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Flagellar Biogenesis of *Xanthomonas campestris* Requires the Alternative Sigma Factors RpoN2 and FliA and Is Temporally Regulated by FlhA, FlhB, and FlgM▽†

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In prokaryotes, flagellar biogenesis is a complicated process involving over 40 genes. The phytopathogen *Xanthomonas campestris* pv. campestris possesses a single polar flagellum, which is essential for the swimming motility. A σ^{54} activator, FleQ, has been shown to be required for the transcriptional activation of the flagellar type III secretion system (F-T3SS), rod, and hook proteins. One of the two *rpoN* genes, *rpoN2*, encoding σ^{54}, is essential for flagellation. RpoN2 and FleQ direct the expression of a second alternative sigma FliA (σ^{28}) that is essential for the expression of the flagellin FliC. FlgM interacts with FliA and represses the FliA regulons. An *flgM* mutant overexpressing FliC generates a deformed flagellum and displays an abnormal motility. Mutation in the two structural genes of F-T3SS, *flhA* and *flhB*, suppresses the production of FliC. Furthermore, FliA protein levels are decreased in an *flhB* mutant. A mutant defective in *flhA*, but not *flhB*, exhibits a decreased infection rate. In conclusion, the flagellar biogenesis of *Xanthomonas campestris* requires alternative sigma factors RpoN2 and FliA and is temporally regulated by FlhA, FlhB, and FlgM.

The flagellar type III secretion system (F-T3SS) is essential for the export of extracytoplasmic components of the flagella (3, 10, 41, 43). In enteric bacteria, the assembly of F-T3SS is initiated by the formation of a membrane-embedded MS ring in which 26 monomers of FliF proteins form a pore at the appropriate position of the inner membrane (69). FliF interacting with the switch protein FliG (23, 40) and FlhAB (32, 44) is required for flagellar rotation and assembly. FlhA, together with FlhB and FliOPQR, residing in the MS ring is essential for flagellar export (44). FlhB controls the choice between secretion of flagellar rod-hook protein and flagellar filament protein (17, 18, 49, 51). FlhA and FlhB form a docking platform for soluble proteins FliI and FliH (50, 71). FliI is an ATPase (28). The energy of ATP hydrolysis by FliI initiates flagellar protein transport (48, 58).

Expression of flagellar genes is generally regulated by several regulators in a hierarchical manner (3, 8, 10, 43). In *Enterobacteriaceae*, which possess multiple peritrichous flagella, the flagellar genes can be classified into three classes: the class I proteins FlhDC expressed from a σ^{70}-directed promoter are the master regulators that regulate the transcription of the class II genes comprising *fliA* (σ^{28}) and the genes encoding F-T3SS, rod, and hook proteins (2, 39). Expression of the class III genes, including those for flagellin proteins, requires an active FliA. Several feedback pathways have been reported to coordinate the gene expression and flagellin assembly. An anti-sigma factor, FlgM, expressed from a class II promoter binds to FliA and inhibits its activity. This inhibition terminates with complete formation of the hook-basal body complex through which FlgM is expelled from the cytoplasm (35). The duration of expression of class III genes is limited due to increased proteolysis of FliA in the absence of FlgM (5). Chaperones, FliT and FlgN, also play roles in shifting from class II to class III promoters. FliT binds directly to FlhC and represses the expression of the class II genes (76). FlgN upregulates *flgM* and represses class III genes (1).

A second type of regulation is represented by a bacterial group, including vibrios and pseudomonads, that possesses single, polar flagella. Two alternative sigma factors (RpoN and FliA) are implicated in the regulation of the transcriptional hierarchy of flagellar genes. In coordination with RpoN, two NtrC-type activators, FlrA/FleQ and FlrBC/FleSR, regulate the transcription of the class II and III promoters (14, 60). The expression of class IV promoters is dependent on FliA under the negative regulation of FlgM (14).

A third type of regulation is found in *Caulobacter crescentus*, which also possesses a single polar flagellum. The production of flagellin (class III) is directly dependent on RpoN and the activator FlbD (a class II protein) (54). The expression of *flbD* is dependent on σ^{73} and the activator CtrA (class I). Regulator FliX binds to FlbD and inhibits its activity (16, 53, 54). After the assembling of an early structure of the basal body, FliX becomes an activator of FlbD, which initiates the transcription of late flagellar genes (52). Similar to the case for FlgM in the enteric bacteria, vibrios, and pseudomonads, FliX regulates a critical checkpoint coupling flagellar gene regulation to assembly.

Xanthomonas campestris pv. campestris, a member of the *Pseudomonadaceae* bearing a single polar flagellum, is a plant-pathogenic bacterium causing black rot in cruciferous plants (74). By examining the genome sequence of *X. campestris* pv.

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† Supplemental material for this article may be found at http://jb.asm.org/.

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TABLE 1. Bacteria and plasmids used in this work

Bacterial strain or plasmid	Description	Reference or source
Xanthomonas campestris pv. campestris strains		
Xc17	Local purified virulent strain	78
XcflhA	*flhA* mutant derived from Xc17, *flhA*::Gm Ap^r Gm^r	This work
XcflhA(c)	XcflhA harboring plasmid pBBADflhA, Ap^r Gm^r Tc^r	This work
XcflhB	*flhB* mutant derived from Xc17, *flhBΔ612-564*::Gm, Ap^r Gm^r	This work
XcflhB(c)	XcflhB harboring plasmid pBBADflhB, Ap^r Gm^r Tc^r	This work
XcfliA	*fliA* mutant derived from Xc17, *fliA*::pOK12 Ap^r Km^r	This work
XcrpoN2	*rpoN2* mutant derived from Xc17, *rpoN2Δ91-1081*::Gm Ap^r Gm^r	This work
XcfleQ	*fleQ* mutant derived from Xc17, *fleQ*::pOK12 Ap^r Km^r	27
XcflgM	*flgM* mutant derived from Xc17, *flgM*::Gm Ap^r Gm^r	This work
XcflgM(c)	XcflgM harboring plasmid pRKflgM, Ap^r Gm^r Tc^r	This work
XcfliC	*fliC* mutant derived from Xc17, *fliCΔ489-948*::Gm Ap^r Gm^r	This work
Escherichia coli strains		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	25
BL21(DE3)	F^- *ompT hsdSB*(r_B^- m_B^-) *gal dcm met*(DE3)	Novagen
Saccharomyces cerevisiae strain Y187	MATα *ura3-52 his3-200 ade2-101 trp1-901 leu2-3,112 gal4*Δ *gal80*Δ *URA3*::*GAL1UAS-GAL1TATA-lacZ*	Clontech
Plasmids		
pOK12	*E. coli* cloning vector, Km^r	72
pBB	*E. coli* cloning vector derived from pOK12, Km^r	This work
Pucgm	Gentamicin resistance gene cassettes donor, Ap^r Gm^r	66
pFY13-9	Promoter-probing vector derived from pRK415 using *lacZ* as the reporter, Tc^r	36
pBBAD22K	Broad-host-range expression vector, Km^r	68
pRK415	Broad-host-range expression vector, Tc^r	31
pET30b	*E. coli* expression vector, Km^r	Novagen
PFYfliC	Transcriptional fusion derivative of pFY13-9 carrying *fliC* promoter (319 bp), Tc^r	This work
PFYflhF	Transcriptional fusion derivative of pFY13-9 carrying *flhF* promoter (1391 bp), Tc^r	27
pBBADflhA	pBBAD22K derivative with total CDS of *flhA* cloned downstream of an *araBAD* promoter	This work
pBBADflhB	pBBAD22K derivative with total CDS of *flhB* cloned downstream of an *araBAD* promoter	This work
PRKflgM	pRK415 derivative with entire CDS of *flgM* and its promoter region	This work
PETfliC	pET30b derivative containing entire *fliC* CDS fused with His tag at both ends	This work
PETfliA	pET30b derivative containing entire *fliA* CDS fused with His tag at both ends	This work
PETrpoN2	pET30b derivative containing entire *rpoN2* CDS fused with His tag at both ends	This work
pACT2	GAL4 activation domain	Clontech
pACT-flgM	pACT2 derivative containing entire *flgM* CDS fused with GAL4 activation domain	This work
pAS2-1	GAL4 DNA-binding domain	Clontech
pAS2-fliA	pAS2-1 derivative containing entire *fliA* CDS fused with GAL4 DNA-binding domain	This work

