INTRODUCTION

Homologous regulatory factors are widely present in bacterial genomes. These homologous regulators may display functional redundancy or different biological functions, and some homologues even have no biological function, for example the two homologous ArsR regulators of Pseudomonas putida KT2440 bind arsenite with similar affinities (Fernandez et al., 2016). AgaR2 and AgaR1 are two novel regulators that are specific for GalN/GalNAc catabolism and have been assigned distinct roles in bacterial infection (Zhang et al., 2015). NagC and Mlc, homologous members of the ROK (repressors, open reading frames [ORFs] and kinases) family of proteins with almost identical helix-turn-helix DNA-binding motifs, specifically regulate genes for the transport and utilization of N-acetylglucosamine and glucose (Brechemier-Baey et al., 2015). Two homologous members of the cyclic-AMP receptor protein (CRP) family of transcription factors (MSMEG_0539 and MSMEG_6189) show differences in cAMP binding affinity, trypsin sensitivity, and binding to a CRP site.
in Mycobacterium smegmatis (Sharma et al., 2014). Three homologous LysR-type transcriptional regulators (MetR, CysR, and HomR) control sulphur amino acid supply in Streptococcus mutans (Sperandio et al., 2010), while rpoN1 and rpoN2 of Xanthomonas citri subsp. citri play different roles in virulence, nutrient utilization, and cell motility (Gicharu et al., 2016). However, some homologous transcription factors still have unknown biological and transcriptional functions. Here, we use Xanthomonas campestris pv. campestris (Xcc) sigma factor 54 (σ54) proteins (XCC RpoN1 and XCC RpoN1) as a model to illustrate the characteristics of homologous transcription factors in a single bacterial genome.

Sigma factor 54, alternatively named RpoN, is also required for the initiation of transcription at specific DNA sequences (Hirschman et al., 1985). The σ factor facilitates transcription at specific DNA sequences by binding to the core RNA polymerase (RNAP) to form the σ-RNAP holoenzyme, recognizing and binding to a specific DNA sequence adjacent to the transcription start site, called the promoter element, and opening the double-stranded DNA to initiate transcription (Barrios et al., 1999; Doucet et al., 2005; Wiesler et al., 2012; Schaefer et al., 2015).

RpoN can regulate the transcription and expression of many genes, and is historically known for its role in nitrogen assimilation (Kohler et al., 1989; Rajeev et al., 2015). This protein has been shown to be involved in the regulation of other important lifestyle-associated functions in bacteria, such as in the regulation of the type III secretion system (Lee et al., 2016) and type VI secretion system (Dong and Mekalanos, 2010; Hayrapetyan et al., 2013; Schaefer et al., 2015). RpoN also affects the growth, virulence, motility, and biofilm formation of bacteria (Hao et al., 2013; Hayrapetyan et al., 2015; Ray et al., 2015). In addition, RpoN can affect the assembly of bacterial flagella (Yang et al., 2009; Schulz et al., 2012), quorum sensing (QS) (Cai et al., 2015), amino acid utilization, the regulation of catalase expression at the transcriptional level (Diep et al., 2015), and carbohydrate metabolism (Stevens et al., 2010; Hayrapetyan et al., 2015).

Analysis of the complete genome sequences of several microorganisms has shown that the rpoN gene (encoding the σ54 factor) is widely distributed among bacteria (Studholme et al., 2000; Studholme and Buck, 2000a, 2000b; Dombrecht et al., 2002; Studholme and Dixon, 2003). A few organisms have two copies of rpoN, such as Ralstonia solanacearum (Ray et al., 2015), Burkholderia pseudomallei (Diep et al., 2015), Bradyrhizobium japonicum (Kullik et al., 1991), and X. campestris (Yang et al., 2009). In B. japonicum, these copies are highly similar and functionally interchangeable (Kullik et al., 1991). The genome of R. solanacearum contains two copies of rpoN that have different functions and are involved in twitching motility, natural competence, growth on nitrate, and virulence (Ray et al., 2015). In contrast, the four different σ54 factors of Rhodobacter sphaeroides are not functionally interchangeable (Poggio et al., 2002), and this unusually high number of rpoN gene copies does not seem to be the result of recent duplication events because the products of these genes show degrees of similarity ranging from 48% to 87% (Poggio et al., 2006). However, why is it that among highly similar RpoNs some are functionally interchangeable and some are not? The molecular determinants that confer this specificity remain unknown.

A majority of bacterial species belonging to the genus Xanthomonas are plant pathogens. Among these species, X. campestris pv. campestris causes black rot disease in crucifers (Zhou et al., 2015a; Wang et al., 2018). Two rpoN homologues genes encoding σ54 were also identified in the genome of X. campestris. To distinguish these two genes, the rpoN gene XCC2802, located in a phosphotransferase system (PTS), was named rpoN1 and the other (XCC1935), located in a large flagellar gene cluster, was named rpoN2 (Figure 1a,b) (da Silva et al., 2002; Yang et al., 2009). The flagellar biogenesis of X. campestris requires the alternative sigma factor RpoN2 (da Silva et al., 2002; Yang et al., 2009). However, the mechanism by which RpoN2 regulates flagellar biogenesis and the role of rpoN1 in X. campestris remain unknown. Whether these two different σ54 factors of X. campestris are functionally interchangeable and the molecular determinants that may confer specificity on RpoN1 and RpoN2 have not yet been investigated.

In this study, we describe for the first time the distinct roles of RpoN1 and RpoN2 and their different regulatory functions in basal metabolism, flagellar biosynthesis, extracellular polysaccharide (EPS) formation, biofilm formation, and virulence in X. campestris. Our study therefore reveals that these functions of the two homologous RpoN proteins are not redundant in X. campestris. Overall, the results of the present study improve the current understanding of RpoN-mediated regulation in X. campestris.

