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Aeromonas hydrophila Lateral Flagellar Gene Transcriptional Hierarchy

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***Aeromonas hydrophila* AH-3 lateral flagella are not assembled when bacteria grow in liquid media; however, lateral flagellar genes are transcribed. Our results indicate that *A. hydrophila* lateral flagellar genes are transcribed at three levels (class I to III genes) and share some similarities with, but have many important differences from, genes of *Vibrio parahaemolyticus*. *A. hydrophila* lateral flagellum class I gene transcription is σ^{70} dependent, which is consistent with the fact that lateral flagellum is constitutively transcribed, in contrast to the characteristics of *V. parahaemolyticus*. The fact that multiple genes are included in class I highlights that lateral flagellar genes are less hierarchically transcribed than polar flagellum genes. The *A. hydrophila lafK-fliEJ*$_L$ gene cluster (where the subscript L distinguishes genes for lateral flagella from those for polar flagella) is exclusively from class I and is in *V. parahaemolyticus* class I and II. Furthermore, the *A. hydrophila flgAMN*$_L$ cluster is not transcribed from the σ^{54}/LafK-dependent promoter and does not contain class II genes. Here, we propose a gene transcriptional hierarchy for the *A. hydrophila* lateral flagella.**

Swarming motility is defined as a rapid multicellular movement of bacteria across a surface that is powered by rotating flagella. Most bacteria that swarm have multiple constitutive flagella distributed randomly on the cell surface (peritrichous flagella) and increase the flagellum number per cell on contact with surfaces (1, 2). On the other hand, polar flagellated bacteria have developed two different strategies to swarm: some bacteria, such as *Pseudomonas aeruginosa*, synthesize an alternative polar flagellar motor that can propel bacteria on surfaces (3, 4), and others, such as *Vibrio parahaemolyticus*, *Aeromonas* spp., and *Rhodospirillum centenum*, have developed lateral flagella distributed randomly on the cell surface which are induced when grown on solid surfaces or in viscous environments (5, 6).

Phylogenetic analysis and organization of lateral flagellar genes suggest that this flagellar system originated in *Betaproteobacteria* and *Gammaproteobacteria* from a duplication of the entire flagellar gene complex in the nonenteric gammaproteobacterial lineage, which was then horizontally transferred to the *Betaproteobacteria* and the enteric bacteria (7). In contrast to polar or peritrichous flagella systems (primary systems), lateral flagellar systems lack *fliO*, and the *fliEFGHIJKLMNPQR* gene cluster is split into two gene clusters (*fliEFGHIJ*$_L$ and *fliMNPQR*$_L$ [where the subscript L distinguishes genes for lateral flagella from those for polar flagella]). The *fliKL*$_L$ (*lafEF*) genes are arranged in the *fliD*$_L$-*motB*$_L$ (*lafB-lafU*) cluster (5, 8).

The best-studied functional lateral flagellar systems are those of *V. parahaemolyticus* and *A. hydrophila*. Both are encoded by 38 genes distributed in six clusters, while *V. parahaemolyticus* genes are distributed in two discontinuous regions on chromosome II (9); *A. hydrophila* genes are distributed in a unique chromosomal region (10, 11). *V. parahaemolyticus flgAMN*$_L$ and *motY*$_L$-*lafK-fliEFGHIJ*$_L$ clusters are transcribed divergently from *flgBCDEFGHIJKL*$_L$, *fliMNPQR*$_L$-*flhBA*$_L$, *lafA*, and *fliDSTKLA*$_L$-*motAB*$_L$. *A. hydrophila* orthologous genes exhibit the same distribution, although only *flgAMN*$_L$ genes are transcribed divergently. Furthermore, *A. hydrophila* does not contain any gene orthologous to *V. parahaemolyticus motY*$_L$ (12), and a modification accessory factor gene, *maf-5* (13), which is independently transcribed, is present between *flgL*$_L$ and *lafA*.

Synthesis and assembly of any flagellar system is regulated coordinately by a transcriptional cascade composed of three or four levels of hierarchy: class I to III or I to IV (14, 15). In polar flagellated *Gammaproteobacteria*, such as *Vibrio*, mesophilic *Aeromonas*, and *Pseudomonas* species, four levels of hierarchy have been described. Transcription of class II and III is σ^{54} dependent, and transcription of class IV is σ^{28} dependent (16, 17, 18). At the top of the hierarchy is a σ^{54}-associated transcriptional activator (FlrA in *A. hydrophila*) which activates σ^{54}-dependent promoters preceding the class II clusters. One of the class II clusters encodes a two-component signal-transducing system (FlrBC in *A. hydrophila*) whose regulator (FlrC) activates class III genes. The class III-transcribed *A. hydrophila* σ^{28} factor, which activates transcription of class IV genes, is class II transcribed in *Vibrio* spp., and flagellar hierarchy is independently transcribed in *P. aeruginosa* (16, 17, 18).

Inducible peritrichous flagella (lateral flagella) of *V. parahaemolyticus* and *A. hydrophila* do not posses an FlhDC master regulator and are σ^{54} dependent (9, 19) as polar flagella. In this work, we investigated the *A. hydrophila* lateral flagellar transcriptional hierarchy by two techniques: promoter-*lacZ* fusion assays (used to measure β-galactosidase activity in several mutant backgrounds) and reverse transcription-PCR (RT-PCR) assays. Until now, little was known about *A. hydrophila*'s transcriptional hierarchy, translational and posttranslational regulatory mechanisms that ensure careful regulation, proper assembly of several different proteins, and whether or not there was a large enough quantity of individual lateral flagellar subunits.

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TABLE 1 Bacterial strains and plasmid used in this study

Strain or plasmid	Genotype and/or phenotype[a]	Reference or source
Strains		
A. hydrophila		
AH-3	*A. hydrophila* wild type, serogroup O:34	45
AH-405	AH-3, spontaneous Rif^r	39
AH-4427	AH-405Δ*flaB*; *flaA*::Km^r	10
AH-5502	AH-405; *rpoN*::Km^r	19
AH-5503	AH-405; *lafK*::Km^r	19
AH-3::*lafS*	AH-405; *lafS*::Km^r	This work
AH-3Δ*lafA*	AH-405Δ*lafA*	This work
E. coli		
DH5α	F$^-$ *endA hdsR17*(r_k^- m_k^+) *supE44 thi-1 recA1 gyr-A96* φ80*lacZ*	25
MC1061λpir	*thi thr1 leu6 proA2 his4 argE2 lacY1 galK2 ara14 xyl5 supE44* λ*pir*	46
Plasmids		
pGEM-T easy	Cloning vector, Ap^r	Promega
pRK2073	Helper plasmid, Sp^r	46
pUC4-KIXX	Source of Tn*5*-derived *nptII* gene (Km^r)	Pharmacia
pDM4	Suicide plasmid, *pir* dependent with *sacAB* genes, *oriR6K*, Cm^r	27
pDM-LafSKm	pDM4 with AH-3 *lafS*::Km, Cm^r Km^r	This work
pDM-LAFA	pDM4 with AH-3Δ*lafA*, Cm^r	This work
pBAD33-Gm	pBAD33 arabinose-induced expression vector with Gm^r	28
pBAD33Gm-LAFK	pBAD33 with AH-3 *lafK* gene, Gm^r	This work
pBAD33Gm-LAFS	pBAD33 with AH-3 *lafS* gene, Gm^r	This work
pDN19 lacΩ	Promoterless *lacZ* fusion vector, Sp^r Sm^r Tc^r	29
pDNlac-*fliM*$_L$p	*fliM*$_L$ promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*lafK*p	*lafK* promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	18
pDNlac-*flgM*$_L$p	*flgM*$_L$ promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*flgA*$_L$p	*flgA*$_L$ promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*flgB*$_L$p	*flgB*$_L$ promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*maf-5*p$_1$	*maf-5* promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*maf-5*p$_2$	*maf-5* promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*lafA*p	*lafA* promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*lafB*p	*lafB* promoter-*lacZ* fusion in pDN19lacΩ, Tc^r	This work
pDNlac-*lafT*p	*lafT* 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.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown on Luria-Bertani (LB) Miller broth and LB Miller agar at 37°C, while *Aeromonas* strains were grown either in tryptic soy broth (TSB) or tryptic soy 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), and tetracycline (20 μg/ml) were added to the media.

