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Polar Flagellum Biogenesis in *Aeromonas hydrophila*

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Mesophilic *Aeromonas* spp. constitutively express a single polar flagellum that helps the bacteria move to more favorable environments and is an important virulence and colonization factor. Certain strains can also produce multiple lateral flagella in semisolid media or over surfaces. We have previously reported 16 genes (*flgN* to *flgL*) that constitute region 1 of the *Aeromonas hydrophila* AH-3 polar flagellum biogenesis gene clusters. We identified 39 new polar flagellum genes distributed in four noncontiguous chromosome regions (regions 2 to 5). Region 2 contained six genes (*flaA* to *maf-1*), including a modification accessory factor gene (*maf-1*) that has not been previously reported and is thought to be involved in glycosylation of polar flagellum filament. Region 3 contained 29 genes (*fliE* to *orf29*), most of which are involved in flagellum basal body formation and chemotaxis. Region 4 contained a single gene involved in the motor stator formation (*motX*), and region 5 contained the three master regulatory genes for the *A. hydrophila* polar flagella (*flrA* to *flrC*). Mutations in the *flaH*, *maf-1*, *fliM*, *flhA*, *fliA*, and *flrC* genes, as well as the double mutant *flaA flaB*, all caused loss of polar flagella and reduction in adherence and biofilm formation. A defined mutation in the *pomB* stator gene did not affect polar flagellum motility, in contrast to the *motX* mutant, which was unable to swim even though it expressed a polar flagellum. Mutations in all of these genes did not affect lateral flagellum synthesis or swarming motility, showing that both *A. hydrophila* flagellum systems are entirely distinct.

Flagellar motility represents an important advantage for bacteria in moving toward favorable conditions or in avoiding detrimental environments, and it allows flagellated bacteria to successfully compete with other microorganisms (21). In addition, motility and flagella play a crucial role in adhesion, biofilm formation, and colonization of several pathogenic bacteria, such as *Pseudomonas aeruginosa* (59), *Salmonella enterica* (13), *Escherichia coli* (51), *Helicobacter pylori* (19), *Vibrio cholerae* (22), and *Aeromonas hydrophila* (43, 53).

Mesophilic *Aeromonas* spp. are ubiquitous waterborne bacteria and pathogens of reptiles, amphibians, and fish (5). They can be isolated as a part of the fecal flora of a wide variety of other animals, including some used for human consumption, such as pigs, cows, sheep, and poultry. In humans, *Aeromonas hydrophila* belonging to hybridization group 1 (HG1) and HG3, *A. veronii* biovar sobria (HG8/HG10), and *A. caviae* (HG4) have been associated with gastrointestinal and extraintestinal diseases, such as wound infections of healthy humans, and less commonly with septicemia of immunocompromised patients (30). The swimming motility of all mesophilic aeromonads has been linked to a single polar unsheathed flagellum, expressed constitutively, which is required for adherence to and invasion of human and fish cell lines (25, 43, 53, 63). Moreover, 50% to 60% of mesophilic aeromonads are able to produce many unsheathed peritrichous lateral flagella when grown in viscous environments or over surfaces (58), which increase bacterial adherence and are required for swarming motility and biofilm formation (23). The expression of two distinct flagellar systems is relatively uncommon, although it has been observed with *Vibrio parahaemolyticus* (39), *Azospirillum brasilense* (45), *Rhodospirillum centenum* (32), *Helicobacter mustelae* (49), and *Plesiomonas shigelloides* (29).

Previous reports described two noncontiguous polar flagellum regions for *Aeromonas*: (i) a polar flagellum region of *A. caviae*, containing five genes that encode two tandem flagellins (FlaA and FlaB), a protein involved in flagellum filament length control (FlaG), a HAP-2 distal capping protein (FlaH), and a putative flagellin chaperone (FlaJ) (53); and (ii) a polar flagellum region of *A. hydrophila*, containing 16 genes which encode chemotaxis (CheV and CheR), hook (FlgE, FlgK, and FlgL), rod (FlgB, FlgC, FlgD, FlgF, FlgG, and FlgJ), L ring (FlgH), and P ring (FlgI) proteins, as well as two specific chaperones (FlgA and FlgN) and the anti-σ^{28} factor (FlgM) (2).

Although some genes have been described, many others are required for the expression and regulation of *Aeromonas* polar flagella. This work employed transposon mutagenesis and mutant complementation to isolate the *A. hydrophila* AH-3 chromosomal regions required for polar flagellum expression. Furthermore, we investigated the distribution of these genes among the mesophilic *Aeromonas* species, characterized several *Aeromonas* strains with defined mutations in different polar flagellar genes, and studied their motility, presence or absence of both types of flagella, adherence to HEp-2 cells, and ability to form biofilms.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown on Luria-Bertani (LB) Miller broth and LB Miller agar at 37°C, *Aeromonas* strains