## 2 | RESULTS

### 2.1 | Two conserved rpoN genes in the X. campestris genome

To investigate the function of RpoN in X. campestris, protein sequence alignments of X. campestris RpoN with RpoN proteins from Escherichia coli (Tucker et al., 2010; Wiesler et al., 2012), Pseudomonas aeruginosa (Shao et al., 2018), and Sinorhizobium meliloti (Iannino et al., 2008) were examined (Figure 1c). The results showed that the X. campestris RpoN1 protein shares 42%, 43%, and 36% identical residues with E. coli RpoN, P. aeruginosa RpoN, and S. meliloti RpoN, respectively. We also aligned the sequence of X. campestris RpoN2 with those of E. coli RpoN, P. aeruginosa RpoN, and S. meliloti RpoN, and the identity values were 40%, 39%, and 35%, respectively. The RpoN1 protein shares 56% identical residues with RpoN2 in X. campestris. We also found that X. campestris RpoN1 and RpoN2 each contain a major RNAP core-binding domain (CBD) and RpoN domain (Yang et al., 2015). Based on these criteria, it seems reasonable that RpoN1 and RpoN2 could be functional σ54 factors that play crucial roles in basal metabolism and virulence in X. campestris.

### 2.2 | Inactivation of rpoN2 caused a deficiency in virulence

X. campestris is the causal agent of black rot disease in cruciferous vegetables. To identify the physiological functions of RpoN1 and
RpoN2 in virulence against host plants, the gene knockout strains ΔrpoN1 and ΔrpoN2 and the double-mutant strain ΔrpoN1N2, in which both the rpoN1 (XCC2802) and rpoN2 (XCC1935) genes were deleted, were constructed by a two-step homologous recombination approach. To further evaluate whether RpoN contributes to the virulence of X. campestris, a leaf-clipping virulence assay using a susceptible cabbage variety (Brassica oleracea 'Jingfeng No. 1') was conducted. The average lesion length caused by wild-type strain Xc1 on a cabbage leaf was 14.1 mm at 10 days after inoculation (Figure 2a,b). The gene knockout strains ΔrpoN2 and ΔrpoN1N2 exhibited significantly reduced average lesion lengths (2.4 and 1.9 mm, respectively) (Figure 2a,b), but the lesion length of the ΔrpoN1 strain was not significantly different compared with that of the wild-type strain (12.6 mm). Under similar test conditions, complementation with rpoN2 restored the virulence of ΔrpoN2 and ΔrpoN1N2 against host plants, and the average lesion lengths with the complemented strains were 6.9 and 8.9 mm, respectively (Figure 2a,b).

The bacterial population in planta was also measured. After inoculation, the bacterial populations of ΔrpoN2 and ΔrpoN1N2 were markedly decreased in planta (Figure 2c). The complemented strains ΔrpoN2/N2 and ΔrpoN1N2/N2, containing a plasmid-borne rpoN2, retained the wild-type colonization ability in planta (Figure 2c). This result indicates that rpoN2, but not rpoN1, is required for the virulence of X. campestris, indicating that RpoN1 and RpoN2 are not functionally interchangeable in terms of the pathogenicity of X. campestris.

FIGURE 1 Identification and sequence characterization of RpoN in Xanthomonas campestris. (a) Chromosomal region of X. campestris surrounding the rpoN1 gene. ptsA, lipopolysaccharide export system protein LptA; yhbG, ABC transporter ATP-binding protein; rpoN1, RNA polymerase $\sigma^{54}$ factor, XCC2803, $\sigma^{54}$ modulation protein; ptsN, nitrogen regulatory IIA protein; ptsK, HP-kinase/phosphorylase; rapZ, RNase adapter protein RapZ; XCC2807, PTS, ascorbate-specific IIA component; ptsH, phosphotransferase system HPr enzyme; ptsI, phosphotransferase system enzyme I. (b) Chromosomal region of X. campestris surrounding the rpoN2 gene. HP, hypothetical protein; viaA, nucleotide sugar transaminase; fleQ, transcriptional regulator; XCC1934, response regulator; rpoN2, RNA polymerase $\sigma^{54}$ factor; XCC1936, response regulator; HP, hypothetical protein; filL, flagellar protein; filD, flagellar protein; filIC, flagellin; filGL, flagellar hook-associated protein FlgL; filGK, flagellar hook-associated protein FlgK; filJ, flagellar rod assembly protein/muraridase FlgJ. (c) Alignment of X. campestris, Pseudomonas aeruginosa, and Sinorhizobium meliloti RpoN sequences. Alignment was performed with ClustalW based on identical residues. CBD and RpoN are highly conserved among $\sigma^{54}$ proteins from different species.
Transcriptomic profiling of the rpoN deletion strains revealed the regulatory role of this gene in basal metabolism and flagellar synthesis