campestris ATCC 33913 (GenBank accession no. NC_003902), more than 40 genes have been predicted to be involved in flagellar biogenesis and motility. It is known that expression of the flagellin gene (*fliC*) is upregulated by Clp, a homolog of the cyclic AMP receptor protein (36). Our previous studies have demonstrated that *fleQ*, encoding a σ^{54}-cognate activator, is essential for normal flagellation and for the transcription of the promoters upstream of *fliE*, *fliL*, *fliQ*, *flgB*, *flgG*, and *flhF* (27). In this work, we show that the expression of flagellar genes of *X. campestris* pv. campestris is regulated in a three-tier hierarchy. Alternative sigma factors, RpoN2 (σ^{54}) and FliA (σ^{28}), are required for the expression of class II and class III flagellar promoters, respectively. FlgM represses the activity of a FliA-dependent promoter via protein-protein interaction. The effects of mutations in two F-T3SS structural genes, *flhA* and *flhB*, on flagellar genes expression and virulence are also examined.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise specified, Luria-Bertani (LB) broth and LB agar plates (47) were employed as the media for the growth of *X. campestris* pv. campestris and *Escherichia coli* at 28 and 37°C, respectively. *Saccharomyces cerevisiae* Y187 was grown in a yeast-peptone-dextrose (YPD) medium, and the transformed Y187 strains were selected and maintained in synthetic dropout (SD/−) media as described in the Clontech Matchmaker III yeast two-hybrid system manual. Antibiotics added for selection were ampicillin (100 μg/ml), kanamycin (50 μg/ml), gentamicin (50 μg/ml), and tetracycline (10 μg/ml).

Recombinant DNA techniques. The standard methods for DNA manipulation were those described by Sambrook et al. (65). Restriction enzymes, T4 DNA ligase, and *Taq* DNA polymerase were purchased from Takara (Kyoto, Japan). Chemicals and reagents were purchased from Sigma (St. Louis, MO) and E. Merck (Darmstadt, Germany).

Construction of *flhA*, *flhB*, *rpoN2*, *fliC*, *flgM*, and *fliA* mutants. The *flhA*, *flhB*, *rpoN2*, *flgM*, and *fliC* mutants were constructed by insertional mutagenesis. A gentamicin resistance (Gm^r) gene from pUCGM (66) was inserted into the target gene that had been cloned in suicide plasmid pOK12 (72) or pBB. The orientation of the inserted Gm^r gene was the same as that of the target gene as confirmed by sequencing. The recombinant plasmids were then electroporated into a wild-type *X. campestris* pv. campestris strain, Xc17. Allelic exchange between the chromosomal gene and the mutagenized plasmidic copy was achieved by double crossover. The mutant strains were selected by antibiotic sensitivity and checked by PCR. A *fliA* mutant was constructed by inserting the plasmid pOK12 into the *fliA* gene via single crossover. The plasmids and oligonucleotide primers used in this work are listed in Tables 1 and 2. The details of the construction of recombinant suicide plasmids are provided as below.

TABLE 2. Oligonucleotides used in this work

Oligonucleotide	Sequence (5′→3′)[a]	Enzyme site added
flhAw-F	GGTACCagcgcatgagcgcccaacc	KpnI
flhAw-R	AAGCTTccggctcagctaatcgtgcc	HindIII
flhBw-F	GGTACCcaatgtccgagtccgaaga	KpnI
flhBw-R	AAGCTTgggcgctcatgcgctggccc	HindIII
flhB_PstI-F	cgaaggcggcCTGCAGcacc	PstI
flhB_PstI-R	ggaactggCTGCAGaagctg	PstI
flgM-F	tgttgtcggccaatgacc	
flgM-R	Cagtgcccagttcacctc	
fliA-F	AGATCTgacgagatccgtcgg	BglII
fliA-R	AAGCTTagcgacagcaccagc	HindIII
fliAw-F	CATATGatgagtaccgccacc	NdeI
fliAw-R	CTCGAGatcgtcaatttcgac	XhoI
fliCw-F	CATATGgcacaggtaatcaaca	NdeI
fliCw-R	GCGGCCGCctgcagcaggctcagcac	NotI
fliCp-F	CTCGAGttgcgcgcaaaaacccgt	XhoI
fliCp-R	TCTAGAttttgatatctcctctaa	XbaI
flgMw-F	AGATCTGatgagccagaaaatcgaaggc	BglII
flgMw-R	GGATCCttgcccgccagttgctggttc	BamHI
rpoN2w-F	CATATGaagacgaccatctctg	NdeI
rpoN2w-R	CTCGAG tcccgcccgggcaagcag	XhoI
fliA_YB-F	CATATGaagatcaaacgctttgttgccc	NdeI
fliA_YB-R	GGATCCtcttcaaggcgaagaacgagac	BamHI
flgM_YA-F	CATATGagtaccgccaccgcaacgaccg	NdeI
flgM_YA-R	GGATCCatcgtcaatttcgacaccggca	BamHI

[a] Uppercase letters indicate the restriction enzyme site.

(i) *flhA*. A 2,105-bp DNA fragment containing the entire coding sequence (CDS) of *flhA* of Xc17 was amplified by PCR with primer pair flhAw-F/-R and cloned into pBB to obtain pBBflhAw. A Gm^r gene was inserted into the BamHI site of pBBflhAw to form pBBflhA::Gm.

