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Transcriptional Hierarchy of *Aeromonas hydrophila* Polar-Flagellum Genes▽

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Aeromonas hydrophila **polar-flagellum class I gene transcription is σ^{70} dependent, which is consistent with the fact that the *A. hydrophila* polar flagellum is constitutively expressed. In contrast to other bacteria with dual flagellar systems such as *Vibrio parahaemolyticus*, the *A. hydrophila* LafK protein does not compensate for the lack of the polar-flagellum regulator FlrA (*V. parahaemolyticus* FlaK homologue). This is consistent with the fact that the *A. hydrophila* FlrA mutation abolishes polar-flagellum formation in liquid and on solid surfaces but does not affect inducible lateral-flagellum formation. The results highlight that the polar- and lateral-flagellum interconnections and control networks are specific and that there are differences between the dual flagellar systems in *A. hydrophila* and *V. parahaemolyticus*. Furthermore, our results indicate that the *A. hydrophila* polar-flagellum transcriptional hierarchy (also in class II, III, and IV genes) shares some similarities with but has many important differences from the transcriptional hierarchies of *Vibrio cholerae* and *Pseudomonas aeruginosa*. The *A. hydrophila flhF* and *flhG* genes are essential for the assembly of a functional polar flagellum because in-frame mutants fail to swim in liquid medium and lack the polar flagellum. In *Vibrio* and *Pseudomonas flhG* disruption increases the number of polar flagella per cell, and *Pseudomonas flhF* disruption gives an aberrant placement of flagellum. Here, we propose the gene transcriptional hierarchy for the *A. hydrophila* polar flagellum.**

Flagellum motility represents an important advantage for bacteria in moving toward favorable conditions or in avoiding detrimental environments, and it allows flagellated bacteria to successfully compete with other microorganisms (14). Flagellum morphogenesis is a complex cascade of events that requires coordinate expression of more than 50 genes encoding structural subunits, regulatory proteins, and chemo-sensor machinery. These genes have been categorized in relation to their temporal requirement during the assembly process into three groups: early, middle, and late genes (1, 10). Early genes encode regulatory proteins that control the expression of the entire regulon. Middle genes include structural components of the hook, the basal body, the export apparatus, and regulatory proteins that couple late-gene expression; and late genes include the filament, motor force generators, and chemotactic proteins. The expression of these genes is an energetically expensive process for the bacterium, and all flagellar systems are highly regulated. Regulation of flagellum biogenesis involves a combination of transcriptional, translational, and post-translational mechanisms (1). In relation to their transcriptional hierarchy, the flagellar clusters of different bacterial species are transcribed from three different promoter classes, whose differential expression is coordinated by the activity of transcriptional regulators which include alternative sigma factors and anti-sigma factors (10, 25). The coordinated expression of these promoters cluster gene transcription in three or four levels of hierarchy: classes I to III or I to IV. In peritrichous flagellated bacteria, such as *Escherichia coli* and *Salmonella*, three levels of hierarchy have been described (22). Transcription of class I and II genes requires the housekeeping sigma factor 70 (σ^{70}). The single class I promoter responds to a number of global regulatory factors (23) and transcribes the genes for the FlhDC master activator, required for expression of all class II σ^{70}-dependent promoters (43). A class II promoter transcribes the gene for the sigma factor 28 (σ^{28}), which directs transcription of class III genes (31). Class III promoters are negatively regulated by the anti-sigma factor FlgM (9). However, inducible peritrichous flagella (lateral flagella) of *Vibrio parahaemolyticus* and *Aeromonas hydrophila* do not posses an FlhDC master regulator and are sigma factor 54 (σ^{54}) dependent (8, 39). Polar flagellated *Gammaproteobacteria*, such as *Vibrio* and *Pseudomonas*, show four transcriptional levels, where classes II and III are σ^{54} dependent and class IV is σ^{28} dependent (12, 33). At the top of the hierarchy is a σ^{54}-associated transcriptional activator (FlrA of *Vibrio cholerae* and FleQ of *Pseudomonas aeruginosa*) which activates class II σ^{54}-dependent promoters. Class II promoters encode a two-component signal-transducing system (FlrBC of *V. cholerae* and FleSR in *P. aeruginosa*) whose regulator (FlrC/FleR) activates class III σ^{54}-dependent promoters. Moreover, in *Vibrio* spp. class II promoters also encode the σ^{28} factor which activates transcription of class IV genes (11, 12, 33).

Mesophilic *Aeromonas* is a ubiquitous aquatic microorganism that constitutively expresses a single polar flagellum although about 60% of strains most commonly associated with

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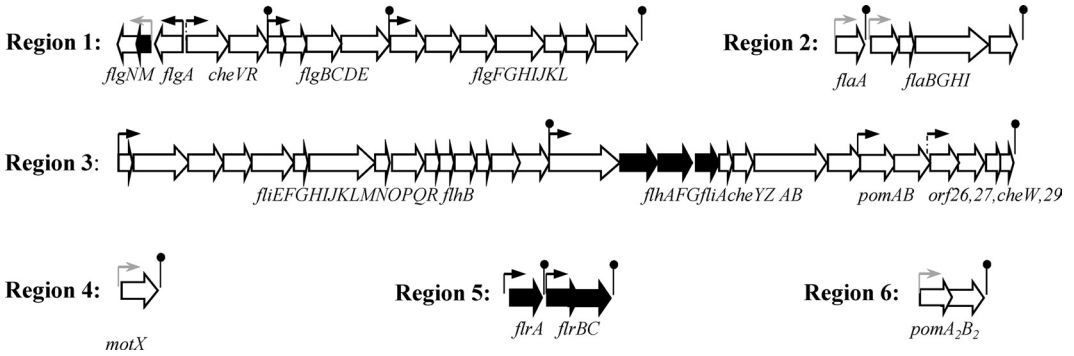


FIG. 1. Organization of *A. hydrophila* AH-3 polar-flagellum chromosomal regions. Arrows indicate the direction of transcription and the extent of coding sequence for each gene. Black arrows indicate regulatory genes. Small arrows indicate *in silico* predicted promoters (black, σ^{54} promoters; gray, σ^{28} promoters; dotted line, undetermined promoter). Lollipops indicate predicted rho-independent transcriptional terminators.

diarrhea (20) are also able to express many lateral flagella when grown in viscous environments or on surfaces (37). The *Aeromonas* polar-flagellum genes are organized in different clusters distributed in six chromosomal regions (Fig. 1). These genes encode structural, regulatory, and chemotaxis proteins, as well as enzymes involved in flagellin glycosylation (2, 7, 44). The regulatory genes are localized in region 1, 3, and 5 (2, 7). Region 1 contains the gene encoding the anti-σ^{28} transcription factor FlgM. Region 3 contains three regulatory genes: the σ^{28} transcription factor (*fliA*) and two genes found only in polar-flagellum systems, *flhF* and *flhG*, which encode proteins that play a regulatory role in *V. cholerae* and *P. aeruginosa* flagellum biosynthesis (11, 30). Region 5 encodes three proteins homologous to the FlrA transcriptional activator and the FlrBC two-component signal-transducing system of *V. cholerae*. In addition, outside the polar-flagellum regions is RpoN (σ^{54}) that is essential for polar- and lateral-flagellum system transcription (8). Given the critical role these genes play in regulating polar-flagellum expression in different bacterial species (11, 12, 30, 33), we investigated the aeromonad polar-flagellum transcriptional hierarchy by two techniques: measurement of the β-galactosidase activity of promoter-*lacZ* fusions in several mutant backgrounds and reverse transcription-PCR (RT-PCR) assays.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown on Luria-Bertani (LB) Miller broth and LB Miller agar at 37°C, while *Aeromonas* strains were grown in either tryptic soy broth (TSB) or agar (TSA) at 30°C. When required, ampicillin (50 μg/ml), kanamycin (50 μg/ml), rifampin (100 μg/ml), spectinomycin (50 μg/ml), chloramphenicol (25 μg/ml), gentamicin (10 μg/ml), and tetracycline (20 μg/ml) were added to the medium.

Motility assays (swarming and swimming). Freshly grown bacterial colonies were transferred with a sterile toothpick into the center of swarm agar (1% tryptone, 0.5% NaCl, 0.5% agar) or swim agar (1% tryptone, 0.5% NaCl, 0.25% agar). The plates were incubated face up for 16 to 24 h at 25°C, and motility was assessed by examining the migration of bacteria through the agar from the center toward the periphery of the plate. Moreover, swimming motility was assessed by light microscopy observations in liquid medium.

TEM. Bacterial suspensions were placed on Formvar-coated grids and negatively stained with a 2% solution of uranyl acetate, pH 4.1. Preparations were observed on a Hitachi 600 transmission electron microscope (TEM).

DNA techniques. DNA manipulations were carried out essentially according to standard procedures (35). DNA restriction endonucleases and *E. coli* DNA polymerase Klenow fragment were obtained from Promega. T4 DNA ligase and alkaline phosphatase were obtained from Invitrogen and GE Healthcare, respectively. PCR was performed using BioTaq DNA polymerase (Ecogen) in a Gene Amplifier PCR System 2400 thermal cycler (Perkin Elmer).