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 media.

DNA techniques. DNA manipulations were carried out essentially according to standard procedures (20). 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 and a PerkinElmer 2400 thermal cycler.

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-stranded DNA sequencing was performed by using the Sanger dideoxy-chain termination method (21) with the BigDye Terminator v3.1 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) (22). The Terminator search program in the GCG Wisconsin package was used to search for factor-independent transcriptional terminators. Neural Network Promoter Prediction, PromScan (23), and PRODORIC (24) were used to search promoter sequences.

Total RNA extraction and RT-PCR. Total RNA was isolated, by RNA Protect bacterial reagent (Qiagen) and an RNeasy Minikit (Qiagen), from *A. hydrophila* AH-3 and *rpoN*, *lafK*, and *lafS* mutant strains grown in liquid medium (TSB), viscous medium (TSB plus 18% [wt/vol] Ficoll), or solid agar (TSA). To ensure that RNA was devoid of contaminating DNA, the preparation was treated with RNase-free TurboDNase 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 DNase-digested RNA. The reaction mixtures were incubated at 25°C for 10 min, 37°C for 120 min, and 75°C for 15 min. Control reaction mixtures lacking reverse transcriptase were used 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 (Invitrogene), specific oligonucleotides, and 30 PCR cycles. Amplicons were analyzed by agarose gel electrophoresis with ethidium bromide staining. *A. hydrophila* ribosomal 16S primers were used as a control for the 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 *fliM*$_L$, *lfgA*$_L$, *lafK*, *lafB*, and *lafT* transcription start sites by RACE PCR. Amplifications of the *A. hydrophila* AH-3 *fliM*$_L$, *lfgA*$_L$, *lafK*, *lafB*, and *lafT* cDNA 5′ ends were performed using the 5′ random amplification of cDNA ends (RACE) 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 *fliM*$_L$, *lfgA*$_L$, *lafK*, *lafB*, and *lafT* internal primers GSP1-FLIM$_L$ (5′-ATCTTGCAAGGTGT G-3′), GSP1-LfgA$_L$ (5′-GAGCTTGGAACAAATC-3′), GSP1-LafK (5′-G ATATAACGAGCCAGTC-3′), GSP1-LafB (5′-TTTCGACAAACTTCTT G-3′), and GSP1-LafT (5′-AATTATCGATGATGAAAC-3′), respectively, 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, as well as tailing of purified cDNA using terminal deoxynucleotidyl transferase and dCTP, was done according to the 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 10 μM each primer, the 5′ RACE abridged anchor primer (AAP) that binds to the tailed cDNA sequence, and GSP2-FLIM$_L$ (5′-AGATGTCGACCTGATATTGG-3′), GSP2-FLGA$_L$ (5′-ATCTCCGGTACGAATGGT-3′), GSP2-LafK (5′-TTCATTAACCA GGATGAC G-3′), GSP2-LafB (5′-GCATTCTCCAACCCACTAT-3′), and GSP2-LafT (5′-TTCAT CGAGTGCCTTCAT-3′), which bind to the respective internal gene sequences. The PCR program applied was 94°C for 1 min and then 35 cycles of 94°C for 45 s, 55°C for 30 s, and 72°C for 1 min, followed by an 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-FLIM$_L$, GSP2-FLGA$_L$, GSP2 LafK, GSP2-LafB, or GSP2-LafT primer.

Construction of defined mutants. To obtain the *A. hydrophila* AH-3:: *lafS* mutant, *lafS* was amplified by PCR with 5′-CGCGGATCCAACCCA AGCCAGAGTTGAG-3′ and 5′-CGCGGATCCATGAAACACCAGGAC ACA-3′ (the BamHI site is underlined), ligated into vector pGEMTeasy (Promega), and transformed into *E. coli* DH5α (25). The Tn*5*-derived kanamycin resistance cartridge (*nptII*) from pUC4-KIXX was obtained by SmaI digestion, and the cassette was inserted into the XbaI blunt-ended restriction internal site of *lafS*. The cartridge contains an outward-reading promoter that ensures the expression of downstream genes when inserted in the correct orientation; however, such insertion will alter the regulation of those genes (26). 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 to suicide vector pDM4 (27), electroporated into *E. coli* MC1061λpir, and plated on chloramphenicol-kanamycin plates at 30°C to obtain the pDM-LafSKm plasmid.

The chromosomal in-frame *lafA* deletion mutant *A. hydrophila* AH-3Δ*lafA* was constructed by allelic exchange as described by Milton et al. (27). Briefly, DNA regions flanking the *lafA* gene were amplified using the primers A (5′-CGCGGATCCTTTGGTGTCGACTTCTCCT-3′), B (5′-CCCATCCACTAAACTTAAACAAGAGTTCAGCTGGTTCTGG-3′), C (5′-TGTTTAAGTTTAGTGGATGGGAGCACCAATATGACCAAGAA-3′), and D (CGCGGATCCCAGCACCATGTTGACCTT-3′) in two sets of asymmetric PCRs to amplify DNA fragments of 779 (pair AB) and 733 (pair CD) bp, respectively. DNA fragments AB and CD were annealed at their overlapping regions (double-underlined letters in primers B and C) and amplified as a single fragment using primers A and D. The fusion product was purified, BamHI digested (the BamHI site is underlined in primers A and D), ligated into BglII-digested and phosphatase-treated pDM4 vector (27), electroporated into *E. coli* MC1061λpir, and plated on chloramphenicol plates at 30°C to obtain pDM-LAFA plasmid. Plasmid pDM-LafSKm or pDM-LAFA was transferred into an *A. hydrophila* AH-405 rifampin-resistant (Rif^r) strain by triparental matings using *E. coli* MC1061λpir containing the insertion constructs and the mobilizing strain HB101/pRK2073. Transconjugants were selected on plates containing chloramphenicol, kanamycin, and rifampin or containing chloramphenicol and rifampin. PCR analysis confirmed that the vector had integrated correctly into the chromosomal DNA. After sucrose treatment, transconjugants that were Rif^r, Km^r, and Cm^s or were Rif^r and Cm^s were chosen and confirmed by PCR.