(ii) *flhB*. A 1,140-bp DNA fragment containing the entire CDS of *flhB* of Xc17 was amplified by PCR with primer pair flhBw-F/-R and cloned into pBB to obtain pBBflhBw. Two PstI sites were introduced into the *flhB* gene in pBBflhBw by inverse PCR with flhB_PstI-F/-R. After digestion with PstI, the PCR fragment was self-ligated to form pBBflhB-PstI. A Gm^r gene was inserted into the created PstI site to form pBBflhB::Gm.

(iii) *rpoN2*. A 2,661-bp PstI-XhoI fragment containing the entire CDS of *rpoN2* of Xc17 was obtained by chromosomal walking and cloned in pOK12. A Gm^r was inserted into the cloned *rpoN2* gene to replace an internal 996 bp of a SphI fragment. The derived plasmid was named pOKrpoN2::Gm.

(iv) *flgM*. An 847-bp DNA fragment containing the entire CDS of *flgM* of Xc17 was amplified by PCR with primer pair flgM-F/-R and cloned into pBB to obtain pBBflgM. A Gm^r gene was inserted into the sole PstI site of *flgM* to form pBBflgM::Gm.

(v) *fliC*. A 1,194-bp DNA fragment containing the entire CDS of *fliC* of Xc17 was amplified by PCR with primer pair fliCw-F/-R and cloned into pBB to obtain pBBfliCw. A Gm^r gene was inserted into pBBfliCw to replace an internal 459 bp of a SalI fragment of *fliC* to form pBBfliC::Gm.

(vi) *fliA*. A 347-bp DNA fragment containing the internal region of *fliA* of Xc17 was amplified with primer pair fliA-F/-R and cloned into pOK12 to form pOKfliA.

Construction of *flhA*, *flhB*, and *flgM* complementary plasmids. The entire CDSs of *flhA* and *flhB* were cut from pBBflhAw and pBBflhBw with KpnI and HindIII and then subcloned into the broad-host-range expression vector pBBAD22K (68) to obtain complementary plasmids pBBADflhA and pBBADflhB, respectively. Expression of the cloned genes was under the control of an arabinose-inducible promoter. An EcoRI/HindIII fragment of 548 bp containing the entire *flgM* gene and the upstream promoter region was cut from pBBflgM and subcloned into a broad-host-range vector pRK415 (31) to form pRKflgM.

Antibody preparation and Western blotting. For antibody preparation, His-tagged antigen proteins were overexpressed from pET30b (Novagen, Madison, WI)-derived plasmids in *E. coli* strain BL21(DE3) after 4 h of induction with 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The proteins were purified on an Ni-nitrilotriacetic acid resin column (Pharmacia), and ca. 1 mg of each protein was used for four injections to immunize a rabbit or five mice at intervals of 1 week. Overexpression plasmids were cloned as described below.

(i) *fliA*. The entire CDS (765 bp) of *fliA* was amplified by PCR with primers fliAw-F/-R and cloned into pBB to obtain pBBfliAw. The CDS region was then subcloned into the NdeI/XhoI sites of pET30b to generate pETfliA.

(ii) *fliC*. The entire CDS of *fliC* in pBBfliCw was subcloned into the NdeI/NotI sites of pET30b to generate pETfliC.

(iii) *rpoN2*. The entire CDS (1,401 bp) of *rpoN2* was amplified by PCR with primers rpoN2w-F/-R and cloned into pBB to obtain pBBrpoN2w. The CDS region was subcloned into the NdeI/XhoI sites of pET30b to generate pETrpoN2.

(iv) Western blotting. Unless otherwise specified, 10 μg of crude extract prepared from test strains was separated on a sodium dodecyl sulfate (SDS)-12% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Perkin-Elmer Inc.). After hybridization with the specific polyclonal antisera and alkaline phosphatase-conjugated goat anti-rabbit antibodies (or alkaline phosphatase-conjugated anti-mouse antibodies), membranes were developed with appropriate substrates (Zymed Laboratories Inc.).

Determination of promoter activity. The upstream promoter-containing regions of the *fliC* gene were PCR amplified with primers fliCp-F/R and cloned into pFY13-9, a promoter-probing vector using promoterless *lacZ* as the reporter (36), to form transcriptional fusion constructs. Promoter activity was monitored by measuring the β-galactosidase activities of the flagellar promoter-*lacZ* fusion constructs in the different strains as previously described (27).

Motility assay. Motility assays were carried out by inoculating 3 μl (ca. 10^5 CFU) of mid-log-phase cultures onto the surfaces of a freshly prepared semisolid XOL medium (19) containing 0.3% agar. The subsurface distribution of bacteria, an indication of movement, was observed at 48 h postinoculation.

TEM. Bacteria were grown in LB medium without agitation to avoid breakage of flagella. Cells were harvested by centrifugation at 4,000 × *g* for 5 min at 4°C. The cells were washed twice and resuspended in cold deionized water, which was then deposited onto grids coated with Formva (Standard Technology). The grids with the cells were floated on a drop of 2% uranyl acetate for 30 s for staining. After air drying for 15 min, transmission electron microscopy (TEM) characterization was performed in a JEOL JEM-1200CXII electron microscope.

Pathogenicity tests. Pathogenicity tests were carried out by inoculating the *X. campestris* pv. campestris cells from overnight cultures onto leaves, which had been cut with a pair of scissors, of 6-week-old potted cabbage plants (77). Statistical results for 15 rounds of independent inoculation experiments are presented.

Yeast two-hybrid assay. Protein-protein interaction assay was performed by using the Matchmaker III yeast two-hybrid system (Clontech) with a 312-bp fragment of *flgM* CDS (amplified with primers flgM_YA-F/-R) cloned into the NdeI/BamHI sites of pACT2 to form pACTflgM and a 768-bp fragment of *fliA* CDS (amplified with primers fliA_YB-F/-R) cloned into the NdeI/BamHI sites of pAS2-1 to form pAS2-fliA. The recombinant and parental plasmids were transformed into yeast strain Y187. Cotransformants were selected on a synthetic complete dropout medium (SD/−Trp/−Leu). Protein interaction was detected by β-galactosidase activity using the colony lift filter approach as described in the Clontech manual.

EPS assay. Cells of *X. campestris* pv. campestris were grown in LB or XOLN medium (20) (40 ml in 250-ml flasks) for 72 h. The cells were removed by centrifugation (12,000 × *g* for 15 min at 4°C), and the exopolysaccharide (EPS) in the culture supernatants was precipitated in the presence of 0.5 M NaCl and 70% ethanol. The amounts of EPS were determined by the anthrone method as described previously (38).

