Aeromonas hydrophila Lateral Flagellar Gene Transcriptional Hierarchy

Markus Wilhelms, Victor Gonzalez, Juan M. Tomás and Susana Merino


Updated information and services can be found at:
http://jb.asm.org/content/195/7/1436

REFERENCES

These include:

This article cites 45 articles, 29 of which can be accessed free at:  http://jb.asm.org/content/195/7/1436#ref-list-1

CONTENT ALERTS

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article),  more»
Aeromonas hydrophila lateral flagellar gene transcriptional hierarchy

Markus Wilhelms, Victor Gonzalez, Juan M. Tomás, Susana Merino
Departamento de Microbiología, Facultad de Biología, Universidad de Barcelona, Barcelona, Spain

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 \( \sigma^{70} \) 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 \( \text{lafK-flfEIJ} \) 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 \( \text{flgAMN} \) cluster is not transcribed from the \( \sigma^{54} \)/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 \( \text{fliO} \), and the \( \text{fliEFGHIJKLMNPQR} \) gene cluster is split into two gene clusters (\( \text{fliEFGHIJ} \), and \( \text{fliMNPR} \) where the subscript L distinguishes genes for lateral flagella from those for polar flagella). The \( \text{flikL} \) (\( \text{lafEF} \)) genes are arranged in the \( \text{fliD}_L\)-\( \text{motB}_L \) (\( \text{lafB-lafU} \) 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 \( \text{flgAMN} \) and \( \text{motY}_L\)-\( \text{lafK-fliEIJ} \) clusters are transcribed divergently from \( \text{fliBCEFHIJK} \), \( \text{fliMNPR} \), \( \text{fliBA} \), \( \text{lafA} \), and \( \text{fliDSTKL} \)-\( \text{motAR} \). A. hydrophila orthologous genes exhibit the same distribution, although only \( \text{flgAMN} \) genes are transcribed divergently. Furthermore, A. hydrophila does not contain any gene orthologous to V. parahaemolyticus \( \text{motY}_L \) (12), and a modification accessory factor gene, \( \text{maf-5} \) (13), which is independently transcribed, is present between \( \text{flgL}_L \) and \( \text{lafA} \).

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 \( \sigma^{54} \) dependent, and transcription of class IV is \( \sigma^{28} \) dependent (16, 17, 18). At the top of the hierarchy is a \( \sigma^{54} \)-associated transcriptional activator (FlrA in A. hydrophila) which activates \( \sigma^{54} \)-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 \( \sigma^{28} \) 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 possess an FlhDC master regulator and are \( \sigma^{54} \) dependent (9, 19) as polar flagella. In this work, we investigated the A. hydrophila lateral flagellar transcriptional hierarchy by two techniques: promoter-\( \text{lacZ} \) fusion assays (used to measure \( \beta \)-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.

Received 17 October 2012 Accepted 11 January 2013
Published ahead of print 18 January 2013
Address correspondence to Juan M. Tomás, jtomas@ub.edu
Copyright © 2013, American Society for Microbiology. All Rights Reserved.
doi:10.1128/JB.01994-12

