that of \( \Delta rpoN2/\Delta vemR/\Delta fleQ \) (Figure 3a,b).

The diameter of the swimming zone of \( \Delta ravA \) was 10.3 mm, while those of \( \Delta rpoN2/\Delta ravA \) and \( \Delta fleQ/\Delta ravA \) double mutants increased to 20.7 and 23 mm, respectively (Figure 3c,d). For swimming, the swimming zone diameter of \( \Delta ravA \) was 23 mm, and that of \( \Delta vemR/\Delta ravA \) increased to 35 mm. In contrast, the swimming ability was lost in \( \Delta rpoN2/\Delta ravA \) and \( \Delta fleQ/\Delta ravA \), similar to the result for \( \Delta rpoN2 \) or \( \Delta fleQ \) (Figure 3e,f). The motilities (including swimming and swimming) of the triple mutants \( \Delta rpoN2/\Delta vemR/\Delta ravA, \Delta vemR/\Delta fleQ/\Delta ravA, \) and \( \Delta rpoN2/\Delta fleQ/\Delta ravA \) and the quadruple mutant \( \Delta rpoN2/\Delta vemR/\Delta fleQ/\Delta ravA \) were also similar to those of \( \Delta rpoN2 \) (Figure 3c–f). The above results show that VemR, RpoN2, and FleQ are epistatic to RavA in the regulation of Xcc virulence and motility.
2.5 | RavA can interact with and phosphorylate VemR

The function of RRs is usually determined by their phosphorylation state, which is controlled by cognate HKs (Galperin, 2006). Although RavA is the cognate HK of RavR, ravA and ravR deletion did not result in the same phenotypes (Figure 3), implying that RavA might regulate other RR activities. VemR, which contains only a REC domain and lacks any output domain (Qian et al., 2008), was reported as the cognate RR of HpaS (Li, Wang, et al., 2020). However, deletion of hpaS and vemR did not result in the same phenotypes (Li, Wang, et al., 2020), indicating that VemR activity may be regulated by other HKs.
As our above genetic data showed that vemR is epistatic to ravA in the regulation of Xcc motility and virulence, and that ΔvemR and ΔravA have highly similar phenotypes, we hypothesized that RavA is also a cognate HK of VemR. We used B2H assays to analyse whether RavA interacts with VemR. The strains expressing both VemR and RavA grew and turned blue on the detection plates, which was similar to the result for the positive control strain (T18CZIP/T25ZIP), demonstrating that VemR can interact with RavA in E. coli cells. When the histidine residue at position 164 of the RavA phosphorylation site was substituted with alanine (H164A), the interaction decreased (Figure 4a,b). Similarly, the D11K and D11K/D56A substitutions in VemR also weakened its interaction with RavA, but the D56A substitution had little effect, showing that the phosphorylatable H164 of RavA and D11 of VemR might be required, but not essential, for their interaction, while the D56 phosphorylation site of VemR does not significantly influence its interaction with RavA.

Nevertheless, these data demonstrated that RavA interacts with VemR in E. coli cells. We also confirmed the interaction of RavA and VemR by pull-down tests because MBP-RavA, not sole MBP, pulled down His-VemR in vitro (Figure 4c). Taken together, our data demonstrate that RavA and VemR interact with each other and might form a TCS.

To confirm our hypothesis that RavA/VemR is a TCS, we performed phosphotransfer assays. When VemR-His was expressed in ΔvemR and ΔravA/ΔvemR, the phosphorylation of VemR-His could be...
detected in both strains, but the levels were much higher in ΔvemR than in ΔravA/ΔvemR (Figure 4d), suggesting that RavA phosphorylates VemR in Xcc. In E. coli, Flag-RavA and VemR-His coexpression resulted in detectable phosphorylation states of VemR, but expression of only VemR did not have this effect, indicating that RavA phosphorylates VemR in E. coli (Figure 4d). Moreover, we performed in vitro phosphorylation assays and found that phosphorylated glutathione S-transferase (GST)-RavA transferred the phosphate group to His-VemR (Figure 4d). These data demonstrate that RavA interacts with and phosphorylates VemR.