TABLE 1. Bacterial strains and plasmids 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	42
AH-405	AH-3; spontaneous Rif^r	2
AH-4422	AH-405; *maf-1*::miniTn*5*Km-1	This work
AH-4423	AH-405; *flaH*::Km^r	This work
AH-4424	AH-405; *maf-1*::Km^r	This work
AH-4425	In-frame AH-405 Δ*flaB* mutant	This work
AH-4426	AH-405; *flaA*::Km^r	This work
AH-4427	AH-4425; *flaA*::Km^r	This work
AH-4440	AH-405; *flhA*::miniTn*5*Km-1	This work
AH-4441	AH-405; *fliM*::Km^r	This work
AH-4442	AH-405; *flhA*::Km^r	This work
AH-4443	AH-405; *fliA*::Km^r	This work
AH-4444	AH-405; *pomB*::Km^r	This work
AH-4460	AH-405; *motX*::miniTn*5*Km-1	This work
AH-4461	AH-405; *motX*::Km^r	This work
AH-4462	AH-405; *flrC*::Km^r	This work
E. coli		
DH5α	F$^-$ *endA hdsR17*(r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96* ϕ80*lacZ*	27
XL1-Blue	*endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1 lac* (F′ *proAB lacIZΔM15* Tn*10*)	Stratagene
S17-1λ*pir*	*thi thr-1 leu tonA lacy supE recA*::RP4-2 (Tc::Mu) Km^r λ*pir* with miniTn*5*Kml	16
MC1061λ*pir*	*thi thr-1 leu-6 proA2 his-4 argE2 lacY1 galK2 ara-14 xyl-5 supE44* λ*pir*	55
Vibrio cholerae		
KKV98	Δ*flrC1* Δ*lacZ*	35
KKV59	Δ*flrA1* Δ*lacZ*	35
Plasmids		
pGEMT	Cloning vector; Ap^r	Promega
pBCSK	Cloning vector with *lacZ* gene; Cm^r	Stratagene
pLA2917	Cosmid vector; Tc^r Km^r	1
pACYC184	Plasmid vector; Cm^r Tc^r	56
pRK2073	Helper plasmid; Sp^r	55
pFS100	pGP704 suicide plasmid, *pir* dependent; Km^r	55
pDM4	Suicide plasmid, *pir* dependent with *sacAB* genes, oriR6K; Cm^r	44
COS-FLG	pLA2917 with AH-3 polar flagellum region 1 (*flgA* to *flgL*); Tc^r	2
pLA-FLA	pLA2917 with AH-3 *flaA* to *maf-1*; Tc^r	This work
pLA-FLIP1	pLA2917 with AH-3 *fliE* to *cheY*; Tc^r	This work
pLA-FLIP2	pLA2917 with AH-3 *fliQ* to *orf29*; Tc^r	This work
pLA-MOTX	pLA2917 with AH-3 *motX*; Tc^r	This work
pLA-FLR	pLA2917 with AH-3 *flrABC*; Tc^r	This work
pACYC-MAF	pACYC184 with AH-3 *maf-1*; Tc^r	This work
pACYC-FLR1	pACYC184 with AH-3 *flrBC*; Tc^r	This work
pFS-FLAA	pFS100 with internal fragment of AH-3 *flaA* gene; Km^r	This work
pFS-MAFP	pFS100 with internal fragment of AH-3 *maf-1* gene; Km^r	This work
pFS-POMB	pFS100 with internal fragment of AH-3 *pomB* gene; Km^r	This work
pFS-MOTX	pFS100 with internal fragment of AH-3 *motX* gene; Km^r	This work
pFS-FLRC	pFS100 with internal fragment of AH-3 *flrC* gene; Km^r	This work
pDM-FLAH	pDM4 with AH-3 *flaH*::Km; Cm^r Km^r	This work
pDM-FLIM	pDM4 with AH-3 *fliM*::Km; Cm^r Km^r	This work
pDM-FLHA	pDM4 with AH-3 *flhA*::Km; Cm^r Km^r	This work
pDM-FLIA	pDM4 with AH-3 *fliA*::Km; Cm^r Km^r	This work
pDM-FLAB	pDM4 Δ*flaB* of AH-3; Cm^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.

were grown in either tryptic soy broth (TSB) or tryptic soy agar (TSA) at 30°C, and *Vibrio cholerae* strains were grown in either LB or LB agar with 2 mM glutamine at 37°C. When required, ampicillin (50 μg/ml), kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), rifampin (100 μg/ml), spectinomycin (50 μg/ml), and tetracycline (20 μg/ml) were added to the different 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, 0.6% agar) or swim agar (1% tryptone, 0.5% NaCl, 0.25% agar). Agar plates consisting of LB with 0.3% agar and 2 mM glutamine were used to measure *Vibrio cholerae* motility. The plates were incubated face up for 16 to 24 h at 30°C, and motility was assessed by examining the migration of bacteria through the agar from the center towards the periphery of the plate. Moreover, swimming motility was assessed by light microscopy observations with liquid media.

Transmission electron microscopy (TEM). Bacterial suspensions were placed on Formvar-coated grids and negative stained with a 2% solution of uranyl

acetate, pH 4.1. Preparations were observed with a Hitachi 600 transmission electron microscope.

MiniTn*5*Km-1 mutagenesis. Conjugal transfer of transposition element miniTn*5*Km-1 from *E. coli* S17-1λ*pir*Km-1 (16) to *A. hydrophila* AH-405 (AH-3, rifampin resistant) was carried out in a conjugal drop incubated for 6 h at 30°C with the ratio 1:5:1, corresponding to S17-1λ*pir*Km-1, AH-405, and HB101 pRK2073 (helper plasmid), respectively. Serial dilutions of the mating mix were plated on TSA supplemented with rifampin and kanamycin in order to select mutants.

DNA techniques. DNA manipulations were carried out essentially as previously described (56). DNA restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerase Klenow fragment, and alkaline phosphatase were used as recommended by the suppliers. PCR was performed using *Taq* DNA polymerase (Invitrogen) in a gene amplifier PCR system 2400 thermal cycler (Perkin-Elmer). Colony hybridizations were carried out by colony transfer onto positive nylon membranes (Roche) and then lysed according to the manufacturer's instructions. Probe labeling with digoxigenin, hybridization, and detection (Amersham) were carried out as recommended by the suppliers.

Cloning of DNA flanking miniTn*5*Km-1 insertions. Chromosomal DNA of miniTn*5*Km-1 mutants was digested with EcoRI, PstI, and EcoRV, purified, ligated into the vector pBCSK (Stratagene), and introduced into *E. coli* XL1-Blue. Recombinant plasmids containing the transposon with flanking insertions were selected in LB plates supplemented with kanamycin and chloramphenicol. The miniTn*5*Km-1 flanking sequences were obtained by using primers specific to the I and O ends of miniTn*5*Km-1 (5′-AGATCTGATCAAGAGACAG-3′ and 5′-ACTTGTGTATAAGAGTCAG-3′, respectively), as well as M13for and T3 primers from the plasmid vector used.

Nucleotide sequencing and computer sequence analysis. Plasmid DNA for sequencing was isolated with 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 (57) with an ABI Prism dye terminator cycle sequencing kit (Perkin-Elmer). Custom-designed primers used for DNA sequencing were purchased from Amersham Biosciences.