Aeromonas hydrophila
Plasmids

**Plasmids**

g|G|A|C|T|
---|---|---|---|---|
**pGEM-T easy** | Cloning vector, Ap<sup>R</sup> | Promega |
**pUC4-KDXX** | Helper plasmid, Sp<sup>R</sup> |  |
**pDM** | Suicide plasmid, pir dependent with sacB genes, oriV6K, Cat<sup>R</sup> | Pharmacia |
**pDM-LaKSm** | pDM4 with AH-3 ΔlacKm<sup>R</sup> | Cat<sup>R</sup> |
**pDM-LAFA** | pDM4 with AH-3 Δlafl<sup>R</sup> | this work |
**pBAD33-Gm** | pBAD33 arabinose-induced expression vector with Gm<sup>R</sup> | this work |
**pBAD33Gm-LAFK** | pBAD33 with AH-3 lafl<sup>R</sup> gene, Gm<sup>R</sup> | this work |
**pBAD33Gm-LAFS** | pBAD33 with AH-3 lafs<sup>R</sup> gene, Gm<sup>R</sup> | this work |
**pDN19 lacfl** | Promoterless lacZ fusion vector, Sp<sup>R</sup> | Sm<sup>R</sup> |
**pDNac-flmLp** | flmL promoter-lacZ fusion in pDN19lacfl, Tc<sup>R</sup> | this work |
**pDNac-lafsKp** | lafl promoter-lacZ fusion in pDN19lacfl, Tc<sup>R</sup> | this work |
**pDNac-flgAlp** | flgAl promoter-lacZ fusion in pDN19lacfl, Tc<sup>R</sup> | this work |
**pDNac-flgBkp** | flgB promoter-lacZ fusion in pDN19lacfl, Tc<sup>R</sup> | this work |
**pDNac-mafLp** | mafL promoter-lacZ fusion in pDN19lacfl, Tc<sup>R</sup> | this work |
**pDNac-mafSp<sub>1</sub>Lp** | maf<sub>1</sub> promoter-lacZ fusion in pDN19lacfl, Tc<sup>R</sup> | this work |
**pDNac-mafSp<sub>2</sub>Lp** | maf<sub>2</sub> promoter-lacZ fusion in pDN19lacfl, Tc<sup>R</sup> | this work |
**pDNac-lafsKp** | lafs promoter-lacZ fusion in pDN19lacfl, Tc<sup>R</sup> | this work |
**pDNac-lafsBp** | lafb promoter-lacZ fusion in pDN19lacfl, Tc<sup>R</sup> | this work |
**pDNac-lafTp** | laft promoter-lacZ fusion in pDN19lacfl, Tc<sup>R</sup> | this work |

**Strains**

**A. hydrophila**

| Strain | Genotype and/or phenotype<sup>a</sup> | Reference or source |
---|---|---|
AH-3 | A. hydrophila wild type, serogroup O:34 | 45 |
AH-405 | A. hydrophila spontaneous Rif<sup>R</sup> | 39 |
AH-4427 | A. hydrophila Δlafl<sup>R</sup> lafB<sup>Km</sup> | 10 |
AH-5502 | A. hydrophila Δlafl<sup>R</sup> lafK<sup>Km</sup> | 19 |
AH-5503 | A. hydrophila Δlafl<sup>R</sup> lafS<sup>Km</sup> | 19 |
AH-3:lafl<sup>+</sup> | This work |
AH-3:lafl<sup>R</sup> | This work |
AH-3:lafl<sup>−</sup> | This work |

**E. coli**

| Gene | Promoter and/tail sequences |
---|---|
DH5α | F<sup>−</sup> endA hsdR17 (rK<sup>−</sup> m<sup>−</sup>) supE44 thi-1 recA1 gyrA156 galK1 araD1217 galE19 thi-1 |
MC1061kpir | this work |