2.6 | RavA/VemR regulates the expression of the flagellar gene fliC by regulating the expression and activity of RpoN2/FleQ

To test whether RavA regulates rpoN2- vemR- fleQ operon expression, rpoN2p-gusA was also transformed into the ΔravA and WT strains to analyse rpoN2 promoter activity. The GUS staining assays showed that the colonies of the ΔravA mutant containing rpoN2p-gusA were bluer than those of the WT strain containing the same plasmid (Figure S2). Compared with WT, ΔravA showed a 668% increase
in rpoN2 promoter activity, similar to ΔvemR (Figures 2a and S2). These results suggest that RavA down-regulates the transcription of rpoN2-vemR-fleQ, similar to VemR.

The expression of the flagellin-encoding gene flIC is regulated by multiple genes, including rpoN2 and fleQ (Figure S1) (Yang et al., 2009). To understand whether VemR and RavA play roles in the regulation of flIC expression, we used promoter-reporter constructs to detect flIC expression levels. We constructed a flICp-lacZ fusion strain based on Δlac8 (Wang et al., 2018) that could directly reflect the expression levels of flIC on plates containing X-gal (5-bromo-4-chloro-3-indolyl β-d-galactoside). When rpoN2 or fleQ was deleted, the colonies did not exhibit a blue colour (Figure S4). After deletion of vemR, the colonies turned blue and showed detectable β-galactosidase activity. β-Galactosidase activity could not be detected in the double mutants (ΔrpoN2/ΔvemR, ΔvemR/ΔfleQ, and ΔrpoN2/ΔfleQ) or the triple mutant (ΔrpoN2/ΔvemR/ΔfleQ) (Figures 5a,b and S4). These data imply that RpoN2 and FleQ positively regulate flIC transcription, while VemR inhibits the
transcription of this gene via RpoN2 and FleQ. Similar to ΔvemR, the fliC promoter activity increased 253% and 284% in ΔvemR and ΔvemR/ΔravA, respectively (Figure 5a,c). In addition, ΔrpoN2/ΔravA and ΔfleQ/ΔravA did not exhibit fliC promoter activity (Figure 5a,c). This suggests that RavA/VemR regulates the expression of the flagellin-encoding gene fliC via RpoN2/FleQ.

2.7 | RavA/VemR regulates fliC expression via FliA

FliA is essential for the transcription of fliC (Figure S1) (Kan et al., 2018). The expression of the fliF-fleN-fliA operon is dependent on RpoN2/FleQ and is down-regulated by FliA (Yang et al., 2009). As RavA and VemR play a crucial role in the transcription of fliC (Figure 5), we asked whether this regulation requires FliA. First, we transformed fliFp-gusA into the WT, ΔravA, ΔrpoN2, ΔvemR, and ΔfleQ strains to analyse fliF promoter activity. Only ΔravA and ΔvemR containing fliFp-gusA produced a dark blue product (Figure S2), and the activity of the fliF promoter increased by approximately 75% in these strains compared with that in the WT strain (Figure 6a), demonstrating that RavA/VemR down-regulates the transcription of the fliF-fleN-fliA operon. Then, we constructed five fliA-related mutants (ΔfliA, ΔravR/ΔfliA, Δrava/ΔfliA, ΔvemR/ΔfliA, and ΔfleQ/ΔfliA) and analysed their virulence and motility. Deletion of fliA did not affect the infection ability of the WT, ΔravR, and ΔfleQ strains but significantly increased the virulence of the ΔravA and ΔvemR strains (Figures 6b and S5), indicating that FliA acts as a negative regulator of virulence in the RavA/VemR-mediated signalling pathway rather than the RavA/RavR-mediated signalling pathway.