To investigate whether there are new genes/functions that are controlled by rpoN1 and rpoN2 in X. campestris, we performed transcriptomic analyses (RNA-Seq) of the wild-type and rpoN mutant strains. The examination of each sample was repeated twice. The results show that compared to the wild type, a total of 385 genes were significantly differentially altered at the transcriptional level in the ΔrpoN1 mutant strain, with 274 genes up-regulated and 111 genes down-regulated (Figure 3a and Table S3). We also analysed and compared the transcriptomes of the ΔrpoN2 mutant strain and wild-type strain using RNA-Seq. Differential gene expression analysis showed that 283 genes were up-regulated and 179 genes were down-regulated in the ΔrpoN2 mutant compared with the wild-type strain, whereas 468 genes were up-regulated and 346 genes were down-regulated in the ΔrpoN1N2 double mutant compared with the wild-type strain (Figure 3a, Tables S4 and S5). We also compared the transcriptome profiles using Venn diagrams, which showed the overlap of 67 up-regulated and three down-regulated genes in different mutant backgrounds (Figure 3a). These results were further partly confirmed by quantitative reverse transcription PCR (RT-qPCR) analysis of eight randomly selected candidate genes (Figure S1). Further bioinformatics analyses showed that the products of these differentially expressed genes (DEGs) belong to three major functional categories. In addition, each major category contains rather diverse subfunctional groups (Figure S2a–c). Notably, the rpoN mutants induced significant changes in mRNA abundance in the genes involved in basal metabolism and flagellar synthesis, as indicated by functional annotation analysis (Figure 3b–d). Many genes involved in basal metabolism were differentially expressed with the wild-type strain, whereas 468 genes were up-regulated and 346 genes were down-regulated in the ΔrpoN1N2 double mutant compared with the wild-type strain (Figure 3a, Tables S4 and S5).
FIGURE 3  Differentially expressed genes (DEGs) involved in basal metabolism and flagellar synthesis. (a) Venn diagram showing overlap analysis of the up-regulated and down-regulated DEGs in the wild-type Xc1, ΔrpoN1, ΔrpoN2, and ΔrpoN1N2. The Venn diagrams were drawn with the "Venn Diagram" package in R. (b) Categories of 385 significant genes assigned in the KEGG database in the ΔrpoN1 mutant compared to the wild-type strain: the 385 significant (p < .05, ≥ ±1 log₂-fold change [FC]) genes were classified into 11 different categories with diverse cellular functions. (c) Categories of 462 significant genes assigned in the KEGG database in the ΔrpoN2 mutant compared to the wild-type strain: the 462 significant (p < .05, ≥ ±log₂FC) genes were classified into seven different categories with diverse cellular functions. (d) Categories of 814 significant genes assigned in the KEGG database in the ΔrpoN1N2 double mutant compared to the wild-type strain: the 814 significant (p < .05, ≥ ±log₂FC) genes were classified into 12 different categories with diverse cellular functions. (e) Hierarchical cluster analysis applied to the 121 DEGs in the amino acid metabolism, carbohydrate metabolism, and lipid metabolism pathways in different mutant backgrounds. The transcriptional profiles in terms of relative gene expression values (log₂ scale of microarray values) were analysed using the heatmap command of R. Red and blue represent up-regulated and down-regulated genes, respectively. (f) Hierarchical cluster analysis applied to the 84 DEGs in the bacterial chemotaxis and flagellar assembly pathway in different mutant backgrounds.
in the $\Delta rpoN1$ mutant and $\Delta rpoN1N2$ double mutant (Figure 3e). Additionally, the transcript levels of 35 flagellar assembly pathway genes and 49 chemotaxis-related genes were significantly altered in the $\Delta rpoN2$ mutant and $\Delta rpoN1N2$ double mutant (Figure 3f). Taken together, our RNA-Seq data reveal that the transcripts of genes involved in basal metabolism and flagellar synthesis were substantially affected by the $rpoN1$ and $rpoN2$ genes.

### 2.4 Deletion of $rpoN2$ affected the swimming ability and flagellar synthesis of X. campestris

Xcc $rpoN2$ (XCC1935) is located in a flagellar synthesis gene cluster (Figure 1a). A series of flagellar synthesis-related genes ($fliDCES$, $flhAB$) and chemotaxis-related genes ($cheABDRWY$, $motAB$) were highlighted in the RNA-Seq data, and all of these genes were downregulated in the $\Delta rpoN2$ mutant (Table S4). We hypothesized that an in-frame deletion mutant of $rpoN2$ affected flagellar biogenesis. To further determine whether deletion of $rpoN2$ affected flagellar biogenesis in X. campestris, the single polar flagellum of various X. campestris strains was observed by transmission electron microscopy (TEM). The electron micrographs demonstrated that the $\Delta rpoN2$ and $\Delta rpoN1N2$ mutants lost the typical single polar flagellum, and recovery to wild-type levels was observed in the $rpoN2$-complemented strains but not in the $rpoN1$-complemented strains (Figure 4c). TEM observation showed that the $\Delta rpoN1$ mutant strain had a normal flagellar morphology compared with the wild-type strain (Figure 4c). These results indicate that RpoN2 is necessary for flagellar biogenesis and motility in X. campestris and suggest that the function of RpoN2 in flagellar synthesis cannot be performed by RpoN1.

The function of $rpoN1$ and $rpoN2$ in flagellum-dependent motility was investigated. We then tested the swimming motility of the $\Delta rpoN1$, $\Delta rpoN2$, and $\Delta rpoN1N2$ mutants on 0.3% semisolid agar plates. Compared with the wild-type strain, deletion of $rpoN2$ abolished swimming motility in X. campestris, suggesting that the motility was significantly impaired (Figure 4a,b). The $\Delta rpoN1$ mutant remained as motile as the wild-type strain. The motility defect phenotypes

**FIGURE 4** Deletion of $rpoN2$ affected Xanthomonas campestris swimming ability and flagellar synthesis. (a) and (b) Assay of swimming motility of the wild-type Xc1; mutants $\Delta rpoN1$, $\Delta rpoN2$, and $\Delta rpoN1N2$; and complemented strains $\Delta rpoN1/N1$, $\Delta rpoN1/N2$, $\Delta rpoN2/N1$, $\Delta rpoN2/N2$, $\Delta rpoN1N2/N1$, and $\Delta rpoN1N2/N2$. The swimming zones were recorded after bacterial growth for 4 days on semisolid plates at 28 °C. Error bars, means ± SD (n = 3). Different letters indicate significant differences between treatments with the least significant difference at $p = .05$. All experiments were repeated three times with similar results. (c) Observation of flagella using transmission electron microscopy.
of the ΔrpoN2 and ΔrpoN1N2 mutants could be restored to wild-type levels by introducing rpoN2 into ΔrpoN2 and ΔrpoN1N2, respectively. Moreover, overexpression of rpoN1 in the ΔrpoN2 and ΔrpoN1N2 mutants resulted in no substantial difference between the mutant and complemented strains of X. campestris in terms of swimming motility (Figure 4a,b). These results suggest that RpoN2, but not RpoN1, is required for the swimming motility of X. campestris.