The DNA sequence was translated in all six frames, and all open reading frames (ORFs) greater than 100 bp were inspected. Deduced amino acid sequences were inspected in the GenBank, EMBL, and SwissProt databases by using the BLASTX, BLASTP, or PSI-BLAST network service at the National Center for Biotechnology Information (NCBI) (3). A protein family profile was performed using the Protein Family Database Pfam at the Sanger Center (6). Determination of possible terminator sequences was done by using the Terminator program from the Genetics Computer Group package (Madison, Wisconsin). Other online sequence analysis services were also used.

RT-PCR. Total RNA was isolated from *A. hydrophila* AH-3 grown in liquid medium (TSB) and plates by Trizol reagent (Invitrogen). To ensure that RNA was devoid of contaminating DNA, the preparation was treated with RNase-free DNase I, amplification grade (Invitrogen). The isolated RNA was used as a template in reverse transcriptase (RT) PCR, utilizing a Thermoscript RT-PCR system (Invitrogen) according to the manufacturer's instructions. PCR without reverse transcriptase was also performed to confirm the absence of contaminating DNA in the RNA samples. RT-PCR amplifications were performed at least twice with total RNA preparations obtained from minimum of two independent extractions. The RT-PCR and PCR products were analyzed by gel electrophoresis.

Mutant construction. To obtain single defined insertion mutants with mutations in genes *flaA*, *maf-1*, *pomB*, *motX*, and *flrC*, we used a method based on suicide plasmid pFS100 (55). Briefly, an internal fragment of the selected gene was amplified by PCR, ligated into pGEM-Teasy (Promega), and transformed into *E. coli* XL1-Blue. The DNA insert was recovered by EcoRI restriction digestion and was ligated into EcoRI-digested and phosphatase-treated pFS100. The ligation product was transformed into *E. coli* MC1061 (λ*pir*) and selected for kanamycin resistance (Km^r). Triparental mating with the mobilizing strain HB101/pRK2073 was used to transfer the recombinant plasmid into *A. hydrophila* AH-405 rifampin-resistant (Rif^r) strains to obtain defined insertion mutants, selecting for Rif^r and Km^r.

To obtain strains with mutations in *flaH*, *fliM*, *flhA*, and *fliA*, the genes were amplified by PCR, ligated into vector pGEMTeasy (Promega), and transformed into *E. coli* XL1-Blue. The Tn*5*-derived kanamycin resistance cartridge (*nptII*) from pUC4-KIXX was inserted into each of these genes. The cartridge contains an outward-reading promoter that ensures the expression of downstream genes when inserted in the correct orientation (9); however, such an insertion will alter the regulation of such genes. The SmaI-digested cassette was inserted into a restriction site internal to each gene, and the presence of a single HindIII site in the SmaI-digested cassette allowed its orientation to be determined. Constructs containing the mutated genes were ligated into suicide vector pDM4 (44), electroporated into *E. coli* MC1061 (λ*pir*), and plated on chloramphenicol plates at 30°C. Plasmids with mutated genes were transferred into *A. hydrophila* AH-405 rifampin-resistant (Rif^r) strains by triparental mating using *E. coli* MC1061 (λ*pir*) containing the insertion constructs and the mobilizing strain HB101/pRK2073. Transconjugants were selected on plates containing chloramphenicol, kanamycin, and rifampin. PCR analysis confirmed that the vector had integrated correctly into the chromosomal DNA. To complete the allelic exchange, the integrated suicide plasmid was forced to recombine out of the chromosome by addition of 5% sucrose to the agar plates. The pDM4 vector contains *sacB*, which produces an enzyme that converts sucrose into a product that is toxic to gram-negative bacteria. Transconjugants surviving on plates with 5% sucrose (Rif^r, Km^r, and chloramphenicol sensitive [Cm^s]) were chosen and confirmed by PCR.

The chromosomal in-frame *flaB* deletion mutant *A. hydrophila* AH-4425 was constructed by allelic exchange as described by Milton et al. (44). Briefly, DNA regions flanking the *flaB* gene were amplified using the primer pairs A (5′-CGC GGATCCCCTGGTCAAGAGATGGAA-3′) and B (5′-CCCATCCACTAA ACT TAAACAGGCGTTCAGTGATGAAGT-3′) and C (5′-TGTTTAAGT TTAGTGGATGGGTCCCTGCTGGGTTAACAG-3′) and D (CGCGGATCC CTGGGCATTGGTCTT TTT-3′) in two sets of asymmetric PCRs to amplify DNA fragments of 592 (AB) and 646 (CD) bp, respectively. DNA fragment AB contains nucleotide 1194, outside *flaB*, to nucleotide 1786, corresponding to the 14th codon of *flaB*. DNA fragment CD contains nucleotide 2636, corresponding to the first base in the 328 codon of *flaB*, to nucleotide 3282, inside the *flaH* gene. DNA fragments AB and CD were annealed at their overlapping regions (underlined letters in primers B and C) and amplified as a single fragment by using primers A and D. The fusion product was purified, BamHI digested (the BamHI site is double underlined in primers A and D), ligated into BglII-digested and phosphatase-treated pDM4 vector (44), electroporated into *E. coli* MC1061 (λ*pir*), and plated on chloramphenicol plates at 30°C to obtain plasmid pDM-FLAB. Introduction of plasmid pDM-FLAB into the *A. hydrophila* AH-405 Rif^r strain was performed as previously described. Transconjugants were selected on plates containing chloramphenicol and rifampin. PCR analysis confirmed that the vector had integrated correctly into the chromosomal DNA. After sucrose treatment, transformants (Rif^r and Cm^s) were chosen and confirmed by PCR.