Nucleotide sequencing and computer sequence analysis. Plasmid DNA for sequencing was isolated by a Qiagen plasmid purification kit (Qiagen, Inc. Ltd.) as recommended by the suppliers. Double-strand DNA sequencing was performed by using the Sanger dideoxy-chain termination method (36) with a BigDye Terminator, version 3.1, cycle sequencing kit (Applied Biosystems). Custom-designed primers used for DNA sequencing were purchased from Sigma-Aldrich. The DNA sequences were inspected in the GenBank and EMBL databases at the National Center for Biotechnology Information (NCBI) (3). The Terminator search program in the GCG Wisconsin package was used to search for factor-independent transcriptional terminators. The Neural Network Promoter Prediction, PromScan (40), and PRODORIC (29) programs were used to search promoter sequences.

Total RNA extraction and RT-PCR. Total RNA was isolated, by means of an RNA Protect Bacteria Reagent (Qiagen) and an RNeasy Mini Kit (Qiagen), from *A. hydrophila* AH-3, and *flrA*, *flrBC*, *flhF*, *flhG*, and *fliA* mutants were grown in liquid (TSB) or solid agar (TSA) medium. To ensure that RNA was devoid of contaminating DNA, the preparation was treated with RNase-free Turbo DNase I (Ambion). First-strand cDNA synthesis was carried out with Moloney murine leukemia virus (M-MuLV) reverse transcriptase (New England BioLabs) and random oligonucleotides (Promega) on 5 μg of total RNA, DNase digested. The reaction mixtures were incubated at 25°C for 10 min, 37°C for 120 min, and 75°C for 15 min. Control reactions lacking reverse transcriptase were performed to confirm that RNA samples were not contaminated with genomic DNA (RT negative controls). PCR, second-strand synthesis, and subsequent DNA amplification were carried out using the Accuprime TaqDNA polymerase (Invitrogen) and specific oligonucleotides. Amplicons were analyzed by agarose gel electrophoresis with ethidium bromide staining. *A. hydrophila* ribosomal 16S primers were used as a control for cDNA template. RT-PCR amplifications were performed at least twice, with total RNA preparations obtained from a minimum of two independent extractions.

Mapping the *A. hydrophila* AH-3 *flrA* and $pomA_2$ transcription start sites by 5′ RACE PCR. Amplification of the *A. hydrophila* AH-3 *flrA* and $pomA_2$ cDNA 5′ ends was performed using a 5′ RACE (random amplification of cDNA ends) System, version 2.0 (Invitrogen). Total RNA extraction from *A. hydrophila* AH-3 was performed as mentioned above. First-strand cDNA was synthesized using the entire volume of DNase-digested total RNA (5 μg), the *flrA* internal primer GSP1-FlrA (5′-GAGAGAGCTCGTGAAT-3′), or the $pomA_2$ internal primer GSP1-$PomA_2$ (5′-GCGCCATACAGAGTA-3′) and the Thermoscript RT-PCR system (Invitrogen) at 45°C for 45 min. Reverse transcriptase was deactivated at 85°C for 5 min, and 1 μl of RNase H was then added and incubated at 37°C for 20 min. Purification of cDNA with S.N.A.P. columns (Invitrogen Life Technologies), as well as tailing of purified cDNA using terminal deoxynucleotidyl transferase and dCTP, was done according to 5′ RACE System, version 2.0 instructions. Confirmation of cDNA was performed after each step by PCR with nested primers. Tailed cDNA was amplified by primary PCR using a 10 μM concentration of each primer, the 5′ RACE abridged anchor primer (AAP) that binds to the tailed cDNA sequence, and GSP2-FlrA (5′-CCTGACAGAAGTG CAGATG-3′) or GSP2-$PomA_2$ (5′-TTTCATGAAGGCATTTGG-3′) that binds to an internal gene sequence. The PCR program applied was 94°C for 1 min, followed by 35 cycles of 94°C for 45 s, 55°C for 30 s, and 72°C for 1 min, with a

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or description[a]	Reference or source
Strains		
A. hydrophila		
AH-3	*A. hydrophila* wild type, serogroup O:34	26
AH-405	AH-3, spontaneous Rif^r	2
AH-5502	AH-405 *rpoN*::Km^r	8
AH-4443	AH-405 *fliA*::Km^r	7
AH-3 *flrA*	AH-405; *flrA*::Km^r	This work
AH-3 *flrBC*	AH-405 *flrB*::pSF, Km^r	This work
AH-3 Δ*flhF*	AH-405 with in-frame Δ*flhF*	This work
AH-3 Δ*flhG*	AH-405 with in-frame Δ*flhG*	This work
E. coli		
DH5α	λ^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	16
MC1061λpir	*thi thr1 leu6 proA2 his4 argE2 lacY1 galK2 ara14 xyl5 supE44* λ *pir*	34
Plasmids		
pGEMT-easy	Cloning vector, Ap^r	Promega
pRK2073	Helper plasmid, Sp^r	34
pDM4	Suicide plasmid, *pir* dependent with *sacAB* genes, oriR6K, Cm^r	28
pDM-FlhF	pDM4 with Δ*flhF* fragment, Cm^r	This work
pDM-FlhG	pDM4 with Δ*flhG* fragment, Cm^r	This work
pDM-FlrAKm	pDM4 with AH-3 *flrA*::Km, Cm^r Km^r	This work
pFS100	pGP704 suicide plasmid, *pir* dependent, Km^r	34
pFS-FlrB	pFS100 with a AH-3 *flrB* internal fragment, Km^r	This work
pBAD33	Arabinose-induced expression vector, ori_{p15} P_{BAD} Cm^r	15
pBAD33-FLHF	pBAD33 with AH-3 *flhF* gene, Cm^r	This work
pBAD33-FLHG	pBAD33 with AH-3 *flhG* gene, Cm^r	This work
pBAD33-Gm	pBAD33 arabinose-induced expression vector with Gm^r	
pBAD33Gm-FLIA	pBAD33 with AH-3 *fliA* gene, Gm^r	This work
pBAD33Gm-FLRA	pBAD33 with AH-3 *flrA* gene, Gm^r	This work
pACYC184	Plasmid vector; Cm^r Tc^r	35
pACYC-RPON	pACYC184 with AH-3 *rpoN* gene, Tc^r	8
pACYC-FLR1	pACYC184 with AH-3 *flrBC* genes, Tc^r	7
pDN19 lacΩ	Promoterless *lacZ* fusion vector; Sp^r Sm^r Tc^r	42
pDNlac-*flaA*p	*flaA* promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*flaB*p	*flaB* promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*flgA*p	*flgA* promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*flgB*p	*flgB* promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*flgF*p	*flgF* promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*flgM*p	*flgM* promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*flhA*p	*flhA* promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*fliE*p	*fliE* promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*flrA*p	*flrA* promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*flrB*p	*flrB* promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*lafK*p	*lafK* promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*motX*p	*motX* promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*pomA*p	*pomA* promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-$pomA_2$p	$pomA_2$ promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work

[a] Tc^r, tetracycline resistant; Km^r, kanamycin resistant; Ap^r, ampicillin resistant; Rif^r, rifampin resistant; Cm^r, chloramphenicol resistant; Sp^r, spectinomycin resistant; Sm^r, streptomycin resistant; Gm^r, gentamicin resistant.

final extension at 72°C for 5 min. Nested PCR was performed for $pomA_2$ transcription start site amplification using the primary PCR product diluted 1:100 as a template and 10 mM (each) nested primer abridged by a universal amplification primer (AUAP) and GSP3-$PomA_2$ (5′-GGCATTTGGCACTTCG-3′). The PCR program applied was 94°C for 1 min, followed by 35 cycles of 94°C for 45 s, 55°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products were analyzed by agarose gel electrophoresis, and amplified bands were excised from the gel, purified, and sequenced with GSP2-FlrA or GSP3-$PomA_2$ primer.

Construction of defined mutants. The chromosomal in-frame *flhF* and *flhG* deletion mutants, *A. hydrophila* AH-3 Δ*flhF* and AH-3 Δ*flhG*, respectively, were constructed by allelic exchange as described by Milton et al. (28). Briefly, DNA regions upstream (fragment AB) and downstream (fragment CD) of the *flhF* and *flhG* genes were independently amplified using two sets of asymmetric PCRs for each gene to amplify DNA fragments of 807 (FlhF-AB) and 570 (FlhF-CD) bp for the *flhF* in-frame deletion and of 641 (FlhG-AB) and 576 (FlhG-CD) bp for the *flhG* in-frame deletion. DNA fragment FlhF-AB contains 687 bp upstream of *flhF* and the first 39 codons of *flhF*. DNA fragment FlhF-CD contains from the first base in codon 448 of *flhF* to 496 bp downstream of *flhF*. DNA fragment FlhG-AB contains 583 bp upstream *flhG* and the first 18 codons of *flhG*. DNA fragment FlhG-CD contains from the first base in codon 270 of *flhG* to 502 bp downstream of *flhG*. DNA fragments AB and CD of each gene were annealed at the overlapping regions provided by the primers B and C and amplified as a single fragment using primers A and D (Table 2). The fusion products were purified, BglII digested (the BglII site is present in primers A and D), ligated into BglII-digested and phosphatase-treated pDM4 vector (27), electroporated into *E. coli* MC1061 (λ*pir*), and plated on chloramphenicol plates at 30°C to obtain pDM-FlhF and pDM-FlhG plasmids. Plasmids with mutated genes were trans-

TABLE 2. Primers used in the construction of chromosomal in-frame *flhF* and *flhG* deletion mutant