Plasmid constructions. Plasmids pBAD33Gm-LAFK and pBAD33Gm-LAFS, containing the complete *lafK* and *lafS* genes of *A. hydrophila* AH-3, respectively, under the arabinose promoter (P$_{BAD}$) on pBAD33-Gm (28) were obtained. Oligonucleotides 5′-G*GATATC*TAATGATAGCGGGGT TAC-3′ and 5′-CCCAAGCTTCATCAGCTTGTTTCGCACCT-3′ generate a band of 1,841 bp containing the *lafK* gene, and oligonucleotides 5′-TCCCCCGGGAACCCAAGCCAGAGTTGAG-3′ and 5′-CCCAAGC TTATGAAACACCAGCACACA-3 generate a band of 1,162 bp containing the *lafS* gene (the EcoRV site is in italics, the SmaI site is double underlined, and the HindIII site is underlined). The amplified bands were digested with EcoRV or SmaI and HindIII and ligated into SmaI- and HindIII-digested pBAD33-Gm vector (19) to construct the pBAD33Gm-LAFK and pBAD33Gm-LAFS recombinant plasmids. Plasmids were independently introduced into *E. coli* DH5α (25) and sequenced.

Construction of flagellar promoter-*lacZ* fusions. Oligonucleotide primer pairs for the *A. hydrophila* AH-3 promoter regions of the *fliM$_L$*, *flgM$_L$*, *flgA$_L$*, *flgB$_L$*, *maf-5*, *lafA*, *lafB*, and *lafT* genes (19) are listed in Table 2. Primers were designed to amplify fragments of 493 to 1,560 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 (25). DNA inserts containing *fliM$_L$*, *flgM$_L$*, *flgA$_L$*, *maf-5*, and *lafA* promoters were recovered by EcoRI/BamHI restriction digestion, inserts containing *lafB* and *flgB$_L$* promoters were recovered by EcoRI/BglII restriction digestion, and the insert containing *lafT* promoter was recovered by SmaI/BamHI restriction digestion. The BglII restriction site in the *lafB* insert is 158 bp downstream from the *lafB* start codon. The EcoRI restriction sites come from the pGEM-T Easy plasmid. Digested fragments were ligated into plasmid pDN19lacΩ EcoRI/BamHI-digested or EcoRI blunt-ended BamHI (29), transformed into *E. coli* DH5α (25), and selected for tetracycline resistance (Tc^r). The final constructs were confirmed by DNA sequencing.

TABLE 2 Primers used for lateral flagellar promoter-*lacZ* fusion construction

Promoter	Primer sequence[a]	Site[b]
*fliM$_L$*p	5′-TGCAACAGAGAGCAAACCG-3′	−486 *fliM$_L$*
	5′-CGGGATCCTGAGTTGTTCTCGGTCTG-3′	+114 *fliM$_L$*
*flgM$_L$*p	5′-AGCAAGAGCAATCGGAATC-3′	−325 *flgM$_L$*
	5′-CGGGATCCCATCCGATGTAGTTGCCAAT-3′	+168 *flgM$_L$*
*flgA$_L$*p	5′-GGAAGATCTTCCCGGTGATTTTCATATTC-3′	−494 *flgA$_L$*
	5′-CGGGATCCCGTGCATTCAGCCAGATAG-3′	+386 *flgA$_L$*
*flgB$_L$*p	5′-CGGGATCCCGTGCATTCAGCCAGATAG-3′	−419 *flgB$_L$*
	5′-GGAAGATCTTCCCGGTGATTTTCATATTC-3′	+361 *flgB$_L$*
*maf-5*p$_1$	5′-GAGCTCTGCGCAAAGAAA-3′	−521 *maf-5*
	5′-CGGGATCCTCAAGTGCAAGACCAGAGC-3′	+339 *maf-5*
*maf-5*p$_2$	5′-CGTTGACCCGAGAAGTCA-3′	−1490 *maf-5*
	5′-CGGGATCCTCGATCCAGCCTTGAAA-3′	−523 *maf-5*
*lafA*p	5′-TGTATGGCACTGGGTTGG-3′	−1519 *lafA*
	5′-GCGGATCCGGTGGTCATGGAAGCAAA-3′	+41 *lafA*
*llafB*p	5′-TCTGCTGAAAACCGGTGG-3′	−553 *lafB*
	5′-TTCTGCGCCTGTAAATTG-3′	+194 *lafB*
*lafT*p)	5′-TCCCCCGGGTGAGTTTGACACATCACCC-3′	−732 *lafT*
	5′-CGCGGATCCATGAAACACCAGGACACA-3′	+53 *lafT*

[a] Underlined letters show the BamHI restriction site. Double-underlined letters show the BglII restriction site. Italicized letters show the SmaI restriction site.
[b] Numbers are numbers of nucleotides upstream (−) or downstream (+) from the start site for the indicated gene.

Transmission electron microscopy (TEM). Three independent samples of bacterial suspensions grown in TSB or TSB medium with 18% (wt/vol) Ficoll at 25°C 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.

Immunoblotting assays. *A. hydrophila* grown on plates (TSA), in viscous medium (TSB plus 18% [wt/vol] Ficoll), or in liquid cultures (TSB) at 25°C was used to analyze lateral flagellins by Western blotting. For analysis of cytoplasmic fractions, cells grown on plates were collected with 20 mM $MgCl_2$ in 100 mM Tris (pH 8.0) and harvested by centrifugation (5,000 × *g*). Cells grown in liquid or viscous medium were collected by centrifugation (5,000 × *g*). Both were suspended in 20 mM $MgCl_2$ in 100 mM Tris (pH 8.0) and diluted to an optical density at 600 nm of 0.8. Flagella were removed from the cells by shearing in a vortex with a glass bar for 3 to 4 min and then passing repetitively (minimum of six times) through a syringe. Cells without flagella on their surface were collected by centrifugation at 8,000 × *g* for 30 min, resuspended in the same cold buffer, and subjected to French press cell lysis. After shearing, the supernatants were also collected for analysis. The lysates were centrifuged at 5,000 × *g* to remove unbroken cells. After centrifugation at 4°C for 1 h at 115,000 × *g*, the soluble fraction (cytoplasmic fraction) remained in the supernatant while the insoluble fraction (membrane-enriched fraction) was retained in the pellet. The cytoplasmic fraction was analyzed by SDS-PAGE and transferred to nitrocellulose membranes, and the membranes were blocked with bovine serum albumin (3 mg/ml) and probed with

polyclonal rabbit anti-polar or anti-lateral flagellin antibodies (1:1,000) that were previously obtained (11). The unbound antibody was removed by three washes in phosphate-buffered saline (PBS), and a goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (1:1000) was added. The unbound secondary antibody was removed by three washes in PBS. The bound conjugate was then detected by the addition of 5-bromo-4-chloroindolylphosphate disodium-nitroblue tetrazolium. Incubations were carried out for 1 h, and washing steps with 0.05% Tween 20 in phosphate-buffered saline were included after each incubation step.

For analysis of whole cells and bacterial supernatants before shearing, cells grown in liquid or viscous medium were collected at an optical density at 600 nm of 0.8, and cells grown in plates were collected with 20 mM $MgCl_2$ in 100 mM Tris (pH 8.0) and diluted to an identical optical density at 600 nm. Both were harvested by centrifugation (5,000 × *g*), and cell suspensions as well as supernatants were analyzed by Western blotting with anti-lateral flagellin antibodies (1:1,000).

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 or TSB medium with 18% (wt/vol) Ficoll at 25°C to an optical density at 600 nm of 0.6 or 0.8. Bacterial cells were permeabilized with chloroform and sodium dodecyl sulfate (SDS) overnight and assayed for β-galactosidase activity as described by Miller (30). All experiments were performed at least 3 separate times.