---

**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 assayed 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 dyeoxy-chain termination method (21) with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Custom-designed primers used for DNA sequencing 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 RNAeasy MiniKit (Qiagen), from A. hydrophila AH-3 and pBo, laf<sup>R</sup>, and laf<sup>S</sup> mutants grown in liquid medium (TSB), viscous medium (TSB plus 18% wt/vol) Ficol, or solid agar (TSA). To ensure that RNA was devoid of contaminating DNA, the preparation was treated with RNase-free TurboDnase 1 (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 DNA-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 (Invitrogen), specific oligonucleotides, and 30 PCR cycles. Amplions 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 flmL<sup>R</sup>, ifgAl<sup>R</sup>, lafK<sup>R</sup>, lafB<sup>R</sup>, and lafT<sup>R</sup> transcription start sites by RACE PCR.** Amplifications of the A. hydrophila AH-3 flmL<sup>R</sup>, ifgAl<sup>R</sup>, lafK<sup>R</sup>, lafB<sup>R</sup>, and lafT<sup>R</sup> 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 DNA-digested total RNA (5 μg), the flmL<sup>R</sup>, ifgAl<sup>R</sup>, lafK<sup>R</sup>, lafB<sup>R</sup>, and lafT<sup>R</sup> internal primers GP51-FILM (5′-ATCGTGCAAGGTGT G-3′), GP51-LfAl<sup>R</sup> (5′-GAGCTGTAAGAAATAC-3′), GP51-LaK<sup>R</sup> (5′-GATATACGAGAGCTG-3′), GP51-LaB<sup>R</sup> (5′-TGGTCGAAACTTCTT G-3′), and GP51-LaT<sup>R</sup> (5′-AATTATGAGTAAGAAA-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 GP52-FILM (5′-AGATGTGCCAGCTATTTG-3′), GP52-FLGA<sup>L</sup> (5′-ATCTCAGTAGCGAATGTTG-3′), GP52-LaA<sup>L</sup> (5′-GCATTACCAAGATGAC G-3′), GP52-LaB<sup>L</sup> (5′-GACCTCAGACCCACT-3′), and GP52-LaT<sup>L</sup> (5′-CTTCATGACCGTCAT-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 GP2-FLM1, GP2-FLG1, GP2-LafK, GP2-LafB, or GP2-LaT primer.

**Construction of defined mutants.** To obtain *A. hydrophila AH-3*: 

- *laf*S mutant, *laf*S was amplified by PCR with 5’-CCGGGATCCACACCATACAAGCCAGAGTGTGAG-3’ and 5’-CCGGGATCCACTGATTGCAACGAC-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 Small digestion, and the cassette was inserted into the XbaI blunt-ended restriction internal site of *laf*S. 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 BlpII site in the Smal-digested cassette allowed its orientation to be determined. Constructs containing the mutated genes were ligated to suicide vector pDM4 (27), electroporated into *E. coli* MC1061Ap1, and plated on chloramphenicol plates at 30°C to obtain the pDM-LafSKm plasmid.

- The chromosomal in-frame *laf*S deletion mutant *A. hydrophila AH-3*Δ*lafS* was obtained by allelic exchange as described by Milton et al. (27). Briefly, DNA fragments flanking the *laf*S gene were amplified using the primers A (5’-CCGGGATCCATGAAACACCAGGAC-3’) and B (5’-CCCAAGCTTCATCAGCTTGTTTCGCACCTGACTCCCT-3’), B (5’-CATTATGAAACACCAGACACA-3’), C (5’-GTGTTATGATGTGTAAGGCAACGACGACATGATGACAA-3’), and D (CCGGGATCCACACCATACAAGCCAGAGTGTGAG-3’). C (5’-GTGTTATGAAACACCAGACACA-3’), D (CCGGGATCCACACCATACAAGCCAGAGTGTGAG-3’), E (CCGGGATCCATGAAACACCAGGAC-3’), and F (CCGGGATCCATGAAACACCAGGAC-3’) in two sets of asymmetric primers to amplify DNA fragments of 779 (pair A) and 733 (pair CD) bp, respectively. DNA fragments AB and CD were annealed at 85°C and the insert containing the wild-type *laf*S gene was ampliminated using the primers A (5’-CCGGGATCCATGAAACACCAGGAC-3’) and F (CCGGGATCCATGAAACACCAGGAC-3’). The amplified fragment was isolated, ligated into vector pGEMTeasy (Promega), and transformed into *E. coli* DH5α (25), and transformed into *E. coli* DH5α (25).