Target and primer	Primer sequence[a]	Amplified fragment
flhF mutant		
A primer	5′-**AGATCT**CCCAGACCCTCGGTTATAC-3′	FlhF-AB
B primer	5′-CCCATCCACTAAACTTAAACATATCTCCACCCCACCTGTG-3′	
C primer	5′-TGTTTAAGTTTAGTGGATGGGGAGCGGGAAACAGAAGAAC-3′	FlhF-CD
D primer	5′-**AGATCT**GATGGAGGTAGGCTCATCG-3′	
flhG mutant		
A primer	5′-**AGATCT**CGGTTTGCCATGAAGTATG-3′	FlhG-AB
B primer	5′-CCCATCCACTAAACTTAAACAGTTTTGACGCATTTTGCG-3′	
C primer	5′-TGTTTAAGTTTAGTGGATGGGGGCGGTCATCTCGAATTTT-3′	FlhG-CD
D primer	5′-**AGATCT**GTTCGTCAAACCCGCTGT-3′	

[a] Underlining indicates overlapping regions. BglII restriction sites are shown in boldface.

ferred into an *A. hydrophila* AH-405 rifampin-resistant (Rif^r) strain by triparental matings using the *E. coli* MC1061 (λ*pir*) containing the insertion constructs and the mobilizing strain HB101/pRK2073. Transconjugants were selected on plates containing chloramphenicol and rifampin. PCR analysis confirmed that the vector had integrated correctly into the chromosomal DNA. After sucrose treatment, transconjugants that were rifampin resistant (Rif^r) and chloramphenicol sensitive (Cm^s) were chosen and confirmed by PCR.

To obtain the *A. hydrophila* AH-3 *flrA* mutant, the *flrA* gene was amplified by PCR with 5′-GCTCTAGATTGTCCTCGTTGCGATG-3′ and 5′-GCTCTAGACCTTGAACAAAGGCGTCA-3′ (XbaI sites are underlined), ligated into the vector pGEM-T Easy (Promega), and transformed into *E. coli* DH5α (16). The Tn*5*-derived kanamycin resistance cartridge (*nptII*) from pUC4-KIXX was obtained by SmaI digestion (5), and the cassette was inserted into the SmaI restriction internal site of *flrA*. The presence of a single BglII site in the SmaI-digested cassette allowed its orientation to be determined. Constructs containing the mutated genes were ligated into the XbaI-digested and phosphatase-treated pDM4 suicide vector (28), electroporated into *E. coli* MC1061 (λ*pir*), and plated on chloramphenicol and kanamycin plates at 30°C to obtain the pDM-FlrAKm plasmid. Introduction of pDM-FlrAKm plasmid into *A. hydrophila* AH-405 was performed as previously described, and transconjugants were selected on plates containing chloramphenicol, kanamycin, and rifampin. PCR analysis confirmed that the vector had integrated correctly into the chromosomal DNA. After sucrose treatment, transconjugants that were Rif^r, kanamycin resistant (Km^r), and Cm^s were chosen and confirmed by PCR.

To obtain the *A. hydrophila* AH-3 *flrBC* mutant, we used a single defined insertion in the *flrB* gene by a method based on suicide plasmid pFS100 (34). Briefly, an internal fragment of *flrB* was amplified by PCR (5′-CTGACCGAA ACCCGCAAAC-3′ and 5′-GAACGACAGGGTAAAGCAG-3′), ligated into pGEM-T Easy (Promega), and transformed into *E. coli* DH5α (16). The DNA insert was recovered by EcoRI restriction digestion and was ligated into EcoRI-digested and phosphatase-treated pFS100 (pFS-FlrB). Ligation was transformed into *E. coli* MC1061 (λ*pir*) and selected for kanamycin resistance (Km^r). Triparental mating with the mobilizing strain HB101/pRK2073 was used to transfer recombinant plasmid into the *A. hydrophila* AH-405 rifampin-resistant (Rif^r) strain to obtain defined insertion mutants, selecting for Rif^r and Km^r.

Plasmid constructions. Plasmid pBAD33-FLHF and pBAD33-FLHG containing the complete *flhF* and *flhG* genes of *A. hydrophila* AH-3, respectively, under the arabinose promoter (P_{BAD}) on pBAD33 (15) and plasmids pBAD33Gm-FLIA and pBAD33Gm-FLRA containing the complete *fliA* and *flrA* genes of *A. hydrophila* AH-3, respectively, under the arabinose promoter (P_{BAD}) on pBAD33-Gm (17) were obtained. Oligonucleotides 5′-TCC**CCCGGG**TGCCTG ATGACAAGCAA-3′ and 5′-GCTCTAGACAGGACATGGGAGAGGTTG-3′ generated a band of 1,723 bp containing the *flhF* gene, and oligonucleotides 5′-*GATATC*GAGCGGGAAACAGAAGAAC-3′ and 5′-GCTCTAGACCTCG GTATCACGAGCAT-3′ generated a band of 1,303 bp (the SmaI site is in boldface, the XbaI site is underlined, and the EcoRV site is in italics) containing the *flhG* gene. The amplified bands were digested with SmaI and XbaI or with EcoRV and XbaI and ligated into SmaI- and XbaI-digested pBAD33 vector to construct the pBAD33-FLHF and pBAD33-FLHG recombinant plasmids. Oligonucleotides 5′-GGAATTCCGGTCATCTCGAATTTTTCC-3′ and 5′-TCC**C CCGGG**TTGCGGATTATGCCTTAGAG-3′ generate a band of 840 bp containing the *fliA* gene, and oligonucleotides 5′-GGAATTCCGTGGCTAGACCACAG ATC-3′ and 5′-CCC*AAGCTT*CTGGCTATTGGGTCAGGTT-3′ generate a band of 1,580 bp containing the *flrA* gene (the EcoRI site is underlined, the SmaI site is in boldface, and the HindIII site is in italics). The amplified bands were digested with EcoRI and SmaI or EcoRI and HindIII and ligated into EcoRI- and SmaI-digested or EcoRI- and HindIII-digested pBAD33-Gm vector (17) to construct the pBAD33Gm-FLIA and pBAD33Gm-FLRA recombinant plasmids. Plasmids were independently introduced into the *E. coli* DH5α (16) and sequenced.

Construction of flagellar promoter-*lacZ* fusions. Oligonucleotide primer pairs for the *A. hydrophila* AH-3 promoter regions of the *flaA*, *flaB*, *flgB*, *flgF*, *flgM*, *flhA*, *fliE*, *flrA*, *flrB*, *pomA*, *motX*, $pomA_2$, and *lafK* genes (2, 7, 8, 44) are listed in Table 3. Primers were designed to amplify a fragment of 216 to 1,732 bp that encompassed regions both upstream and downstream of the predicted start codon. Restriction sites were added to some primers for cloning purposes. Promoter fragments were PCR amplified from *A. hydrophila* AH-3 genomic DNA, ligated into pGEM-T Easy (Promega), and transformed into *E. coli* DH5α (16). DNA inserts containing *flaA*, *flgM*, *flgB*, *fliE*, *flrA*, *motX*, *pomA*, and $pomA_2$ promoters were recovered by EcoRI/BamHI restriction digestion; inserts containing *flaB*, *flgF*, *flhA*, and *flrB* promoters were recovered by EcoRI/BglII restriction digestion; and the insert containing the *flgA* promoter was recovered by NotI-blunt ended/BamHI restriction digestion. The BamHI restriction site in the *flrA* insert is 86 bp downstream from the *flrA* start codon, and the BglII restriction sites in the *flaB* and *flrB* inserts are 146 and 223 bp downstream from the *flaB*

TABLE 3. Primers used for flagellar promoter-*lacZ* fusion constructions

Promoter	Primer sequence[a]	Site[b]
*flaA*p	5′-AATGGCTGCCTGCAAAAG-3′	−357 *flaA*
	5′-CGGGATCCCAGGCGGGTGTAGGAAGTA-3′	+96 *flaA*
*flaB*p	5′-GCTGCATCGACCATACTGT-3′	−650 *flaB*
	5′-TCCGCAGTCTGAGCAACA-3′	+235 *flaB*
*flgA*p	5′-AAGAATCGTCTGCCACCAG-3′	−705 *flgA*
	5′-CGGGATCCGTGAGAAATACCGCAAAA-3′	+52 *flgA*
*flgB*p	5′-CTTTCGCCCTTGATGACTC-3′	−781 *flgB*
	5′-CGGGATCCAACACTCAGCGCGTATTGA-3′	+53 *flgB*
*flgF*p	5′-AAGATGATCACGCCGACTAC-3′	−679 *flgF*
	5′-GGA**AGATCT**ATGCTGCAGGTTCTGACC-3′	+203 *flgF*
*flgM*p	5′-TGACTATCTCAGCGATCCG-3′	−360 *flgM*
	5′-CGGGATCCGAGGTCGCTGGTTTGGTAT-3′	+89 *flgM*
*flhA*p	5′-AGCTCAAGATGACCAAGCAG-3′	−586 *flhA*
	5′-GGA**AGATCT**GAAAGCGCAATATTGAAGGA-3′	+160 *flhA*
*fliE*p	5′-TTGTCGCATGGTACTGCTC-3′	−1010 *fliE*
	5′-CGGGATCCGACCATCTTGTTACGCACC-3′	+281 *fliE*
*flrA*p	5′-CCCTCTGTTGCTCGATTG-3′	−579 *flrA*
	5′-CCAGCTCGTTCTCGACTATC-3′	+120 *flrA*
*flrB*p	5′-TACTATCGCCTCAATGTCTTCC-3′	−670 *flrB*
	5′-TCATCTTCCACGACCAGAAT-3′	+1062 *flrB*
*lafK*p	5′-GGGCAAGTTGGGCCAATAT-3′	−997 *lafK*
	5′-CGGGATCCTTTCCATTTGATAACGCAGG-3′	+98 *lafK*
*motX*p	5′-GCCACTCTGAAAGCCGATA-3′	−172 *motX*
	5′-CGGGATCCGTGGTCAGCAAACAAGCAA-3′	+44 *motX*
*pomA*p	5′-CGGGTCAAGGAAATATCGC-3′	−746 *pomA*
	5′-CGGGATCCAGGATCAGGCCAAACAT-3′	+24 *pomA*
$pomA_2$p	5′-ATGGTTTCCAGCTCTTCCA-3′	−283 $pomA_2$
	5′-CGGGATCCCAGCACTATGCCAAT-3′	+37 $pomA_2$