Plate assays for extracellular hydrolytic enzymes. The ability of the *X. campestris* pv. campestris strains to secrete extracellular enzymes was tested on LB plates containing skim milk (1%), starch (0.2%), polygalacturonic acid (PGA) (1%), or carboxymethylcellulose (0.5%). The cells (ca. 1× 10^7 CFU in 10 μl) were deposited on the surface of the plates. After 48 h of incubation, the plates were examined either by direct visualization or by being treated with appropriate reagents. Protease activity was judged by the appearance of clear zones surrounding the colonies on milk plates. In amylase assays, starch plates were stained with iodine (5% in ethanol). PGA plates were developed with 1% hexadecyl-trimethyl-ammonium bromide. The region exhausted of PGA by secreted pectinase displayed a clear zone. Carboxymethylcellulose plates were stained with 0.1% Congo red for 5 min, rinsed once with water, and washed twice with 20 ml of 1.0 M NaCl. Cellulase-positive colonies manifested pale-yellow clear zones against a red background.

Bioinformatic analysis. The FliA-dependent promoters in *X. campestris* pv. campestris were predicted using the tools in RSAT (http://rsat.scmbb.ulb.ac.be/rsat/). A weight matrix was generated based on 25 published FliA-dependent promoter sequences (see Table S1 in the supplemental material). A genome-scale Patser search on *X. campestris* pv. campestris strain ATCC 33913 was

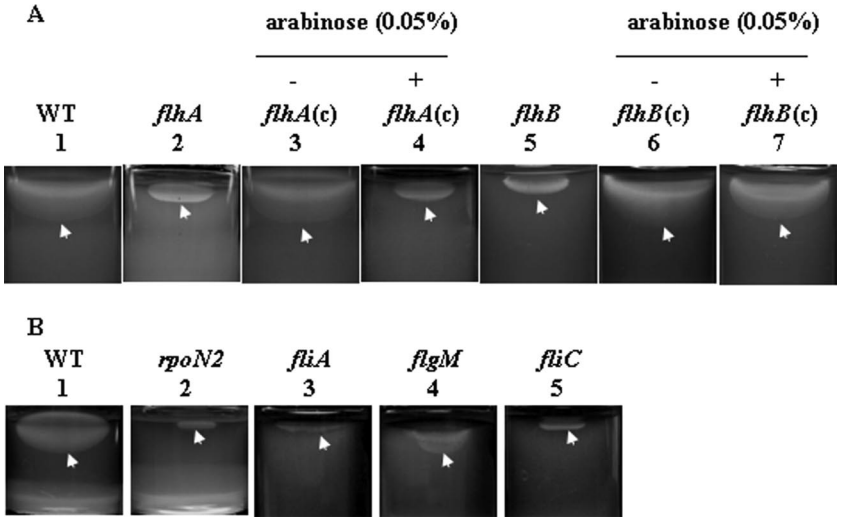

FIG. 1. Swimming motility assay of Xc17 and Xc17-derived mutant strains. WT, wild-type strain Xc17; *flhA*(c), *flhA* mutant complemented with pBBADflhA; *flhB*(c), *flhB* mutant complemented with pBBADflhB. For each tube, 3 μl (ca. 10^5 CFU) mid-log phase culture was inoculated onto the surface of freshly prepared semisolid (0.3% agar) XOL medium and observed after 48 h of incubation.

performed using the FliA matrix (see Table S2 in the supplemental material), and the 21 best-hit sequences were selected (see Table S3 in the supplemental material).

RESULTS

The *X. campestris* pv. campestris *flhA* and *flhB* genes are essential for motility and flagellar biogenesis. Putative F-T3SS genes *flhA* (XCC1909) and *flhB* (XCC1910) were identified in the genome of *X. campestris* pv. campestris ATCC 33913. Modeling of data using the program TMHMM 2.0 (33) suggested that the N-terminal parts of FlhA and FlhB contained seven and four transmembrane helices, respectively. To study the function of *flhA* and *flhB* in flagellum formation, *flhA* (XcflhA) and *flhB* (XcflhB) mutants of Xc17, a virulent *X. campestris* pv. campestris strain exhibiting high sequence homology with *X. campestris* pv. campestris ATCC 33913, were constructed by insertional mutagenesis. Motility assays were carried out in 0.3% agar medium. The results revealed that both XcflhA and XcflhB were nonmotile (Fig. 1A). Electron micrographs demonstrated that XcflhA and XcflhB lost the typical single, polar flagellum (Fig. 2).

To confirm the effect of mutation, plasmids pBBADflhA and pBBADflhB were used to complement XcflhA and XcflhB, respectively. The motility assay demonstrated that the complementary strain XcflhB(c) regained motility in the presence or absence of 0.05% arabinose (Fig. 1A). However, the motility of XcflhA(c) was restored only in a soft-agar medium without arabinose (Fig. 1A). This suggested that the addition of arabinose might induce overexpression of FlhA that impaired the motility. Based on this observation, all tested strains in the following experiments were cultured in media without arabinose. TEM observation showed that mutant strains complemented in *trans* had a normal flagellar morphology (Fig. 2).

Production of FliC protein is suppressed in *flhA* and *flhB* mutants. To elucidate the effects of *flhA* and *flhB* on the production of flagellin, the flagellin FliC was detected by Western blotting using polyclonal anti-FliC antibodies. A *fliC* (XCC1941) mutant, XcfliC, was used as a negative control. As shown in Fig. 1B, XcfliC was nonmotile, and it did not have a flagellum (data not shown). The Western blot results showed a band of approximately 40.4 kDa, which corresponded to the FliC protein, in the wild-type strain Xc17 but not in the *flhA*

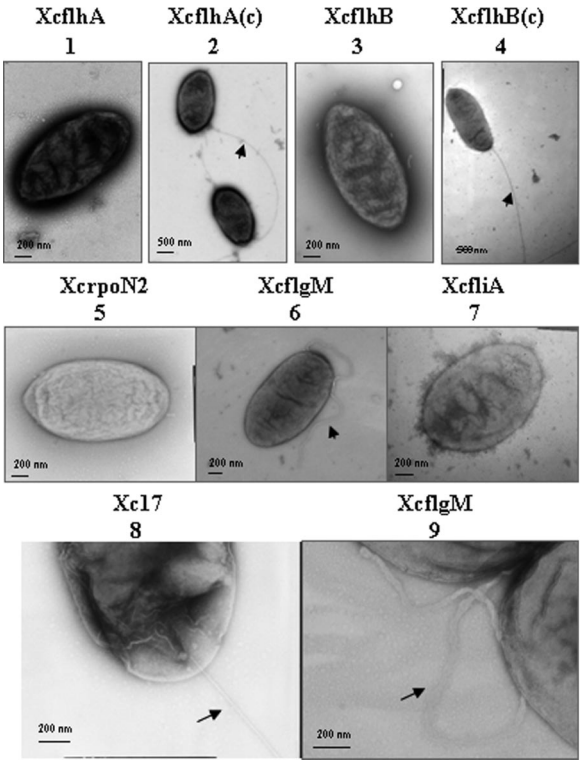

FIG. 2. TEM of Xc17 and Xc17-derived mutant strains. 1, *flhA* mutant; 2, *flhA* mutant complemented with pBBADflhA; 3, *flhB* mutant; 4, *flhB* mutant complemented with pBBADflhB; 5, *rpoN2* mutant; 7, *fliA* mutant; 6 and 9, *flgM* mutant; and 8, Xc17. The cells of Xc17 and complementary strains of *flhA* and *flhB* mutants exhibit a long, straight polar flagellum. Mutations in *flhA*, *flhB*, *rpoN2*, and *fliA* abolish the flagellation. An *flgM* mutant has a truncated flagellum with an abnormal structure (9).