Mutation of fliA in the WT, ΔravR, Δrava, ΔvemR, or ΔfleQ strains did not change the swimming motility of these strains (Figures 6c and S5). In contrast, the swimming ability of ΔfliA, ΔravR/ΔfliA, Δrava/ΔfliA, and ΔvemR/ΔfliA decreased 75%-91%, similar to that of ΔfleQ (Figures 6d and S5), whose swimming ability was almost completely lost. The activity of the fliC promoter decreased significantly in the ΔravR/ΔfliA, Δrava/ΔfliA, and ΔvemR/ΔfliA double mutants (Figures 6e and S5). The above data suggest that fliC expression regulated by RavA/VemR is dependent on RpoN2/FleQ and FliA.
2.8 clp is epistatic to ravA/vemR in Xcc virulence, not motility, regulation

Clp is a global transcriptional regulator that regulates extracellular enzymes, EPS, motility, and virulence in Xcc (He et al., 2007). As the virulence of the ravA/vemR deletion mutant was similar to that of the clp mutant, we studied the relationship between these genes. First, we constructed five clp-related mutants: Δclp, ΔravR, Δrava, ΔvemR, ΔfleQ, Δclp/ΔravA, Δclp/ΔvemR, and Δclp/ΔfleQ. (b, c) Swarming and swimming zone diameters of the above strains, respectively. (d) The fliC promoter activity was analysed by measuring the β-galactosidase activity of the above strains. The values given are the means ± SD from triplicate experiments. Significant differences between a single-gene mutant and the corresponding double-gene mutant (ΔravR vs. Δclp/ΔravR, Δrava vs. Δclp/Δrava, ΔvemR vs. Δclp/ΔvemR, and ΔfleQ vs. Δclp/ΔfleQ) are shown. A significant difference between the clp mutant and the WT strain (Δclp vs. WT) is also shown. *p < 0.05, **p < 0.01, ***p < 0.001.

clp is epistatic to ravA/vemR in the regulation of Xanthomonas campestris pv. campestris (Xcc) virulence but not motility. (a) Lesion lengths (14 days postinoculation) of broccoli leaves infected with wild type (WT), Δclp, ΔravR, Δrava, ΔvemR, ΔfleQ, Δclp/ΔravA, Δclp/ΔvemR, and Δclp/ΔfleQ. (b) The fliC promoter activity was analysed by measuring the β-galactosidase activity of the above strains. The values given are the means ± SD from triplicate experiments. Significant differences between a single-gene mutant and the corresponding double-gene mutant (ΔravR vs. Δclp/ΔravR, Δrava vs. Δclp/Δrava, ΔvemR vs. Δclp/ΔvemR, and ΔfleQ vs. Δclp/ΔfleQ) are shown. A significant difference between the clp mutant and the WT strain (Δclp vs. WT) is also shown. *p < 0.05, **p < 0.01, ***p < 0.001.
In the genome of Xcc, \( \text{rhoN2} \), \( \text{vemR} \), and \( \text{fleQ} \) are expressed as a transcriptional operon (Tao & He, 2010; Wu et al., 2019). \( \text{vemR} \) deletion resulted in a significant decrease in the virulence and adaptation of Xcc, but single or double deletion of \( \text{fleQ} \) and \( \text{rhoN2} \) did not affect these behaviours (Figure 1). Interestingly, the phenotypes of \( \Delta \text{rhoN2}/\Delta \text{vemR} \) and \( \Delta \text{vemR}/\Delta \text{fleQ} \) were at an intermediate level between those of \( \Delta \text{vemR} \) and \( \Delta \text{rhoN2} \) or \( \Delta \text{fleQ} \), while those of \( \Delta \text{rhoN2}/\Delta \text{fleQ} \) and \( \Delta \text{rhoN2}/\Delta \text{vemR}/\Delta \text{fleQ} \) were similar to those of \( \Delta \text{rhoN2} \) or \( \Delta \text{fleQ} \) (Figure 1). Notably, our complementation assays showed that \( \text{rhoN2}, \text{vemR}, \) and \( \text{fleQ} \) rescued the phenotypes of the corresponding mutants (Figure 1), demonstrating that there were no alternative promoters in the \( \text{rhoN2} \) and \( \text{vemR} \) coding regions, and that deletion of \( \text{rhoN2} \) or \( \text{vemR} \) had no polar effect. Therefore, we suggest that \( \text{fleQ} \) and \( \text{rhoN2} \) are epistatic to \( \text{vemR} \) and that \( \text{vemR} \) may directly or indirectly regulate \( \text{fleQ} \) and \( \text{rhoN2} \) activities.