[a] BamHI restriction sites are underlined; BglII restriction sites are in boldface.
[b] Nucleotide position from the gene start codon.

and *flrB* start codons, respectively. The EcoRI or NotI restriction site comes from the pGEM-T Easy plasmid. Digested fragments were ligated into plasmid pDN19lacΩ EcoRI/BamHI-digested or EcoRI-blunt ended/BamHI (42), transformed into *E. coli* DH5α (16), and selected for tetracycline resistance (Tc^r). The final constructs were confirmed by DNA-sequencing.

β-Galactosidase assays. The promoter-*lacZ* fusion plasmids described above were introduced into several *A. hydrophila* strains (Table 1). The cultures were grown in TSB medium at 25°C to an optical density of 0.4 to 0.8 at 600 nm. Bacterial cells were permeabilized with chloroform and sodium dodecyl sulfate (SDS) overnight and assayed for β-galactosidase activity as described by Miller (27). All experiments were performed at least three separate times.

Statistical analysis. The data obtained for the β-galactosidase assays were analyzed by the *t* test using Microsoft Excel software.

RESULTS

The polar-flagellum σ^{28} factor (FliA) does not control $pomA_2B[r]_2$ stator motor transcription. Transcription of the *V. parahaemolyticus* and *P. aeruginosa* polar-flagellum late genes (class IV) are σ^{28} dependent (11, 12, 33). In *A. hydrophila* mutation of FliA abolishes polar-flagellum formation (7), and both polar flagella and swimming motility were restored by complementation with the pBAD33Gm-FLIA plasmid in the presence of 0.2% L-arabinose. *In silico* sequence analysis of the *A. hydrophila* AH-3 polar-flagellum regions show putative σ^{28} promoter sequences upstream of *flaA*, *flaB*, *flgM*, *motX* (7), and $pomA_2$ (44), which is redundant to *pomA* located in polar-flagellum region 3. In order to study whether any of these *A. hydrophila* promoters are σ^{28} dependent, we independently transferred the promoter-*lacZ* fusion plasmids pDNlac-*flaA*p (*flaA*p-*lacZ*), pDNlac-*flaB*p (*flaB*p-*lacZ*), pDNlac-*flgM*p (*flgM*p-*lacZ*), pDNlac-*motX*p (*motX*p-*lacZ*), and pDNlac-$pomA_2$p ($pomA_2$p-*lacZ*) into *A. hydrophila* AH-405 (AH-3 rifampin-resistant) and the *fliA* mutant (AH-4443) by triparental mating. Transconjugants that were Rif^r Tc^r or Rif^r Km^r Tc^r were chosen, and β-galactosidase activity was measured. Transcription from the *flaA*, *flaB*, *flgM*, and *motX* promoters in the *fliA* mutant background shows a 97, 95, 87, and 93% reduction in activity, respectively, in comparison to the *A. hydrophila* AH-405 value. However, $pomA_2$ expression exhibited comparable values in both strains (Fig. 2A). Furthermore, total RNA from *A. hydrophila* AH-3 and the *fliA* mutant was used to amplify internal fragments of *flaA*, *flaB*, *flgM*, *motX*, and $pomA_2$ transcripts, but no *flaA*, *flaB*, *flgM*, and *motX* amplicons were obtained in the *fliA* mutant, whereas an amplicon was observed for $pomA_2$ (data not shown). These results suggest that *flaA*, *flaB*, *flgM*, and *motX* transcription is σ^{28} dependent but that $pomA_2$ transcription is not.

A. hydrophila AH-3 encodes an alternative σ^{54} sigma factor (RpoN) which is essential for both polar- and lateral-flagellum expression (8). We analyzed the possibility that $pomA_2$ could be transcribed from a σ^{54}-dependent promoter. The pDNlac-$pomA_2$p plasmid was transferred into the *A. hydrophila rpoN* mutant (AH-5502), and β-galactosidase activity was measured. Similar activity was detected in the *rpoN* mutant and the wild type (Fig. 2B).

To identify the $pomA_2$ promoter region, amplification of the *A. hydrophila* AH-3 $pomA_2$ cDNA 5′ end was performed using 5′ RACE as described in Materials and Methods. Primary PCR of tailed cDNA using primers AAP (abridged anchor primer) and GSP2-$PomA_2$ give two very faint bands, but nested PCR using primers AUAP (abridged universal amplification primer) and GSP3-$PomA_2$ showed two DNA bands of approximately 350 and 400 bp (Fig. 3). The smaller band shows enhanced intensity compared to the larger band on the agarose gel, suggesting that $pomA_2B[r]_2$ is more actively transcribed from the promoter region closer to the $pomA_2$ start codon. DNA sequences of the amplified bands indicate that both were tailed with G residues, and therefore $pomA_2B[r]_2$ is transcribed from two promoter regions. The $pomA_2$ transcription starts were located −41 nucleotides (nt) and −107 nt upstream from the $pomA_2$ translation start site. DNA sequence upstream of the −41-nt transcription start contains a σ^{70} promoter sequence (TTGCCG-N14-GCAGAAAAT), and sequence upstream of the −107-nt transcription start contains a σ^{28} promoter sequence (TAAA-N14-GCCGATAA) (Fig. 3). Using the same techniques, we were able to identify a σ^{70} promoter sequence and upstream of it a σ^{28} promoter sequence in strains *A. hydrophila* ATCC 7966^T and *A. caviae* Sch3N (data not shown).

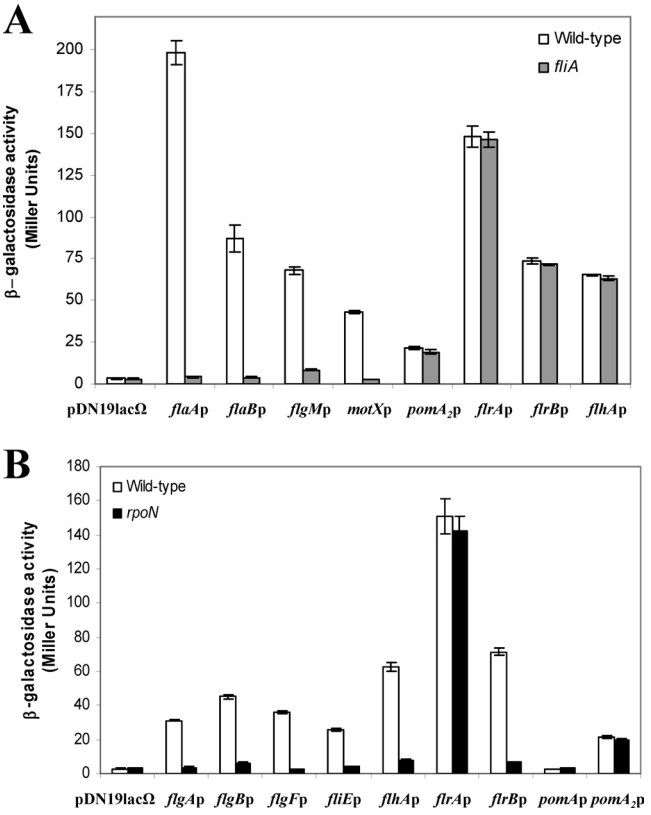
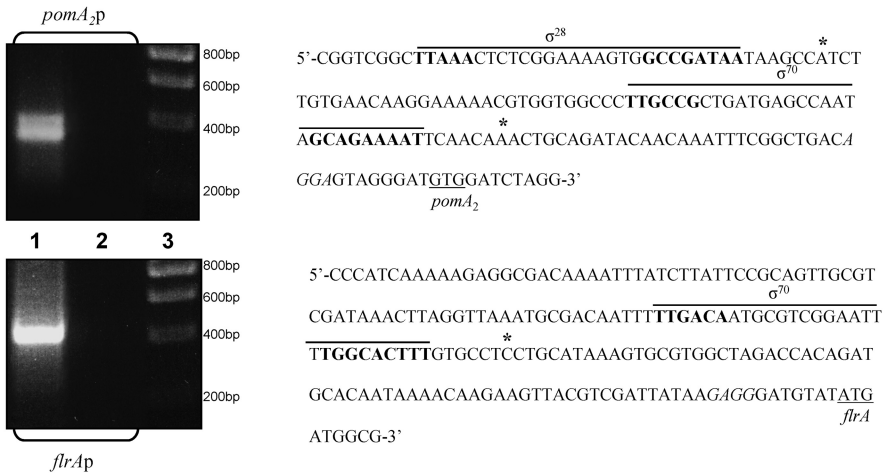


FIG. 2. Analysis of β-galactosidase activity. (A) pDNlac-*flaA*p, pDNlac-*flaB*p, pDNlac-*flgM*p, pDNlac-*motX*p, pDNlac-$pomA_2$p, pDNlac-*flrA*p, pDNlac-*flrB*p, and pDNlac-*flhA*p plasmids in *A. hydrophila* wild-type (AH-405) and *fliA* mutant (AH-4443) after growth in TSB at 25°C. (B) pDNlac-*flgA*p, pDNlac-*flgB*p, pDNlac-*flgF*p, pDNlac-*fliE*p, pDNlac-*flhA*p, pDNlac-*flrA*p, pDNlac-*flrB*p, pDNlac-*pomA*, and pDNlac-$pomA_2$p plasmids in *A. hydrophila* wild type (AH-405) and *rpoN* mutant (AH-5502) after growth in TSB at 25°C. As a control we also measured the pDN19lacΩ promoterless plasmid. The results shown are representative of three independent experiments. Bars represent standard deviations.

RpoN is not involved in *flrA* transcription in *A. hydrophila* AH-3. RpoN is involved in *A. hydrophila* polar- and lateral-flagellum formation (8), and *in silico* analysis of *A. hydrophila*

A



B

Gene	Promoter sequence determined *in silico*	Distance upstream of start codon
flaA	**TTAA**GTCCTGCGAGAATGT**GCCGATAA**	147
flaB	**TAAA**GCTTGTGTCCATGCG**GCCGTTAA**	178
flgM	**TAAA**GTTCTGACGACCGTC**ACCGATAA**	39
motX	**TAAG**GGGATGGGCCTGGCT**GCCGATAA**	25
σ^{28} consensus	**TAAA-** N_{15}**-GCCGATAA**	
flgA	T**TGGCAC**TAATC**ATGC**A	108
flgB	T**TGGCAC**ACCTC**TTGC**T	26
flgF	T**TGGCAC**TCAAA**TTGC**A	38
fliE	T**TGGCAC**TCTAA**TTGC**T	40
flhA	T**TGGCTT**GATGT**TTGC**T	40
flrB	A**TGGCAT**GGAAA**TTGA**A	35
σ^{54} consensus	T**GGCAC**-N_4-T**TTGC**A/T	

FIG. 3. (A) Amplification of the *A. hydrophila* AH-3 $pomA_2$ and *flrA* cDNA 5′ end performed using the 5′ RACE System, version 2.0 (Invitrogen). An amplicon was obtained by nested PCR using primers AUAP (abridged universal amplification primer) and GSP3-$PomA_2$ ($pomA_2$p) and by primary PCR using primers AAP (abridged anchor primer) and GSP2-FlrA (*flrA*p). Lanes 1, primary PCR template; lanes 2, PCR negative control; and lanes 3, molecular size standard (Ecogen). Underlined sequences show start codons, italics indicate the ribosome binding sites, asterisks show locations of the transcriptional start sites, and bold nucleotides show potential consensus sequences. (B) Alignment *in silico* of σ^{28} and σ^{54} promoter elements in *A. hydrophila* polar-flagellum promoters. The consensus σ^{28} sequence is from Kutsukake (22). The consensus σ^{54} sequence is from Barrios et al. (4).

AH-3 polar-flagellum regions shows putative σ^{54} promoter sequences upstream of *flgA*, *flgB*, *flgF*, *flhA*, *fliE*, *flrA*, *flrB*, and *pomA* genes (2, 7). Furthermore, transcription of *Vibrio* and *Pseudomonas* polar-flagellum class II and III genes, which include structural components of the hook, the basal body, the export apparatus, and regulatory proteins, is σ^{54} dependent (12, 33). Therefore, we investigated the effect of *rpoN* mutation on the transcription of different *Aeromonas* polar-flagellum promoters. β-Galactosidase activity of *A. hydrophila* wild type and the *rpoN* mutant (AH-5502) carrying the polar-flagellum gene promoter-*lacZ* fusion plasmids pDNlac-*flgA*p, pDNlac-*flgB*p, pDNlac-*flgF*p, pDNlac-*fliE*p, pDNlac-*flhA*p, pDNlac-*flrA*p, pDNlac-*flrB*p, and pDNlac-*pomA*p was measured. No significant β-galactosidase activity was detected from pDNlac-*flgA*p, pDNlac-*flgB*p, pDNlac-*flgF*p, pDNlac-*flhA*p, pDNlac-*fliE*p, and pDNlac-*flrB*p in the *rpoN* mutant background in comparison to the wild-type activity levels, suggesting that they are σ^{54}-dependent promoters (Fig. 2B). However, β-galactosidase activity from pDNlac-*flrA*p plasmid is not affected by loss of σ^{54} factor, as no reduction in activity in comparison to the wild type was observed (Fig. 2B). Surprisingly, no β-galactosidase activity was detected from the *in silico* predicted *pomA* promoter in either the wild type or the mutant strain tested. To determine whether *pomA* is *flhA* cotranscribed, total RNA from *A. hydrophila* AH-3 was subjected to RT-PCR using primer pairs which amplified the *flhA-flhF*, *flhF-fliA*, *fliA-cheZ*, *cheZ-cheA*, *cheA-cheB*, and *cheB-pomA* genes. Amplicons were obtained with all primer pairs used (data not shown), suggesting that *pomA* transcription is under the control of the *flhA* promoter.

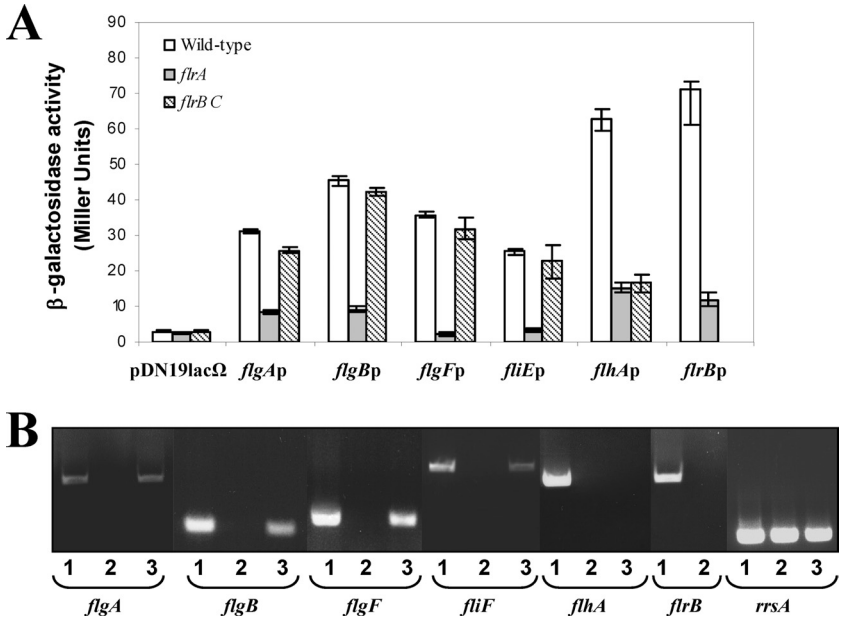


FIG. 4. (A) Analysis of β-galactosidase activity of pDNlac-*flgA*p, pDNlac-*flgB*p, pDNlac-*flgF*p, pDNlac-*fliE*p, pDNlac-*flhA*p, and pDNlac-*flrB*p plasmids in *A. hydrophila* wild-type (AH-405) and the AH-3 *flrA* or AH-3 *flrBC* mutant after growth in TSB at 25°C. As a control we also measured the pDN19lacΩ promoterless plasmid. The results shown are representative of three independent experiments. (B) RT-PCR amplification of *flgA*, *flgB*, *flgF*, *fliF*, *flhA*, and *flrB* from cDNA of the AH-3 (lanes 1) strain and the AH-3 *flrA* (lanes 2) and AH-3 *flrBC* (lanes 3) mutants. *A. hydrophila* ribosomal 16S (*rrsA*) amplification was used as a control for cDNA template. RT-PCR amplifications were performed at least twice, with total RNA preparations obtained from a minimum of two independent extractions.

Given that *flrA* transcription was σ^{54} independent, we performed 5′ RACE, as described in Materials and Methods, to further analyze the *flrA* promoter region. Primary PCR of tailed cDNA using primers AAP (abridged anchor primer) and GSP2-FlrA gave a unique band of approximately 400 bp (Fig. 3). DNA sequence of the amplified band indicates that it was tailed with G residues. The *flrA* transcription start was located −68 nt upstream from the *flrA* translation start site, and DNA sequence upstream of the transcription start contains a σ^{70} promoter sequence (TTGACA-N14-TGGCACTTT) (Fig. 3). Using the same techniques, we were able to identify a σ^{70} promoter sequence upstream of *flrA* in strains *A. hydrophila* ATCC 7966^T and *A. caviae* Sch3N (data not shown).