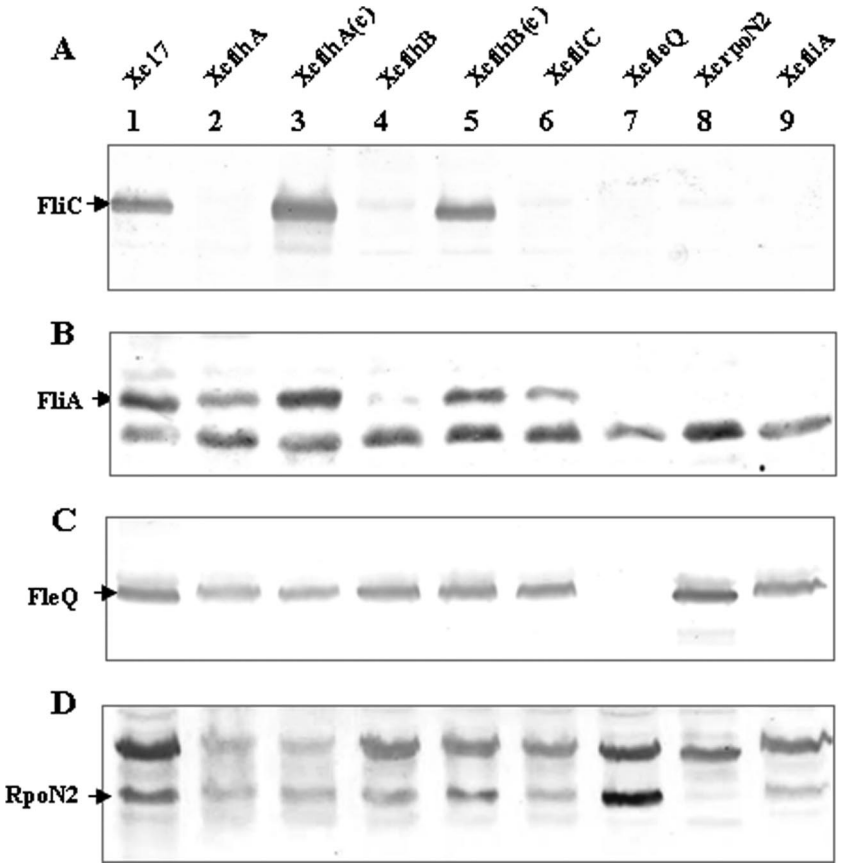

FIG. 3. Western blotting, using polyclonal antibodies raised against the flagellin (FliC) (A), σ^{28} (FliA) (B), activator (FleQ) (C), and σ^{54} (RpoN2) (D), of whole-cell extracts from *X. campestris* pv. campestris wild-type Xc17 and Xc17-derived mutant strains. Lane 1, Xc17; lane 2, *flhA* mutant; lane 3, *flhA* mutant complemented with pBBADflhA; lane 4, *flhB* mutant; lane 5, *flhB* mutant complemented with pBBADflhB; lane 6, *fliC* mutant; lane 7, *fleQ* mutant; lane 8, *rpoN2* mutant; lane 9, *fliA* mutant. The positions of FliC, FliA, FleQ, and RpoN2 are indicated.

and *flhB* mutants (Fig. 3A). Production of FliC protein was restored in XcflhA(c) and XcflhB(c) (Fig. 3A). These results demonstrated that *flhA* and *flhB* were required for the synthesis of flagellin FliC.

The *rpoN2*, *fliA*, and *flgM* genes are essential for motility and normal flagellar biogenesis. Two *rpoN* homologs encoding σ^{54} were identified in the genome of *X. campestris* pv. campestris ATCC 33913. To distinguish these two genes, *rpoN* (XCC2802), located in a conserved region in most bacteria, was named *rpoN1* and the other was named *rpoN2* (XCC1935). Genes encoding the σ^{28} factor (*fliA*, XCC1906) and the anti-σ^{28} factor (*flgM*, XCC1955), as well as *rpoN2*, were located in a large flagellar gene cluster (27).

To elucidate the function of these genes in flagellation, *rpoN2*, *fliA*, and *flgM* mutants were constructed by insertional mutagenesis. Motility assay revealed that *rpoN2* and *fliA* mutants were nonmotile on soft-agar medium (Fig. 1B). Electron micrographs revealed that *rpoN2* and *fliA* mutants did not have any flagellum present (Fig. 2). A mutation of *flgM* severely reduced motility (Fig. 1B). TEM observation demonstrated that XcflgM had a short, immature flagellum (Fig. 2). Upon further investigation, we noticed that a normal flagellum had a smooth, long whip-like structure, while in XcflgM, the flagellum was short with an abnormal structural appearance (Fig. 2). These observations suggested that the two sigma factors, σ^{54} and σ^{28}, were necessary for flagellar biogenesis and motility. The anti-sigma factor FlgM was essential for normal flagellar structure formation and full motility.

Synthesis of FliC is regulated by a cascade of three sigma factors: RpoD, RpoN2, and FliA. To elucidate the hierarchy of regulation between RpoN2/FleQ and FliA, Western blotting was carried out using antibodies raised against FliA, RpoN2, FleQ, and FliC. FliC protein was absent in *fliA*, *fleQ*, and *rpoN2* mutants (Fig. 3A). Consequently, FliA, FleQ, and RpoN2 were necessary for the production of FliC. A 28-kDa FliA protein could be detected in the total cell lysates of Xc17 and the *fliC* mutant XcfliC but was undetectable in the *fliA*, *fleQ*, and *rpoN2* mutants (Fig. 3B). Therefore, RpoN2 and FleQ were required for the production of FliA. A 51.2-kDa RpoN2 protein and a 54.4 kDa FleQ protein were detected in the cell lysates of Xc17, XcfliC, and XcfliA, while they were absent in the respective mutants (Fig. 3C and D). This suggested that the regulation of the synthesis of both FleQ and RpoN2 was independent of FleQ, RpoN2, FliA, and FliC.