**Plasmid constructions.** Plasmids pBAD33Gm-LafK and pBAD33Gm-LafB, containing the complete *lafK* and *lafB* genes of *A. hydrophila AH-3*, respectively, under the arabinose promoter (PBAD) on pBAD33-Gm (28), were transferred into mutant *A. hydrophila AH-3*Δ*lafS* (25). Briefly, DNA regions flanking the *lafK* and *lafB* genes were amplified using the primers A (5’-CGCGGATCCTTTGGTGTCGACTTCTCCT-3’) and B (5’-CCCAAGCTTCATCAGCTTGTTTCGCACCTGACTCCCT-3’), and D (CCGGGATCCACACCATACAAGCCAGAGTGTGAG-3’). C (5’-GTGTTATGAAACACCAGACACA-3’), and E (CCGGGATCCATGAAACACCAGGAC-3’), and F (CCGGGATCCATGAAACACCAGGAC-3’) in two sets of asymmetric primers to amplify DNA fragments of 779 (pair A) and 733 (pair CD) bp, respectively. DNA fragments AB and CD were annealed at 85°C and the insert containing the wild-type *lafS* gene was ampliminated using the primers A (5’-CCGGGATCCATGAAACACCAGGAC-3’) and F (CCGGGATCCATGAAACACCAGGAC-3’). The amplified fragment was isolated, ligated into vector pGEMTeasy (Promega), and transformed into *E. coli* DH5α (25), and transformed into *E. coli* DH5α (25).

**Construction of flagellar promoter-lacZ fusions.** Oligonucleotide primer pairs for the *A. hydrophila AH-3* promoter regions of the flmI, flmJ, flmK, flmL, flmM, flmN, 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 flmI, flmJ, flmK, flmL, maf-5, and *lafA* promoters were recovered by EcoRI/BamHI restriction digestion, inserts containing *lafB* and *lafB* promoters were recovered by EcoRI/BglII restriction digestion, and the insert containing *lafT* promoter was recovered by Small/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 vector and the promoter fragment was ligated into pDM-LAFA plasmid. Digestion fragments were ligated into plasmid pDN19αC EcoRI/BamHI-digested or EcoRI blunt-ended BamHI (29), transformed into *E. coli* DH5α (25), and selected for tetracycline resistance (Tc). The final constructs were confirmed by DNA sequencing.

**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.

**Immunoblot 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 flagellar fractions, cells grown on plates were collected with 20 mM MgCl₂, 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 MgCl₂, 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 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

---

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

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Primer sequence</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>flmI</td>
<td>5’-TGCAACAGGAGCAACAGGAC-3’</td>
<td>486 flmI</td>
</tr>
<tr>
<td>flmJ</td>
<td>5’-CCGGGATCTGTTGAGTCCTCGGCTTC-3’</td>
<td>+114 flmJ</td>
</tr>
<tr>
<td>flmK</td>
<td>5’-ACGAGAAGACCTGGAATC-3’</td>
<td>-325 flmK</td>
</tr>
<tr>
<td>flmL</td>
<td>5’-CCGGGATCCTCCGAGGATGCCGATACATGAAT-3’</td>
<td>+168 flmL</td>
</tr>
<tr>
<td>flmM</td>
<td>5’-GGAAGATCCCTCGGATTTTCTATTACATC-3’</td>
<td>-494 flmM</td>
</tr>
<tr>
<td>flmN</td>
<td>5’-CCGGAATCCCTCGGATTTTCTATTACATC-3’</td>
<td>+386 flmM</td>
</tr>
<tr>
<td>maf-5</td>
<td>5’-GAGTCTTGCAGAGGAAGAAA-3’</td>
<td>-521 maf-5</td>
</tr>
<tr>
<td>maf-5</td>
<td>5’-CCGGATCTCTAGTGCAAGGACGACGAC-3’</td>
<td>+359 maf-5</td>
</tr>
<tr>
<td>lafA</td>
<td>5’-GTGATGACGCTTGGTTGACGATGCTGCTG-3’</td>
<td>-1490 maf-5</td>
</tr>
<tr>
<td>lafB</td>
<td>5’-CGGAGATCCCTGATCAGGCCGATGTTGAAT-3’</td>
<td>+523 maf-5</td>
</tr>
<tr>
<td>lafC</td>
<td>5’-TTCCTCGGAGTTGCAAGGAC-3’</td>
<td>-1519 lafA</td>
</tr>
<tr>
<td>lafD</td>
<td>5’-TCCCTGGGGATTCATGACATG-3’</td>
<td>+41 lafA</td>
</tr>
<tr>
<td>lafE</td>
<td>5’-TCTTGGGATTGTAAATG-3’</td>
<td>-553 lafB</td>
</tr>
<tr>
<td>lafF</td>
<td>5’-TGTCTTGGGAACCAATCGGAAT-3’</td>
<td>+194 lafB</td>
</tr>
<tr>
<td>lafG</td>
<td>5’-TCCCGGCGGTAGTTGATGACATGACATGAC-3’</td>
<td>-732 lafT</td>
</tr>
<tr>
<td>lafH</td>
<td>5’-CCGGGATCCATGAAACACCAGGACGAC-3’</td>
<td>+53 lafT</td>
</tr>
</tbody>
</table>