\( \text{vemR} \) is an atypical RR that contains a CheY-like receiver but lacks an output domain (Qian et al., 2008), suggesting that its function may require its interaction with other proteins. Thus, we tested whether \( \text{vemR} \) can interact with \( \text{FleQ} \) and \( \text{rhoN2} \), and found that \( \text{vemR} \) physically binds \( \text{FleQ} \) but not \( \text{rhoN2} \) (Figure 2). As \( \text{FleQ} \) acts as a transcriptional activator and may interact with \( \text{rhoN2} \) to control gene expression (Dasgupta et al., 2002), the regulatory function of \( \text{vemR} \) in Xcc virulence and motility might depend on its interaction with \( \text{FleQ} \). Notably, \( \text{vemR} \) was recently reported to interact with \( \text{rhoN2} \) in \( \text{X. citri} \) subsp. \( \text{citri} \) and regulate bacterial virulence and swimming (Wu et al., 2019), but we did not find this interaction in Xcc (Figure S3). Because \( \Delta \text{rhoN2}/\Delta \text{vemR} \) and \( \Delta \text{vemR}/\Delta \text{fleQ} \) have similar phenotypes, \( \text{vemR} \) may also have the ability to interact with \( \text{rhoN2} \) in Xcc, but alternative analysis methods are required to explore this further, as our B2H assays could not verify this hypothesis. Moreover, a recent study showed that \( \text{vemR} \) directly interacts with the flagellum protein \( \text{FliM} \), HpaS/VemR regulates bacterial swimming via direct interaction with the flagellum protein \( \text{FliM} \). HpaS phosphorylates \( \text{HrpG} \) and regulates the expression of the type III secretion system (T3SS) and Xcc virulence. \( \_ \_ \) positive regulation; \( \_ \_ \) negative regulation; solid lines, having experimental evidence; dotted lines, supposed relationship. Components of a given pathway are indicated in the same colour.

3 | DISCUSSION

The function of an RR is typically controlled by its cognate HK, but bioinformatics analysis could not predict the cognate HK of \( \text{vemR} \) because no HK has been annotated in the genomic context of \( \text{vemR} \) (Qian et al., 2005). When HpaS was used as bait to screen for its interacting proteins, \( \text{vemR} \) was identified. Further yeast two-hybrid, pull-down, and phosphorylation assays showed that HpaS directly interacts with and phosphorylates \( \text{vemR} \), indicating that HpaS and \( \text{vemR} \) form a TCS (Li, Wang, et al., 2020). Interestingly, mutation of \( \text{hpaS} \) did not affect the swimming ability of Xcc but significantly reduced its swimming ability. Additionally, double mutation of \( \text{hpaS} \) and \( \text{vemR} \) increased the swimming ability to the WT levels but caused the swimming phenotype to be similar to that of the \( \text{vemR} \) mutant (Li, Wang, et al., 2020). These data indicate that \( \text{vemR} \) is epistatic to \( \text{hpaS} \) in Xcc swimming regulation, but \( \text{vemR} \) and \( \text{hpaS} \) have antagonistic effects on swimming regulation. Therefore,
VemR is not the downstream partner of HpaS in the regulation of Xcc swimming. Further study is needed to determine how HpaS and VemR coregulate Xcc motility.

As the vemR/ravA double mutant has similar phenotypes, including swimming, swarming, and virulence, as the vemR single mutant, vemR has an epistatic relationship with ravA (Figure 3), leading us to ask whether RavA and VemR interact directly or indirectly. Our B2H, pull-down, and phosphorylation assays showed that RavA interacted with and phosphorylated VemR (Figure 4). We also found that changes in the key residues for the phosphorylation of RavA and VemR might influence their interaction in the B2H assays (Figure 4). Together, these genetic and biochemical analyses indicate that RavA and VemR also form a TCS that regulates Xcc motility and virulence.