Plasmid construction. Plasmid pACYC-MAF, containing the complete *maf-1* gene, and plasmid pACYC-FLR1, containing *flrB* and *flrC* genes from *A. hydrophila* AH-3, were obtained by PCR amplification of genomic DNA using oligonucleotides 5′-GGGGTGGGATTAGGATACC-3′ and 5′-GGGATACAT GAGCCTACGG-3′ to generate a band of 1,829 bp and 5′-TGGAGCACAGTG CCAGTTA-3′ and 5′-TCCCCCTGCTCTACCAATA-3′ to generate a band of 2,857 bp, respectively. The amplified bands were ligated into pGEM-Teasy (Promega) and transformed into *E. coli* XL1-Blue. The DNA insert was recovered by EcoRI restriction digestion and ligated into EcoRI phosphatase-treated pACYC184 vector (56), introduced into *E. coli* DH5α to generate plasmid pACYC-MAF.

Whole-cell protein preparation and immunoblotting. Whole-cell proteins were obtained from *Aeromonas* strains grown at 30°C. Equivalent numbers of cells were harvested by centrifugation, and the cell pellet was suspended in 50 to 200 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer and boiled for 5 min. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer to nitrocellulose membranes, the membranes were blocked with bovine serum albumin (3 mg/ml) and probed with either polyclonal rabbit anti-polar or anti-lateral flagellin antibody (1:1,000), previously obtained (23). The unbound antibody was removed by three washes in phosphate-buffered saline (PBS), and a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:1,000) was added. The unbound secondary antibody was removed by three washes in PBS. The bound conjugate was then detected by the addition of 2 ml 0.5% 4-chloro-1-naphthol (Sigma) prepared in methanol and diluted in 8 ml PBS containing 50 μl of 30% H_2O_2.

Adherence assay to HEp-2 cells. Tissue culture was maintained as described by Thornley et al. (63). The adherence assay was conducted as a slight modification of that described by Carrello et al. (12). Bacteria were grown statically in brain heart infusion broth at 37°C, harvested by gentle centrifugation (1,600 × *g* for 5 min), and resuspended in PBS (pH 7.2) at approximately 10^7 CFU/ml (A_{600} of ~0.07). The monolayer was infected with 1 ml of the bacterial suspension for 90 min at 37°C in 5% CO_2. Following infection, the nonadherent bacteria were removed from the monolayer by three washes with PBS. The remaining adherent bacteria and the monolayers were then fixed in 100% methanol for 5 min. Methanol was removed by washing monolayers and bacteria with PBS, and the HEp-2 cells with the adherent bacteria were stained for 45 min in 10% (vol/vol) Giemsa stain (BDH) prepared in Giemsa buffer. The coverslips were air dried,

mounted, and viewed by oil immersion under a light microscope. Twenty HEp-2 cells/coverslips were randomly chosen, and the number of bacteria adhering to HEp-2 cells was recorded. Assays were carried out in duplicate or triplicate.

Biofilm formation. Quantitative biofilm formation was performed in a microtiter plate as described previously (51), with minor modifications. Briefly, bacteria were grown on TSA and several colonies were gently resuspended in TSB (with or without the appropriate antibiotic); 100-μl aliquots were place in a microtiter plate (polystyrene) and incubated for 48 h at 30°C without shaking. After the bacterial cultures were poured out, the plate was washed extensively with water, fixed with 2.5% glutaraldehyde, washed once with water, and stained with 0.4% crystal violet solution. After solubilization of the crystal violet with ethanol-acetone (80/20, vol/vol), the absorbance was determined at 570 nm.

Statistical analysis. The differences in adherence to HEp-2 cells or biofilm formation in vitro between the wild-type and mutant strains were analyzed by the *t* test using Microsoft Excel software.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been submitted to the GenBank/EMBL database under accession numbers DQ119104, DQ124696, DQ124697, and DQ124698.

RESULTS

Isolation and characterization of *A. hydrophila* AH-3 mutants with reduced swimming phenotype in plates. In order to find the chromosomal regions of *A. hydrophila* AH-3 involved in polar flagellum biogenesis, we performed miniTn*5*Km-1 mutagenesis using *A. hydrophila* AH-405 (AH-3, rifampin resistant) as the recipient strain, and transconjugants were screened for reduced swimming motility in swim agar. From 7,000 transconjugants analyzed by light microscopy, 23 transposon insertion mutants exhibited a reproducible reduction in swim agar and inability to move in liquid media. Transposon insertion mutants unable to swim were analyzed by transmission electron microscopy after growth in liquid media and on plates; these were subsequently divided in two groups on the basis of their ability to produce polar flagella. The first group was unable to produce polar flagella but was able to produce lateral flagella, and the second group was unable to swim but showed both flagellum types.

As no EcoRV restriction sites were present in the transposon, all altered-motility mutants were analyzed for the presence of the transposon by Southern hybridization of EcoRV chromosomal DNA digestions. A single band was detected in every mutant, indicating that each mutant had a single copy of the mini-transposon in its genome.

Sequence analysis of miniTn*5*Km-1 interrupted genes. The DNA flanking the transposon was isolated and cloned into pBCSK (see Materials and Methods). From 22 mutants of the first transposon insertion mutant group, 18 ORFs that shared homology with different *A. hydrophila* AH-3 polar flagellum structural genes in the *flg* locus (2) were revealed: two mutants showed the transposon inserted in *flgN*, two in *flgM*, four in *flgB*, three in *flgE*, three in *flgK*, and four in *flgL* (2). However, the encoded product of sequences flanking the insertion of four more mutations had not previously been described for *Aeromonas*. The predicted amino acid sequences from mutant AH4422 encoded a product similar to an uncharacterized protein of *Shewanella oneidensis* (GenBank no. SO3259), *Clostridium acetobutylicum* (CAC2196), and *Campylobacter jejuni* (Cj1337), a group of proteins thought to be involved in phase-variable flagellum-mediated motility and flagellin modification (33). Of the other ORF products, AH-4440 shared homology with FlhA of *Shewanella oneidensis* and *V. parahaemolyticus*, a component of the polar flagellar export machinery (60). The only representative mutant of the second mutant group, AH-4460, revealed an ORF whose predicted amino acid sequence shared homology with the sodium-type flagellar motor component MotX of *Vibrio alginolyticus*.