Identification of σ^{54} *A. hydrophila* promoters that are FlrA or FlrC dependent. Promoters recognized by the σ^{54} holoenzyme require specialized enhancer-binding proteins, which bind specific sequences located in a relatively remote position from the transcription start site (6). Two σ^{54}-dependent regulators are required to direct polar-flagellum class II and III gene transcription in *V. cholerae* and *P. aeruginosa* (12, 33). In *A. hydrophila* the mutation of *flrA* or *flrC*, which encodes σ^{54} enhancer-binding proteins homologous to *V. cholerae* FlrA/FlrC and *P. aeruginosa* FleQ/FleR (7), abolishes polar-flagellum formation, and the ability of the mutants to synthesize polar-flagellum formation and swimming motility was restored by complementation with the pBAD33Gm-FLRA plasmid in the presence of 0.2% L-arabinose and plasmid pACYC-FLR1 (7), respectively. To investigate which of the *A. hydrophila* σ^{54} polar-flagellum promoters are FlrA or FlrC dependent, β-galactosidase activity of the *A. hydrophila* wild type and the mutant strains AH-3 *flrA* and AH-3 *flrBC* carrying the promoter-*lacZ* fusion plasmids pDNlac-*flgA*p, pDNlac-*flgB*p, pDNlac-*flgF*p, pDNlac-*fliE*p, pDNlac-*flhA*p, and pDNlac-*flrB*p was measured. Transcription from *flgA*, *flgB*, *flgF*, *fliE*, and *flrB* promoters appeared to be affected by the *flrA* mutation, showing a 73, 79, 93, 86, and 83% reduction of β-galactosidase activity, respectively, in the AH-3 *flrA* strain compared to the wild type, and no significant variations were obtained in the AH-3 *flrBC* mutant (Fig. 4A). However, transcription from the *flhA* promoter showed β-galactosidase activity reduction in both mutant strains (76% in AH-3 *flrA* and 74% in AH-3 *flrBC*), suggesting that the *flhA* promoter is FlrC dependent. Furthermore, RT-PCRs to compare *flgA*, *flgB*, *flgF*, *fliF*, and *flhA* gene transcription levels in the wild-type as well as in the *flrA* and *flrBC* mutants produced *flgA*, *flgB*, *flgF*, and *fliF* amplicons in the wild type and the *flrB* mutant, whereas the *flhA* amplicon was found only in the wild type (Fig. 4B). Transcription of *flrB* was tested in the wild type and the *flrA* mutant, detected amplicons in the wild type only (Fig. 4B).

***A. hydrophila flhF* and *flhG* are involved in polar-flagellum biosynthesis and regulation.** It has been reported that FlhF and FlhG are unique to polar-flagellated bacteria and regulate the number and distribution of flagella in *Vibrio* and *Pseudomonas* (11, 21, 32). *A. hydrophila* contains within the polar-flagellum *flhA-cheW* gene cluster two genes whose encoded proteins showed 51% and 70% identity to FlhF and FlhG of *Vibrio* spp. (Fig. 1) (7). *A. hydrophila* AH-3 *flhF* and *flhG* in-frame mutants were constructed, as described in Materials and Methods, and transcription of the downstream gene *fliA* was investigated by RT-PCR (data not shown). Both in-frame mutants showed expression of *fliA*, which is located downstream of *flhG*, and they also exhibited growth in TSB similar

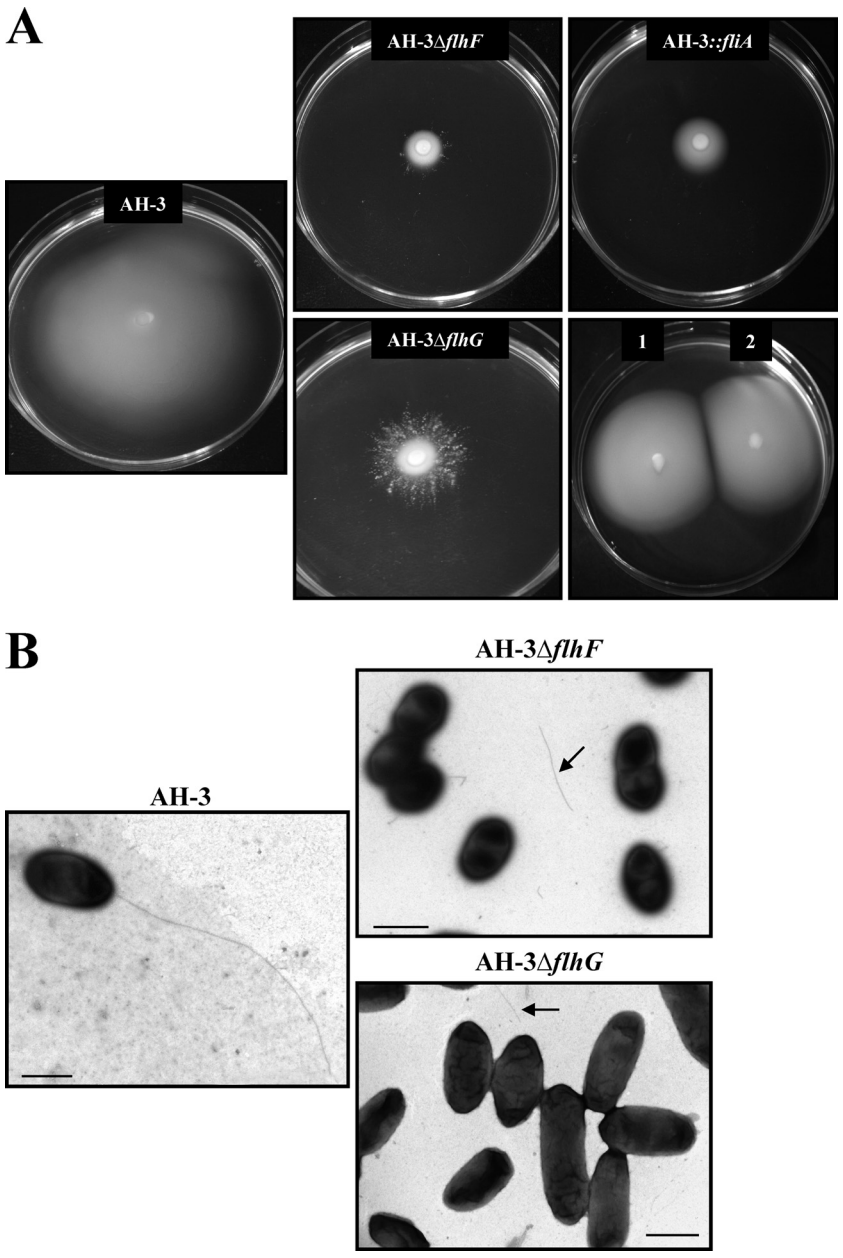


FIG. 5. Phenotypes of FlhF and FlhG mutants. (A) Swarming motility observed for *A. hydrophila* AH-3, AH-3 Δ*flhF*, AH-3 Δ*flhG*, and AH-3 *fliA* (AH-4443), as well as complemented AH-3 Δ*flhF* (1) and AH-3 Δ*flhG* (2) strains with the pBAD33-FLHF and pBAD33-FLHG plasmids, respectively, in the presence of 0.2% L-arabinose. (B) Transmission electron microscopy of *A. hydrophila* AH-3, AH-3 Δ*flhF*, and AH-3 Δ*flhG* grown at 25°C on liquid medium. Arrows show flagellum fragments. Bacteria were gently placed onto Formvar-coated copper grids and negatively stained using 2% uranyl acetate. Scale bar, 1 μm.

to that of the wild type. Unlike the wild type, both mutants failed to swim in liquid medium, as determined by light microscopy observation, and they also showed a reduced spread in semisolid agar plates, similar to that observed for polar-flagellum mutants such as *flaH*, *flhA*, or *fliA* (7). However, the spread of these two mutants in semisolid plates did not constitute homogeneous concentric rings, especially in the *flhG* mutant, but clotted and branching rings were observed (Fig. 5). Electron microscopic observations of the *flhF* and *flhG* mutants grown in liquid medium revealed that both mutants lacked a polar flagellum although approximately 10% of bacteria showed flagellum fragments on their surface, and some flagellum structures were observed in the medium (Fig. 5). Swimming motility was restored by complementation of the *flhF* and *flhG* mutants with the pBAD33-FLHF and pBAD33-FLHG plasmids, respectively, in the presence of 0.2% L-arabinose (Fig. 5). These results indicate that *flhF* and *flhG* are required for *A. hydrophila* assembly of a functional polar flagellum.