* Underlined letters show the BglII restriction site. Double-underlined letters show the BglII restriction site.
* Italized letters show the Small restriction site.
* Numbers are bases of nucleotides upstream (−) or downstream (+) from the start site for the indicated gene.
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).

**RESULTS**

Lateral flagellar genes of *A. hydrophila* AH-3 are transcribed, but lateral flagella are not expressed in liquid medium. *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$^{44}$-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-lafKp (lafKp-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-lafAp (lafAp-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$_1$, flgB$_1$, 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$_1$, flgB$_1$, 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$_2$ and flgB$_2$, which are divergently transcribed genes, eliminating the possibility of residual DNA in the RNA samples from liquid cultures.

The presence of lateral flagellin (LafA) in the cytoplasmic fraction of *A. hydrophila* AH-3 and the mutants AH-4427 (without polar flagellin), AH-3ΔlafA (without lateral flagellin), and AH-5502 (ropN) 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). AH-3 and AH-4427 mutant cytoplasmic fractions obtained after growth in liquid medium, 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.

**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-lafKp, pDNlac-lafAp, pDNlac-flgA$_1$, pDNlac-flgB$_1$, and pDNlac-laflBp 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 pDN19lacC1 promoterless plasmid. The results shown are representative of three independent experiments. (C) RT-PCR amplification of lafK, lafA, flgA$_1$, flgB$_1$, 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.
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. Non-lateral 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 show 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 plasmids pDNlac-flgM, pDNlac-flgA, pDNlac-lafB, and lafT promoters exhibited comparable values for both strains (Fig. 3A). The maf-5-lacZ cluster does not contain any promoter region, 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 flgM, flgA, fliM, and lafT promoters exhibited comparable values for both strains (Fig. 3A). The maf-5-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 70 promoter sequence 331 bp upstream of flgA using the 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 of the lafK translation start site, and DNA sequence upstream of the transcription start contains a σ^{28} 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^{28} promoter sequence 331 bp upstream of the lafK start codon (5'-TTGAAT-N16-TATGAT-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 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% 3,4-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 flgM1 (19), putative σ^{28} promoter sequences upstream of flgM2, flgA1, flgB1, 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-flgM, pDNlac-flgA, pDNlac-lafB, pDNlac-maf-5, pDNlac-lafT, 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 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 flgM, flgA, fliM, and lafT promoters exhibited comparable values for both strains (Fig. 3A).
Identification of *Aeromonas hydrophila* LfK-dependent $\sigma^{\text{II}}_5$ lateral flagellar promoters. Promoters recognized by the $\sigma^{\text{II}}_5$ 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 $\sigma^{\text{II}}_5$ enhancer-binding protein, LfK (34), which is required for *V. parahaemolyticus* lateral flagellum transcription (9). In *A. hydrophila* the LfK 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 $\sigma^{\text{II}}_5$ and LfK dependent, $\beta$-galactosidase activities of the *A. hydrophila* wild type and the *lafK* (AH-5503) and *rpoN* (AH-5502) mutant strains. $\beta$-Galactosidase activities of the *A. hydrophila* wild type and the *lafK* (AH-5503) mutant strains. $\beta$-Galactosidase activities of the *A. hydrophila* wild type and the *lafK* (AH-5503) mutant strains. $\beta$-Galactosidase activities of the *A. hydrophila* wild type and the *lafK* (AH-5503) mutant strains. P DNlac-fliM p, pDNlac-flgM p, pDNlac-flgA p, pDNlac-flgB p, pDNlac-maf-5 p, pDNlac-lafAp, pDNlac-lafBp, and pDNlac-lafTp plasmids in *A. hydrophila* wild-type (AH-405) and lafS mutant (AH-3::lafS) strains. P DNlac-maf-5 p, pDNlac-lafK p, pDNlac-flgAp, pDNlac-flgBp, pDNlac-lafBp, and pDNlac-lafTp plasmids in the *A. hydrophila* wild type (AH-405) and rpoN (AH-5502) and lafK (AH-5503) mutant strains. P DNlac-lafKp was not analyzed in the lafK mutant. As a control, we measured the pDN19lac 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, maf-5, lafA, lafB, and ltf 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.