Organization of *A. hydrophila* AH-3 polar flagellum region 2. A cosmid genomic library of *A. hydrophila* AH-3 (41) was screened by colony blotting using a DNA probe to the *maf*-like transposon flanking sequences from mutant AH-4422. Several positive recombinant clones were identified, of which clone pLA-FLA was chosen because it was able to complement mutant AH-4422.

Nucleotide sequencing of pLA-FLA revealed six complete ORFs (Fig. 1) transcribed in the same direction, five of which are related to *Aeromonas salmonicida* and *A. caviae* polar flagella (53, 65). Flanking the 3′ end of *fla* polar flagella loci, 70 bp downstream of ORF6, and transcribed in the opposite direction was the stop codon of a incomplete ORF whose amino acid sequence exhibited homology to the OrfB transposase of *Pseudomonas putida*, as well as transposases of other bacteria. ORF1 (*flaA*) was separated from ORF2 (*flaB*) by 585 bp, and ORF5 (*flaJ*) was separated from ORF6 (*maf-1*) by 1,060 bp. The other ORFs were located one behind the other, with intergenic regions less than 80 bp. Sequences defining putative ribosome binding sites were found upstream of each of the ORF's start codons. Data summarizing the locations of the six complete ORFs are shown in Table 2. Sequence analysis in silico showed three possible transcriptional terminator rho-independent sequences downstream of ORF1 (*flaA*), ORF5 (*flaJ*), and ORF6 (*maf-1*) and putative σ^{28} promoter sequences upstream of ORF1 (*flaA*), ORF2 (*flaB*), and ORF6 (*maf-1*), as well as putative σ^{54} promoter sequences upstream of ORF2 (*flaB*) (Fig. 1). RT-PCR using specific primers and total RNA from *A. hydrophila* AH-3 grown in liquid (TSB) and solid (TSA) media demonstrated amplifications between ORFs 2 and 5 (*flaB* to *flaJ*) under both conditions. However, no amplifications were obtained with oligonucleotide pairs from ORFs 1 (*flaA*) to 2 (*flaB*) and ORFs 5 (*flaJ*) to 6 (*maf-1*), confirming that *flaA* and *maf-1* are transcribed independently (Fig. 1).

Table 2 summarizes the characteristics of the individual proteins and predicted functions analyzed using the BLASTP program (3) of the NCBI database. ORFs 1 to 5 (*flaA* to *flaJ*) had the same organization and were similar to those previously reported for the polar flagellins of *A. salmonicida* and *A. caviae* (53, 65). However, downstream of ORF5 (*flaJ*) was ORF6, whose deduced amino acid sequence contained the DUF115 protein family domain and exhibited homology to the motility accessory factor Maf-1 of *Campylobacter jejuni* (26% identity), thought to be involved in a slipped-strand mispairing mechanism of phase variation (33).

Organization of *A. hydrophila* AH-3 polar flagellum region 3. The cosmid library of *A. hydrophila* AH-3 (48) was screened by colony blotting using DNA probes to the *A. hydrophila* AH-3 *flhA*-like gene. Several positive recombinant clones were identified, of which pLA-FLIP1 and pLA-FLIP2 were selected, as they were able to complement the AH-4440 mutant.

Nucleotide sequencing of the overlapping cosmids pLA-FLIP1 (ORFs 1 to 20) and pLA-FLIP2 (ORFs 13 to 29) revealed 29,799 bp which contained 29 ORFs transcribed in the same direction (Fig. 1), most of which encoded homologues

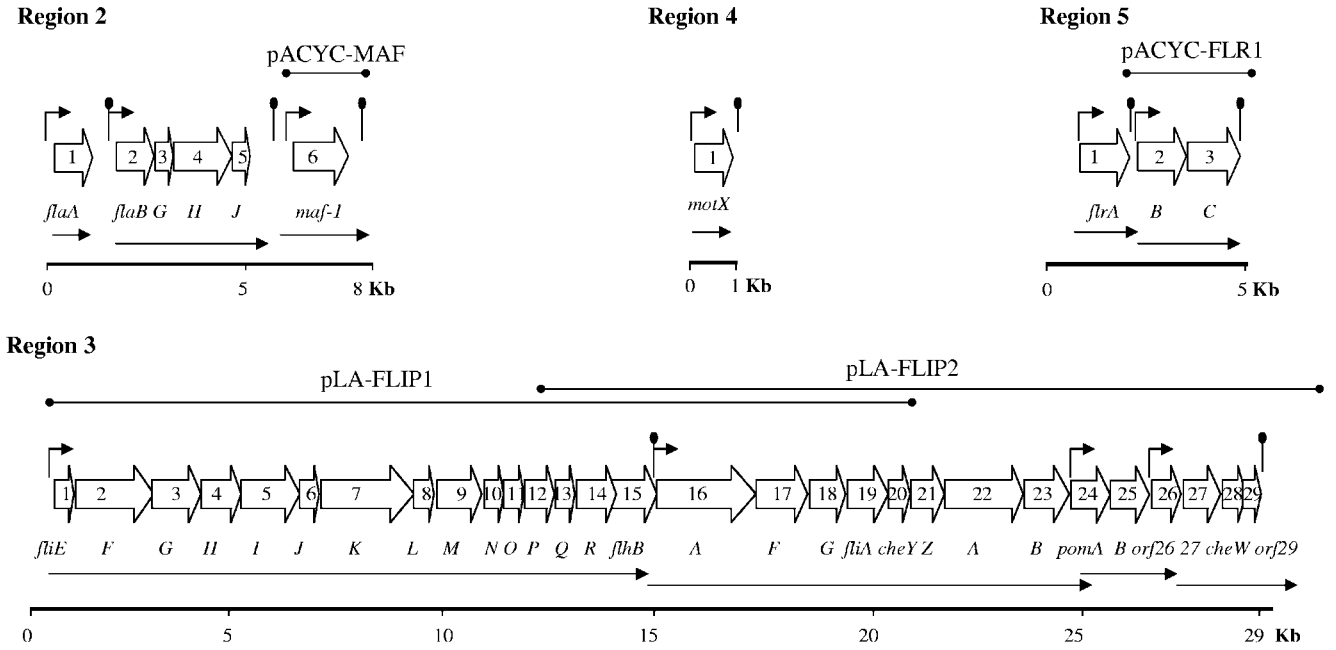

FIG. 1. Genetic organization of *A. hydrophila* AH-3 polar flagellum regions 2, 3, 4, and 5. ORFs and their transcriptional directions are indicated by large open arrows and named after their homologues in other bacterial species. Black arrows above the ORFs indicate the locations of putative promoter sequences. Lollipop structures depict the approximate positions of the putative transcriptional rho-independent terminators. We also show the clusters determined by RT-PCR in the sequenced regions (black arrows below the ORFs) and genes contained in different plasmids.