To further demonstrate the biological function of RpoN, we performed electrophoretic motility gel shift assays (EMSA) without a core RNAP (Cannon et al., 1993). The RpoN1 and RpoN2 proteins were then expressed in E. coli BL21 (DE3), and the N-terminal-His-tagged versions of the proteins were purified with nickel chelate chromatography (Figure S3). To determine the direct link between RpoN and flagellar biogenesis genes, RpoN-regulated candidate genes of X. campestris were selected for further investigation. Deletion of rpoN2 resulted in down-regulation of Xcc fliC (Figure 5a), which suggests that RpoN might bind directly to the promoter region of Xcc fliC to regulate transcription, in turn affecting the flagellar synthesis in X. campestris. To test this hypothesis, the putative promoter DNA fragment covering 409 bp upstream of the Xcc fliC translational start site, namely, pXCC fliC, was cloned and analysed using EMSA. Addition of purified RpoN2 protein, at concentrations ranging from 0 to 8 μM, to the reaction mixtures (20 μl, 28 °C, 25 min) caused a shift in the mobility of the pXCC fliC DNA fragment, which suggested that an RpoN2-pXCC fliC complex was formed (Figure 5c). However, addition of purified RpoN1 protein indicated weak binding to the promoter of Xcc fliC (Figure 5d). RT-qPCR showed that the expression of Xcc fliC in the ΔrpoN2 and ΔrpoN1N2 mutant strains was compromised compared with that in the wild-type X. campestris strain, indicating that RpoN2 positively regulates the expression of Xcc fliC (Figure 5b). To further study regulation by RpoN, we constructed pFliC-lacZ reporter systems in rpoN mutant strains. Consistent with the RNA-Seq and RT-qPCR results, deletion of rpoN2 resulted in reduced expression levels of XCC fliC (Figure 5e). We found that RpoN2 can also bind the promoter of Xcc fliQ (Figure S4). Taken together, these results indicate that RpoN2 positively regulates the Xcc fliC and Xcc fliQ genes by directly binding to the corresponding promoters to regulate flagellar synthesis in X. campestris.

2.6 | Deletion of rpoN1 affected the synthesis of branched-chain fatty acids and the production of diffusible signalling factor family signals in X. campestris

Xcc rpoN1 (XCC2802) is located in a phosphotransferase gene cluster (Figure 1a). The transcription of genes involved in the basal metabolism pathway, including genes involved in carbon metabolism, biosynthesis of secondary metabolites, starch and sucrose metabolism, pentose and glucuronate interconversion, biosynthesis of amino acids, QS and other pathways was changed in the ΔrpoN1 mutant cells. To test the function of RpoN in basal metabolism, we analysed the fatty acid composition of the total lipid extracts from Xcc rpoN mutant strains grown in nutrient yeast glycerol (NYG) medium at 28 °C by gas chromatography-mass spectrometry (GC-MS). Compared to the fatty acid composition of the wild-type strain Xc1 grown in NYG medium at 28 °C, the ΔrpoN1 mutant strain and ΔrpoN1N2 double-mutant strain exhibited significantly decreased amounts of branched-chain fatty acids (BCFAs), especially iso-C15:0 and anteiso-C15:0 fatty acids, and increased the amount of penta-decanoic acid (p-C15:0). The fatty acid profile of Xcc ΔrpoN2 did not differ from that of wild-type strain Xc1 under these temperature conditions (Figure 7a–c). These results suggest that Xcc RpoN1 is crucial for fatty acid synthesis in strain Xc1.

To further evaluate the function of RpoN in basal metabolism, we also tested the production of diffusible signalling factor (DSF) family signals by rpoN mutant strains grown in NYG medium using high-performance liquid chromatography (HPLC). As the Xc1 rpfC deletion strain produced high levels of DSF family signals (Zhou et al., 2015b), sufficient for analysis, we constructed the mutant strains ΔrpfCΔrpoN1, ΔrpfCΔrpoN2, and the double-mutant strain ΔrpfCΔrpoN1N2 by deleting rpoN1 and rpoN2 from the parental strain Xc1 ΔrpfC. DSF family signal production was further studied in NYG medium. All the strains produced DSF family signals, namely, cis-11-methyl-2-dodecanoic acid (DSF), cis-2-dodecanoic acid (BDSF), and cis-10-methyl-2-dodecanoic acid (iDSF), but DSF was the main signal produced under these conditions (Figure 7d–f), consistent with the result of a previous report (Zhou et al., 2015b; Li et al., 2017). However, the amounts of each DSF family signal

2.5 | RpoN2 is involved in biofilm formation and EPS production

Bacterial biofilms are involved in adaptation to complex environments and are used by pathogenic bacteria to colonize host cells (Tao et al., 2010). To test whether RpoN plays a role in biofilm formation, three independent assays were carried out in the first assay, the frequently used crystal violet (CV) staining approach was applied. The results showed that the ΔrpoN2 and ΔrpoN1N2 mutant strains exhibited 5.8-fold and 14.9-fold reductions, respectively, in biofilm formation on the polystyrene surface after staining with CV, compared with the wild type (Figure 6a); however, inactivation of rpoN1 had no effect on biofilm formation (Figure 6a). These data reveal that RpoN2 is required for full biofilm formation.