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

RESULTS

Lateral flagellar genes of *A. hydrophila* AH-3 are transcribed, but lateral flagella are not expressed in liquid media. *A. hydrophila* and *V. parahaemolyticus* both have dual flagellar systems (polar and lateral flagella) but do not share structural or regulatory genes, and both contribute to motility in semisolid plates (9, 18, 19). *A. hydrophila* polar flagellum is constitutive; however, lateral flagella are induced in highly viscous media or on surfaces. Both flagellar types have σ^{54}-dependent response regulators, FlrA and LafK, which are essential for polar and lateral flagellum generation, respectively (10, 19). Furthermore, despite *A. hydrophila* FlrA and LafK showing 57% similarity to each other, their C-terminal domains might recognize different DNA binding regions, and LafK is unable to compensate for the FlrA mutation and *vice versa* (18, 19). In order to know whether viscosity conditions regulate transcription of lateral flagella, we measured the β-galactosidase activity of pDNlac-*lafK*p (*lafK*p-*lacZ*) after growth in liquid (TSB) and viscous media (TSB with 18% [wt/vol] Ficoll or 3% [wt/vol] gelatin) as well as on solid plates (TSA) (Fig. 1B). Data showed similar β-galactosidase values after growth in liquid and viscous media or on solid plates. Lateral flagellin (LafA) transcription was analyzed by measuring β-galactosidase activity of pDNlac-*lafA*p (*lafA*p-*lacZ*). Lateral flagellin transcription in liquid media shows a very slight reduction compared to transcription in viscous media or on solid plates, although lateral flagella are not produced in liquid media (Fig. 1A and B). However, the TEM assays showed lateral flagella in 85% of bacterial cells grown in TSB with Ficoll and in 80% of cells grown in TSA. Levels of β-galactosidase activity from other lateral flagella promoters, such as $flgA_L$, $flgB_L$, and *lafB* promoters, were also analyzed, with all of them being similar in liquid and viscous media (Fig. 1B). In addition, RT-PCR assays showed that *lafK*, *lafA*, $flgA_L$, $flgB_L$, and *lafB* transcription are viscosity/surface independent, since they are transcribed in liquid and viscous media as well as on solid plates (Fig. 1C). No RT-PCR product was obtained with primer pairs for $flgA_L$ and $flgB_L$, which are divergently transcribed genes, eliminating the possibility of residual DNA in the RNA samples from liquid cultures.

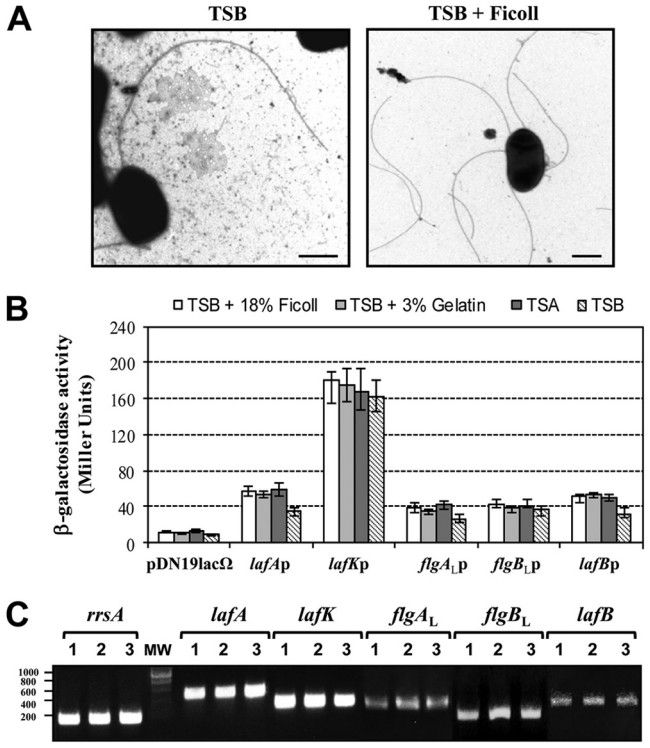


FIG 1 (A) Transmission electron microscopy of *A. hydrophila* AH-3 (wild type) grown at 25°C in TSB and TSB with 18% (wt/vol) Ficoll. Bacteria were gently placed onto Formvar-coated copper grids and negatively stained using a 2% solution of uranyl acetate. Bar, 0.5 μm. (B) Analysis of β-galactosidase activity of pDNlac-*lafK*p, pDNlac-*lafA*p, pDNlac-$flgA_L$p, pDNlac-$flgB_L$p, and pDNlac-*lafB*p plasmids in *A. hydrophila* AH-405 after growth in liquid (TSB) medium, viscous medium (TSB plus 18% [wt/vol] Ficoll or 3% [wt/vol] gelatin), and solid agar (TSA) at 25°C. As a control, we also measured the pDN19lacΩ promoterless plasmid. The results shown are representative of three independent experiments. (C) RT-PCR amplification of *lafK*, *lafA*, $flgA_L$, $flgB_L$, and *lafB* from cDNA of AH-3 after growth in liquid medium (TSB), viscous medium (TSB plus 18% [wt/vol] Ficoll), or solid agar (TSA) (lanes 1, 2, and 3, respectively). *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. The Ecogen molecular weight marker (MW) was used.

The presence of lateral flagellin (LafA) in the cytoplasmic fraction of *A. hydrophila* AH-3 and the mutants AH-4427 (without polar flagellin), AH-3Δ*lafA* (without lateral flagellin), and AH-5502 (*rpoN*) grown in liquid or viscous medium or on solid plates was analyzed by Western blotting with anti-lateral flagellin (1:1,000) polyclonal antibodies (Fig. 2A). We analyzed the presence of polar flagellins by Western blotting with anti-polar flagellin (1:1,000) polyclonal antibodies in these strains (Fig. 2B). AH-3 and AH-4427 mutant cytoplasmic fractions obtained after growth in viscous media or on solid plates showed positive reactions with anti-lateral flagellin serum but were unable to react with similar fractions grown in liquid media. AH-3Δ*lafA* and AH-5502 (*rpoN*) cytoplasmic fractions were unable to react with anti-lateral flagellin serum. AH-3 and AH-3Δ*lafA* cytoplasmic fractions reacted positively with polar flagellin serum and were unable to react with AH-4427 and AH-5502 (*rpoN*) cytoplasmic fractions in all growth

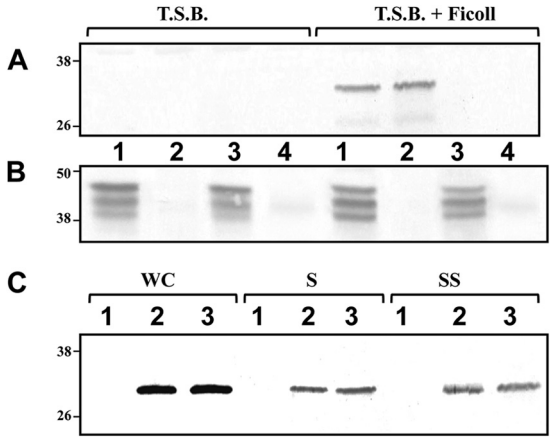


FIG 2 (A) Western blot analysis with anti-lateral flagellin (1:1,000) polyclonal antibodies of cytoplasmic fractions of *A. hydrophila* AH-3 (wild type), mutant AH-4427 (without polar flagellin), AH-3Δ*lafA* (without lateral flagellin), and AH-5502 (*rpoN*) (lanes 1, 2, 3, and 4, respectively) of bacteria grown in liquid (TSB) or viscous medium (TSB plus 18% [wt/vol] Ficoll) at 25°C in TSB. (B) Western blot analysis with anti-polar flagellin (1:1,000) polyclonal antibodies of cytoplasmic fractions of the same strains and grown conditions as those described for panel A. (C) Western blot analysis with anti-lateral flagellin (1:1,000) polyclonal antibodies of whole cells (WC) and supernatant before shearing (S) and after shearing (SS) of *A. hydrophila* AH-3 grown in liquid medium (TSB), viscous medium (TSB plus 18% [wt/vol] Ficoll), or an agar plate (TSA) (lanes 1, 2, and 3, respectively).