of proteins involved polar flagellum biosynthesis and chemotaxis in different bacteria, such as *Photobacterium profundum*, *Shewanella oneidensis*, and *V. parahaemolyticus* (41, 60). ORFs 1 to 23 (*fliE* to *cheB*) and ORFs 26 to 29 had the same organization as region 2 of the *V. parahaemolyticus* polar flagellum system (Fig. 2). However, unlike *V. parahaemolyticus*, *A. hydrophila* lacked the *flaF* to *flaM* genes in this chromosomal region but contained the *pomAB* genes (ORFs 24 to 25) which are located in region 3 of the *V. parahaemolyticus* polar flagellum system (Fig. 2). Upstream of *A. hydrophila* ORF1 (*fliE*) was a truncated ORF transcribed in the opposite direction, with homology to different bacterial regulatory proteins related to the AraC family, and downstream of ORF29 was another truncated ORF transcribed in the same direction, which shared homology with the CcmA ATP-binding subunit of a putative ABC-type heme exporter. Sequences defining putative ribosome binding sites were found upstream of each ORF's start codons. Most ORFs were overlapping or with intergenic regions less than 80 bp. Data summarizing the locations of the 29 complete ORFs are shown in Table 3. Sequence analysis in silico showed possible transcriptional terminator rho-independent sequences downstream of ORF15 (*flhB*) and ORF29.

TABLE 2. Characteristics of *A. hydrophila* AH-3 polar flagellum gene regions 2, 4, and 5

ORF and region	Nucleotide positions	Protein size (aa)[a]	Molecular mass (kDa)	pI	Predicted function	Homologous gene	Identity/ similarity (%)
Region 2							
1	248–1160	304	32.4	8.99	Flagellin	*flaA* of *Aeromonas salmonicida*	81/84
2	1745–2648	301	31.8	8.86	Flagellin	*flaB* of *Aeromonas salmonicida*	83/85
3	2689–3127	146	16	4.85	Filament length control	*flaG* of *Aeromonas salmonicida*	69/71
4	3157–4564	469	49.9	8.89	HAP-2	*flaH* of *Aeromonas caviae*	60/76
5	4592–5012	140	15.9	5.29	Chaperone	*flaJ* of *Aeromonas caviae*	58/79
6	6072–7404	444	50.9	5.61	Motility accessory factor	*maf-1* of *Campylobacter jejuni*	26/41
Region 4							
1	171–1104	311	32.8	5.31	Stator	*motX* of *Vibrio alginolyticus*	54/73
Region 5							
1	811–2235	475	53.2	5.24	Flagellum regulator	*flrA* of *Vibrio cholerae*	53/65
2	2345–3379	344	37.2	6.18	Two-component sensor kinase	*flrB* of *Vibrio cholerae*	40/53
3	3376–4716	447	49.4	5.16	Two-component response kinase	*flaM* of *Vibrio parahaemolyticus*	60/73

[a] aa, amino acids.