EPS is a critical virulence factor for pathogenicity (Rai et al., 2012; Cai et al., 2017). Yu et al. found that RpoN (σ25) is required for floc formation but not for EPS biosynthesis in a floc-forming Aquincola tertiaricarbonis strain (Yu et al., 2017). To further investigate the function of Xcc RpoN in EPS production, we examined EPS synthesis in the rpoN mutants. As shown in Figure 6b, the EPS production ability of the ΔrpoN2 and ΔrpoN1N2 mutants was significantly decreased, but EPS production by the ΔrpoN1 strain was not significantly different compared with that by the wild-type strain. These findings suggest that RpoN2 is required for full EPS production.
FIGURE 5  The effects of RpoN2 on binding to the promoter of Xanthomonas campestris pv. campestris fliC. (a) Relative expression of fliC as determined by RNA-Seq. (b) Relative expression of fliC as determined by quantitative reverse transcription PCR. (c) and (d) Gel shift assay showing that RpoN2 and RpoN1 directly regulate fliC. RpoN2 and RpoN1 (0, 1, 2, 4, or 8 μM) were added to reaction mixtures containing 50 ng of probe DNA, and the reaction mixtures were separated on polyacrylamide gels. (e) The effect of RpoN on fliC gene expression was measured by assessing the β-galactosidase activity of the fliC-lacZ transcriptional fusions in the Xc1 wild-type, ΔrpoN1, ΔrpoN2, and ΔrpoN1N2 strains. Error bars, means ± SD (n = 3). *p < .05, **p < .01, ****p < .0001, assessed by one-way analysis of variance. All experiments were repeated three times with similar results.
RpoN has been shown to be involved in the regulation of many bacterial functions, such as nitrogen metabolism, flagellar biosynthesis, biofilm formation, motility, colonization, lipoprotein biosynthesis, and the activity of a type III secretion system (Kohler et al., 1989; Yang et al., 2009; Dong and Mekalanos, 2012; Schulz et al., 2012; Hao et al., 2013; Sana et al., 2013; Hayrapetyan et al., 2015; Rajeev et al., 2015; Ray et al., 2015; Lee et al., 2016). RpoN also affects bacterial QS (Cai et al., 2015). Although RpoN regulates bacterial virulence and shares similar functions in gram-negative bacteria, the regulons of RpoN vary among bacteria (Schaefer et al., 2015; Shao et al., 2018; Xu et al., 2019). In this study, we demonstrate that RpoN1 and RpoN2 play different roles in regulation of basal metabolism, flagellar biosynthesis and virulence traits in X. campestris.

Our data show that in-frame deletions of the rpoN2 coding sequence caused a significant decrease in bacterial virulence and in planta growth in a susceptible cabbage variety (B. oleracea ‘Jingfeng No. 1’) (Figure 2), whereas the ΔrpoN1 mutant did not cause a substantial decrease in virulence or in planta growth. However, why does the rpoN2 mutation cause a decrease in X. campestris virulence? To understand the underlying mechanisms, the role of rpoN2 in X. campestris virulence was examined. We evaluated several pathogenicity-related virulence factors produced by the rpoN mutant strains. The activities of extracellular cellulase, amyrase, and protease enzymes produced by the rpoN mutant strains were not significantly different from those of the enzymes produced by the wild-type strain Xc1 (Figure S5). However, we evaluated EPS production in the rpoN mutant strains, and found that the ability of the ΔrpoN2 and ΔrpoN1N2 mutants to produce EPS was significantly decreased (Figure 6b). EPS act as a matrix material and is essential for biofilm formation in most gram-negative bacteria (Pratt and Kolter, 1999; Donlan, 2002; Singh et al., 2006). Does this finding indicate that the rpoN2 mutant also affects biofilm formation in X. campestris? We tested the biofilm formation ability of ΔrpoN1, ΔrpoN2, and ΔrpoN1N2, and found that ΔrpoN2 and ΔrpoN1N2 exhibited significantly reduced biofilm formation compared with the wild type (Figure 6a). Bacterial biofilms are involved in adaptation to complex environments and are used by pathogenic bacteria to colonize host cells (Tao et al., 2010). Thus, we hypothesized that reduction in EPS and biofilm formation was one of the reasons why X. campestris exhibited impaired virulence against host plants. However, further investigation is required to understand these mechanisms in greater detail.

RpoN has been implicated in the regulation of flagellar and motility genes in many species. We also showed that the absence of native RpoN2 significantly reduced motility-associated phenotypes (Figure 4a–c). For many bacteria, motility is essential for survival, growth, virulence, biofilm formation, and intra/interspecies interactions and allows the bacteria to position themselves in appropriate locations at appropriate times (Nan and Zusman, 2016; Kilmur and Burrows, 2018). In prokaryotes, flagellation is essential for swimming motility. Flagellar biogenesis is a complex process that involves over 40 genes. The phytopathogen X. campestris possesses a single polar flagellum (Yang et al., 2009). RNA-Seq analysis revealed that the ΔrpoN2 and ΔrpoN1N2 mutations affected a group of genes associated with flagellar assembly, and 55.6% and 100% of these genes, respectively, were down-regulated (Tables S4 and S5). This result suggests that the ΔrpoN2 mutant could be disruptive to the flagellar system with subsequent disorganization of flagellar assembly. To confirm the effect of the ΔrpoN2 mutation on X. campestris flagellation, we used TEM to observe the filaments of the Xc1, ΔrpoN1, ΔrpoN2, and