Results from a series of *lacZ* transcriptional fusion experiments indicated that the upstream regions of *fliA* and *fleN* did not have promoter activity (data not shown). Hence, the expression of *fliA*, *fleN*, and *flhF* was dependent on the promoter

TABLE 3. β-Galactosidase activities of the *fliC* promoter-*lacZ* and *flhF* promoter-*lacZ* fusions in Xc17 and flagellar mutants

Promoter	Time (h)	Mean β-galactosidase activity (Miller units) ± SD in strain[a]				
		Xc17	*rpoN2* mutant	*fleQ* mutant	*fliA* mutant	*flgM* mutant
fliC	1	70.4 ± 2.6	19.3 ± 4.7 (28)	18.8 ± 1.1 (27)	34.1 ± 5.6 (48)	168.7 ± 32.8 (240)
	4	50.1 ± 6.9	13.8 ± 2.8 (28)	14.9 ± 4.6 (30)	29.9 ± 6.4 (60)	111.8 ± 8.7 (223)
	7	30.6 ± 2.8	10.0 ± 1.4 (33)	8.6 ± 1.7 (28)	19.6 ± 3.8 (64)	81.4 ± 6.2 (266)
fliF	1	68.4 ± 7.0	9.5 ± 2.9 (14)	18.0 ± 0.4 (26)[b]	102.0 ± 14.2 (149)	32.4 ± 1.1 (47)
	4	49.9 ± 5.4	2.9 ± 4.4 (6)	12.2 ± 5.6 (24)[b]	69.8 ± 11.2 (140)	24.5 ± 6.0 (49)
	7	30.3 ± 2.9	3.4 ± 5.8 (11)	8.3 ± 2.5 (27)[b]	45.6 ± 3.9 (150)	22.8 ± 3.7 (75)

[a] Values in parentheses are percent activity in the mutant strains relative to that in Xc17.
[b] Data are from reference 28.

region upstream of *flhF*. The transcription levels of *fliC* and *fliA* were monitored by β-galactosidase activity of promoter-*lacZ* fusion constructs pFYfliC and pFYflhF (27) in Xc17 and flagellar mutants at 3-h intervals from an initial optical density at 550 nm of 0.35 (Table 3). The data demonstrated that the expression of *fliC* in *rpoN2*, *fleQ*, and *fliA* mutants was reduced to 28 to 33, 27 to 30, and 48 to 64%, respectively, of that in Xc17. The promoter activity of the upstream region of *flhF* decreased to 6 to 14 and 24 to 27% in *rpoN2* and *fleQ* mutants, respectively, but increased to 140 to 150% in a *fliA* mutant (Table 3). These results suggested that RpoN2, FleQ, and FliA were required for the transcription of *fliC*. Moreover, the transcription of the *flhF* promoter was dependent on RpoN2/FleQ and was moderately autoregulated by FliA.

In conclusion, the synthesis of FliC is under a hierarchical regulation of the two alternative sigma factors RpoN2/FleQ and FliA at the transcriptional level. An RpoN-dependent promoter was identified upstream of *flhF-fleN-fliA* (27), and a FliA-dependent promoter was identified upstream of *fliC* (see Table S3 in the supplemental material). According to the promoter type, flagellar genes could be classified into three classes: the first class consisted of RpoD-dependent genes, the second class included the RpoN2/FleQ regulons, and genes in the FliA regulons belonged to the third class.

The Western blot data also showed that expression of RpoN2 and FleQ was not significantly affected by the mutation of *flhA* or *flhB* (Fig. 3C and D). FliA production was slightly decreased in the *flhA* mutant and significantly reduced in the *flhB* mutant (Fig. 3B). The defect in expression was rescued after complementation in *trans* by plasmid-borne *flhA* and *flhB* genes, respectively (Fig. 3B). This indicated that *flhB* was involved not only in the regulation of class III genes but also in that of class II genes.

The anti-sigma factor FlgM negatively regulates the production of FliC via an interaction with FliA. To detect the expression of FliC in XcflgM, different amounts of cell lysate of XcflgM were prepared, separated on an SDS-polyacrylamide gel (Fig. 4B), and then immunoblotted with anti-FliC antiserum (Fig. 4A). The blotting results showed that the FliC protein was overproduced about fivefold in an *flgM* mutant in comparison with Xc17 (Fig. 4A). The promoter activity of *fliC* was enhanced to 223 to 266% in XcflgM compared to the parental strain (Table 3), indicating the expression of *fliC* was negatively regulated by FlgM at the transcriptional level. The quantity of FliA did not change significantly in XcflgM (Fig. 4C), even though the transcription level of the *flhF* promoter underwent a 25% decrease at the late log phase, compared to Xc17 (Table 3).

To detect the interaction between FliA and FlgM, the entire coding regions of *fliA* and *flgM* were cloned in an in-frame translational fusion with the DNA-binding domain and activating domain of the yeast activator GAL4 in plasmids pAS2-1 and pACT2, respectively, followed by transforming into yeast strain Y187. Cotransformants were selected on a synthetic complete dropout medium without leucine and tryptophan. Yeast clones harboring only one of the recombinant plasmids (pAS2-fliA or pACT-flgM) or the parental plasmids, pAS2-1

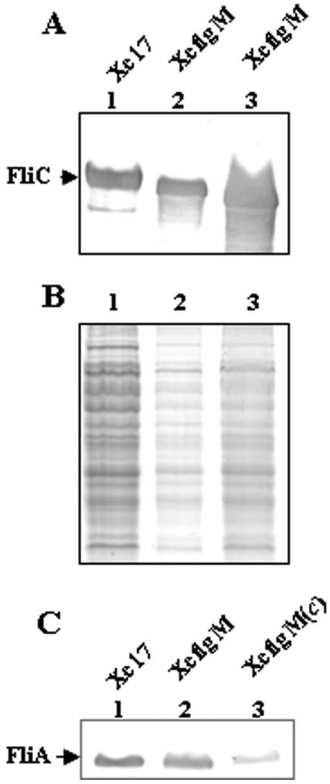

FIG. 4. (B) Different amounts of whole-cell extracts were separated on an SDS-polyacrylamide gel. Lane 1, 10 μg Xc17; lane 2, 2 μg *flgM* mutant; lane 3, 8 μg *flgM* mutant. (A) This was followed by Western blotting with polyclonal antibodies raised against the flagellin (FliC). (C) Western blotting, using polyclonal antibodies against σ^{28} (FliA), of the cell extract. Lane 1, Xc17; lane 2, *flgM* mutant; lane 3, *flgM* mutant complemented with pRKflgM.

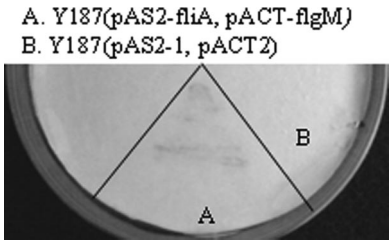

FIG. 5. Colony filter lift assay to identify the specificity of interaction between FliA and FlgM. *S. cerevisiae* Y187 carrying recombinant plasmids pAS2-fliA and pACT-flgM (sector A) and parental plasmids pAS2-1 and pACT2 (sector B) were grown on yeast synthetic dropout medium (−Trp/−Leu) and transferred to a Whatman no. 5 paper filter. Cells were permeabilized by freeze-thaw treatment of the filters. The filters carrying the cells were then placed over filters presoaked with Z-buffer–X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution. A blue color revealed within 30 min was considered positive. Assays were repeated three times with at least four independent transformants.

and pACT2, were also used as controls. Protein interaction was evaluated by β-galactosidase activity using the colony lift filter approach. As shown in sector A of Fig. 5, the yeast clone cotransformed by the two recombinant plasmids had β-galactosidase activity (blue color), while the clones harboring pAS2-fliA or pACT-flgM (data not shown) or the two parental plasmids (white color; Fig. 5, sector B) displayed insignificant activity. The results suggested possible cross talk between FliA and FlgM.