conditions tested. Furthermore, we analyzed the presence of lateral flagellin by Western blotting in whole cells of AH-3 before shearing, as well as in the supernatants before and after shearing, when grown in liquid or viscous medium or on solid plates. Nonlateral flagellin was detected in whole cells or supernatants from liquid cultures before and after shearing (Fig. 2C). These data suggest that lateral flagellin is not translated in liquid media, although it is transcribed.

The *A. hydrophila* AH-3 σ^{54} factor (RpoN) is not involved in *lafK* transcription. *A. hydrophila* AH-3 encodes an alternative σ^{54} sigma factor (RpoN) which is essential for both polar and lateral flagellum expression (10, 19). Lateral flagellar clusters contain only one gene that encodes a σ^{54}-dependent response regulator, LafK (10, 38, 43). *In silico* analysis of the *A. hydrophila* upstream *lafK* sequence did not shown putative σ^{54} promoter sequences. In order to establish if there was any relationship between the σ^{54} factor (RpoN) and *lafK* transcription, we measured the β-galactosidase activity of *A. hydrophila* wild-type and *rpoN* mutant (AH-5502) strains carrying the lateral flagella gene promoter-*lacZ* fusion plasmid pDNlac-*lafK*p. Similar β-galactosidase activities were detected in both strains (Fig. 3B). In addition, RT-PCR assays showed *lafK* transcription in both the wild type and the *rpoN* mutant (Fig. 3C).

Given that *lafK* transcription was σ^{54} independent, we performed 5′ RACE, as described in the Materials and Methods, to further analyze the *lafK* promoter region. Primary PCR of tailed cDNA using primers AAP and GSP2-LafK gave a unique DNA band of 620 bp (Fig. 4). Sequence of the amplified band indicates that it was tailed with G residues. The *lafK* transcription start was located 335 nucleotides (nt) upstream from the *lafK* translation start site, and DNA sequence upstream of the transcription start contains a σ^{70} promoter sequence (TTGAAT-N16-TATGAT) (Fig. 4). Furthermore, *in silico* analysis of the lateral flagellar region of *A. caviae* Sch3N (also showing dual flagellar systems) allowed us to identify a σ^{70} promoter sequence 331 bp upstream of the *lafK* start codon (5′-TTGAAT-N16-TATCAT-3′).

Identification of *A. hydrophila* lateral flagellum σ^{28}-dependent promoters. Transcriptions of polar and peritrichous flagellar late genes are σ^{28} dependent (16, 17, 18, 31). The *lafB-U* cluster of the *A. hydrophila* lateral flagellar chromosomal region contains a gene, *lafS*, which encodes a sigma factor orthologous to the *V. parahaemolyticus* σ^{28} factor $FliA_L$ and homologous to the *A. hydrophila* σ^{28} factor FliA (38/54 and 34/54% identity/similarity, respectively) (10, 11, 23). In *A. hydrophila*, mutation of LafS abolishes lateral flagellum formation (11), and lateral flagella were restored by complementation with the pBAD33Gm-LAFS plasmid in the presence of 0.2% L-arabinose. *In silico* sequence analysis of *A. hydrophila* AH-3 lateral flagellum genes show a putative σ^{28} promoter sequence upstream of the anti-σ^{28} factor $flgM_L$ (19), putative σ^{54} promoter sequences upstream of $fliM_L$, $flgA_L$, $flgB_L$, *maf-5*, *lafB*, and the lateral flagellin gene, *lafA* (11, 19), and several putative promoter sequences upstream of the motor gene *lafT*. In order to study which of these lateral flagella genes were σ^{28} dependent, we independently transferred the promoter-*lacZ* fusion plasmids pDNlac-$fliM_L$p ($fliM_L$p-*lacZ*), pDNlac-$flgM_L$p ($flgM_L$p-*lacZ*), pDNlac-$flgA_L$p ($flgA_L$p-*lacZ*), pDNlac-$flgB_L$p ($flgB_L$p-*lacZ*), pDNlac-*maf-5*p_1 (*maf-5*p_1-*lacZ*), pDNlac-*lafA*p (*lafA*p-*lacZ*), pDNlac-*lafB*p (*lafB*p-*lacZ*), and pDNlac-*lafT*p (*lafT*p-*lacZ*) into *A. hydrophila* AH-405 (AH-3 with rifampin resistance) and the *lafS* mutant (AH-3::*lafS*). Transconjugants were chosen and β-galactosidase activity measured. Transcription from the $flgM_L$ and *lafA* promoters appeared to be highly affected by the *lafS* mutation, since 90 and 98% reduction of β-galactosidase activity, respectively, was found in the AH-3::*lafS* mutant compared to that of the wild-type AH-405. However, the activity of the *lafB* promoter was only slightly affected in the AH-3::*lafS* mutant (31% reduction), and activities from $fliM_L$, $flgA_L$ $flgB_L$, *lafB*, and *lafT* promoters exhibited comparable values for both strains (Fig. 3A). The *maf-5*p_1-*lacZ* cluster does not contain any promoter region, since β-galactosidase activity in the wild-type AH-405 was similar to that obtained with the pDN19lacΩ promoterless plasmid (data not shown). Sequence analysis of the *maf-5* upstream region found a pseudogene which encodes an incomplete flagellin fragment homologous (77/79% identity/similarity) to the C-terminal region of ASA_0374 of *A. salmonicida* A449 (32). We amplified the region both upstream and downstream of the predicted pseudogene start codon and cloned it into the plasmid pDN19lacΩ to generate the promoter-*lacZ* fusion plasmid pDNlac-*maf-5*p_2 (*maf-5*p_2-*lacZ*). This plasmid was transferred by triparental conjugation into *A. hydrophila* AH-405 and the AH-3::*lafS* mutant. The measurement of β-galactosidase activity from the *maf-5*p_2 promoter is significant only for wild-type AH-405 and was similar to the one obtained with the pDN19lacΩ promoterless plasmid in the *lafS* mutant (Fig. 3A).

Total RNAs from *A. hydrophila* AH-3 and the *lafS* mutant were used to amplify internal fragments of $fliM_L$, $flgM_L$, $flgA_L$, $flgB_L$, *maf-5*, *lafA*, *lafB*, and *lafT* transcripts, but no *lafA* and *maf-5* amplicons were obtained from the *lafS* mutant (Fig. 3C). Furthermore, analysis of $flgM_L$-$flgA_L$ transcription in the wild type by RT-PCR showed that these two genes were cotranscribed. The results suggest that $flgM_L$, *maf-5*, and *lafA* transcription are σ^{28} dependent, and $flgM_L$ is also transcribed from the $flgA_L$ promoter.