![Figure 6](image-url) Effects of rpoN genes on the production of biofilms and extracellular polysaccharide (EPS) in Xanthomonas campestris. (a) Deletion of rpoN2 in X. campestris Xc1 affects biofilm formation in nutrient yeast glycerol (NYG) broth, as determined by crystal violet (CV) staining. (b) Deletion of rpoN2 in X. campestris affects EPS production in NYG broth. Error bars, means ± SD (n = 3). ***Significant differences (p < .0001, assessed by one-way analysis of variance). All experiments were repeated three times with similar results.
FIGURE 7 Deletion of rpoN1 affected the synthesis of branched-chain fatty acids and increased the production of diffusible signalling factor (DSF) family signals in Xanthomonas campestris. (a) Fatty acid composition of the total lipid extracts from the wild-type Xc1 and \( \Delta rpoN1 \) mutant strains grown in nutrient yeast glycerol (NYG) medium. (b) Fatty acid composition of the total lipid extracts from the Xc1 and \( \Delta rpoN2 \) mutant strains grown in NYG medium. (c) Fatty acid composition of the total lipid extracts from the Xc1 and \( \Delta rpoN1N2 \) double-mutant strains grown in NYG medium. Total lipids were extracted and transesterified to fatty acid methyl esters, and the products were identified by gas chromatography-mass spectrometry (GC-MS). Values are percentages of total fatty acid levels and are means ± SD of three independent experiments. \( n-C_{14:0} \), 3-hydroxytetradecanoic acid; \( n-C_{14:0} \), tetradecanoic acid; \( iso-C_{15:0} \), 13-methyl-tetradecanoic acid; \( anteiso-C_{15:0} \), 12-methyl-tetradecanoic acid; \( n-C_{15:0} \), pentadecanoic acid; \( n-C_{16:1} \), cis-9-hexadecenoic acid; \( n-C_{16:0} \), hexadecanoic acid; \( iso-C_{17:1} \), cis-9, cis-9-15-methyl-hexadecenoic acid; \( n-C_{18:1} \), cis-11-octadecenoic acid; \( n-C_{18:0} \), octadecanoic acid. (d) DSF signals produced by the rpoN1 deletion mutant \( \Delta rpoN1 \) grown in nutrient broth (NB) medium. Supernatants (50 ml) of strain \( \Delta rpfC\Delta rpoN1 \) grown in NB medium for 36 hr were collected and DSF signals were detected. (e) DSF signals produced by the rpoN2 deletion mutant \( \Delta rpoN2 \) grown in NB medium. Supernatants (50 ml) of the strain \( \Delta rpfC\Delta rpoN2 \) grown in NB medium for 36 hr were collected and DSF signals were detected. (f) DSF signals produced by the rpoN1 and rpoN2 double-mutant \( \Delta rpoN1N2 \) grown in NB medium. Supernatants (50 ml) of strain \( \Delta rpfC\Delta rpoN1N2 \) grown in NB medium for 36 hr were collected and DSF signals were detected. DSF, cis-11-methyl-2-dodecenoic acid; BDSF, cis-2-dodecenoic acid; IDSF, cis-10-methyl-2-dodecenoic acid. Error bars, mean ± SD (\( n = 3 \)). *\( p < .05 \), **\( p < .01 \), ***\( p < .001 \), assessed by one-way analysis of variance. All experiments were repeated three times with similar results.
ΔrpoN1N2 strains and the rpoN1- and rpoN2-complemented strains. TEM observation showed that deletion of rpoN2 led to defects in flagellar morphology compared with the wild-type strain (Figure 4a). We found that RpoN1 and RpoN2 are not functionally interchangeable for X. campestris flagellation (Figure 4a). This discovery can provide experimental reference information for analysis of the biological functions of RpoN2 for X. campestris flagellar assembly in the future. RpoN binds to cognate promoters containing a TGGCANCCTTGCCW motif (Barrios et al., 1999; Doucleff et al., 2005; Wiesler et al., 2012; Schaefer et al., 2015). To determine the genome-wide variation caused by X. campestris RpoN1, the conserved RpoN consensus binding motif was also used to search the complete genomic sequences of X. campestris to identify the conserved GC sequences in the promoter of Xcc fliC. We hypothesized that RpoN2 binds to the promoter of Xcc fliC to regulate X. campestris flagellation. We demonstrated by EMSA that RpoN2 could positively regulate the expression of Xcc fliC by directly binding to the promoter of this gene, but RpoN1 exhibited weak binding to the Xcc fliC promoter (Figure 5). We found that RpoN2 can also bind to the promoter of Xcc fliQ (Figure S4). Taken together, these results suggest that RpoN2 might be a transcriptional activator that modulates bacterial flagellation by directly binding to the promoters of Xcc fliC and Xcc fliQ in X. campestris. However, further details need to be studied in the future.

Fatty acids and DSF family QS signals are critical secondary metabolites for bacterial physiological metabolism (Zhou et al., 2015a; Li et al., 2017; Hu et al., 2018). In this study, in-frame deletion of rpoN1 led to significantly reduced production of branched-chain amino acids (BCAAs) and increased production of DSF family QS signaling molecules, especially cis-11-methyl-2-dodecenoic acid. The precursors of the DSF family signals originate from the fatty acid synthesis pathway (Yu et al., 2016; Li et al., 2017). However, BCAAs are the primary precursors for the synthesis of BCFAs (Zhu et al., 2005). RpoN also affects amino acid utilization and carbohydrate metabolism (Stevens et al., 2010; Diep et al., 2015; Hayrapetyan et al., 2015). Therefore, we speculate that rpoN1 directly regulates the biosynthesis of amino acids and basal metabolism to affect BCFA and DSF synthesis. RNA-Seq showed that the rpoN1 mutation significantly affected the expression of a valine, leucine, and isoleucine biosynthesis-related gene (leuA) and a long-chain fatty acid-CoA ligase gene (rpfB) (Figure S6 and Tables S3 and S5). These results suggest that RpoN1 and RpoN2 might play different regulatory roles in the basal metabolism of X. campestris. However, elucidation of the detailed mechanism requires further investigation.

Based on our results, we propose a schematic model, as shown in Figure 8. In summary, X. campestris RpoN2 modulates biofilm formation and EPS production, and regulates bacterial flagellation via direct binding to the promoters of Xcc fliC and Xcc fliQ. These factors together cause weakening of X. campestris virulence. Moreover, RpoN1 could regulate fatty acid synthesis and DSF production in X. campestris.

4 | EXPERIMENTAL PROCEDURES

4.1 | Materials

The antibiotics were obtained from Sigma-Aldrich. Takara Biotechnology Co. provided the molecular biology reagents. Novagen provided the pET vectors. Ni-agarose columns were obtained from Sigma-Aldrich. Bio-Rad provided Quick Start Bradford dye reagent. All other reagents were of the highest available quality. Genscript (Nanjing, Jiangsu, China) synthesized the oligonucleotide primers.

4.2 | Bacterial strains, plasmids, and growth conditions

The strains and plasmids used in this study are listed in Table S1. E. coli strains were grown in Luria Bertani medium (10 g/L tryptone,
5 g/L yeast extract, 10 g/L NaCl, pH 7.0) at 37 °C. *X. campestris* strains were grown at 28 °C in NYG medium (5 g/L peptone, 3 g/L yeast extract, 20 g/L glycerol, pH 7.0) or nutrient broth agar (NA) (5 g/L peptone, 3 g/L beef extract, 10 g/L sucrose, 1 g/L yeast extract, pH 7.0). For culture medium preparation, tryptone, peptone, beef extract, and yeast extract were purchased from Sangon Biotech. When required, antibiotics were added at the following concentration: 100 µg/ml sodium ampicillin, 30 µg/ml kanamycin sulphate, and 30 µg/ml gentamycin for *E. coli* or 50 µg/ml rifampicin for *X. campestris*.

### 4.3 Protein expression and purification

Protein expression and purification were performed as described previously (Li et al., 2017). To clone the Xcc rpoN1 and Xcc rpoN2 genes, genomic DNA extracted from *X. campestris* was used for PCR amplification with *Pfu* DNA polymerase using the primers listed in Table S2. PCR products were inserted into pET-28b(+) to produce the plasmids pET-rpoN1 and pET-rpoN2. The Xcc rpoN1 and Xcc rpoN2 genes were verified via nucleotide sequencing by Genscript. Xcc rpoN1 and Xcc rpoN2 with vector-encoded His6-tagged N-termini were expressed in *E. coli* BL21 (DE3) and purified with Ni-NTA agarose (Sigma-Aldrich) using a nickel-iron affinity column. Protein purity was monitored by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

### 4.4 Deletion of Xcc rpoN genes and complementation

To disrupt the Xcc rpoN1 and Xcc rpoN2 genes, the pK18mobsacB-borne in-frame deletion suicide plasmids pK18-ΔrpoN1 and pK18-ΔrpoN2 were constructed. The 500-bp DNA fragments flanking the Xcc rpoN1 and Xcc rpoN2 genes were amplified with *Pfu* DNA polymerase using *X. campestris* genomic DNA as a template, and either rpoN1 BamHI and rpoN1 up1 (for Up rpoN1), rpoN1 down1 and rpoN1 HindIII (for Down rpoN1), rpoN2 BamHI and rpoN2 up1 (for Up rpoN2), or rpoN2 down1 and rpoN2 HindIII (for Down rpoN2), as primers (Table S2). Fragments were purified and joined by overlap PCR. The fused fragment was digested with BamHI and HindIII, and inserted into pK18mobscaB (Schafer et al., 1994) to obtain the plasmids pK18-ΔrpoN1 and pK18-ΔrpoN2. The resulting constructs were transferred into *X. campestris* by electroporation and kanamycin was used to select for integration of the nonreplicating plasmid into the recipient chromosome. A single-crossover integrant colony was spread on NYG medium without kanamycin at 28 °C for 36 hr, and after appropriate dilution the culture was spread on NYG plates containing 15% sucrose. Colonies sensitive to kanamycin were screened by PCR using the primers listed in Table S2, and the Xcc rpoN1 and Xcc rpoN2 deletion strains (ΔrpoN1 and ΔrpoN2) were obtained. For complementation of the Xcc rpoN1 and Xcc rpoN2 mutants, the coding regions of Xcc rpoN1 and Xcc rpoN2 were amplified by PCR and cloned into the versatile pBBR1MCS5 plasmid (Kovach et al., 1995). The resulting plasmid was transferred into the *X. campestris* strain by electroporation. The ΔrpoN1/N1 and ΔrpoN2/N2 strains were obtained. The ΔrpoN1N2 double-mutant and complemented strains ΔrpoN1/N2, ΔrpoN2/N1, ΔrpoN1N2/N1, and ΔrpoN1N2/N2 were obtained by the same method.

### 4.5 Pathogenicity assays

Plant inoculation and virulence assays were conducted as previously described (Cai et al., 2017). In brief, 6-week-old plants of the cabbage cultivar *B. oleracea* ‘Jingfeng No. 1’ were used as host plants. The wild-type strain Xc1 and sterile 10 mM MgCl2 were used as positive and negative controls, respectively. All bacterial strains were cultured overnight in NYG medium containing appropriate antibiotics. Cells were collected and washed with 10 mM MgCl2, and the cell densities were adjusted to OD600 = 0.1 before inoculation into plant leaves using sterile scissors. Lesion lengths were measured 10 days after inoculation on 20 leaves for each strain tested.

### 4.6 Quantitative reverse transcription PCR

RT-qPCR was carried out as described in previous studies (Cui et al., 2018). The bacterial cells were collected when the cell optical density (OD600) reached 1.0 in NYG medium. Total RNA was extracted using the TRIzol-based method (Life Technologies). RNA quality control was performed by several steps: (a) RNA degradation degree and potential contamination were monitored on 1% agarose gels; (b) RNA purity (A260/A280, A260/A230) was checked using a NanoPhotometer spectrophotometer (IMPLEN); and (c) RNA integrity was measured using a Bioanalyzer 2,100 (Agilent). The primers used in this assay are listed in Table S2. Quantification of gene expression and melting curve analysis were completed using QuantStudio 6 Flex real-time PCR system (Applied Biosystems), and TransStart Top Green qPCR SuperMix (TransGen Biotech) was used according to the manufacturer’s instructions. As a control, RT-qPCR was similarly applied to analyse 16S rDNA expression. The relative expression levels of target genes were calculated using the 2−ΔΔCt method for comparative quantitation.

### 4.7 RNA-Seq

The RNA-Seq assay was performed as described previously (Yang et al., 2017; Zhou et al., 2017). Briefly, the wild-type Xc1, ΔrpoN1, ΔrpoN2, and ΔrpoN1N2 mutant strains were grown in NYG medium, and their cells were collected when the OD600 reached 1.0 based on the growth curve. The collected cells were used for RNA extraction by the TRIzol-based method (Life Technologies), and RNA degradation and contamination were monitored on 1% agarose gels. Clustering and sequencing were performed by Genedenovo Biotechnology Co.,
Ld (Guangzhou, Guangdong, China). To analyse the DEGs between the wild-type Xc1, ΔrpoN1, ΔrpoN2, and ΔrpoN1N2 mutant strains, the gene expression levels were further normalized using the fragments per kilobase of transcript per million (FPKM) mapped reads method to eliminate the influence of different gene lengths and amount of sequencing data on the calculation of gene expression. The edgeR package (http://www.r-project.org/) was used to determine DEGs across samples with fold changes ≥2 and a false discovery rate-adjusted \( p \) value <.05. DEGs were then subjected to enrichment analysis of gene ontology (GO) functions and KEGG pathways, and \( q \) values were corrected using <.05 as the threshold.