The FlhA mutant has an attenuated virulence. FlhA and FlhB are F-T3SS proteins. To determine the effects of FlhAB on pathogenicity, a virulence assay was carried out in cabbage leaves by wound inoculation with Xc17 and *flhA* and *flhB* mutants. The results showed that XcflhB and the complementary strain XcflhB(c) exhibited the same virulence as the parental strain (15 successful infections/15 inoculations). Conversely, XcflhA produced a low infection rate, i.e., 33%, and the rate increased to 80% in the complementation strain XcflhA(c). The production of important virulence factors, i.e., extracellular enzymes and EPSs, was assayed to determine the cause of the low infection rate. The diameters of digested zones for amylases, cellulases, proteases, and pectinases were about 2.15 to 2.21, 1.67 to 1.84, 1.62 to 1.80, and 0.75 to 0.85 cm, respectively, for Xc17, XcflhA, XcflhB, and their complementary strains (Table 4). The EPS productivity was about 195 to 255 and 2,480 to 3,110 μg/ml for all tested strains grown in LB and XOLN media (Table 4). In conclusion, there was no significant difference in the activities of extracellular enzymes and the productivities of EPS for all tested strains.

DISCUSSION

The regulation of flagellar biogenesis in *X. campestris* pv. campestris is a simplified form of that in vibrios/pseudomonads. The biogenesis of the bacterial flagellum is a very complex process involving the temporal and spatial coordination of gene expression and protein assembly. As shown in this work, the expression of the three classes of flagellar promoters of *X. campestris* pv. campestris is dependent on σ^{70}, σ^{54}, and σ^{28}, respectively, as commonly seen in vibrios and pseudomonads (14, 60). Nevertheless, two-tier RpoN-cognate activators, FlrA/FleQ and FlrBC/FleSR, are involved in flagellar gene expression in vibrios/pseudomonads (14, 60), whereas in *X. campestris* pv. campestris, FleQ appears to be the sole RpoN regulator involved in the flagellation (26). A similar model has been reported for *Helicobacter pylori*, except that only the FleSR-type regulator was identified (29, 57). A proposed model of the flagellar regulation pathway in *X. campestris* pv. campestris is illustrated in Fig. 6.

The early flagellar genes are RpoN2/FleQ dependent. The *rpoN* gene, encoding the σ^{54} factor, is widely present in the microbial genome. RpoN is involved not only in nitrogen assimilation but also in many unrelated functions, such as utilization of carbon sources, nitrogen fixation, motility, alginate biosynthesis, and virulence, in different species (45, 61, 67). Most bacteria have only one *rpoN* gene, yet exceptions have been found in some nitrogen fixation bacteria and plant pathogens. The functions of the *rpoN* homologs in the same genome could be redundant or irrelevant (34, 46, 59). An allelic-exchange mutation in *rpoN2* totally abolishes flagellar biogenesis and motility. This suggests that the two *rpoN* genes in *X. campestris* pv. campestris work independently. Further study is necessary to determine the mechanism of the specificity of the two RpoN proteins. Our previous data have characterized five RpoN/FleQ-dependent promoters encoding three types of proteins (Fig. 6): (i) regulators (FliA, FlhF, and FleN); (ii) secretion system (*fliEFGHIJK*, *fliLMNOP*, and *fliQR*); and (iii) rod and hook (*flgBCDEF* and *flgGHIJKL*). In *Pseudomonas aeruginosa*, FlhF and FleN play a role in the early stage of flagellation by regulating the localization and number of flagella (15, 55). A putative RpoN-dependent GG-N_{10}-GC promoter (aGGaacaccacttGCa) is identified upstream of *flhBA*, indicating that it may also belong to the class II genes (Fig. 6).

TABLE 4. Activities of extracellular enzymes and production of EPS in the *flhA* and *flhB* mutants, their complementary strains, and Xc17

Strain	Mean diameter (cm) of digestive zone ± SD for extracellular enzyme[a]				Mean EPS production (μg/ml) ± SD in medium	
	Amylase	Celluloses	Pectinase	Protease	LB	XOLN
Xc17	2.21 ± 0.59 (100)	1.84 ± 0.27 (100)	0.85 ± 0.01 (100)	1.62 ± 0.08 (100)	252 ± 51	2,914 ± 24
XcflhA	2.17 ± 0.61 (98)	1.76 ± 0.31 (95)	0.77 ± 0.11 (91)	1.80 ± 0.08 (110)	230 ± 7	2,968 ± 34
XcflhA(c)	2.17 ± 0.57 (98)	1.70 ± 0.31 (92)	0.76 ± 0.15 (89)	1.72 ± 0.13 (106)	233 ± 36	3,110 ± 48
XcflhB	2.16 ± 0.62 (98)	1.74 ± 0.33 (94)	0.83 ± 0.13 (97)	1.74 ± 0.08 (107)	255 ± 15	2,480 ± 75
XcflhB(c)	2.15 ± 0.64 (97)	1.74 ± 0.33 (94)	0.84 ± 0.12 (99)	1.64 ± 0.02 (101)	195 ± 8	2,597 ± 51

[a] Diameters of clearing zones were measured in plates containing the appropriate substrate. Values in parentheses are percent diameter for the mutant strains relative to that for Xc17.