Previous studies have shown that the expression of flagellar genes is regulated by cascade signal transduction pathways (Figure S1). RpoN2/FleQ regulates the expression of the class II gene operon flhF-fliA. FliA can regulate the expression of its own operon and the fliC gene (Yang et al., 2009). Through analysis of fliC promoter activity, RpoN2 and FleQ were found to activate flic transcription, and RavA and VemR inhibited flic transcription via RpoN2/FleQ (Figures 5 and S4). Analysis of the transcriptional expression of fliH-fleN-fliA also revealed that RavA/VemR can down-regulate the transcription of this operon and that RavA/VemR regulates flic expression via a mechanism dependent on FliA (Figures 6 and S5). Based on the above results, we have discovered a new signalling pathway that regulates the expression of the flagellar gene flic (Figure 8). RavA senses an unknown signal (which might be an intracellular cue) and undergoes autophosphorylation, followed by transfer of the phosphate group to VemR. VemR interacts with the transcription activator FleQ and regulates FleQ activity and its own transcription. RpoN2 and FleQ regulate the expression of flhF-fleN-fliA and influence flic expression, thus affecting the swimming ability of Xcc (Figure 8). Simultaneously, FliA regulates the expression of genes involved in c-di-GMP turnover and thus indirectly influences Xcc swimming ability (Yang et al., 2009) (Figure S1). As RavR is important for c-di-GMP metabolism and Xcc swimming (Tao et al., 2014), we also analysed the role of FliA in RavR-mediated flic expression and found that FliA was also vital for RavR signalling (Figure 6). Therefore, FliA is required for the effects of both VemR and RavR in the regulation of Xcc swimming (Figure 8).

In addition to VemR, RavA can also form a TCS with RavR to regulate bacterial motility and virulence (Tao et al., 2014). Moreover, RavS/RavR is also an important TCS for Xcc adaptation and virulence (He et al., 2007). Although we uncovered the functions of these TCSs in Xcc, the relationship among RavA/RavR, Rava/VemR, and RavS/RavR remains unclear. Because RavS is a transmembrane HK while RavA is a cytoplasmic HK, they might sense extracellular signals, respectively (Figure 8), and promote different signal transduction pathways. Similarly, HpaS is also a transmembrane HK that may also transduce extracellular signals to regulate VemR, HrpG, or HrpR2 activity. However, how and when HpaS phosphorylates these proteins is unknown. Uncovering the complex regulatory network by these newly identified branched TCSs (Rava/RavR, Rava/VemR, RavS/RavR, HpaS/VemR, HpaS/HrpG, and HpaS/HrpR2) in Xcc will deepen our understanding of bacterial adaptation to environments (Figure 8). To best understand these complex cross-talk networks, two aspects should be considered in future studies: (a) the affinities and phosphoryl-transfer efficiencies, and (b) the subcellular locations and concentrations of an HK (RavS, RavA, HpaS) and its cognate RR (RavR, VemR, HrpG, HpaR2) in different environmental and cellular conditions.

RavA/RavR and RavS/RavR are involved in c-di-GMP signalling, which is important for bacterial motility, biofilm formation, EPS production, and virulence (He et al., 2007; Tao et al., 2014). These behaviours are also regulated by Clp (He et al., 2007), RavA, and VemR (Tao & He, 2010; Tao et al., 2014). Because Clp acts as a c-di-GMP receptor (He et al., 2007), we also analysed the relationship between RavA/VemR and Clp. Notably, the regulation of swimming by Clp is independent of VemR and flagellar regulators, including RpoN2 and FliA, but requires FleQ (Figure 7). However, virulence regulation by VemR is partly dependent on Clp. The results indicate that Clp and VemR are involved in different regulatory pathways of Xcc swimming but have an epistatic relationship in regulating the virulence of Xcc. They also suggest that the Clp-mediated c-di-GMP signalling pathway participates in cross-talk with RavA/VemR signalling. Because RavA/RavR is a crucial regulatory module in c-di-GMP signalling that regulates Xcc motility (Tao et al., 2014), RavA might regulate RavR activity to change cellular c-di-GMP levels and thus affect Clp activity. Although mutation of clp in the rav mutant significantly altered bacterial virulence and swimming, swarming and flic promoter activity were not regulated by Clp in the ΔravR strains. Thus, RavA influences VemR and RavR activities, thereby regulating Clp function in an unknown manner (Figure 8).