To determine whether *A. hydrophila* FlhF and FlhG affect

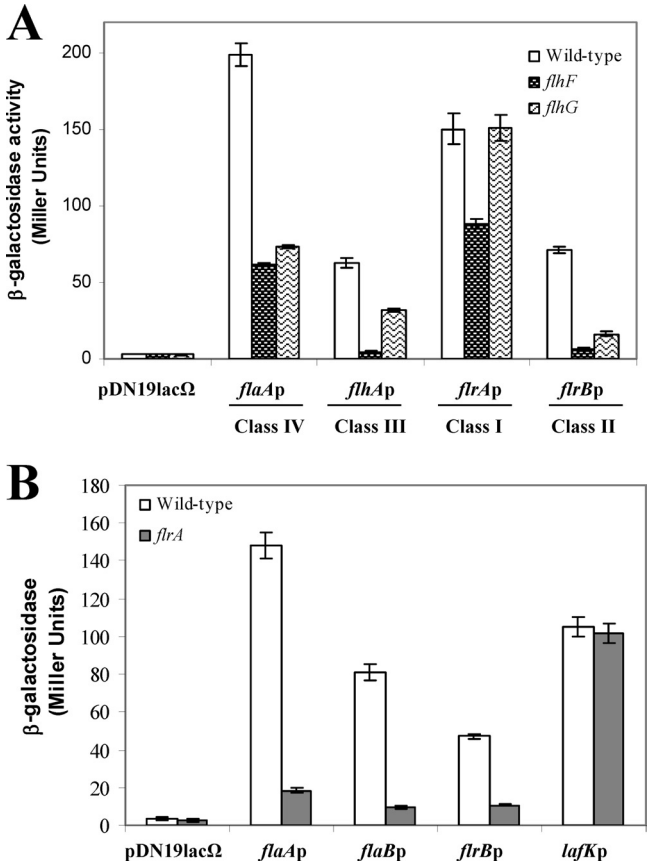


FIG. 6. Analysis of β-galactosidase activity. (A) pDNlac-*flaAF*p, pDNlac-*flhA*p, pDNlac-*flrA*p, and pDNlac-*flrB*p plasmids in *A. hydrophila* wild type (AH-405) and AH-3 Δ*flhF* and AH-3 Δ*flhG* mutants after growth in TSB at 25°C. (B) pDNlac-*lafK*p, pDNlac-*flaA*p, pDNlac-*flaB*p, and pDNlac-*flrB*p plasmids in *A. hydrophila* wild-type (AH-405) after growth in plates at 25°C. As a control we also measured the pDN19lacΩ promoterless plasmid. The results shown are representative of three independent experiments.

polar-flagellum gene transcription, plasmids containing promoters belonging to different classes of the flagellum hierarchy fused to *lacZ* were transferred into the *A. hydrophila flhF* and *flhG* mutants. Analysis of the β-galactosidase activity of pDNlac-*flaA*p, pDNlac-*flhA*p, pDNlac-*flrA*p, and pDNlac-*flrB*p plasmids in the *flhF* mutant showed reduced activity of 68, 92, 41, and 91% from the *flaA*, *flhA*, *flrA*, and *flrB* promoters, respectively, in comparison to the wild-type values, suggesting that FlhF positively regulates all classes of *A. hydrophila* polar-flagellum genes (Fig. 6A). The *flhG* mutant showed an *flrA* promoter transcription level similar to that of the wild type and a significant reduction of 63, 48 and 77% in the activity of the *flaA*, *flhA*, and *flrB* promoters versus the wild-type values, respectively. The data suggest that FlhG positively regulates *A. hydrophila* polar-flagellum gene classes II, III, and IV (Fig. 6A).

FlrA cannot be substituted for the lateral-flagellum regulator LafK. *A. hydrophila* as well as *V. parahaemolyticus* has dual flagellum systems (polar and lateral flagella) which do not share structural or regulatory genes, and both contribute to motility in semisolid plates. One open reading frame of lateral-flagellum clusters of both species encodes a σ^{54}-dependent response regulator, LafK, essential for lateral-flagellum generation (8, 39). *V. parahaemolyticus* LafK is able to compensate for mutation of the polar-flagellum σ^{54}-dependent response regulator, FlaK (19). The *A. hydrophila* FlrA mutation abolished polar-flagellum formation but did not affect lateral-flagellum-dependent swarming motility on semisolid medium; furthermore an *A. hydrophila* LafK mutation that abolished lateral-flagellum formation did not affect polar-flagellum biogenesis (7, 8). β-Galactosidase activity of pDNlac-*lafK*p (*lafK*p-*lacZ*) was measured after growth in semisolid plates (inducing conditions for lateral flagella) to ensure that LafK was transcribed. Analyses show that *lafK* was transcribed similarly in the wild-type and the *flrA* mutant strains in semisolid medium. We measured the β-galactosidase activity of pDNlac-*flaA*p, pDNlac-*flaB*p, and pDNlac-*flrB*p plasmids in the *A. hydrophila* wild type and the *flrA* mutant after growth on semisolid medium. β-Galactosidase values were reduced in the *flrA* mutant versus the wild-type background (Fig. 6B). These results suggest that LafK cannot supplement FlrA function and that FlrA is required for polar-flagellum production and swimming motility.

DISCUSSION

Mesophilic *Aeromonas* produces a single polar flagellum constitutively expressed in both liquid and solid media. In addition, 50 to 60% of strains also have an inducible lateral-flagellum system that is expressed in high-viscosity medium. Polar-flagellum genes are organized in different clusters distributed in six chromosomal regions (Fig. 1). The measurement of promoter-*lacZ* fusion activities and RT-PCR assays in defined flagellum-regulatory mutants allowed us to analyze the *A. hydrophila* polar-flagellum cluster transcription hierarchy. *A. hydrophila* polar-flagellum expression, as in other mono-flagellated bacteria, seems to be organized in four transcriptional levels (classes I to IV), where each level contains the activator for the subsequent transcriptional level.

Class I. *A. hydrophila* polar-flagellum-regulatory cascade class I seems to include only the *flrA* gene since its mutation reduced the β-galactosidase activity of most promoter-*lacZ* fusions tested (Fig. 4). *A. hydrophila* FlrA looks like the master regulator of the polar-flagellum regulon, and the analysis of *flrA*p-*lacZ* fusion in the *A. hydrophila rpoN* and *fliA* mutants (AH-5502 and AH-4443, respectively) showed β-galactosidase activity levels similar to the level determined in the wild type (Fig. 2), suggesting that *flrA* is transcribed in a σ^{54}- and σ^{28}-independent manner. Amplification of the *flrA* cDNA 5′ ends by 5′ RACE allowed us to obtain a DNA sequence upstream of the *flrA* transcription start which contained a σ^{70} promoter sequence (TTGACA-N14-TGGCACTTT) (Fig. 3). This result indicated that *A. hydrophila* polar-flagellum class I gene transcription is σ^{70} dependent in several strains (Fig. 7) and is consistent with the fact that the *A. hydrophila* polar flagellum is constitutively expressed, which is in contrast to other bacteria with dual flagellum systems, such as *V. parahaemolyticus* (24). Our data are similar to those for flagellum class I gene transcription of *P. aeruginosa* (13) although the σ^{54} factor is possibly involved in the transcription regulation of other pseudomonads (38). In *Vibrio* spp. class I gene transcription is σ^{54}

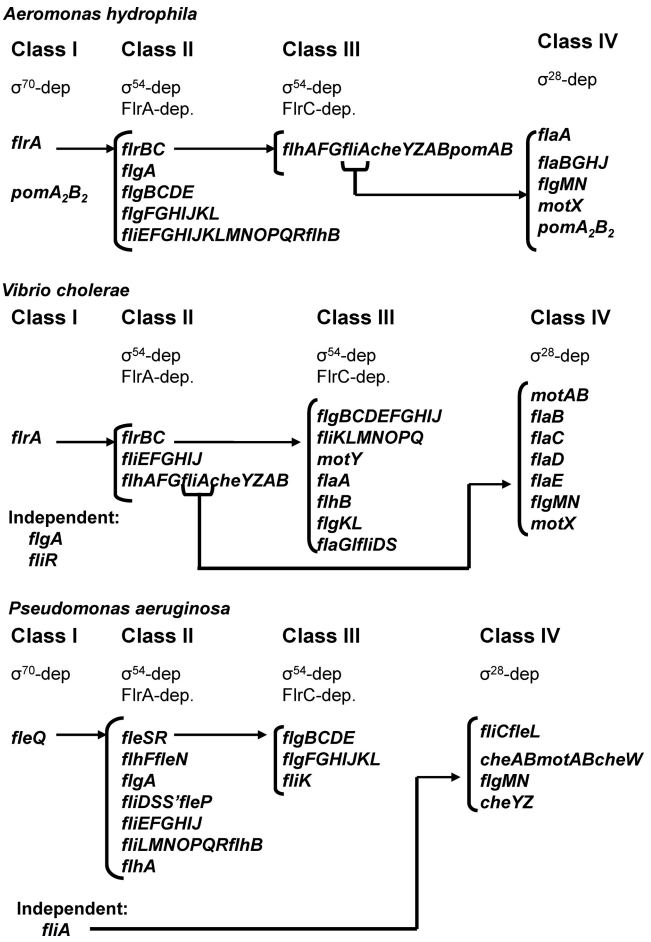


FIG. 7. Comparative proposed *A. hydrophila*, *V. cholerae* (41), and *P. aeruginosa* (12) polar-flagellum gene transcription hierarchies.

and σ^{28} independent, but no specific motifs for σ^{70} binding were found in their promoter sequences (33).

V. parahaemolyticus and some *A. hydrophila* strains express dual flagellum systems, and LafK is the lateral-flagellum master regulator (8, 39). In *V. parahaemolyticus* LafK is able to compensate for the mutation of the polar-flagellum σ^{54}-dependent response regulator FlaK (19). Data obtained are consistent with the fact that the *A. hydrophila* FlrA mutation abolishes polar-flagellum formation in liquid and solid media but does not affect inducible lateral-flagellum formation (7). These results suggest that despite the 57% similarity between *A. hydrophila* LafK and FlrA, their C-terminal domains might recognize different DNA binding regions which do not coexist in polar-flagellum class II promoter sequences. It is tempting to suggest that *V. parahaemolyticus* polar-flagellum class II promoter sequences should contain binding sites for LafK and FlaK. Therefore, results highlight that polar- and lateral-flagellum interconnections and control networks are specific and different for the dual flagellum systems of *A. hydrophila* and *V. parahaemolyticus*.