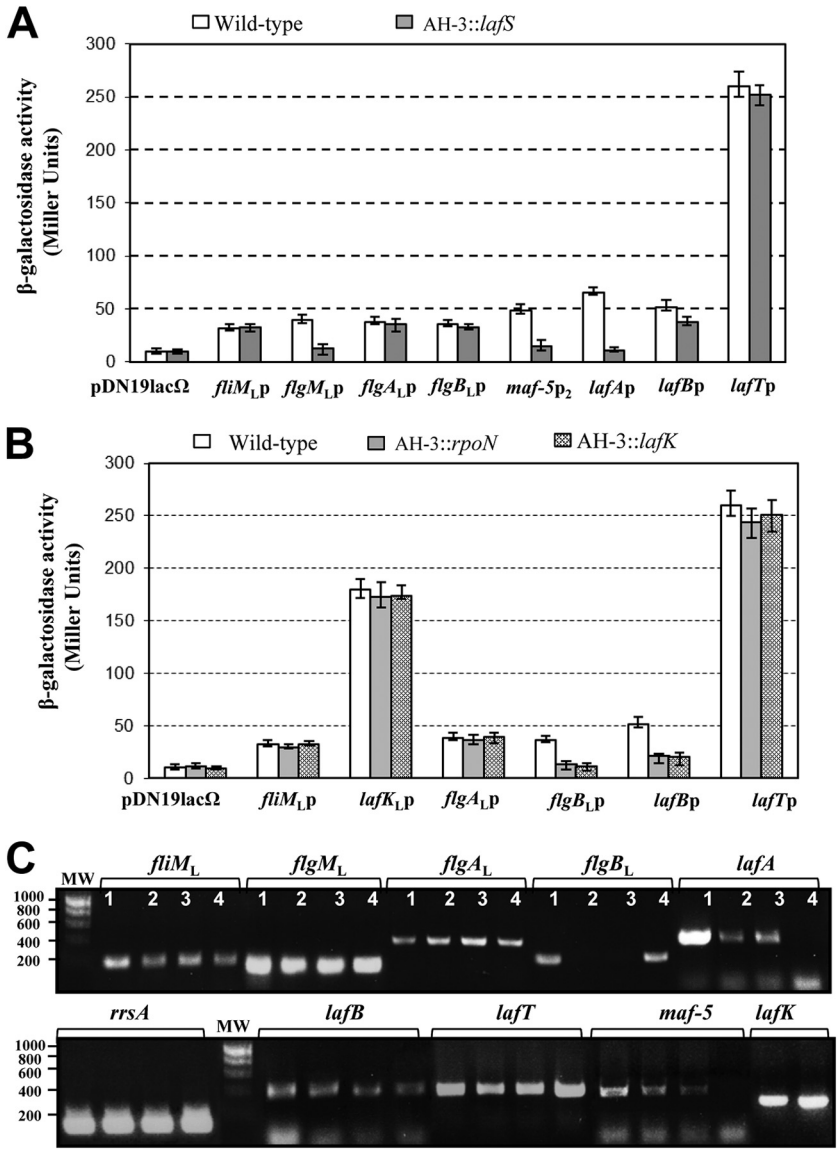
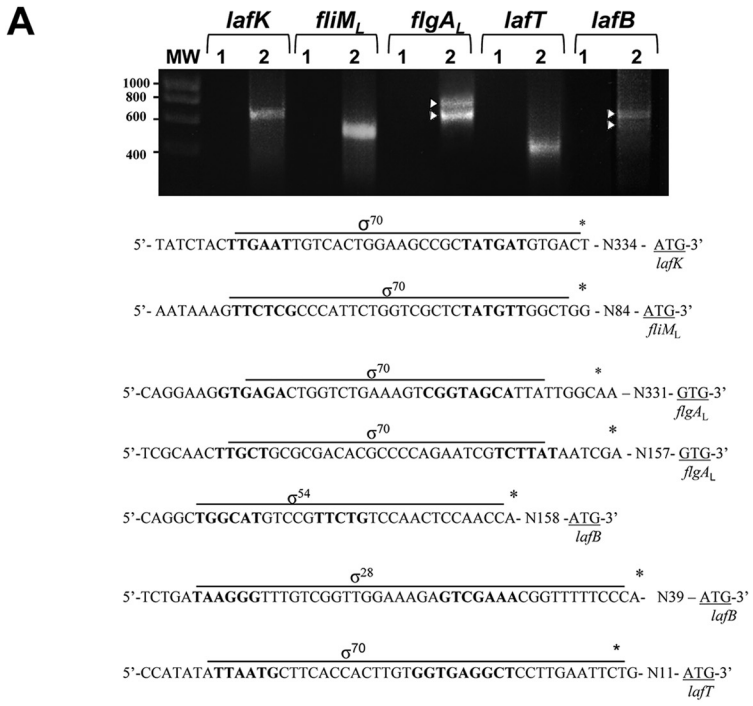


FIG 3 Analysis of β-galactosidase activity after growth in TSB with 18% Ficoll at 25°C. (A) pDNlac-*fliM*$_L$p, pDNlac-*flgM*$_L$p, pDNlac-*flgA*$_L$p, pDNlac-*flgB*$_L$p, pDNlac-*maf-5*p$_2$, pDNlac-*lafA*p, pDNlac-*lafB*p, and pDNlac-*lafT*p plasmids in *A. hydrophila* wild-type (AH-405) and *lafS* mutant (AH-3::*lafS*) strains. (B) pDNlac-*fliM*$_L$p, pDNlac-*lafK*p, pDNlac-*flgA*$_L$p, pDNlac-*flgB*$_L$p, pDNlac-*lafB*p, and pDNlac-*lafT*p plasmids in the *A. hydrophila* wild type (AH-405) and *rpoN* (AH-5502) and *lafK* (AH-5503) mutant strains. pDNlac-*lafK*p was not analyzed in the *lafK* mutant. As a control, we measured the pDN19lacΩ promoterless plasmid. The results shown are representative of three independent experiments. Bars represent standard deviations. (C) RT-PCR amplification of *fliM*$_L$, *lafK*, *flgM*$_L$, *flgA*$_L$, *flgB*$_L$, *maf-5*, *lafA*, *lafB*, and *lafT* from cDNA of AH-3 (lane 1), AH-3::*rpoN* (2), AH-3::*lafK* (3), and AH-3::*lafS* (4) mutants. *A. hydrophila* ribosomal 16S (*rrsA*) amplification was used as a control for the cDNA template. RT-PCR amplifications were performed at least twice with total RNA preparations obtained from a minimum of two independent extractions. The Ecogen molecular weight marker (MW) was used.

Identification of *A. hydrophila* LafK-dependent σ^{54} lateral flagellar promoters. 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 (33). Lateral flagellar clusters only contain one gene that encodes a σ^{54} enhancer-binding protein, LafK (34), which is required for *V. parahaemolyticus* lateral flagellum transcription (9). In *A. hydrophila* the LafK mutation abolishes lateral flagellum formation and swarming motility (19), as the wild-type phenotype is restored by complementation with the pBAD33Gm-LAFK plasmid in the presence of 0.2% L-arabinose. To investigate which of the *A. hydrophila* lateral flagellar clusters are σ^{54} and LafK dependent, β-galactosidase activities of the *A. hydrophila* wild type and the *lafK* (AH-5503) and *rpoN* (AH-5502) mutants carrying the promoter-*lacZ* fusion plasmids pDNlac-*fliM*$_L$p (*fliM*$_L$p-*lacZ*), pDNlac-*lafK*p (*lafK*p-*lacZ*), pDNlac-*flgA*$_L$p (*flgA*$_L$p-*lacZ*), pDNlac-*flgB*$_L$p (*flgB*$_L$p-*lacZ*), pDNlac-*lafB*p (*lafB*p-*lacZ*), and pDNlac-*lafT*p (*lafT*p-*lacZ*) were measured. Activities from *flgB*$_L$ and *lafB* promoters appeared to be affected in both LafK and RpoN mutant strains. The *flgB*$_L$ promoter showed a reduction of 93% in both mutants, and the *lafB* promoter show a reduction of 75 and 73% in the RpoN and LafK mutants, respectively (Fig. 3B).