In conclusion, our results indicate that VemR controls bacterial motility and virulence by interacting with the transcriptional activator FleQ. This interaction may affect the activity and expression of RpoN2/FleQ to regulate the transcription of flagellum-related genes (Figure 8). Overall, our findings illustrate that the RavA/VemR TCS plays a key regulatory role in Xcc motility and virulence.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains, plasmids, media, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1. The Xcc strains were grown in nutrient-yeast-glycerol (NYG) medium (Daniels & Barber, 1984), NY medium (NYG medium without glycerol), or minimal medium 4 (M4) modified (M4M) medium (each litre of which contained 10.5 g Na2HPO4, 4.5 g NaH2PO4, 1 g (NH4)2SO4, 0.5 g C6H5OH, 0.2 g MgSO4, 8 g casein enzymatic hydrolysate, and 1 g glucose) at 28°C. E. coli strains were incubated in Luria Bertani (LB) medium (Miller, 1972) or minimal medium 63 (M63) (each litre of which contained 100 mM KH2PO4, 75 mM KOH, 15 mM (NH4)2SO4, 1 mM MgSO4, 3.9 μM FeSO4, and 22 mM glucose). Antibiotics at the following final concentrations were used: 25 μg/ml kanamycin (Kan), 50 μg/ml rifampicin (Rif), 100 μg/ml ampicillin (Amp), 50 μg/ml spectinomycin (Spc), 5 μg/ml gentamicin (Gm), and 25 μg/ml chloramphenicol (Cm),
4.2 | Construction and complementation of the deletion mutant strains

The promoter of fliC was fused with the lacZ gene and integrated into the $\beta$-galactosidase-deficient strain Δlac8 to construct the reporter strain Δlac8-flic-lacZ (Wang et al., 2018). To construct the ravA deletion mutant, c.500 bp fragments flanking ravA were amplified and then cloned together into the HindIII/XbaI sites of pk18mobsacB (Schäfer et al., 1994). The recombinant plasmid pk18mobsacB-ravA was introduced into Δlac8-flic-lacZ (WT) by triparental conjugation (Li & Wang, 2011). After two cycles of selection, the primer set ravA-FF/ravA-RR was used to identify the ΔravA mutant. The same genetic manipulation method was used to obtain other mutants. The primers used in this study are listed in Table S2.

For complementation assays, the promoter and the coding sequence of the given gene were amplified and cloned into pX664G (Lee et al., 2012) to generate complementation plasmids. The complementation plasmids were introduced into the corresponding mutants by electroporation.

4.3 | Pathogenicity assays

Six-week-old plants of broccoli cv. Wenxin were used as host plants. The virulence of Xcc strains was determined by leaf clipping, as described elsewhere (Qian et al., 2005). Lesion lengths were measured 2 weeks after inoculation on 10 leaves for each strain.

4.4 | Motility assays

Swarming motility was determined on NY plates containing 2% glucose and 0.6% agar. Swimming motility was assessed on 0.28% agar plates containing 0.03% Bacto peptone and 0.03% yeast extract as described previously (Qi et al., 2020).

4.5 | $\beta$-Galactosidase activity assays

$\beta$-Galactosidase (LacZ) activity analysis was carried out as previously described (Griffith & Wolf, 2002; Smale, 2010; Wang et al., 2018). For plate assays, 1 μl of diluted cell culture was inoculated on NYG plates containing 40 mg/L X-gal. After incubating at 28°C for 2 days, the plates were checked for the appearance of blue colonies. For quantitative assays of $\beta$-galactosidase activity, cells (logarithmic growth phase) were collected by centrifugation. Equal amounts (OD$_{600}$) of culture (in ml), and OD$_{600}$ = optical density of the culture used.

4.6 | $\beta$-Glucuronidase activity assays

Bacteria (logarithmic growth phase) were collected by centrifugation, washed twice with double deionized water (ddH2O), and resuspended in ddH2O (OD$_{600}$ = 1.0). One hundred microlitres of the bacterial suspension was mixed with 200 μl of GUS staining solution (50 mM NaH$_2$PO$_4$, 50 mM Na$_2$HPO$_4$, 10 mM Na$_2$EDTA, 0.1% Triton X-100, 0.5 mM K$_4$[Fe(CN)$_6$], 0.5 mM K$_3$[Fe(CN)$_6$], 20 mM X-Gluc), incubated at 37°C for 4 h, and the colour change was observed (Kosugi et al., 1990). The method for quantification of GUS activity was almost the same as that used for measuring $\beta$-galactosidase activity. The differences were as follows: (a) the lysis buffer contained 50 mM NaH$_2$PO$_4$, 50 mM Na$_2$HPO$_4$, 10 mM Na$_2$EDTA, 0.1% Triton X-100, 0.1% SDS, and 10 mM β-ME, (b) the reaction substrate was 2 mM Methylumbellifery-β-D-glucuronide (4-MUG), and (c) the fluorescence excitation wavelength was 365 nm and the emission wavelength detected was 455 nm.