### 4.8 | Electrophoretic mobility gel shift assays

EMSAs were performed as described (Hirakawa et al., 2015; Shao et al., 2018). For RpoN gel shift assays, we used DNA fragments that included the pXCC fliC (408 bp) and pXCC fliQ (497 bp) promoter regions as probes. The probe DNA (50 ng) was mixed with protein in a 20 \( \mu \)l reaction mixture containing 10 mM Tris (pH 7.5), 50 mM KCl, 1 mM dithiotorretol, and 0.4% glycerol. After incubation for 25 min at 28 °C, the samples were electrophoresed on a 5% nondenaturing acrylamide gel in 0.5 × Tris-borate-EDTA (TBE) buffer at 4 °C. The gel was soaked in 10,000-fold-diluted SYBR Green I nucleic acid dye (Sangon Biotech), and the DNA was visualized at 300 nm.

### 4.9 | Measurement of extracellular enzymatic activity and swimming motility

The relative activities of extracellular enzymes were assayed as described previously (Wei et al., 2007; Yu et al., 2016). Two microlitres of each X. campestris strain culture (OD\(_{600} = 1.0\)) was spotted onto NYG agar plates containing 1% (wt/vol) skim milk (for protease), 0.5% (wt/vol) carboxymethylcellulose (for cellulase), or 0.1% (wt/vol) starch (for amylase) and incubated at 28 °C for 24–48 hr. The plates of each strain culture (2 ml, OD\(_{600} ≈ 1.0\)) was spotted onto NYG agar plates containing 1% (wt/vol) skim milk (for protease), 0.5% (wt/vol) carboxymethylcellulose (for cellulase), or 0.1% (wt/vol) starch (for amylase) and incubated at 28 °C for 24–48 hr. The plates were stained where necessary as previously described (Wei et al., 2007). The zones of clearance around the spots due to degradation of the substrate were photographed. Three plates were inoculated in each experiment, and each experiment was repeated three times. The relative enzymatic activity was indicated by the diameter of the clearance zone.

Swimming motility was determined on semisolid agar (0.3%). Bacteria were inoculated into the centres of NYG plates containing 0.3% agarose. The plates were incubated at 28 °C for 48 hr before the colony diameters were measured.

### 4.10 | EPS formation assay

EPS production was measured as described previously (Yu et al., 2016). Each X. campestris strain culture (2 ml, OD\(_{600} = 1.0\)) was used to inoculate 100 ml of NYG medium containing 4% glucose in a 250 ml flask and kept at 28 °C with shaking at 180 rpm for 4 days. The EPS was precipitated from the culture supernatant by the addition of 4 volumes of ethanol. The pelleted EPS was washed with 70% ethanol, air dried, and weighed. Three flasks were inoculated in each experiment, and each experiment was repeated three times.

### 4.11 | Biofilm formation assay

A biofilm formation assay was performed as described previously with some modifications (Wang et al., 2018). Briefly, bacterial cells were cultured in NYG medium to a final OD\(_{600}\) of 1.0. Then, 3 ml of the cell suspension was added to sterilized polystyrene tubes. These tubes were kept in a humidified chamber at 28 °C for 7 days without shaking. The cultures were then moved and the tubes were washed three times in tap water. Biofilm formation on the tubes was visualized by staining with 0.1% CV, followed by washing three times in tap water. The CV-stained biofilm in polystyrene tubes was dissolved in methanol-acetic acid-water (4:1:5, vol/vol/vol) and quantified by measuring the \( A_{575} \) using an 8,453 UV-visible spectrophotometer (Agilent). The average of three replicates was used for quantitative measurement. The biofilm assays were repeated three times and showed similar results with three replicates each time.

### 4.12 | Detection of DSF signal components in the X. campestris culture supernatant

The protocol for extraction and purification of DSF family components was described previously (Zhou et al., 2015a). X. campestris strains were cultured in liquid medium for 36 hr and 50 ml of the bacterial supernatant was collected by centrifugation at 4,000 × g for 15 min at 4 °C. The pH of the supernatants was adjusted to 4.0 by adding hydrochloric acid prior to two extractions with an equal volume of ethyl acetate. The ethyl acetate fractions were collected, and the solvent was removed by rotary evaporation to dryness at 42 °C. The residue was dissolved in 100 µl of methanol. The crude extract was subjected to 0.22-µm Mini-star filtration, and the filtrate was concentrated to 100 µl. The extract (10 µl) was injected into a C18 reversed-phase HPLC column (4.6 × 250 mm, Agilent Technologies, Inc.) and eluted with water in methanol–acetic acid–water (23:77 vol/vol, 0.1% formic acid) at a flow rate of 1 ml/min in an HPLC E2695 system (Waters) with a UV220 detector.

### 4.13 | Analysis of fatty acid composition

Bacterial cultures were grown aerobically for 2–4 days. Cells were harvested and washed three times with sterile water. Fatty acid methyl esters were synthesized and extracted as described
4.14 Statistical analyses

The experimental datasets were subjected to analyses of variance using GraphPad Prism 7.0. The significance of the treatment effects was determined by the F value (p = .05). If a significant F value was obtained, separation of means was accomplished by Fisher’s protected least significant difference at p ≤ .05.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Guichun Wu https://orcid.org/0000-0002-9520-7615
Fengquan Liu https://orcid.org/0000-0001-9325-1500

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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