B

Gene	Promoter sequences determined *in silico*	Distance upstream of start codon
$flgM_L$	5'-**GTGAT**CGAGAAAGGTGAA**GTCGAAA**-3'	87
maf-5	5'-**TCAAGT**GGATGAAGGGCAT**GCCGAGAA**-3'	683
lafA	5'-**CCCAT**TCCCGCCTTTTTGA**GCCGATCA**-3'	106
σ^{28} consensus	5'-**TAAA**-N15-**GCCGATAA**-3'	
$flgB_L$	5'-**TGGCAT**GAGTC**TTGAT**-3'	41
σ^{54} consensus	5'-T**GGCAC**-N4-**TTTGC**(A/T)-3'	

FIG 4 (A) Amplification of the *A. hydrophila* AH-3 *lafK*, $fliM_L$, $flgA_L$, *lafB*, and *lafT* cDNA 5′ end was performed using the 5′ RACE system, version 2.0 (Invitrogen). Amplicons were obtained by PCR using primers AAP and GSP2-LafK (*lafK*p), GSP2-$FLIM_L$ ($fliM_L$p), GSP2-$FLGA_L$ ($flgA_L$p), GSP2-LAFB (*lafB*p), and GSP2-LAFT (*lafT*p). Lanes: 1, PCR negative control; 2, primary PCR template; MW, molecular weight standard (Ecogen). Underlined sequences show start codons, asterisks show locations of the transcriptional start sites, and boldface nucleotides show potential consensus sequences. (B) Alignment *in silico* of σ^{28} and σ^{54} promoter elements in *A. hydrophila* lateral flagellar promoters. The consensus σ^{28} sequence is from Kutsukake (47). The consensus σ^{54} sequence is from Barrios (48).

No significant variations were obtained from $fliM_L$, *lafK*, $flgA_L$, and *lafT* promoters in any of these mutants. Furthermore, RT-PCRs to compare $fliM_L$, $flgA_L$, $flgB_L$, *lafB*, and *lafT* gene transcription in the wild type and the RpoN and LafK mutants showed $fliM_L$, $flgA_L$, *lafB*, and *lafT* amplicons in the wild type and both mutants, whereas no $flgB_L$ amplicon was found in the RpoN and LafK mutants (Fig. 3C). These results suggest that the $flgB_L$ promoter is σ^{54} and LafK dependent.

To identify the $fliM_L$, $flgA_L$, *lafB*, and *lafT* promoter regions, amplification of the *A. hydrophila* AH-3 $fliM_L$, $flgA_L$, *lafB*, and *lafT* cDNA 5′ ends was performed using 5′ RACE as described in Materials and Methods. Primary PCR of tailed cDNA using primers AAP and GSP2-$FLIM_L$ or GSP2-LafT give amplicons of 537 and 428 bp, respectively. However, primers AAP and GSP2-$FLGA_L$ or GSP2-LAFB render two amplicons of 650 and 827 bp and 700 and 588 bp, respectively. DNA sequence of the amplified bands indicates that amplicons were tailed with G residues. The $fliM_L$ and *lafT* transcription starts were located 85 and 12 nt upstream from the $fliM_L$ and *lafT* translation start sites, respectively. The $flgA_L$ transcription starts were located 158 and 332 nt upstream from the $flgA_L$ start site. The *lafB* transcription starts were located 39 and 158 nt upstream from the *lafB* start site. We were able to identify σ^{70} promoter sequences in DNA regions upstream of the transcription starts of $fliM_L$, $flgA_L$, and *lafT*. Both σ^{28} and σ^{54} promoter sequences were found upstream of the *lafB* transcription start (Fig. 4A).

DISCUSSION

Functional lateral flagellar systems, whose flagella are randomly distributed over the cell surface in a manner similar to that of the peritrichous flagella of *Enterobacteriaceae*, were reported in polar flagellated bacteria with dual flagellar systems, such as *A. hydrophila* and *V. parahaemolyticus* (5). Lateral flagellar systems of these two species are encoded by 38 genes, 37 of which are orthologous and do not share either structural or regulatory genes with polar flagellar systems. Despite these similarities, *A. hydrophila* lateral

flagellum genes are distributed in a unique chromosomal region, whereas *V. parahaemolyticus* lateral flagellar genes are distributed in two discontinuous chromosomal regions (9, 11, 19). The presence of two active flagellar systems implies a high energetic cost for a bacterium, therefore lateral flagellar synthesis should be carefully regulated in response to different environmental conditions. Different environmental conditions have been associated with lateral flagellar induction (35, 36), but the most extensive association is growth in viscous media or on a solid surface, which reduces polar flagellum motility. However, while *V. parahaemolyticus* and *Azospirillium brasilense* defects in polar flagellum formation or motility allow lateral flagellum expression (37, 38, 39), *Aeromonas* sp. polar flagellum defects do not induce constitutive lateral flagella (10, 40). This difference suggests that *Aeromonas* polar flagella do not act as mechanosensors and that lateral flagellar regulation is not linked to polar flagella.

The *V. parahaemolyticus* lateral flagellar system is the best studied at the regulatory level, and it has been demonstrated that its viscosity/surface-dependent expression is transcriptionally regulated (41). Transcription of *V. parahaemolyticus* lateral flagellar genes is organized into 3 levels (class I to III), where the first level contains the unique lateral flagellum σ^{54}-associated transcriptional activator, *lafK*, and the third level contains the lateral flagellin gene, *lafA*. Lateral flagella of *A. hydrophila* are also expressed in viscous media or on solid surfaces (Fig. 1A), but their regulatory mechanisms have not been determined yet. Our data show that *A. hydrophila* lateral flagellar genes are transcribed in liquid and viscous media or on solid surfaces, although lateral flagellin is not transduced in liquid media. All of the data suggest that lateral flagella expression should be regulated by transductional mechanisms.

Two transcriptional hierarchy models in *Gamma-* and *Alphaproteobacteria* lateral flagella have been described (7, 9, 42). Until now, *V. parahaemolyticus* lateral flagellar transcriptional hierarchy has represented the *Gammaproteobacteria* model. In this model, the σ^{54}-associated transcriptional activator, LafK, controls transcription of class II lateral flagellum genes which contain the σ^{28} factor ($fliA_L$) involved in transcription of class III lateral flagellum genes (9). In order to establish the *A. hydrophila* lateral flagellar cluster transcription hierarchy, promoter-*lacZ* fusions with lateral flagellum promoters were analyzed in defined *A. hydrophila lafK*, *lafS*, and *rpoN* mutants. Furthermore, transcription analysis of genes in different lateral flagellar clusters in these *A. hydrophila* mutants were tested by RT-PCR assays.