FIG 3 Analysis of $\beta$-galactosidase activity after growth in TSB with 18% Ficoll at 25°C. (A) P DNlac-fliM p, P DNlac-flgM p, P DNlac-flgA p, P DNlac-flgB p, P DNlac-maf-5 p, P DNlac-lafAp, P DNlac-lafBp, and P DNlac-lafTp plasmids in *A. hydrophila* wild-type (AH-405) and lafS mutant (AH-3::lafS) strains. (B) P DNlac-maf-5 p, P DNlac-lafK p, P DNlac-flgAp, P DNlac-flgBp, P DNlac-lafBp, and P DNlac-lafTp plasmids in the *A. hydrophila* wild type (AH-405) and rpoN (AH-5502) and lafK (AH-5503) mutant strains. P DNlac-lafKp was not analyzed in the lafK mutant. As a control, we measured the pDN19lac 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, maf-5, lafA, lafB, and ltf 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 *Aeromonas hydrophila* LfK-dependent $\sigma^{\text{II}}_5$ lateral flagellar promoters. Promoters recognized by the $\sigma^{\text{II}}_5$ 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 $\sigma^{\text{II}}_5$ enhancer-binding protein, LfK (34), which is required for *V. parahaemolyticus* lateral flagellum transcription (9). In *A. hydrophila* the LfK 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 $\sigma^{\text{II}}_5$ and LfK dependent, $\beta$-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 p (fliM Lp-lacZ), pDNlac-lafK p (lafK p-lacZ), pDNlac-flgA p (flgA Lp-lacZ), pDNlac-flgB p (flgB Lp-lacZ), pDNlac-lafB p (lafB p-lacZ), and pDNlac-lafTp (lafTp-lacZ) were measured. Activities from flgB L and lafB promoters appeared to be affected in both LfK 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 LfK mutants, respectively (Fig. 3B).
No significant variations were obtained from fliM<sub>L</sub>, flgA<sub>L</sub>, and lafT promoters in any of these mutants. Furthermore, RT-PCRs to compare fliM<sub>L</sub>, flgA<sub>L</sub>, flgB<sub>L</sub>, lafB, and lafT gene transcription in the wild type and the RpoN and LafK mutants showed fliM<sub>L</sub>, flgA<sub>L</sub>, lafB, and lafT amplicons in the wild type and both mutants, whereas no flgB<sub>L</sub> amplicon was found in the RpoN and LafK mutants (Fig. 3C). These results suggest that the flgB<sub>L</sub> promoter is RpoN and LafK dependent.

To identify the fliM<sub>L</sub>, flgA<sub>L</sub>, lafB, and lafT promoter regions, amplification of the A. hydrophila AH-3 lafK, fliM<sub>L</sub>, flgA<sub>L</sub>, 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<sub>p</sub>), GSP2-FLIM<sub>L</sub> (fliM<sub>L</sub><sub>p</sub>), GSP2-FLGAL (flgA<sub>L</sub><sub>p</sub>), GSP2-LAFB (lafB<sub>p</sub>), and GSP2-LAFT (lafT<sub>p</sub>). 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<sup>28</sup> and σ<sup>54</sup> promoter elements in A. hydrophila lateral flagellar promoters. The consensus σ<sup>28</sup> sequence is from Kutsukake (47). The consensus σ<sup>54</sup> sequence is from Barrios (48).
flagellum genes are distributed in a unique chromosomal region, whereas \textit{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 \textit{V. parahaemolyticus} and \textit{Azospirillum brasilense} defects in polar flagellum formation or motility allow lateral flagellum expression (37, 38, 39), \textit{Aeromonas} sp. polar flagellum defects do not induce constitutive lateral flagella (10, 40). This difference suggests that \textit{Aeromonas} polar flagella do not act as mechanosensors and that lateral flagellar regulation is not linked to polar flagella.