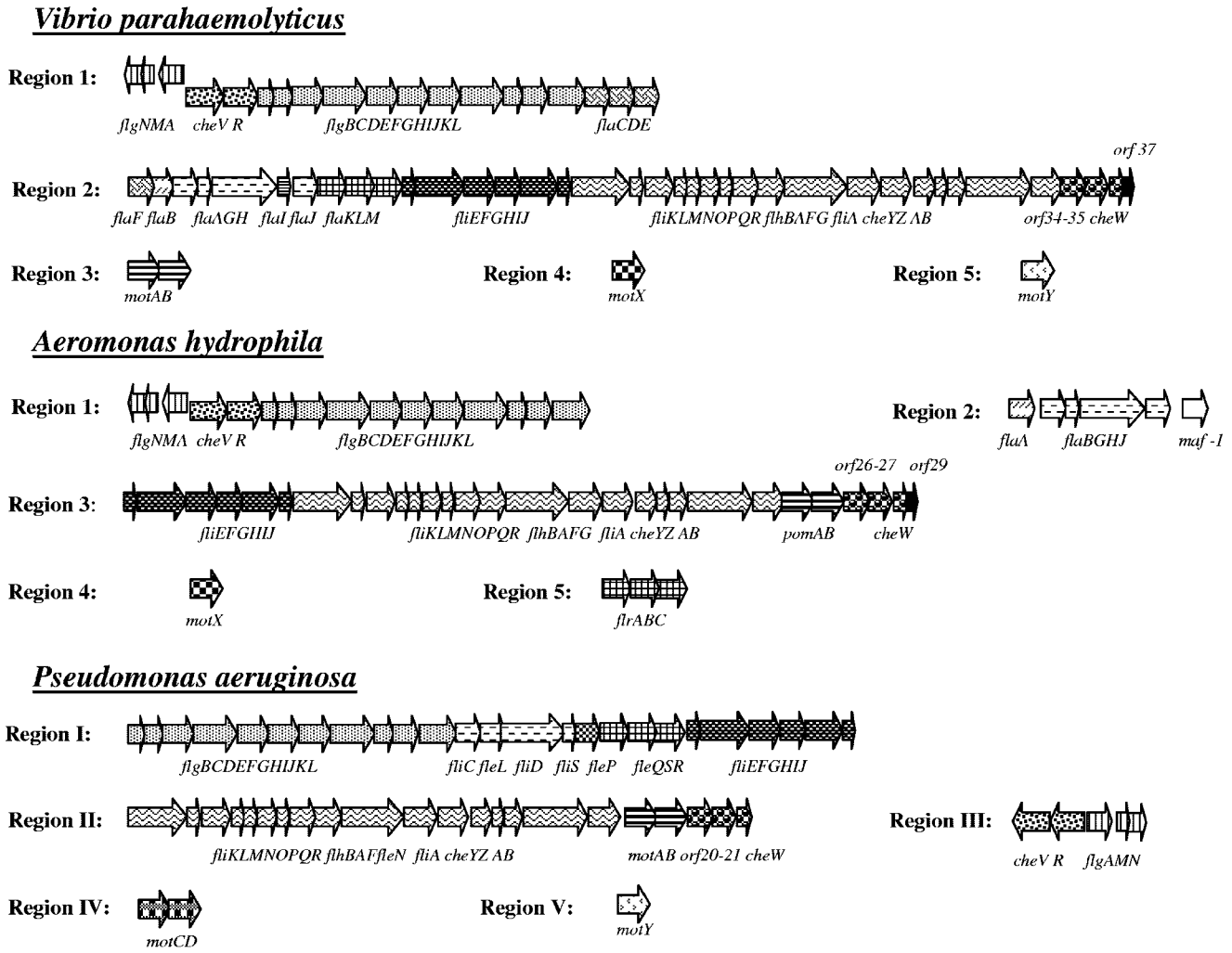

FIG. 2. *A. hydrophila* AH-3 polar flagellum genetic regions compared to polar flagellum genetic regions of *V. parahaemolyticus* and *P. aeruginosa*. Arrows with the same shading correspond to homologous genes among these bacteria.

Putative σ^{54} promoter sequences were found upstream of ORF1 (*fliE*), ORF16 (*flhA*), and ORF24 (*pomA*), and a putative promoter sequence was found upstream of ORF26 (Fig. 1). RT-PCRs using specific primers showed amplifications between ORFs 1 and 15 (*fliE* to *flhB*), ORFs 16 and 27 (*flhA* to *cheB*), ORFs 24 and 25 (*pomAB*), and ORFs 26 to 29. However, no amplifications were obtained with oligonucleotide pairs from ORFs 15 (*flhB*) to 16 (*flhA*), ORFs 23 (*cheB*) to 24 (*pomA*), and ORFs 25 (*pomB*) to 26. The RT-PCR results showed that *fli* and *flh A. hydrophila* polar genes are distributed in four clusters, although no rho-independent terminator sequences were found downstream of ORF23 (*cheB*) and ORF25 (*pomB*).

Table 3 shows the characteristics of the individual proteins and their protein homologues analyzed using the BLASTP program (3) of the NCBI database. ORF1, ORF2, ORF7, and ORF8 encoded proteins that exhibited more than 40% identity to different polar flagellum basal body and hook proteins FliE (57% identity) of *Vibrio vulnificus*, FliF (50% identity) of *S. oneidensis*, FliK (43% identity) of *V. parahaemolyticus*, and FliL (55% identity) of *P. profundum*, respectively. ORF3, ORF9, and ORF10 predicted amino acid sequences exhibited high homology (>70% identity) to the polar flagellum motor switch proteins FliG, FliM, and FliN of different bacteria, respectively. ORFs 4 to 6 and ORFs 11 to 16 exhibited homologies to different export and assembly polar flagellum proteins, with ORF4 and ORF6 having 38% identity to the ATP synthase-negative regulator FliH of *Photobacterium profundum* and 39% identity to a potential nonspecific chaperone FliJ of *Pseudomonas syringae*, respectively. ORF5, which is homologous to FliI, a polar flagellum ATP synthase, and ORFs 11 to 16 (FliOPQR and FlhBA homologues) displayed homologies greater than 43% identity to *Vibrio* spp., *S. oneidensis*, and *P. profundum* export and assembly proteins. ORFs 17 and 18 matched with a pair of proteins that could be involved in the control of site selection of flagellum insertion as well as flagellum number. ORF17 (FlhF homologue) showed homology to FlhF of *V. vulnificus* and *V. parahaemolyticus* (50% identity), a GTP-binding signal recognition particle pathway protein required for flagellar synthesis in *Bacillus subtilis* (38), *Campylo-*