Class II and III. Transcription of the *Pseudomonas* and *V. cholerae* polar-flagellum genes included in the class II and III levels, as well as the dually flagellated *V. parahaemolyticus*, is σ^{54} dependent. Their transcriptions require specialized enhancer-binding proteins (FleQ, FlrA, and FlaK, respectively) involved in class II and class III (FleS, FlrC, and FlaM, respectively) transcription (12, 18, 33, 41). Transcription of *A. hydrophila* polar-flagellum promoters, whose sequences *in silico* contain conserved nucleotides that could represent σ^{54}-dependent consensus regions, was analyzed in the *A. hydrophila* wild type and the AH-5502 (*rpoN* mutant) (8), AH-3 *flrA*, and AH-3 *flrBC* mutants by promoter-*lacZ* fusions and RT-PCR. Each of these three mutants restored swimming motility after being complemented with the mutated gene. A previous study suggested that *A. hydrophila pomAB*, in polar-flagellum region 3 (Fig. 1), constituted an independent transcriptional unit, and a putative σ^{54}-dependent promoter upstream of *pomA* was found (7); however, no β-galactosidase activity was detected in the *pomA*p-*lacZ* fusion in either the wild type or mutant strains tested (Fig. 2B). The data obtained by both techniques indicate that *pomAB* are not independently transcribed and constitute a unique transcript from an *flhA* promoter previously identified although this fact does not completely eliminate the possibility of additional promoters. The data obtained suggest that *flgA*, *flgB*, *flgF*, *fliE*, and *flrB* promoters are σ^{54} and FlrA dependent, whereas the *flhA* promoter is σ^{54} and FlrC dependent. Furthermore, analysis of *flrB*p-*lacZ* and *flhA*p-*lacZ* fusions representing class II and class III promoters in the *A. hydrophila fliA* mutant showed β-galactosidase activities similar to the activity determined in the wild type (Fig. 2).

Therefore, the *A. hydrophila* polar-flagellum class II transcription level includes the following: *flgA*, which is necessary for P-ring addition; *flgB-E* (operon comprising *flgB* and *flgE* and the genetic material between the two genes) and *flgF-L*, which encode proteins involved in basal body, L and P rings, and hook formation; *fliE-flhB*, which encode the export/assembly apparatus, the MS ring-switch complex, and the hook length control protein; and *flrBC*, which encode regulatory proteins. All *A. hydrophila* polar-flagellum genes included in the class III transcription level are localized in a unique cluster, *flhA-pomB*, whose genes encode proteins involved in export/assembly, motor, and chemotaxis, as well as the σ^{28} factor and two flagellum regulators (Fig. 7).

Although *A. hydrophila*, *V. cholerae*, and *P. aeruginosa* polar-flagellum clusters are quite conserved, they differ in their chromosomal distributions and in the transcription hierarchies (12, 25, 33). While genes homologous to *flrBC* and *fliE-J* are transcribed as class II in the three species, greater diversity in the transcriptional hierarchy is shown in the remaining polar-flagellum class II/III genes. *A. hydrophila flgB-E*, *flgF-L*, and *fliK* are class II, but in *P. aeruginosa* and *V. cholerae* they are class III. *A. hydrophila flhA-G* are class III, *P. aeruginosa* and *V. cholerae* homologous genes are class II. *A. hydrophila* and *P. aeruginosa fliL-flhB* and *flgA* are class II, whereas *V. cholerae fliL-flhB* are class III and *flgA* is σ^{54}- and σ^{28}-independently transcribed. The genes transcribed in a more variable hierarchical order are *fliA*, *cheY-B*, and *pomAB*. Thus, *P. aeruginosa fliA* is σ^{54} independent, and *cheY-B pomAB* are class IV genes, whereas *A. hydrophila* and *V. cholerae fliA cheY-B* are transcribed from the *flhA* promoter, being σ^{54} and FlrA dependent in *V. cholerae* (class II) and σ^{54} and FlrC dependent in *A. hydrophila* (class III). *A. hydrophila pomAB* are also transcribed from the *flhA* promoter, but in *V. cholerae* these two genes

constituted a transcriptionally independent, σ^{28}-dependent unit (class IV) located in a different chromosomal region.

Class IV. *A. hydrophila fliA* encodes a σ^{28} homologue that is required for polar-flagellum formation (7). FliA from *Gammaproteobacteria* is involved in transcription of late flagellar genes, such as flagellins, the anti-sigma factor FlgM, and motor components (12, 33). Analysis of β-galactosidase activity of *flaA*p-*lacZ*, *flaB*p-*lacZ*, *flgM*p-*lacZ*, *motX*p-*lacZ*, and $pomA_2$p-*lacZ* fusions in the *A. hydrophila* wild type and the *fliA* mutant showed that all promoter-*lacZ* fusions tested, with the exception of $pomA_2$p-*lacZ*, have a low level of transcription in the *fliA* mutant, suggesting that they are σ^{28} dependent (Fig. 2A). The $pomA_2$ promoter exhibited a similar level of transcription in both strains, as well as in the *rpoN* mutant, suggesting that it is σ^{54} and σ^{28} independent (Fig. 2B). Analysis of $pomA_2$ cDNA 5′ ends by 5′ RACE showed two different transcription start sites at −41 and −107 nt. The DNA fragment upstream of the −41-nt transcription start contained a σ^{70} promoter sequence, and a fragment upstream of the −107-nt transcription start contained a σ^{28} promoter sequence (Fig. 3). Since *A. hydrophila* has a redundant set of motor proteins involved in polar-flagellum rotation, $PomA_2B_2$ and PomAB, with $PomA_2B_2$ being more sensitive to low-level sodium ion variations and having a lower level of transcription than PomAB (44), the results obtained suggest that transcription of these redundant motor proteins is specifically regulated and expressed at different levels of the flagellar hierarchy. Thus, while *pomAB* is transcribed from the *flhA* promoter, which is σ^{54} and FlrC dependent, $pomA_2B[r]_2$ is transcribed independently of the flagellum hierarchy although conditions that increase its transcription remain to be determined (Fig. 7). Homologous motor proteins of *Vibrio* (PomAB) and *Pseudomonas* (MotAB), as well as MotX, are transcribed from σ^{28}-dependent promoters (12, 33, 41).

A. hydrophila, *P. aeruginosa*, and *V. cholerae* polar-flagellum class IV genes include *flgMN* and most flagellin genes, as well as *motX*. Genes that encode the filament length control (*flaG*), the filament cap protein (*flaH*), and a chaperone (*flaJ*) are more diversely transcribed in the hierarchies of these three species. *A. hydrophila flaGHJ* homologues and *P. aeruginosa flaG* homologues are class IV genes, *P. aeruginosa flaHJ* homologues are class II, and *V. cholerae flaGHJ* are class III (Fig. 7). On the other hand, although *flgMN* constitutes a σ^{28}-dependent transcription unit in these three species, in *P. aeruginosa* and *V. cholerae* these genes are also transcribed with *flgA*, whereas in *A. hydrophila* no mRNA containing *flgA* and *flgM* was found (data not shown).

FlhF and FlhG structural and regulatory implications. It has been reported that FlhF and FlhG act together to regulate flagellum placement and number in *Vibrio alginolyticus* and *Pseudomonas* species. *V. cholerae* and *V. alginolyticus flhF* disruption abolishes flagellum formation (11, 21), and *Pseudomonas flhF* disruption gives an aberrant placement of flagella (30, 32). In *Vibrio* and *Pseudomonas*, *flhG* disruption increases the number of polar flagella per cell (11, 12, 21, 30). Our results indicate that *A. hydrophila* FlhF and FlhG are essential for the assembly of a functional polar flagellum because in-frame mutants fail to swim in liquid medium and lack polar flagella although some flagellum fragments were observed in the medium (Fig. 5). Furthermore, the irregular motility in a semi-solid plate (Fig. 5), especially of the *flhG* mutant, together with the presence of some polar-flagellum fragments in the medium, might suggest that some cells assemble an unstable polar flagellum, which allows them to swim for a short time and then constitute a colony after loosing the flagellum.

A. hydrophila FlhF positively regulates all polar-flagellum transcription levels (classes I to IV), whereas FlhG positively regulates classes II, III, and IV. Because of the hierarchical nature of flagellum transcription, the reduction in the transcription of the master regulator FlrA may be the cause for the general reduction in all of the classes of flagellum promoters, suggesting that FlhF exerts its influence by positively regulating the transcription of *flrA*. Furthermore, FlhG may exert its function through the regulation of the activity of FlrA. Among *Aeromonas*, *Vibrio*, and *Pseudomonas* species, FlhF and FlhG homologues do not play the same role in flagellum gene regulation, but they regulate the expression of the majority of flagellum genes directly or indirectly. FlhF increases the expression of *Vibrio* class III genes only (11), whereas there was an increase in expression of all *Aeromonas* polar-flagellum gene classes. *A. hydrophila* FlhG is a positive regulator of class II genes, whereas in *Vibrio* it is a repressor of class I genes, and in *Pseudomonas* it is a repressor of class II genes (11, 13).

Our results indicate that the *A. hydrophila* polar-flagellum transcription hierarchy shares some similarities but many important differences with the transcription hierarchies of *V. cholerae* and *P. aeruginosa* (12, 33, 41).

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