The \textit{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 \textit{V. parahaemolyticus} lateral flagellar genes is organized into 3 levels (class I to III), where the first level is fully regulated (41). Transcription of \textit{Viscosity/surface-dependent expression} is transcriptionally regulated at the regulatory level, and it has been demonstrated that its regulation is not linked to polar flagella.

Class II. Transcription of the \textit{V. parahaemolyticus} lateral flagellar genes included in the class II level are \(\sigma^{54}\) and LafK dependent (9). \textit{A. hydrophila} LafK is essential for lateral flagella formation; however, in contrast to the case for \textit{V. parahaemolyticus}, LafK, flgA, and lafT are transcribed from \(\sigma^{54}\)/LafK-independent promoters and are not class II genes (Fig. 5). Our results also suggest that flgM is transcribed from \(\sigma^{28}\)/LafK-independent promoters and flgB is transcribed from a \(\sigma^{28}\)/LafK-dependent promoter, the sequence of which was predicted \textit{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 \(\sigma^{28}\) promoter sequence (TAAAGG-N17-GTCGAAA) and a \(\sigma^{24}\) promoter sequence (TGGCAT-N5-TTCTG), with the latter being more active (Fig. 4). These results indicate that lafBCXEUSTU is transcribed from two promoters. A similar situation is described for \textit{V. parahaemolyticus}, although it has not been studied at the transcriptional level. The \(\sigma^{28}\) factor lafS is contained in the lafBCXEUSTU cluster, but the lack of LafK does not prevent lafS transcription in \textit{Aeromonas}. RT-PCR assays showed transcription of lafM and lafA in LafK and RpoN mutants (Fig. 3C). Data suggest that lafS is transcribed from a second promoter in an \(\sigma^{54}\)/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 ampli-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5}
\caption{Comparative proposed \textit{A. hydrophila} and \textit{Vibrio parahaemolyticus} 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 \(\sigma^{54}\) dependent (dep.) in \textit{Aeromonas}. One of the class I genes encodes a \(\sigma^{54}\)-associated transcriptional activator (LaF) that activates \(\sigma^{54}\) dependent promoters preceding the class II clusters. One of the class II genes encodes the \(\sigma^{28}\) factor (LafS), which activates the transcription of class III genes. In \textit{Aeromonas}, the \(\sigma^{28}\) factor might also be transcribed from another promoter.}
\end{figure}
regulatory cascade class I genes are known to include more that one gene. Although previous in silico analysis showed putative σ^54 promoter sequences upstream of lafK, fliM, and flgA (19), the data obtained
now show that upstream regions of fliM, flgA, lafT, and lafK contain a 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 motY₁, which encodes a lateral motor protein that does not possess an orthologue in A. hydrophila, and genes of the motY₁-lafK-flfEFGHIJ cluster are classified as class I and II. In addition, the V. parahaemolyticus fliMNPRQ₁-fliAB₁ 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^70-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 pseudominic 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).

ACKNOWLEDGMENTS

This work was supported by Plan Nacional de I + D (Ministerio de Educa-


ción, Ciencia y Deporte and Ministerio de Sanidad, Spain) and Gener-


alitat de Catalunya (Centre de Referència en Biotecnologia).

We thank Maite Polo for her technical assistance and the Servicios Científico-Técnicos from the University of Barcelona.

REFERENCES

tory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Har-
bory, NY.
21. Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain-