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Regulatory Network Controlling Extracellular Proteins in *Erwinia carotovora* subsp. *carotovora*: FlhDC, the Master Regulator of Flagellar Genes, Activates *rsmB* Regulatory RNA Production by Affecting *gacA* and *hexA* (*lrhA*) Expression[▽]

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***Erwinia carotovora* subsp. *carotovora* produces an array of extracellular proteins (i.e., exoproteins), including plant cell wall-degrading enzymes and Harpin, an effector responsible for eliciting hypersensitive reaction. Exoprotein genes are coregulated by the quorum-sensing signal, *N*-acyl homoserine lactone, plant signals, an assortment of transcriptional factors/regulators (GacS/A, ExpR1, ExpR2, KdgR, RpoS, HexA, and RsmC) and posttranscriptional regulators (RsmA, *rsmB* RNA). *rsmB* RNA production is positively regulated by GacS/A, a two-component system, and negatively regulated by HexA (PecT in *Erwinia chrysanthemi*; LrhA [LysR homolog A] in *Escherichia coli*) and RsmC, a putative transcriptional adaptor. While free RsmA, an RNA-binding protein, promotes decay of mRNAs of exoprotein genes, binding of RsmA with *rsmB* RNA neutralizes the RsmA effect. In the course of studies of GacA regulation, we discovered that a locus bearing strong homology to the *flhDC* operon of *E. coli* also controls extracellular enzyme production. A transposon insertion FlhDC$^-$ mutant produces very low levels of pectate lyase, polygalacturonase, cellulase, protease, and *E. carotovora* subsp. *carotovora* Harpin (Harpin$_{Ecc}$) and is severely attenuated in its plant virulence. The production of these exoproteins is restored in the mutant carrying an FlhDC$^+$ plasmid. Sequence analysis and transcript assays disclosed that the *flhD* operon of *E. carotovora* subsp. *carotovora*, like those of other enterobacteria, consists of *flhD* and *flhC*. Complementation analysis revealed that the regulatory effect requires functions of both *flhD* and *flhC* products. The data presented here show that FlhDC positively regulates *gacA*, *rsmC*, and *fliA* and negatively regulates *hexA* (*lrhA*). Evidence shows that FlhDC controls extracellular protein production through cumulative effects on *hexA* and *gacA*. Reduced levels of GacA and elevated levels of HexA in the FlhDC$^-$ mutant are responsible for the inhibition of *rsmB* RNA production, a condition conducive to the accumulation of free RsmA. Indeed, studies with an RsmA$^-$ FlhDC$^-$ double mutant and multiple copies of *rsmB*$^+$ DNA establish that the negative effect of FlhDC deficiency is exerted via RsmA. The FlhDC-mediated regulation of *fliA* has no bearing on exoprotein production in *E. carotovora* subsp. *carotovora*. Our observations for the first time establish a regulatory connection between FlhDC, HexA, GacA, and *rsmB* RNA in the context of the exoprotein production and virulence of *E. carotovora* subsp. *carotovora*.**

Erwinia carotovora subsp. *carotovora* (Ecc), a member of the *Enterobacteriaceae* family, causes soft-rotting disease on a wide variety of plants worldwide. A consortium of plant cell wall-degrading extracellular enzymes comprising pectate lyase (Pel), polygalacturonase (Peh), protease (Prt), and cellulase (Cel) contribute to its plant virulence (4, 15, 19, 61, 71). Among them, extracellular pectinases, including Pel and Peh, play a crucial role in tissue maceration and cell death. In addition, motility and some effectors secreted through the type III secretion system also augment virulence of *E. carotovora* subsp. *carotovora* (12, 37, 65).

The regulation of the extracellular enzymes and proteins including Harpin, the elicitor of the hypersensitive response, in *E. carotovora* subsp. *carotovora* has been extensively studied, and many regulatory genes and factors have been identified. These extracellular proteins are coregulated by plant signals, quorum-sensing signals (39, 62), as well as by an assortment of transcriptional factors and posttranscriptional factors (Fig. 1), including the RsmA-*rsmB* system (13, 23, 48), RsmC (HexY [25, 67]), the GacS/GacA two-component system (20, 28, 30), KdgR (49), RpoS (9, 57), ExpR1 (22), ExpR2 (VirR [8, 21]), Hor (70), and HexA (34, 56). Of these regulators, the post-transcriptional system comprising RsmA and *rsmB* RNA is absolutely critical in the expression of exoprotein genes. Indeed, many of the transcriptional regulators and the quorum-sensing signal, *N*-acyl homoserine lactone (AHL), controlling exoprotein production actually act via RsmA-*rsmB* RNA (Fig. 1). In this system, RsmA, a small RNA-binding protein, promotes mRNA decay (13, 23). *rsmB* specifies an untranslated regulatory RNA that binds RsmA and neutralizes its negative regulatory effect (48). GacS, the putative sensor kinase, and GacA, the cognate response regulator, members of a widely occurring two-component system, control exoprotein produc-

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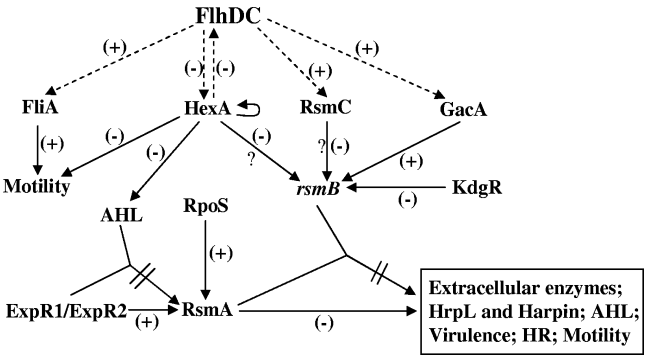

FIG. 1. Model depicting the regulatory network controlling the production of extracellular enzymes, HrpL, Harpin, and AHL, as well as motility, pathogenicity, and the hypersensitive response in *E. carotovora* subsp. *carotovora* (see the text for the details). The regulatory steps indicated by broken lines with arrows are based upon the results presented in this report.

tion in *E. carotovora* subsp. *carotovora*, mainly via regulating *rsmB* (20, 28). HexA negatively controls exoprotein and AHL production, as well as motility (34, 56). Our studies demonstrated that the HexA effect on exoprotein production occurs via *rsmB* (56). In addition, RsmC, a putative transcriptional adaptor, affects exoproteins by modulating the levels of *rsmB* RNA (25), although the underlying regulatory mechanism remains unknown. KdgR, an IcII-like protein, negatively controls exoproteins by inhibiting the transcription of *rsmB* by a novel "road-block" mechanism (49). RpoS, an alternate sigma factor, negatively affects the production of exoprotein by stimulating *rsmA* transcription (57). The two LuxR homologs ExpR1 and ExpR2 activate *rsmA* transcription in the absence of AHL (21, 22). However, ExpR2, but not ExpR1, severely reduces exoprotein production and attenuates virulence, and its effects are neutralized by AHL.

Our findings disclosed a crucial role of GacA in *rsmB* expression (20). However, *gacA* expression itself is regulated, as is apparent from the effects of cultural conditions such as medium composition and growth phase. Surprisingly, despite extensive studies of the GacS/A-mediated regulation in various bacteria (14, 20, 28, 35), little is known of genes and environmental factors controlling *gacS/A* expression. One objective of the present study was to identify regulators controlling the production of GacA. For this, we used a mutagenesis/selection strategy that led to the isolation of mutants that no longer responded to the presence of *gacA* carrying its native promoter. Subsequent studies revealed that one such class of mutants resulted from inactivation of the *flhDC* operon.

Flagellar motility is an accessory virulence determinant in animal pathogens, as well as in several plant pathogens, including *E. carotovora* (31, 37, 40, 44, 52, 53, 59). The FlhDC complex, comprising the products of *flhD* and *flhC*, is the master regulatory operon controlling the expression of flagellar genes in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (5, 7, 18, 53). The flagellum-chemotaxis regulon in *E. coli* and serovar Typhimurium comprises more than 50 genes organized into at least 14 operons. The transcription of these flagellar genes is organized in a three-tier hierarchy: class I, class II, and class III. *flhD* and *flhC* comprise the class I genes, and the products are known to form an FlhD4C2 hexamer complex (72). Both FlhD and FlhC subunits are essential for effective transcription. FlhC protein is the DNA-binding component, and its function is strengthened by FlhD. Claret and Hughes (18) showed that reconstituted FlhD2C2 (= FlhD4C2) complex from purified FlhD and FlhC subunits increases the specificity of DNA binding and also increases the stability of the resultant DNA interaction in vitro. The action of FlhD would ensure that FlhC efficiently locates its multiple target genes and stabilizes the FlhC-DNA complex. FlhDC complex binds promoter regions of the class II genes and activates their transcription. The class II flagellar regulon includes genes that encode proteins for the basal body and the hook of the flagellum, as well as two regulators, FliA and FliM. FliA is an alternative sigma factor (σ^{28}) specific for the flagellar regulon, and FlgM acts as an anti-sigma factor that inhibits FliA-dependent transcription by stripping FliA from the core of RNA polymerase, as well as by preventing the sigma-core interaction (2, 3, 10, 43, 63, 68). FliA is required for the expression of some class II genes and all class III genes. The class III genes encode components for assembly of the flagellar filament, chemotaxis proteins, and motor activity (1, 17, 18, 53).

In addition to the flagellum-chemotaxis regulon for swimming motility, the flagellar master operon FlhDC controls genes for virulence, the type III secretion system, and extracellular enzyme production. For example, it is required for the expression of the extracellular phospholipase gene, as well as swarming motility in *Serratia liquefaciens* (33). Bleves et al. (7) reported that the y*op* regulon is upregulated in an FlhDC$^-$ mutant in *Yersinia enterocolitica*. The genes of the *yop* regulon encode proteins for the Yop virulon, which are secreted via the Yop secretion apparatus designated as the type III secretion system. Yop proteins enable bacteria in close contact with target cells to inject bacterial toxic proteins directly into the cytosol of the target cells. Kapatral et al. (41) determined that FlhDC regulates the expression of genes for enzymes involved in the synthesis and degradation of carbamoylphosphate in *Y. enterocolitica*. In addition to motility, FlhDC is also required for lipolysis, extracellular hemolysis, and full virulence in the insect pathogenic bacterium *Xenorhabdus nematophilus* (32). Remarkably, a recent study by of Park and Forst (60) showed that the FlhDC effect on extracellular enzymes in *X. nematophilus* occurs via FliA. FlhD alone is found to regulate the cell division rate in *E. coli* (64).

As stated above, in *E. carotovora* subsp. *carotovora* the FlhDC operon plays an important role in pathogenicity and controls motility. Hossain et al. (37) reported that the nonmotile state in Δ*fliC* and Δ*motA* mutants of the *E. carotovora* subsp. *carotovora* strain EC1 reduced their ability to cause soft-rot disease on Chinese cabbage, but the mutations had no deleterious effect on the levels of major extracellular enzymes. In addition, Matsumoto et al. (52) showed that the FlhC$^-$ and FlhD$^-$ mutants of *E. carotovora* subsp. *carotovora* strain EC1-N caused a severe reduction in the transcript levels of *fliC* and *fliA*, flagellum synthesis, and virulence in Chinese cabbage and potato. However, how the FlhDC master regulon affected virulence in *E. carotovora* subsp. *carotovora* remained unknown. We report here the characteristics of the *flhDC* operon of *E. carotovora* subsp. *carotovora* strain Ecc71 and the effects

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. carotovora subsp. *carotovora*		
Ecc71	Wild type	73
AC5006	Lac$^-$ derivative of Ecc71	58
AC5047	Nal^r derivative of AC5006	13
AC5070	Km^r, RsmA$^-$ derivative of AC5047	13
AC5140	Km^r, FlhDC$^-$ derivative of Ecc71	This study
AC5141	Km^r, FlhDC$^-$ derivative of AC5006	This study
AC5142	Km^r Sp^r, FlhDC$^-$ RsmC$^-$ derivative of AC5006	This study
AC5143	Km^r Gm^r, FlhDC$^-$ GacA$^-$ derivative of Ecc71	This study
AC5144	Km^r Sp^r, FlhDC$^-$ RsmA$^-$ derivative of AC5006	This study
AC5145	Km^r Sp^r, FlhDC$^-$ HexA$^-$ derivative of Ecc71	This study
E. coli DH5α	ϕ80*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 hsdR17 recA1 endA1 thi-1*	Gibco BRL
Plasmids		
p34S-Gm	Gm^r, source of Gm^r cassette	27
pBluescript SK(+)	Ap^r	Stratagene
pCL1920	Sp^r Sm^r	46
pCL1920Gm^r	Sp^r Gm^r, Gm^r cassette in pCL1920	This study
pMP220	Tc^r, promoter-probe vector	69
pRK415	Tc^r	42
pRK2013	Km^r, Mob+, Tra+	29
pUT mini-Tn*5*-Sp	A lambda-pir vector containing mini-Tn*5*-Sp transposon	26
pUT mini-Tn*5*-Km	A lambda-pir vector containing mini-Tn*5*-Km transposon	26
pAKC781	Ap^r, *peh-1* DNA in pBluescript SK(+)	47
pAKC783	Ap^r, *pel-1* DNA in pBluescript SK(+)	47
pAKC882	Ap^r, *rsmA* coding region in pT7-7	55
pAKC924	Ap^r, $hrpN_{Ecc}$ DNA in pBluescript SK(+)	24
pAKC975	Sp^r, *rsmC* DNA in pCL1920	25
pAKC980	Sp^r Tc^r, inactivated *rsmC* DNA in pRK415	25
pAKC983	Sp^r Tc^r, inactivated *hexA* DNA in pRK415	56
pAKC985	Km^r, *hexA* DNA in pDK6	56
pAKC1004	Sp^r, *rsmB* DNA in pCL1920	48
pAKC1111	Tc^r, $gacA_{DC3000}$ DNA in pLARF5	14
pAKC1034	Ap^r, 200-bp *celV* fragment in pGEM-T Easy	49
pAKC1049	Sp^r, *plac-rsmB*$^+$ in pCL1920	51
pAKC1049Gm^r	Sp^r, Gm^r,Gm^r cassette in pAKC1049	This study
pAKC1057	Gm^r Tc^r, inactivated *gacA* DNA in pRK415	20
pAKC1071	Ap^r, *hrpL* DNA in pBluescript SK(+)	11
pAKC1240	Ap^r Km^r, 6.5-kb ClaI fragment containing mini-Tn*5*-Km^r inactivated *flhDC* in pBluescript SK(+)	This study
pAKC1241	Sp^r, *plac-flhD* in pCL1920	This study
pAKC1242	Sp^r, *plac-flhDC* in pCL1920	This study
pAKC1243	Tc^r, *gacA-lacZ* in pMP220	This study
pAKC1244	Tc^r, *rsmC-lacZ* (containing DNA segment of −684 nt to +57 nt from the putative translational start site) in pMP220	This study
pAKC1245	Sp^r, *plac-gacA* in pCL1920	This study
pAKC1246	Sp^r, *plac-fliA* in pCL1920	This study
pAKC1249	Tc^r Km^r, inactivated *flhDC* in pRK415 from pAKC1240	This study
pAKC1250	Tc^r, *rsmC-lacZ* (containing DNA segment of −323 nt to +57 nt from the putative translational start site) in pMP220	This study

[a] nt, nucleotide.

of this operon on various global regulators known to control extracellular protein production and motility. We show that (i) FlhDC is required for the production of major extracellular enzymes and *E. carotovora* subsp. *carotovora* Harpin ($Harpin_{Ecc}$), as well as virulence in *E. carotovora* subsp. *carotovora*; (ii) as a master regulator, FlhDC controls the expression of several key regulatory genes and sigma factor genes such as *gacA*, *rsmC*, *hexA*, *fliA*, and *hrpL*; (iii) FlhDC activates regulatory *rsmB* RNA production via its effects on GacA and HexA; and (iv) the sigma factor FliA does not affect extracellular protein production.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids in this study are described in Table 1. *E. carotovora* subsp. *carotovora* strains Ecc71 and AC5006 were maintained on LB agar. The strains carrying antibiotic markers were maintained on LB agar containing appropriate antibiotics.

The compositions of LB, nutrient gelatin, and minimal salts media have been

described in previous publications (13, 58). When required, antibiotics were supplemented as follows: ampicillin (Ap), 100 μg/ml; gentamicin (Gm), 10 μg/ml; kanamycin (Km), 50 μg/ml; spectinomycin (Sp), 50 μg/ml; and tetracycline (Tc), 10 μg/ml. Media were solidified by using 1.5% (wt/vol) agar.

The composition of media for agarose plate assays for enzymatic activities was described by Chatterjee et al. (13).

Extracellular enzyme assays. Bacterial cells were grown in minimal salts medium supplemented with sucrose (0.5% [wt/vol]) without or with appropriate drugs to a Klett value of ca. 250, and the cultural supernatants were used for assays. The quantitative extracellular Pel enzymatic assay, as well as the semiquantitative Pel, Peh, Prt, and Cel agarose plate assays, was performed according to previously published procedures (13, 16). Briefly, the reaction mixture for quantitative Pel assays contained 0.24 ml of substrate (0.575% polygalacturonic acid [pH 5.5]), 0.26 ml of reaction buffer (230 mM Tris-HCl [pH 8.5], 0.78 mM $CaCl_2$), and enzyme or water in a total volume of 0.6 ml. The Pel specific activities were measured at an A_{235} in a spectrophotometer. The Pel activities are expressed as units/per ml/A_{600}. One unit of Pel activity is defined as the amount of enzyme that produces 1 μmol of unsaturated digalacturonic acid equivalent per min at 30°C. For semiquantitative assays of Pel, Peh, Cel, and Prt, wells were made in agarose media with a number 2 cork borer, and the bottoms were sealed with molten agarose (0.8% [wt/vol]). Samples were applied to the wells, and the plates were incubated at 28°C. After 16 to 18 h, Pel and Peh assay plates were developed with 4 N HCl, and the Cel assay plates were developed with Congo red and NaCl solutions. Halos around the wells due to protease activity became visible in Prt assay plates within 24 to 36 h without any further treatment.

Plant tissue maceration. The celery petiole assay was performed as described by Murata et al. (58). The extent of tissue maceration was estimated visually.

Determination of nucleotide sequences of Ecc71 *flhDC* and sequence alignment. The chromosomal DNA of the FlhDC$^-$ mutant AC5140 containing mini-Tn*5*-Km^r fragment was digested with ClaI and ligated with ClaI-digested pBluescript SK(+). The ligated DNAs were electroporated into DH5α and selected on LB plus Ap and Km agar to yield pAKC1240. The nucleotide sequence of *flhDC* was determined from pAKC1240 using primers of transposon sequences. Nucleotide sequencing was performed at the DNA Core Facility of University of Missouri-Columbia. The amino acid sequences of FlhDC of different bacteria were obtained from GenBank. Sequence alignment was performed by using CLUSTAL W (www.expasy.ch), and default parameters were used.

DNA techniques. Standard procedures were used in the isolation of plasmids and chromosomal DNA, gel electrophoresis, DNA ligation, transformation, and electroporation (66). Restriction and modification enzymes were obtained from Promega Biotec (Madison, WI). Prime-a-Gene DNA labeling system (Promega Biotec) was used for labeling DNA probes.

Construction of FlhDC$^-$ mutants. The insertion DNA containing inactivated *flhDC* DNA in pAKC1240 was transcloned into pRK415 to yield pAKC1249. The FlhDC$^-$ mutants AC5140 and AC5141 were constructed by marker exchange of Ecc71 and AC5006 with pAKC1249, respectively. The FlhDC$^-$ RsmC$^-$ mutant AC5142 was obtained by marker exchange of AC5141 with pKC980. The FlhDC$^-$ GacA$^-$ mutant AC5143, and the FlhDC$^-$ HexA$^-$ mutant AC5145 was obtained by marker exchange of AC5140 with pAKC1057 and pAKC983, respectively. The procedures for marker exchange have been described in Chatterjee et al. (13). The FlhDC$^-$ RsmA$^-$ mutant AC5144 was constructed by inactivating *rsmA* in AC5141 using mini-Tn*5*-Sp^r. Inactivation of target genes in mutants were confirmed by Northern blot analysis.

Construction of *plac-flhD*, *plac-flhDC*, *plac-gacA*, and *plac-fliA* plasmids, as well as *rsmC-lacZ* and *gacA-lacZ* fusions. DNA fragments containing coding regions of *flhD*, *gacA*, and *fliA* of Ecc71 were PCR amplified by using the primer pairs 71flhD1-71flhD2, 71gacA1-71gacA2, and 71fliA1-71fliA2, respectively (see Table 2 for the primer sequences). The amplified DNA fragments were cloned into pCL1920 to yield pAKC1241, pAKC1245, and pAKC1246. The *plac-flhDC* plasmid pAKC1242 was constructed by cloning a SalI fragment containing *flhDC*$^+$ DNA behind the *lac* promoter in pCL1920. To construct *rsmC-lacZ* and *gacA-lacZ* fusions, PCR-amplified DNA fragments containing *gacA* and *rsmC* upstream DNAs by using the primer pairs 71gacAZ1-71gacAZ2 and 71rsmCZ1-71rsmCZ2 (Table 2) were cloned into pMP220 to yield pAKC1243 and pAKC1244, respectively.

Northern blot and Western blot analyses. Bacterial cultures were grown at 28°C in minimal salts medium supplemented with sucrose (0.5% [wt/vol]). Cells were collected while cultures reached a Klett value of ca. 220. RNA isolation and Northern blot analysis were performed as described by Liu et al. (47). The probes used were the 183-bp NdeI-SalI fragment of *rsmA* from pAKC882, the 314-bp EcoRV-KpnI fragment of *pel-1* from pAKC 783, the 743-bp HindIII fragment of *peh-1* from pAKC781, the 200-bp EcoRI fragment of *celV* from pAKC1034, the 779-bp EcoRV-SmaI fragment of *hrpN* from pAKC924, the 321-bp BamHI-HindIII fragment of *rsmB* from pAKC1004, the 304-bp EcoRV-KpnI fragment of *rsmC* from pAKC975, the 950-bp EcoRI-SalI fragment of *hexA* from pAKC985, the 287-bp BglII-EcoRV fragment of *hrpL* from pAKC1071. and the 811-bp BamHI-HindIII fragment of *fliA* from pAKC1246. The *flhD*, *flhC*, and *gacA* probes were PCR amplified using the primer pairs 71flhD1-71flhD2, 71flhC1-71flhC2, and 71gacA1-71gacA2, respectively. For Western blot analysis, bacterial cells were collected, suspended in 1× sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (66), and boiled. The protein concentrations were determined by using a CB-X protein assay kit (Geno Technology, Inc., St. Louis, MO) according to the manufacturer's specifications. Western blot analysis of the total bacterial protein was performed as described by Mukherjee et al. (55). The antisera raised against $Harpin_{Ech}$ (6) were used as probes.

TABLE 2. Primers used for PCR amplification

Primer	Sequence (5′–3′)
71flhD1	GATGGATTCATAGCCTGTCGGGATGGGAAATATG
71flhD2	GATCTGCAGTCTCCGCCATTACTTATGCCC
71flhC1	GATGGATTCGGGCATAAGTAATGGCGGAGA
71flhC2	GATAAGCTTACAGGCTCAGACTGCGTGTT
71gacA1	GATGGATCCGGAGAATTATTCTTTGATTAGCG
71gacA2	GATAAGCTTGCCGACGCATCGAAATCTTCAC
71fliA1	TGCGGATCCACGCTATTCAGGCGATTGGCTACC
71fliA2	TGCAAGCTTCGCAGCGCAATTAAACATCGTTC
71gacAZ1	TGCAGATCTGATGCGGTGAGCAATAGTGCT
71gacAZ2	TGCGAATTCGGTCATCAACAAGAAAAACGCTAAT
71rsmCZ1	TGCAGATCTGAATTATCAGTGCTGTTATAATGTC
71rsmCZ2	TGCTCTAGACTGAACTGGTTGAGAAAGCATGCC

β-Galactosidase assays. Bacterial constructs were grown at 28°C in minimal salts medium plus sucrose and Tc to a Klett value of ca. 200. The β-galactosidase assays were performed according to the method of Miller (54).

Extracellular enzyme assays, Northern blot and Western blot analyses, β-galactosidase assays, and pathogenicity tests were performed at least twice, and the results were reproducible.

RESULTS AND DISCUSSION

***E. carotovora* subsp. *carotovora flhDC* is closely related to enterobacterial genes.** To identify gene(s) regulating GacA, we screened a library of Tn*5*-insertion mutants of Ecc71. Since GacA is a positive regulator of exoprotein production, a mutant downregulated in GacA expression was predicted to produce reduced levels of exoproteins. We mutagenized with mini-Tn*5*-Km^r, a GacA$^-$ mutant of Ecc71 carrying a plasmid containing *gacA*$^+$ DNA of *Pseudomonas syringae* pv. tomato strain DC3000. Five colonies that did not produce protease on nutrient gelatin agar were selected, and chromosomal DNA fragments carrying mini-Tn*5*-Km^r were cloned from these mutants and sequenced using the mini-Tn*5* primers. The sequence data revealed that in one of those mutants the Tn*5* insertion is located 80 bases upstream of an open reading frame that encodes a homolog of the flagellar transcriptional activator, FlhD. The flagellar transcriptional activator gene, *flhC*, possessing a putative start site three bases after the stop codon of *flhD*, is also present in the mini-Tn*5* containing fragment cloned from this mutant. The plasmid containing this fragment was used for marker exchange with the *E. carotovora* subsp. *carotovora* strains Ecc71 and AC5006 to yield AC5140 and

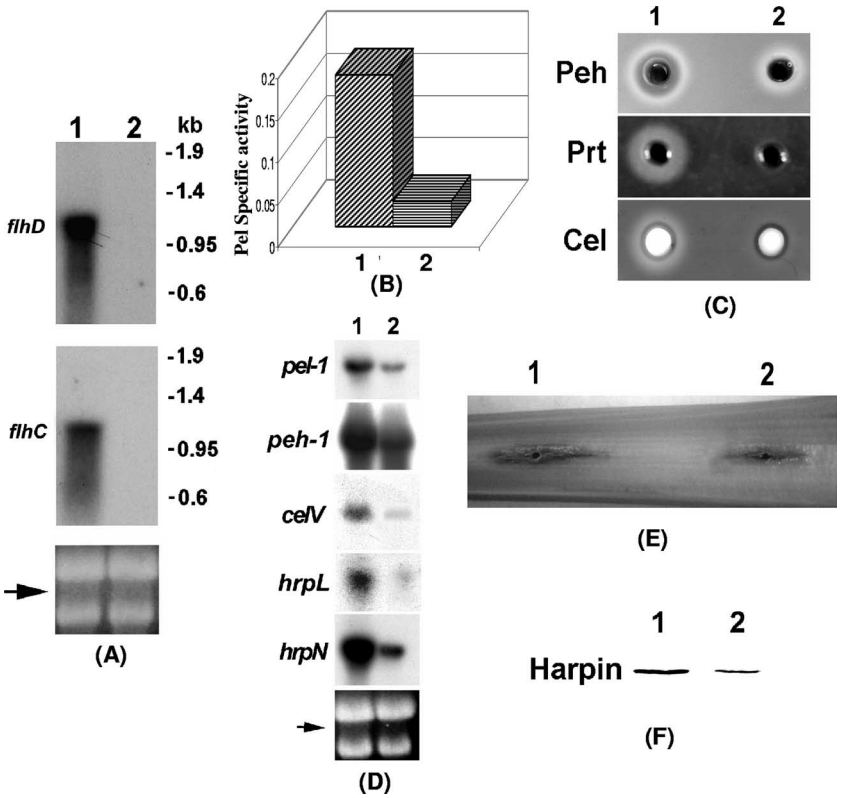

FIG. 2. Characteristics of an FlhDC$^-$ mutant (1). Ecc71 and (2) its FlhDC$^-$ mutant AC5140. (A) Northern blot analysis of *flhD* and *flhC*; (B) Pel activities; (C) agarose plate assays of Peh, Prt, and Cel activities; (D) Northern blot analysis of *pel-1*, *peh-1*, *celV*, *hrpL*, and *hrpN*. For panels A and D, each lane contained 15 μg of total RNA. The arrows show the levels of total RNA as revealed by ethidium bromide staining of denatured agarose gel. (E) Soft-rot disease symptoms in celery petiole; (F) Western blot analysis of $Harpin_{Ecc}$ protein. Each lane contained 20 μg of total protein.

AC5141, respectively. Northern blot analysis with *flhD* or *flhC* DNA as probes revealed that Ecc71 produced ~1.1-kb transcripts (Fig. 2A, lane 1), whereas with those two probes no hybridization signal was detected with RNA of AC5140 (Fig. 2A, lane 2) or the RNA from AC5141 (data not shown). These results indicated that *flhD* and *flhC* are components of one transcriptional unit and that the mutants AC5140 and AC5141 are FlhDC$^-$. The Tn*5* insertions in the other four mutants are located in *ahlI*, a gene that encodes AHL synthase.

flhD and *flhC* genes are linked together and share high homology in different bacteria. As an example, the translational start site of *flhC*, as in *E. carotovora* subsp. *carotovora*, is three bases behind the stop codon of *flhD* in both *E. coli* and *S. enterica* serovar Typhimurium. The sequence alignment results revealed that the deduced amino acid sequences of Ecc71 FlhD and FlhC have strong homology with previously reported FlhD and FlhC proteins of various enterobacterial species. The percent identities of *E. carotovora* subsp. *carotovora* FlhD and FlhC with the cognate proteins, shown parenthetically in that order, are as follows: *E. carotovora* subsp. *atroseptica* strain SCRI1043 (accession number YP_049786 [97% identical] and accession number YP_049787 [99% identical]), *Serratia marcescens* (accession number O85806 [83% identical] and accession number O85807 [88% identical]), *Serratia proteamaculans* strain 568 (accession number YP_001479218 [82% identical] and accession number YP_001479217 [89% identical]), *Y. enterocolitica* subsp. *enterocolitica* strain 8081 (accession number YP_001006784 [81% identical] and accession number YP_001006783 [87% identical]), *E. coli* strain EDL933 (accession number NP_288329 [76% identical] and accession number NP_288328 [83% identical]), and *S. enterica* serovar Typhimurium strain LT2 (accession number NP_460882 [74% identical] and accession number NP_460881 [82% identical]).

FlhDC positively controls extracellular protein production and pathogenicity. (i) Effects on extracellular enzymes. To examine the effects of FlhDC on extracellular enzyme production, the culture supernatants of the FlhDC$^-$ mutant AC5140 and its parent strain, Ecc71, were assayed to determine their enzymatic activities, and the cells were used for the extraction of total RNAs for transcript assays. The levels of extracellular Pel (Fig. 2B) and Peh, Prt, and Cel (Fig. 2C) produced by AC5140 were much lower than those produced by Ecc71. Similarly, the transcript levels of *pel-1*, *peh-1*, and *celV* (Fig. 2D) of the FlhDC$^-$ mutant also were lower than those of the parent.

(ii) Effects on pathogenicity. A positive correlation between the levels of extracellular enzymes and virulence of *E. carotovora* subsp. *carotovora* has been established (4, 59, 71). Since extracellular enzyme production is suppressed in the FlhDC$^-$ mutant AC5140, a reduced level of virulence of the mutant was expected. The pathogenicity test in celery petioles (Fig. 2E) showed that indeed the degree of maceration caused by AC5140 was reduced compared to that caused by Ecc71.

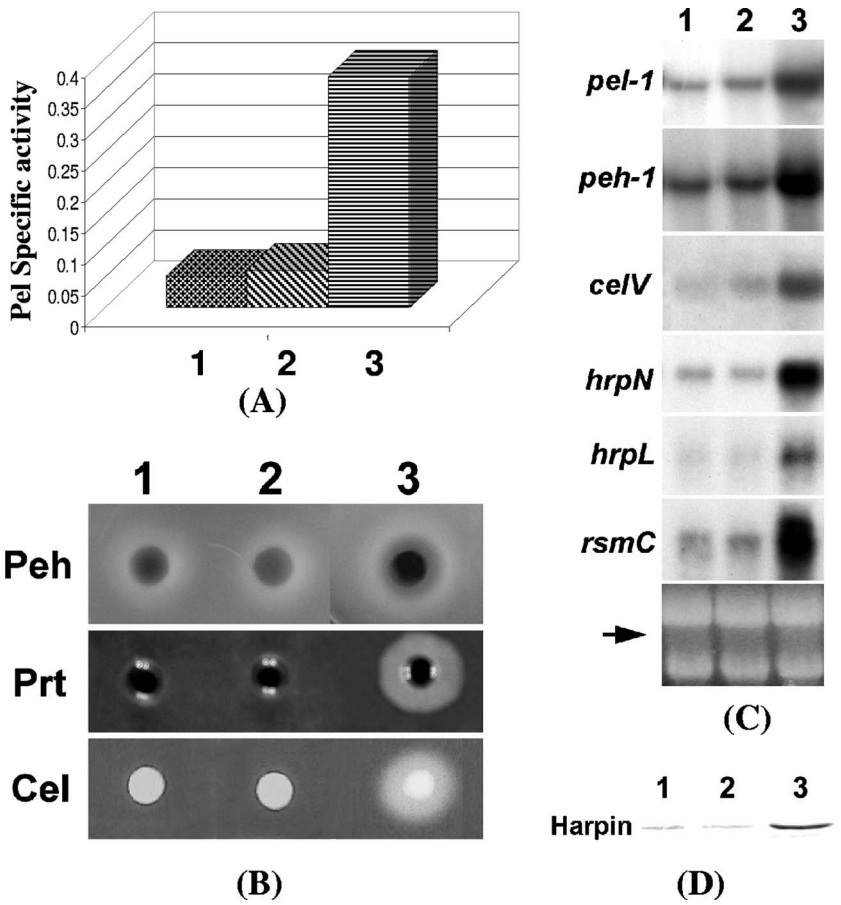

FIG. 3. Reversal of the pleiotropic phenotype of the FlhDC$^-$ mutant by *flhDC*$^+$ DNA. (A) Pel activities; (B) agarose plate assays of Peh, Prt, and Cel activities; (C) Northern blot analysis of *pel-1*, *peh-1*, *celV*, *hrpN*, *hrpL*, and *rsmC*. Each lane contained 15 μg of total RNA. The arrow shows the levels of total RNA as revealed by ethidium bromide staining of denatured agarose gel. (D) Western blot analysis of $Harpin_{Ecc}$ of the FlhDC$^-$ mutant AC5140 carrying pCL1920 (cloning vector) (lane 1), pAKC1241 (*flhD*$^+$) (lane 2), or pAKC1242 (*flhDC*$^+$) (lane 3). Each lane contained 20 μg of total protein.

(iii) Effects on the sigma factor HrpL that controls effector (Harpin) production. We have shown that extracellular enzymes secreted by the type I and type II secretion systems, as well as proteins (effectors) secreted by the type III system, are coregulated in *E. carotovora* subsp. *carotovora* (11, 12, 20, 22, 24, 25, 4, 49, 55, 57). These findings raised the possibility that FlhDC in *E. carotovora* subsp. *carotovora* could control the expression of effector genes as well as the type III secretion system. To test this hypothesis, we first compared the transcript levels of *hrpL*, the gene for an alternate sigma factor known to control the expression of genes that encode effectors secreted via the type III pathway, as well as genes specifying the type III secretion machinery. The results (Fig. 2D) revealed that the level of *hrpL* transcript was much reduced in the FlhDC$^-$ mutant (lane 2) compared to the parent (lane 1). The expression of *E. carotovora* subsp. *carotovora hrpN*, the gene that encodes $Harpin_{Ecc}$, as well as the levels of $Harpin_{Ecc}$ protein, was much lower in AC5140 (Fig. 2D and F, lane 2) compared to Ecc71 (Fig. 2D and F, lane 1). These findings strengthen the notion that FlhDC belongs to the global regulatory network that controls exoprotein production in *E. carotovora* subsp. *carotovora* through the type I, type II, and type III secretion systems.

(iv) Multiple copies of *flhDC* restore exoprotein production in the FlhDC$^-$ mutant. To test whether multiple copies of *flhD* or *flhDC* could restore the extracellular enzyme production and expression of the cognate genes, we cloned *flhD*$^+$ DNA and *flhDC*$^+$ DNA behind the *lac* promoter in vector pCL1920 to yield pAKC1241 and pAKC1242, respectively. Extracellular enzyme levels (Fig. 3A, sample 2, and Fig. 3B, lane 2), as well as the transcript levels of *pel-1*, *peh-1*, and *celV* (Fig. 3C, lane 2), of the FlhDC$^-$ mutant carrying *flhD*$^+$ plasmid were similar to that of the mutant carrying the cloning vector (Fig. 3A, sample 1; Fig. 3B, lane 1; Fig. 3C, lane 1). Similarly, a plasmid carrying *flhC* driven by the *lac* promoter of pCL1920 failed to restore extracellular protein production in the FlhDC$^-$ mutant (data not shown). In contrast, the levels of transcripts of *pel-1*, *peh-1*, and *celV* (Fig. 3C, lane 3) and extracellular enzyme levels (Fig. 3A, sample 3, and Fig. 3B, lane 3) in the FlhDC$^-$ mutant were restored by the *flhDC*$^+$ DNA. These results demonstrate that the regulatory function requires both *flhD* and *flhC* products. In addition to these exracllular enzymes, multiple copies of *flhDC* also restored the levels of *hrpL* and *hrpN* transcripts (Fig. 3C, lane 3), as well as the $Harpin_{Ecc}$ protein (Fig. 3D, lane 3) in the mutant.

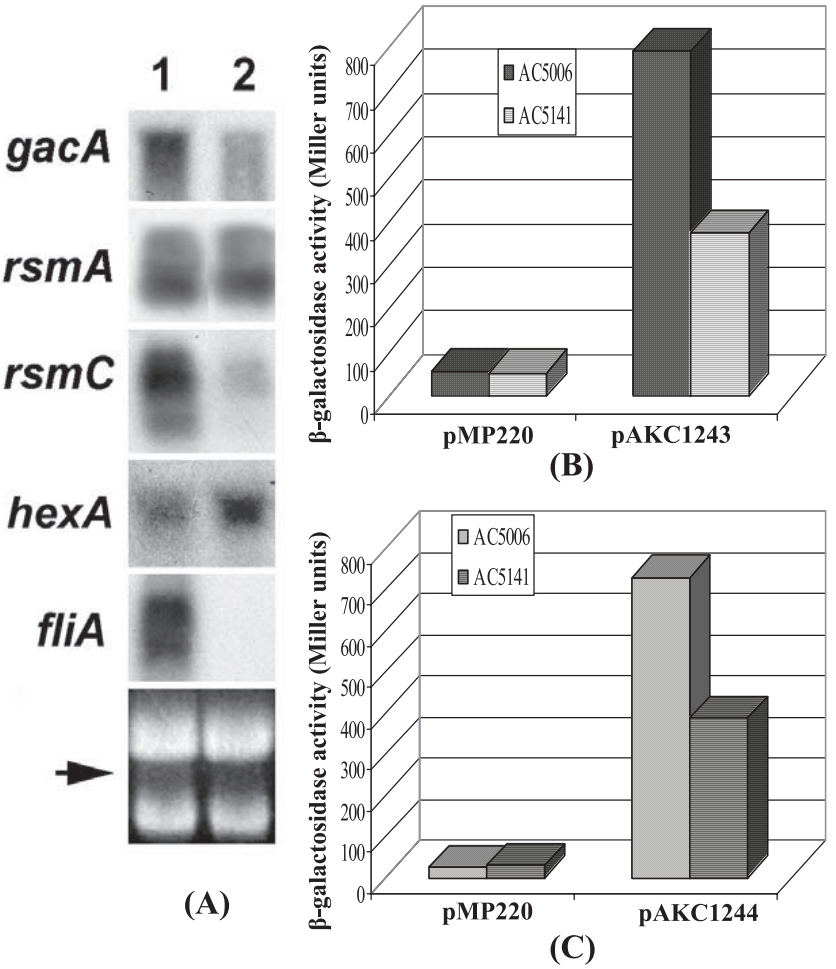

FIG. 4. FlhDC controls regulatory genes for exoprotein production and motility. (A) Northern blot analysis of *gacA*, *rsmA*, *rsmC*, *hexA*, and *fliA* in Ecc71 (lane 1) and its FlhDC$^-$ mutant AC5140 (lane 2). Each lane contained 15 μg of total RNA. The arrow shows the levels of total RNA as revealed by ethidium bromide staining of denatured agarose gel. (B and C) β-Galactosidase activities of transcriptional *gacA-lacZ* fusion pAKC1243 and *rsmC-lacZ* fusion pAKC1244 in AC5006 and AC5141, respectively.

FlhDC controls the expression of several regulatory genes. To understand whether FlhDC controls the expression of exoprotein genes via other regulatory genes, we examined the effects of FlhDC deficiency on expression of several global regulatory genes whose products are known or are predicted to control exoprotein production, motility, and virulence. We present below the evidence for FlhDC effects on the expression of *gacA*, *hexA*, *rsmC*, and *fliA*.

(i) Positive regulation of *gacA*. A Northern blot analysis (Fig. 4A) revealed that the transcript levels of *gacA* were much reduced in the FlhDC$^-$ mutant compared to those in Ecc71. In addition, β-galactosidase assay data (Fig. 4B) demonstrated that expression of a *gacA-lacZ* fusion plasmid pAKC1243 was lower in the FlhDC$^-$ mutant than in the parent. It has been well established that FlhDC complex activates transcription of class II flagellar genes by binding within the promoter regions of target genes and interacting with the C-terminal region of the α subunit of RNA polymerase (50). The consensus FlhDC binding sequences have been identified as GCAATAA and TTATTCC with several variations (17). However, sequence analysis revealed that only one element (TTATTCC) of the FlhDC binding sequence occurs upstream of *gacA*. The significance of this sequence in FlhDC-mediated positive regulation of *gacA* expression is not known.

(ii) Negative regulation of *hexA*. A Northern blot analysis (Fig. 4A) revealed that the FlhDC$^-$ mutant produced higher levels of *hexA* transcript than the parent. In addition, a potential FlhDC binding site (GAAATAA N16 TTATTGA) was found 202 bases upstream of the putative translational start site of the *hexA* gene. Thus, one possible explanation for the negative effects of FlhDC on *hexA* expression is that FlhDC binds sequences upstream of *hexA* and represses its expression.

The effects of FlhDC on exoprotein production are channeled via GacA and HexA, and the evidence for this is discussed below.

(iii) Positive regulation of *rsmC*. Northern blot analysis results (Fig. 4A) revealed that the transcript level of *rsmC* was severely reduced in the FlhDC$^-$ mutant AC5140 compared to the level in Ecc71. Expression of a transcriptional *rsmC-lacZ* fusion plasmid pAKC1244 was lower in the FlhDC$^-$ mutant AC5141 than in the parent AC5006 (Fig. 4C). Furthermore, our data demonstrate that AC5140 carrying the *flhDC*$^+$ plas-

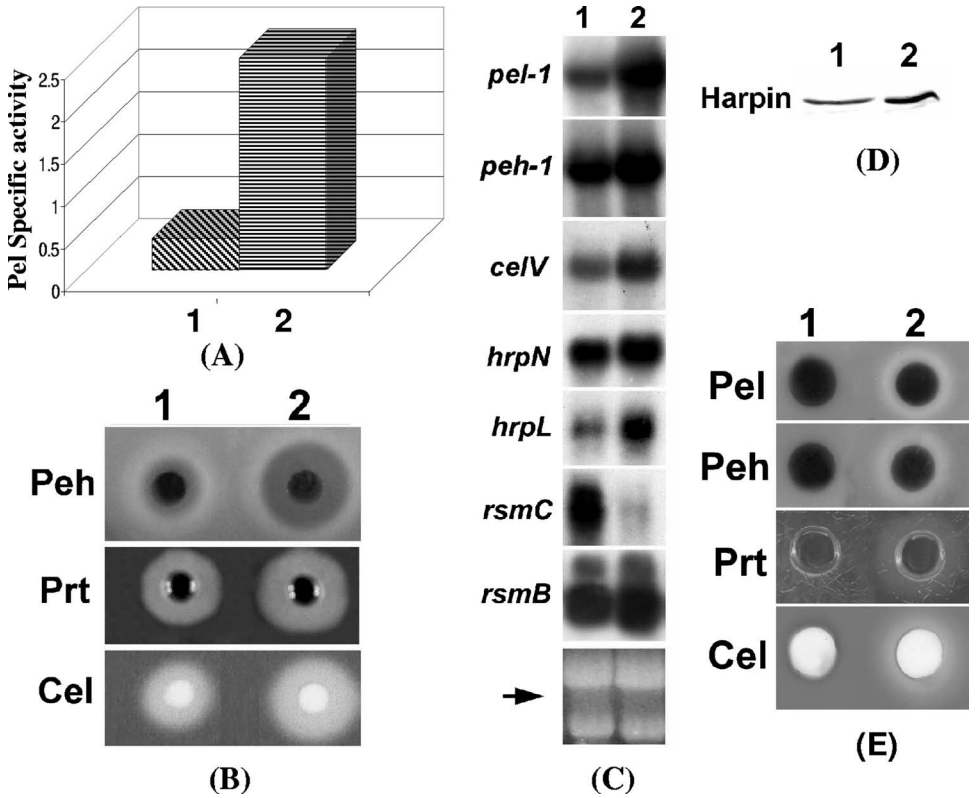

FIG. 5. Reversal of the pleiotropic phenotype of the FlhDC$^-$ mutant by *gacA*$^+$ DNA, as well as by HexA deficiency. (A) Pel activities; (B) agarose plate assays of Peh, Prt, and Cel activities; (C) Northern blot analysis of *pel-1*, *peh-1*, *celV*, *hrpN*, *hrpL*, *rsmB*, and *rsmC*. Each lane contained 15 μg of total RNA. The arrow shows the levels of total RNA as revealed by ethidium bromide staining of denatured agarose gel. (D) Western blot analysis of $Harpin_{Ecc}$ in the FlhDC$^-$ mutant AC5140 carrying pAKC1242 (*flhDC*$^+$) (lane 1) or pAKC1245 (*gacA*$^+$) (lane 2). Each lane contained 20 μg of total protein. (E) Agarose plate assays of Pel, Peh, Prt, and Cel activities in FlhDC$^-$ HexA$^+$ strain AC5140 (column 1) and FlhDC$^-$ HexA$^-$ strain AC5145 (column 2).

mid pAKC1242 (Fig. 3C, lane 3), but not the *flhD*$^+$ plasmid pAKC1241 (Fig. 3C, lane 2), produces higher levels of *rsmC* RNA than the mutant carrying the vector (Fig. 3C, lane 1). A putative FlhDC binding site (GCATAAA N8 TTATTCA) very similar to the consensus sequence was found 564 bp upstream of the predicted *rsmC* translational start site. A transcriptional *rsmC-lacZ* fusion plasmid pAKC1250 which lacks this putative FlhDC binding sequence produced similar β-galactosidase activities in AC5141 and AC5006 (data not shown). These data suggested that FlhDC binds to this putative FlhDC binding sequence and activates the expression of *rsmC*.

To examine the possibility that FlhDC controls exoprotein production via regulating *rsmC* expression, we made an FlhDC$^-$ RsmC$^-$ double mutant AC5142 by marker exchange of AC5141 with pAKC980. In this plasmid *rsmC* is inactivated by inserting an omega (Sp^r) fragment (25). We should recall that RsmC$^-$ mutants of Ecc71 produce much higher levels of exoproteins than the parent (25). Extracellular enzyme assay results revealed that inactivation of RsmC in the FlhDC$^-$ mutant did not restore extracellular enzyme production (data not shown). Thus, RsmC deficiency in the FlhDC$^-$ background has no bearing on exoprotein production. The basis for this response is currently under investigation.

(iv) Positive regulation of *fliA*. A Northern blot analysis revealed that *fliA* RNA was not detectable in the FlhDC$^-$ mutant, whereas high levels of *fliA* transcript were produced by the parent (Fig. 4A). This result was expected since FlhDC is required for the expression of *fliA* in other enterobacteria (17, 50), as well as in *E. carotovora* subsp. *carotovora* strain EC1N (52).

FlhDC effect on extracellular protein production is mediated via *gacA* and *hexA*. The data presented above demonstrated that FlhDC affects the expression of global regulatory genes *gacA* and *hexA*. To examine whether the FlhDC effect on extracellular proteins is mediated via GacA, we compared the effects of *plac-flhDC* plasmid pAKC1242 and *plac-gacA* plasmid pAKC1245 in the FlhDC$^-$ mutant AC5140. The levels of Pel, Peh, Cel, Prt, and $Harpin_{Ecc}$ in AC5140 carrying the *plac-gacA* plasmid pAKC1245 (Fig. 5A, sample 2; Fig. 5B, lane 2; Fig. 5D, lane 2) were higher than in AC5140 carrying *plac-flhDC* plasmid pAKC1242 (Fig. 5A, sample 1; Fig. 5B, lane 1; Fig. 5D, lane 1). The levels of the cognate transcripts also were higher in AC5140/pAKC1245 (Fig. 5C, lane 2) than in AC5140/pAKC1242 (Fig. 5C, lane 1). This difference most likely results from the differential regulatory effects of FlhDC and GacA. As shown above, FlhDC positively regulates both *rsmC* and *gacA* expression, whereas GacA has no apparent effect on *rsmC* (20; also see below). Since the expression of *rsmC* is positively regulated by FlhDC and the FlhDC$^-$ mutant (AC5140) is *rsmC*$^+$, *rsmC* expression in the presence of

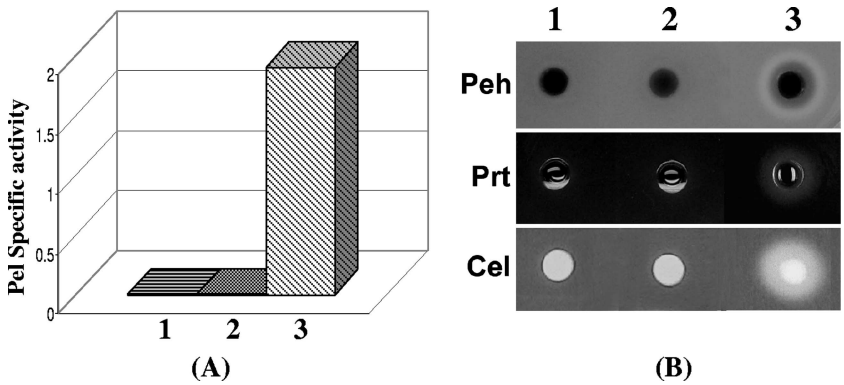

FIG. 6. Restoration of extracellular enzyme production by *gacA* in an FlhDC$^-$ GacA$^-$ double mutant, AC5143. (A and B) Pel activities (A) and agarose plate assays of Peh, Prt, and Cel activities (B) in AC5143 carrying the cloning vector pCL1920 (column 1), the *flhDC*$^+$ plasmid pAKC1242 (column 2), or the *gacA*$^+$ plasmid pAKC1245 (column 3).

flhDC$^+$ plasmid would be activated, leading to inhibition of *rsmB* expression. As expected, Northern blot results revealed that multiple copies of *flhDC* plasmid stimulate the transcript levels of *rsmC* in AC5140 (Fig. 5C, lane 1), whereas GacA has no effect on *rsmC* expression (Fig. 5C, lane 2). Consistent with the differential regulatory effects of GacA and FlhDC on *rsmB* expression are the findings that AC5140 carrying the *plac-gacA* plasmid pAKC1245 produces higher levels of *rsmB* RNA (Flg. 5C, lane 2) than AC5140 carrying the *plac-flhDC* plasmid pAKC1242 (Fig. 5C, lane 1).

HexA was previously determined to negatively control exoprotein production via its effect on *rsmB* RNA (56). To test whether FlhDC controls exoprotein production via its effect on HexA, we examined extracellular enzyme production in an FlhDC$^-$ HexA$^-$ mutant. The results revealed that the absence of HexA in FlhDC$^-$ background partially restored extracellular enzyme production (Fig. 5E). These results suggest that the FlhDC effect on exoprotein production is partly mediated through *hexA*.

FlhDC restores phenotypes in FlhDC$^-$ mutant but not in GacA$^-$ mutant. Extrapolating from the observations described above, we postulated that FlhDC controls *gacA* which, in turn, activates *rsmB* transcription to modulate exoprotein gene expression. This hypothesis places GacA below FlhDC in the regulatory network. If true, FlhDC should have no effect in GacA-deficient bacteria. To verify this possibility, we made FlhDC$^-$ GacA$^-$ double mutant AC5143 and transferred *plac-flhDC* plasmid pAKC1242 and the cloning vector pCL1920 into this double mutant. AC5143 carrying the *plac-gacA* plasmid pAKC1245 was used as a positive control. The *plac-flhDC* plasmid pAKC1242 did not restore extracellular enzyme production in the FlhDC$^-$ GacA$^-$ mutant (Fig. 6A, sample 2, and Fig. 6B, lane 2), whereas AC5143 carrying the *plac-gacA* plasmid pAKC1245 produced extracellular Pel, Peh, Prt, and Cel (Fig. 6A, sample 3, and Fig. 6B, lane 3). These results further prove that FlhDC is located at the top of the FlhDC-GacA-exoprotein regulatory pathway.

GacA and HexA control *rsmB* RNA production. The data presented above proved that the FlhDC controls extracellular protein production through cumulative effects on *hexA* and *gacA*. Previous studies have established that GacA positively regulates *rsmB* expression (20) and, on the other hand, HexA suppresses *rsmB* RNA production (56). Thus, we propose that in the FlhDC$^-$ mutant, low levels of GacA and high levels of HexA result in low levels *rsmB* RNA. *rsmB* RNA binds RsmA protein and the RsmA-*rsmB* RNA complex loses its ability to promote decay of target gene mRNAs. Although FlhDC has no direct effect on expression of *rsmA* (Fig. 4A), low levels of *rsmB* RNA presumably result in an increase in the pool of free RsmA which, in turn, causes decay of the mRNAs of exoprotein genes and consequently suppresses the exoprotein production. To test the hypothesis, we first established that *rsmB* expression driven by the *lac* promoter could restore exoprotein production in the FlhDC$^-$ mutant. We transferred the *plac-rsmB* pasmid pAKC1049 and the cloning vector into the FlhDC$^-$ mutant AC5141. These constructs were grown in minimal salts plus sucrose medium for extracellular enzyme assay. The assay results revealed that pAKC1049 restored the extracellular Pel, Peh, Prt, and Cel production in the FlhDC$^-$ mutant (Fig. 7A, sample 2, and Fig. 7B, lane 2).

The findings of mutant studies (Fig. 8) further demonstrate that FlhDC controls *rsmB* RNA levels through GacA and HexA. For example, the inactivation of *flhDC* or *gacA* severely reduces *rsmB* RNA levels (Fig. 8A, lane 2, and Fig. 8B, lane 1), indicating their positive regulation. In addition, multiple copies of *flhDC* plasmid restored *rsmB* transcript levels in an FlhDC$^-$ GacA$^+$ strain AC5140 (Fig. 8C, lane 3). In contrast, in the

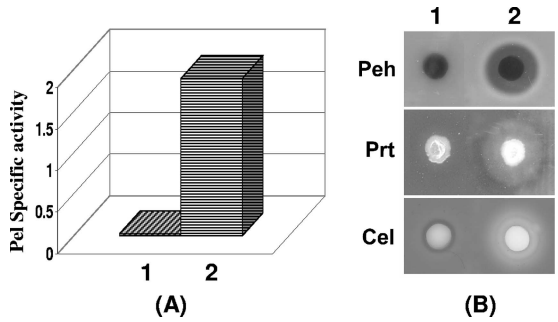

FIG. 7. Reversal of extracellular enzyme production of the FlhDC$^-$ mutant by *rsmB*$^+$ DNA. (A and B) Pel activities (A) and agarose plate assays of Peh, Prt, and Cel activities (B) in FlhDC$^-$ mutant AC5141 carrying pCL1920 (column 1) or pAKC1049 (*rsmB*$^+$) (column 2).

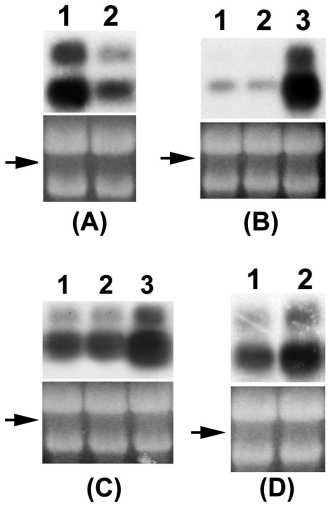

FIG. 8. FlhDC controls *rsmB* RNA via their effects on *gacA* and *hexA*. Northern blot analysis of *rsmB* RNA in wild-type strain Ecc71 (A1) and its FlhDC$^-$ mutant AC5140 (A2); in FlhDC$^-$ GacA$^-$ strain AC5143 carrying pCL1920 (B1), pAKC1242 (*flhDC*$^+$) (B2), or pAKC1245 (*gacA*$^+$) (B3); in FlhDC$^-$ GacA$^+$ strain AC5140 carrying pCL1920 (C1), pAKC1241 (*flhD*$^+$) (C2), or pAKC1242 (C3); and in FlhDC$^-$ HexA$^+$ strain AC5140 (D1) and FlhDC$^-$ HexA$^-$ strain AC5145 (D2). Each lane contained 10 μg of total RNA. The arrows show the levels of total RNA as revealed by ethidium bromide staining of denatured agarose gel.

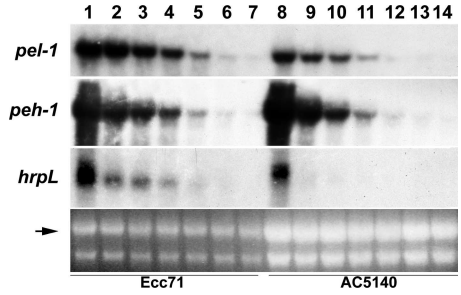

FIG. 9. Stabilities of *pel-1*, *peh-1*, and *hrpL* transcripts in Ecc71 (lanes 1 to 7) and its FlhDC$^-$ mutant AC5140 (lanes 8 to 14). Samples were collected at 0, 2.5, 5, 7.5, 10, 12.5, and 15 min after the addition of rifampin. For lanes 1 to 7, each lane contained 15 μg of total RNA, and for lanes 8 to 14, each lane contained 30 μg of total RNA. The arrow shows the levels of total RNA as revealed by ethidium bromide staining of denatured agarose gel.

FlhDC$^-$ GacA$^-$ mutant, FlhDC does not stimulate *rsmB* RNA production (Fig. 8B, lane 2), whereas GacA reverses the negative effect in this mutant (Fig. 8B, lane 3).

The inhibition of *rsmB* RNA production by HexA in the FlhDC$^+$ wild-type strain Ecc71 (56) could result in two ways. It could be mediated through the negative effect of HexA on *flhDC* expression; the latter is required for *rsmB* expression via GacA as documented above. LrhA, a HexA homolog negatively regulates expression of *flhDC* in *E. coli* (45). We also have observed a similar response in *E. carotovora* subsp. *carotovora* (data not shown).

However, we also considered the possibility that HexA may affect *rsmB* expression by a pathway different from FlhDC-GacA-RsmB. If so, *rsmB* RNA production would be elevated in a HexA$^-$ FlhDC$^-$ strain compared to a HexA$^+$ FlhDC$^-$ strain. The data in Fig. 8D reveal that indeed is the case. Thus, *rsmB* RNA production in *E. carotovora* subsp. *carotovora* is controlled by HexA via the FlhDC pathway, as well as by an FlhDC-independent pathway. We have initiated studies to clarify the events associated with the latter pathway.

The primary action of *rsmB* RNA is to bind RsmA and thereby neutralize its negative effects on gene expression. We therefore predicted that reduced levels of *rsmB* RNA in the FlhDC$^-$ mutant resulted in RsmA-promoted decay of transcripts of exoprotein genes. To test this possibility, we compared mRNA stabilities of *pel-1*, *peh-1*, and *hrpL* genes in Ecc71 and its FlhDC$^-$ mutant AC5140. The results (Fig. 9) clearly demonstrate that the mRNAs of these genes were more stable in the parent (lanes 1 to 7) than in the FlhDC$^-$ mutant (lanes 8 to 14). In addition, we constructed an FlhDC$^-$ RsmA$^-$ mutant (AC5144) by inactivating *rsmA* in AC5141 (FlhDC$^-$ RsmA$^+$) using mini-Tn*5*-Sp^r. This mutant was grown in minimal salts plus sucrose medium along with its parents and an FlhDC$^+$ RsmA$^-$ strain AC5070 for extracellular enzyme assay. The results revealed that RsmA deficiency in the FlhDC$^-$ mutant restored the enzyme production (Fig. 10A, sample 3, and Fig. 10B, lane 3). The levels of extracellular Pel, Peh, Prt, and Cel were higher than those of AC5006 (FlhDC$^+$ RsmA$^+$) (Fig. 10A, sample 1, and Fig. 10B, lane 1) and AC5141 (FlhDC$^-$ RsmA$^+$) (Fig. 10A, sample 2, and Fig. 10B, lane 2) but slightly lower than those of AC5070 (FlhDC$^+$ RsmA$^-$) (Fig. 10A, sample 5, and Fig. 10B, lane 5). Similar results were observed with transcript levels of *pel-1*, *peh-1*, and *celV* of these strains (Fig. 10C). The difference between the FlhDC$^-$ RsmA$^-$ mutant (AC5144) and the FlhDC$^+$ RsmA$^-$ mutant (AC5070) may be due to the levels of *rsmB* RNA. Northern blot analysis revealed that *rsmB* transcript levels of AC5144 (Fig. 10D, lane 2) were lower than that of AC5070 (Fig. 10D, lane 1). We have previously reported that overexpression of *rsmB* has some stimulatory effect in the RsmA$^-$ background (48). To further test this, we transferred a *plac-rsmB* plasmid pAKC1049Gm^r or the cloning vector into AC5144. The assay results showed that AC5144 (FlhDC$^-$ RsmA$^-$) carrying the *plac-rsmB* plasmid (Fig. 10E, sample 2, and Fig. 10F, lane 2) produced levels of extracellular enzymes comparable to those produced by the FlhDC$^+$ RsmA$^-$ strain AC5070 (Fig. 10E, sample 3, and Fig. 10F, lane 3); these levels were higher than those of AC5144 carrying the vector (Fig. 10E, sample 1, and Fig. 10F, lane 1).

FliA does not affect extracellular enzyme production in *E. carotovora* subsp. *carotovora*. FliA is a flagellar sigma factor that is required for the expression of class III flagellar genes. However, it has also been shown that FliA controls several extracellular enzymes such as lipase (XlpA) and protease (XrtA) in *Xenorhabdus nematophila* (60). To test the effect of FliA on extracellular enzymes of *E. carotovora* subsp. *carotovora*, we transferred a *plac-fliA* plasmid pAKC1246 into AC5006 and its FlhDC$^-$ mutant. Northern analysis showed that *fliA* in this plasmid was expressed in both AC5006 and the FlhDC$^-$ mutant (Fig. 11A, lanes 2 and 4). However, the enzyme assay results revealed that pAKC1246 had no effect on extracellular enzyme production in AC5006 (Fig. 11B, lanes 1 and 2), and it did not restore the extracellular enzyme levels in the FlhDC$^-$ mutant (Fig. 11B, lanes 3 and 4). These results collectively demonstrate that FliA is

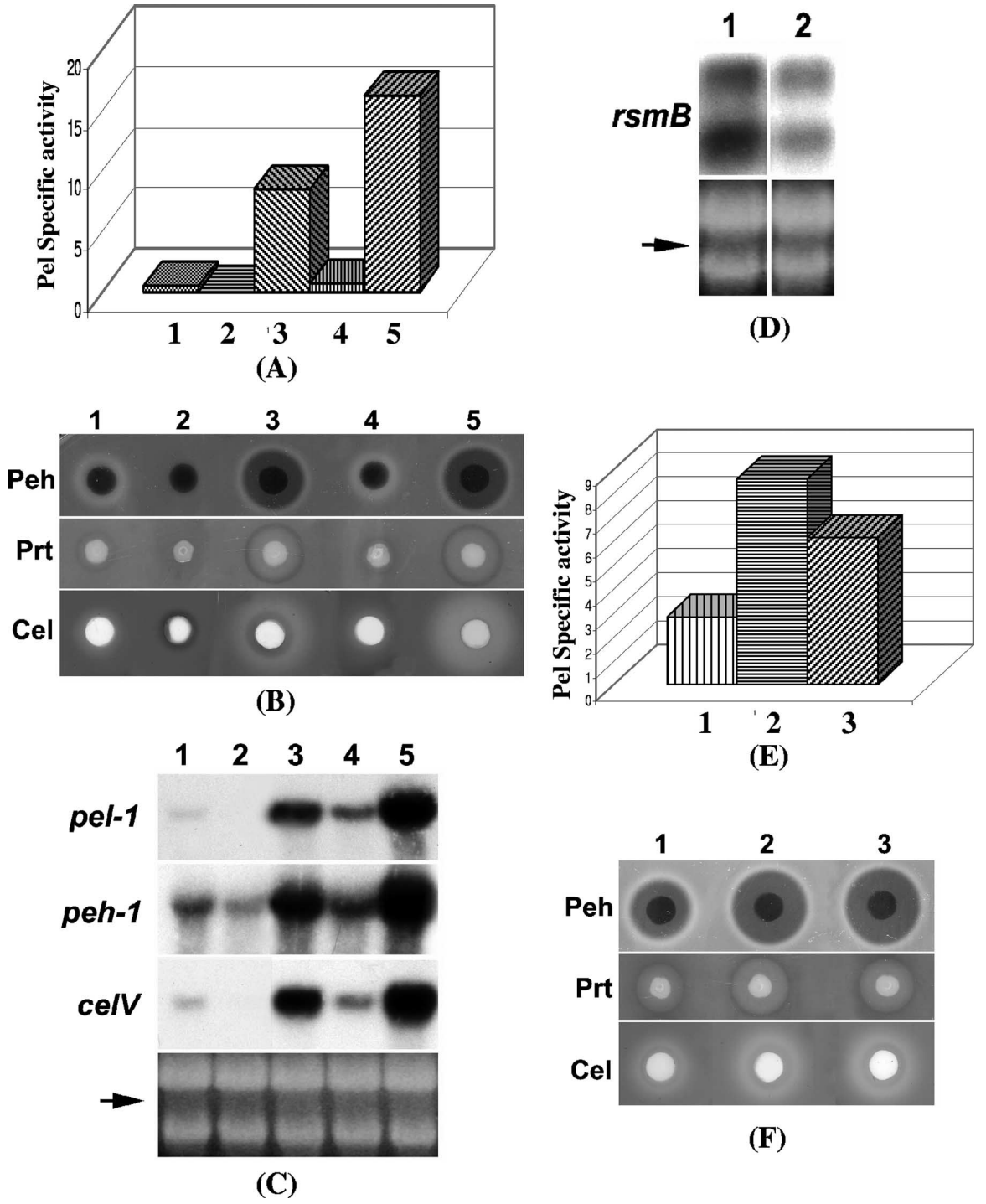

FIG. 10. RsmA is responsible for the pleiotropic phenotype resulting from FlhDC deficiency. (A to C) Pel activities (A), agarose plate assays of Peh, Prt, and Cel activities (B), and *pel-1*, *peh-1*, and *celV* transcripts (C) in FlhDC$^+$ RsmA$^+$ strain AC5006 (column 1), FlhDC$^-$ RsmA$^+$ strain AC5141 (column 2), FlhDC$^-$ RsmA$^-$ strain AC5144 (column 3), FlhDC$^+$ RsmA$^+$ strain AC5047 (column 4), and FlhDC$^+$ RsmA$^-$ strain AC5070 (column 5). (D) *rsmB* RNA levels in AC5070 (column 1) and AC5144 (column 2). (E and F) Pel, Peh, Prt, and Cel activities in AC5144 carrying the cloning vector pCL1920Gm^r (column 1) or the *rsmB*$^+$ plasmid pAKC1049Gm^r (column 2) and AC5070 carrying pCL1920Gm^r (column 3).

not required for extracellular enzyme/protein production in *E. carotovora* subsp. *carotovora*, and the effects of FlhDC on extracellular enzymes/proteins are not mediated through FliA. The consensus sequence for FliA binding has been identified as TAAAGTTT N11 GCCGATAA (17, 36, 38). The absence of these sequences in the promoter regions of *rsmA*, *rsmB*, or *hrpL* or exoprotein genes further supports that hypothesis.

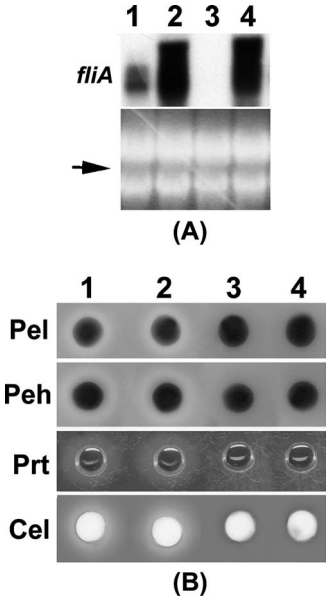

FIG. 11. FliA does not control exoprotein production in *E. carotovora* subsp. *carotovora*. (A) Northern blot analysis of *fliA* and (B) agarose plate assays of Pel, Peh, Prt, and Cel activities in AC5006 (FlhDC$^+$) carrying the cloning vector pCL1920 (column 1) or the *fliA*$^+$ plasmid pAKC1246 (column 2), as well as AC5141 (FlhDC$^-$) carrying pCL1920 (column 3) or pAKC1246 (column 4).

Conclusions. The findings presented here now provide us with a clearer picture of the regulatory network controlling exoprotein production in *E. carotovora* subsp. *carotovora*. As depicted in Fig. 1, we propose that FlhDC, one of the major regulatory components, is located at the top of the network. This master regulator controls the production of FliA, HexA, RsmC, and GacA. These four regulators, in turn, control genes required for bacterial locomotion, exoprotein production, or both. Based upon the available evidence, it is likely that FliA controls the genes for flagellum formation and bacterial movement. In contrast, HexA controls bacterial locomotion by regulating FliA production (34), most likely through its negative effect on FlhDC and exoprotein production by regulating *rsmB* RNA levels via FlhDC, as well as an FlhDC-independent pathway. GacA and RsmC actions are directed primarily toward *rsmB* RNA production (20, 25). The mechanisms underlying these two regulatory events are not yet known. Our working hypotheses postulates that phosphorylated GacA (GacA~P) directly activates transcription of the *rsmB* promoter and that RsmC interacts with another regulator, possibly FlhDC, to reduce the levels of transcription of *gacA* and other targets of FlhDC. A plausible teleological significance of FlhDC-mediated regulation of negative and positive regulators is apparent from the following: as a negative regulator, HexA controls many phenotypes, including bacterial movement, exoprotein production, and the quorum-sensing system (34, 56). On the other hand, FlhDC is a positive regulator of most of these traits. Under conditions that are not conducive to the expression of these traits, bacteria produce HexA, which represses not only these traits but also prevents *flhDC* expression that could potentially counteract the responses triggered by HexA. It is also significant that *hexA* expression is positively autoregulated (34), ensuring the maintenance of an adequate HexA pool size. Activation of *flhDC* expression by environmental conditions, as yet undefined in *E. carotovora* subsp. *carotovora*, would reduce HexA pool and increase GacA and FliA levels, resulting in the activation of genes for motility and *rsmB* RNA controlling exoprotein production. How RsmC fits into this model would become clear as we gain a better understanding of its target and its regulatory mechanism. A well-orchestrated regulation of *flhDC*, *hexA*, *gacA*, *fliA*, *rsmC*, and *rsmB* is apparently geared toward efficient and timely expression of environmentally significant traits under appropriate conditions.

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