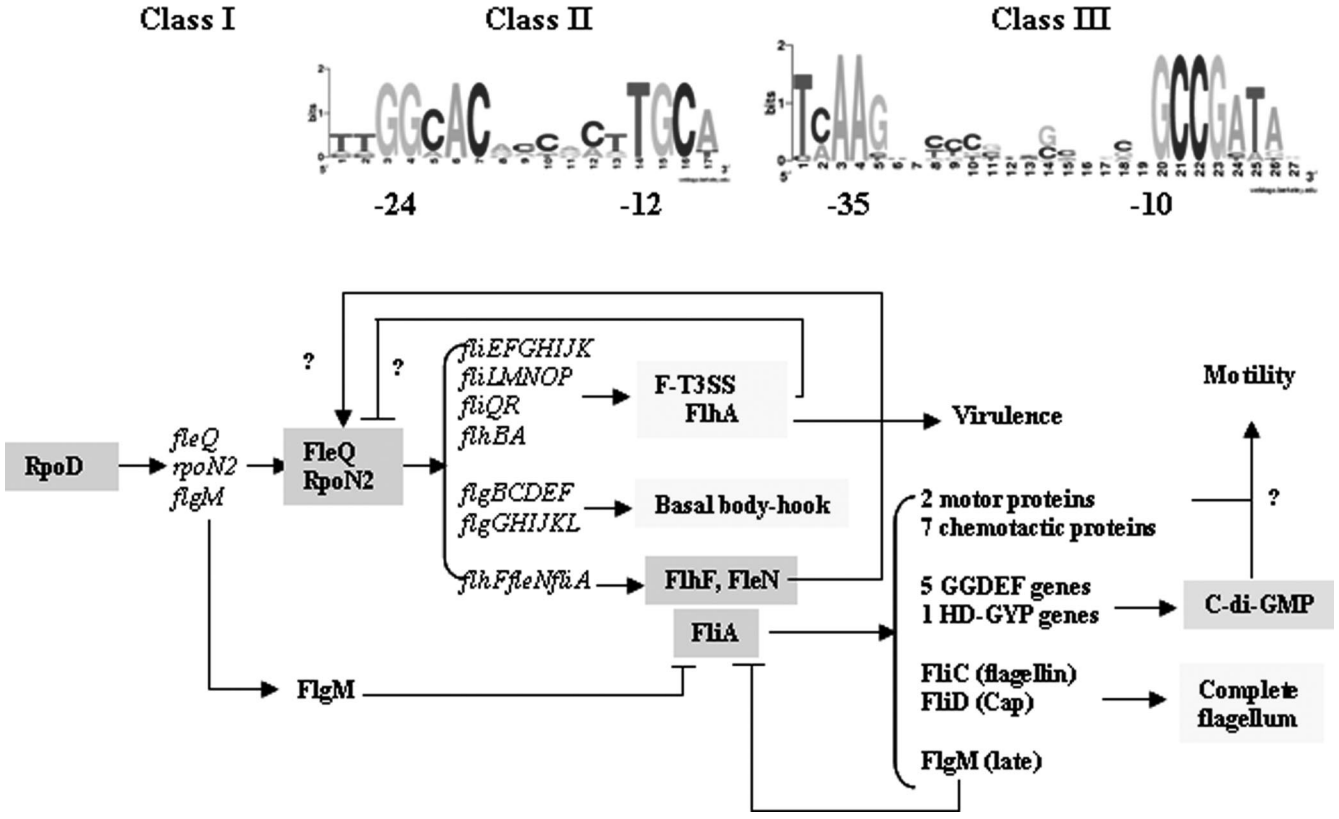

FIG. 6. Model of the flagellar transcriptional cascade in *X. campestris* pv. campestris. Flagellar genes can be placed in three temporal classes according to the type of promoter recognized by the different sigma factors. Class I proteins RpoN2 (σ^{54}) and the cognate activator FleQ are the master regulator directing the expression of class II genes. Early FlgM transcribed from a class I promoter inhibits class II sigma factor FliA until completion of the production of class II proteins and assembly of the F-T3SS–basal body–hook structure. Late (class III) genes belong to the FliA (σ^{28}) regulons. Flagellar filament, cap proteins, and motility regulatory proteins are identified. Late FlgM acts as a final brake to the whole process. The promoter consensus based on the type II and type III flagellar genes of *X. campestris* pv. campestris is indicated at the top.

Late flagellar genes and some motility/chemotaxis and c-di-GMP-signaling-related genes belong to the FliA regulons. FliA is a σ^{70} family protein that recognizes a $-35/-10$-type promoter sequence. Twenty-one putative σ^{28}-dependent genes are predicted in this work (see Table S3 in the supplemental material). Most are highly related to the late stage of flagellar biogenesis (such as *flgM*, *fliC*, and *fliD*) and to motility/chemotaxis (including XCC3653, XCC1891, XCC1727, XCC1870, XCC1871, XCC1883, XCC2315, and XCC3522). The most interesting finding is that five GGDEF domain-containing proteins (XCC0407, XCC1443, XCC1777, XCC3519, and XCC3546) and one HD-GYP domain-containing protein (XCC0350) are likely regulated by FliA. Both the GGDEF and HD-GYP domains are related to the synthesis and breakdown of an important bacterial intracellular secondary messenger, 3′,5′-cyclic diguanylic acid (c-di-GMP) (4, 62, 63). Recently, c-di-GMP has been shown to be involved in EPS production, biofilm formation, quorum sensing, virulence, and flagellar mobility (6, 37, 56, 75). c-di-GMP may be involved in the sigma factor regulatory pathways. In a pathogenic *E. coli* strain, FliA regulates adhesion and invasion via a c-di-GMP-dependent pathway (11). c-di-GMP is also involved in the signaling pathway of the σ^S network (73). In the *X. campestris* pv. campestris genome, 29 proteins with a GGDEF motif and three proteins containing an HD-GYP domain have been identified, and, more importantly, some are involved in motility (64). More experiments are required to evaluate whether the expression of c-di-GMP-related genes is FliA dependent and to elucidate its roles in flagellar biogenesis and motility.

F-T3SS structural proteins FlhB and FlhA are required for the production of FliA and for virulence. FlhA and FlhB are two F-T3SS structural proteins. It is not surprising that the defect in F-T3SS formation results in an accumulation of FlgM and represses expression from a FliA-dependent promoter. The dramatic decrease of FliA protein in an *flhB* mutant suggests that FlhB might play a role in the expression and/or the stability of FliA. The effect of FlhA (and FlhB) on class one or two promoters has been reported for some bacteria. In *Proteus mirabilis*, an *flhA* mutant expresses 10-fold-lower levels of *flhCD* (class 2) transcripts (20). In *H. pylori*, FlhA acts as a master regulator and positively regulates transcription in the RpoN (class 2) and FliA (class 3) regulons (57). In *Campylobacter jejuni*, mutation in either *flhA* or *flhB* reduces the transcription from σ^{54}-dependent flagellar genes (26).

Two classes of T3SS have been identified in gram-negative bacteria (13, 22). Besides F-T3SS, the nonflagellar T3SS (NF-T3SS) is involved in the delivery of protein effectors across eukaryotic cytoplasm to promote pathogenesis (12, 42). F- and NF-T3SS share many structural and functional features, and nine components of these two classes are homologous in se-

quence (13, 70). Furthermore, functional coordination between F- and NF-T3SS has been reported for some species. In *Yersinia enterocolitica*, the virulence-associated phospholipase YpIA can be secreted via F- and NF-T3SS (79, 80). In *C. jejuni*, FlhA coordinately regulates the bacterial motility and virulence (9, 30), and many virulence factors are secreted via F-T3SS (24). In *Bacillus thuringiensis*, an *flhA* mutation results in a defect in flagellar biogenesis, motility, virulence factors secretion, and cytotoxicity (7, 21). Our work demonstrated that the *flhA* mutant has an attenuated virulence, but the production of extracellular enzymes and EPS is not influenced. FlhA is highly homologous (30%) to HrcV (XCC1229), a protein of NF-T3SS. Since the nonmotile strain XcflhB exhibits a normal infection rate, F-T3SS and motility of *X. campestris* pv. campestris are not necessary for pathogenesis. Thus, further experiments will be necessary to determine whether FlhA participates in pathogenicity via NF-T3SS.

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