4.7 | Bacterial two-hybrid assays

The Bacterial Adenylate Cyclase (CyaA) Two-Hybrid System (Karimova et al., 1998) was used to detect protein–protein interactions. VemR, RavA, FleQ, and RpoN2 were fused with the T25 or T18 fragment of CyaA and cotransformed into the reporter strain BTH101. Selection and data analysis were carried out as described in a previous report (Karimova et al., 1998). Briefly, the resulting strains were grown overnight in liquid media containing the corresponding antibiotics and washed twice with ddH$_2$O. Then, the cell suspension was diluted and spotted on LB medium or auxotrophic M63 medium containing 40 mg/ml X-gal and cultured at 30°C for 24 h.

4.8 | Site-directed mutagenesis

A Q5 Site-Directed Mutagenesis Kit (NEB) was used for site-directed mutagenesis. The recombinant plasmid containing the target gene was used as the PCR template. The mutants were obtained by PCR using primer pairs containing the mutant sequences. The amplified product was treated with KLD enzyme mix and transformed into E. coli DH5α. Mutation was confirmed by DNA sequencing.

4.9 | Protein pull-down assays

The PCR-amplified vemR or ravA full-length coding region was digested with NdeI and EcoRI, and cloned into the pMAL-c5x vector (NEB), and the fleQ and vemR PCR fragments were digested with BamHI and HindIII and cloned into the pQE80L vector (Qiagen). The recombinant vectors were transferred into E. coli BL21 (DE3)
cells. Protein synthesis was induced by 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The bacterial cells were collected and lysed by ultrasonication. Both MBP-VemR/FleQ-His and MBP-RavA/VemR-His were incubated with amylose resin overnight at 4°C, followed by multiple washes with Tri-buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) to eliminate the influence of contaminants, and proteins were eluted with 10 mM maltose. Electrophoresis was performed by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting was performed with an anti-MBP antibody and an anti-His antibody. The MBP protein was used as a negative control (Liu et al., 2011).

4.10 Protein phosphorylation analysis

In vivo VemR phosphorylation states were detected in Xcc and E. coli. In E. coli, the strains expressing VemR-His and RavA-Flag/VemR-His were cultured in LB medium to an OD₆₀₀ of 0.6. Then, 0.3 mM IPTG was added to induce protein expression, and the cells were cultured for 5 h at 25°C. Then, the bacterial cells were collected and lysed by ultrasonication to prepare total proteins. VemR-His was purified with Ni-NTA, and 10-μl elution samples were subjected to Phos-tag SDS-PAGE as described previously (Li et al., 2014) except that Zn²⁺ was used instead of Mn²⁺. Protein transfer from the gel to a PVDF membrane and western blotting were performed as described in a previous report (Li et al., 2014). Anti-FLAG and anti-His antibodies were used for protein detection. In Xcc, the plasmids encoding VemR-His were transformed into ΔvemR and ΔravA/ΔvemR. The resulting strains were cultured in M4M medium to an OD₆₀₀ of 0.8, and protein expression was induced by 0.3 mM IPTG. Then, the bacterial cells were collected and lysed by ultrasonication to prepare total proteins. VemR-His protein purification and Phos-tag SDS-PAGE were performed as described above. For in vitro assays, GST-RavA and VemR-His were expressed in E. coli BL21 and then purified. The autophosphorylation of GST-RavA and phosphotransfer assays were performed as described previously (Tao et al., 2014). The phosphorylation states of VemR-His were detected in Phos-tagged gels as described above for in vivo assays.

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DATA AVAILABILITY STATEMENT

The raw data from this study are available from the corresponding author upon reasonable request.

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