Class III. In *V. parahaemolyticus* the σ^{28} factor ($FliA_L$) is involved in transcription of late genes, such as lateral flagellin, the anti-sigma factor $flgM_L$, and motor components ($motAB_L$), but also some middle genes, such as $flgKL_L$ and $fliDSTKLA_L$ (9). Our data show that *A. hydrophila* $flgM_L$, *maf-5*, and *lafA* are transcribed from σ^{28}-dependent promoters, with the *maf-5* promoter being upstream of the pseudoflagelin gene, and $fliM_L$, $flgB_L$, $flgA_L$, and *lafT* are transcribed from σ^{28}-independent promoters (Fig. 3A and C). Furthermore, the slight reduction of β-galactosidase activity of *lafB* promoter fusion and the presence of the *lafB* transcript in the LafS mutant suggest that the *lafBCXEFSTU* cluster is transcribed from different promoters, with one of them being characterized as σ^{28} dependent. A similar situation has been reported in the *V. parahaemolyticus* lateral flagellar orthologous cluster $fliDSTKLA_L$-$motAB_L$ (9) (Fig. 4 and 5).

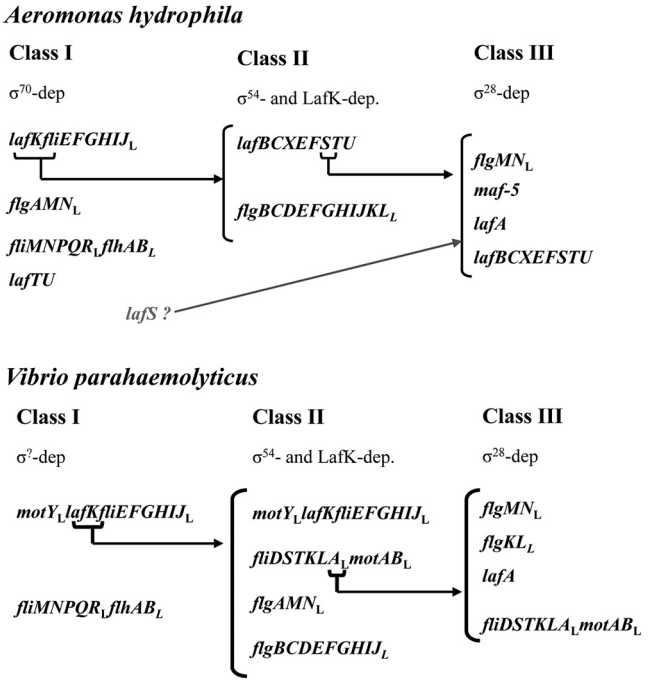


FIG 5 Comparative proposed *A. hydrophila* and *Vibrio parahaemolyticus* (9) lateral flagellar gene transcription hierarchies. Diagrams show the three levels of lateral flagellar hierarchy, class I to III. Class I genes are at the top of the hierarchy, being σ^{70} dependent (dep.) in *Aeromonas*. One of the class I genes encodes a σ^{54}-associated transcriptional activator (LafK) that activates σ^{54}-dependent promoters preceding the class II clusters. One of the class II genes encodes the σ^{28} factor (LafS), which activates the transcription of class III genes. In *Aeromonas*, the σ^{28} factor might also be transcribed from another promoter.

Class II. Transcription of the *V. parahaemolyticus* lateral flagellar genes included in the class II level are σ^{54} and LafK dependent (9). *A. hydrophila* LafK is essential for lateral flagella formation; however, in contrast to the case for *V. parahaemolyticus*, *lafK*, $flgA_L$ and *lafT* are transcribed from σ^{54}/LafK-independent promoters and are not class II genes (Fig. 5). Our results also suggest that $fliM_L$ is transcribed from σ^{54}/LafK-independent promoters and *flgB* is transcribed from a σ^{54}/LafK-dependent promoter, the sequence of which was predicted *in silico*. Furthermore, assays indicate that *lafB* promoter activity was only 73 to 75% reduced in the LafK and RpoN mutants (Fig. 3B), and the amplification of the *lafB* cDNA 5′ ends by 5′ RACE shows two promoter sequences, a σ^{28} promoter sequence (TAAGGG-N17-GTCGAAA) and a σ^{54} promoter sequence (TGGCAT-N5-TTCTG), with the latter being more active (Fig. 4). These results indicate that *lafBCXEFSTU* is transcribed from two promoters. A similar situation is described for *V. parahaemolyticus*, although it has not been studied at the transcriptional level. The σ^{28} factor *lafS* is contained in the *lafBCXEFSTU* cluster, but the lack of LafK does not prevent *lafS* transcription in *Aeromonas*. RT-PCR assays showed transcription of *maf-5* and *lafA* in LafK and RpoN mutants (Fig. 3C). Data suggest that *lafS* is transcribed from a second promoter in a σ^{54}/LafK-independent manner and is less active than the promoter upstream of *lafB*. Amplification of the *lafS* cDNA 5′ ends by 5′ RACE allowed us to obtain an amplicon (data not shown).

Class I. As previously indicated, *A. hydrophila* lateral flagellum

regulatory cascade class I seems to include more that one gene. Although previous *in silico* analysis showed putative σ^{54} promoter sequences upstream of *lafK*, $fliM_L$, and $flgA_L$ (19), the data obtained now show that upstream regions of $fliM_L$, $flgA_L$, *lafT*, and *lafK* contain σ^{70} promoter sequences (Fig. 4). *A. hydrophila* lateral flagellum class I gene transcription is σ^{70} dependent, as has been reported for *A. hydrophila* polar flagellar class I genes. Data are consistent with the fact that *A. hydrophila* lateral flagellar genes are transcribed in liquid and viscous media and on solid medium, in contrast to *V. parahaemolyticus* (41). *A. hydrophila* lateral flagella are not induced by mutation of polar flagellum genes, as happens in *V. parahaemolyticus*. In *V. parahaemolyticus*, the *lafK* promoter is located upstream of $motY_L$, which encodes a lateral motor protein that does not possess an orthologue in *A. hydrophila*, and genes of the $motY_L$-*lafK*-$fliEFGHIJ_L$ cluster are classified as class I and II. In addition, the *V. parahaemolyticus* $fliMNPQR_L$-$flhAB_L$ lateral flagellar cluster is classified as class I (43), as is the case in *A. hydrophila*. In *A. hydrophila*, *lafS* transcription feeds into class I, since LafK mutation does not abolish transcription of σ^{28}-dependent promoters (Fig. 3C). The *A. hydrophila* lateral flagellar transcriptional hierarchy is complex, since many clusters of genes are transcribed independently of LafK. LafK is not strictly the master lateral flagellar regulator in *A. hydrophila*.

Our results indicate that despite *A. hydrophila* lateral flagella only being expressed in viscous media or on solid surfaces, their genes are transcribed in liquid, although lateral flagellin was not detected in liquid media. Recently, it has been described that the *A. hydrophila* AH-3 lateral flagellin is glycosylated (44), although it is nonglycosylated in *V. parahaemolyticus*, and this fact could contribute to the complexity of their lateral flagella transcription hierarchy and the important differences between these two bacteria. An *A. hydrophila* AH-3 in-frame deletion mutant of the pseudaminic acid biosynthetic gene *pseB* homologue resulted in the abolition of lateral flagella formation by posttranscriptional regulation of the flagellin, which was restored by complementation with the wild-type *pseB* homologue or *Campylobacter pseB* (data not shown).

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