TABLE 3. Characteristics of *A. hydrophila* AH-3 polar flagellum gene region 3

ORF	Nucleotide positions	Protein size (aa)[a]	Molecular mass (kDa)	pI	Predicted function	Homologous protein	Identity/ similarity (%)
1	1607–1922	105	11.4	4.76	MS ring/rod adapter	FliE of *Vibrio vulnificus*	57/76
2	1937–3647	570	62.1	4.88	M ring	FliF of *Shewanella oneidensis*	50/67
3	3642–4767	375	41.7	4.78	Switch	FliG of *Shewanella oneidensis*	71/85
4	4773–5584	271	29.6	4.66	Export/assembly	FliH of *Photobacterium profundum*	38/56
5	5620–6949	443	47.7	6.04	Export ATP synthase	FliI of *Vibrio parahaemolyticus*	68/82
6	6978–7416	146	17.0	9.47	Export/assembly	FliJ of *Pseudomonas syringae*	39/62
7	7514–9452	646	66.5	4.97	Hook length	FliK of *Vibrio parahaemolyticus*	43/69
8	9512–10025	171	18.4	6.37	Flagellar protein	FliL of *Photobacterium profundum*	55/73
9	10037–11105	356	40.4	4.81	Switch	FliM of *Photobacterium profundum*	80/91
10	11159–11549	130	14.1	4.39	Switch	FliN of *Photobacterium profundum*	80/90
11	11511–11994	161	13.7	6.69	Export/assembly	FliO of *Vibrio parahaemolyticus*	43/70
12	11983–12760	259	28.3	5.83	Export/assembly	FliP of *Vibrio parahaemolyticus*	63/78
13	12790–13057	89	9.7	4.35	Export/assembly	FliQ of *Vibrio vulnificus*	67/82
14	13112–13934	274	29.5	5.49	Export/assembly	FliR of *Shewanella oneidensis*	51/73
15	14023–15151	376	41.9	9.42	Export/assembly	FlhB of *Shewanella oneidensis*	46/63
16	15261–17361	700	75.4	5.87	Export/assembly	FlhA of *Shewanella oneidensis*	60/73
17	17380–18796	472	51.6	6.71	Polar flagellar site determinant	FlhF of *Vibrio vulnificus*	50/65
18	18791–19673	294	32	8.73	Flagellar number regulator	FlhG of *Shewanella oneidensis*	73/87
19	19668–20385	239	26.6	5.56	σ^{28}	FliA of *Shewanella oneidensis*	66/80
20	20511–20880	123	13.8	6.73	Chemotaxis	CheY of *Shewanella oneidensis*	91/95
21	20895–21444	183	21	6.42	Chemotaxis	CheZ of *Shewanella oneidensis*	58/77
22	21601–23743	714	76.1	4.70	Chemotaxis	CheA of *Photobacterium profundum*	61/69
23	23778–24891	371	39.8	8.94	Chemotaxis	CheB of *Vibrio vulnificus*	67/78
24	24896–25631	245	26.9	5.03	Motor	MotA of *Pseudomonas aeruginosa*	50/69
25	25636–26545	303	33	6.77	Motor	MotB of *Pseudomonas putida*	30/52
26	26552–27344	264	29.6	9.17	Unknown	Soj-like protein of *Photobacterium profundum*	63/83
27	27343–28213	290	32.7	4.53	Unknown	VP2226 of *Vibrio parahaemolyticus*	50/67
28	28285–28771	162	17.9	4.23	Chemotaxis	CheW of *Vibrio parahaemolyticus*	78/93
29	28786–29172	130	14.4	5.28	Unknown	VC2058 of *Photobacterium profundum*	50/73

[a] aa, amino acids.

bacter jejuni (28), and *Helicobacter pylori* (47) and required for polar flagellar placement in *P. putida* (50). Furthermore, it has recently been demonstrated with *V. cholerae* that FlhF can regulate class III flagellar transcription (46). ORF18 (FlhG homologue) encoded a protein with a CbiA domain and is a MinD-related protein involved in septum site determination in *Bacillus subtilis* (11, 38) as well as in regulation of flagellum number in *P. aeruginosa* (14). The predicted amino acid sequence of ORF19 (FliA homologue) exhibited 66% identity to the RNA polymerase σ^{27} factor of *S. oneidensis* and 62% identity to the RNA polymerase σ^{28} factor of *V. vulnificus*. ORFs 20 to 23 and ORF28 shared 58 to 91% identity to different chemotaxis proteins (7, 10, 37). ORFs 20 to 23 and ORF28 matched with CheY, CheZ, CheA, CheB, and CheW, respectively (Table 3). Proteins encoded by ORF24 and ORF25 showed homology (50% and 30% identity, respectively) to the cytoplasmic membrane proteins MotA and MotB (of *Pseudomonas* spp.), respectively, which constitute the stator of the flagellum motor. Together, these proteins are involved in the formation of a proton or sodium-conducting channel to generate rotational motion in the proton-type or sodium-type flagellum motor (31, 61). ORF25 (MotB) contained an OmpA domain at the C-terminal sequence, which is probably involved in peptidoglycan interaction (17). Although ORF26, ORF27, and ORF29 appear not to be involved in polar flagellum formation, their locations, downstream of chemotaxis genes, are conserved in different bacteria that possess polar flagella. ORF26 encodes a putative protein with a CbiA domain that exhibits high homology to Soj-like proteins as well as other ATPase proteins involved in chromosome partitioning (36). The predicted protein for ORF27 had a CheW-like domain which is found in proteins involved in the two-component signaling systems regulating bacterial chemotaxis (7) and matched to VP2226 of *V. parahaemolyticus* (Table 3). ORF29 showed sequence homology (41 to 50% identity) (Table 3) to hypothetical proteins located at the end of polar flagellum regions of different bacteria (41).

Sequence analysis of *A. hydrophila* AH-3 polar flagellum region 4. The *A. hydrophila* AH-3 genomic library (48) was screened by colony blotting using a DNA probe to the *motX*-like gene of *A. hydrophila* AH-3, leading to the identification of clone pLA-MOTX, which was able to complement the AH-4460 mutant. By sequence analysis of pLA-MOTX, we found a complete ORF of 933 bp, encoding a protein of 311 amino acids and a predicted molecular weight of 32.8 kDa, that was homologous to MotX of *V. alginolyticus* (54% identity), involved in the sodium-type motor formation in *V. parahaemolyticus* (35) (Table 2). A putative Shine-Dalgarno sequence is positioned 7 bp upstream of the ATG start codon, and there is also a putative σ^{28} promoter sequence (CTAAG-N15-GCC GATAA) 26 bp upstream of the start codon. The nucleotide sequence GACCCACAGATCTGCATCTGTGGGTC downstream of the *motX*-like TGA stop codon could provide a termination stem-loop structure (Fig. 1). Moreover, 86 nucleotides upstream of the *motX*-like start codon is found a putative terminator sequence, AAGGGGAGCTTCGGCTCC

CCTT, and the stop codon (125 bp upstream) of an incomplete ORF whose deduced amino acid sequence is similar to that of the malate dehydrogenase of *V. cholerae*. Downstream of *motX*, transcribed in the opposite direction, is an incomplete ORF which showed 55% identity to a *Yersinia pestis* flavohemoprotein which plays a central role in the inducible response to nitrosative stress.

Cloning and sequence analysis of *A. hydrophila* AH-3 polar flagellum regulatory region 5. The *A. hydrophila* AH-3 genomic library (48) was transferred by mating into the rifampin-resistant *Vibrio cholerae flrC* in-frame deletion mutant strain KKV98 (35). Transconjugants were selected for rifampin and tetracycline resistance and inoculated onto 0.3% agar LB plates supplemented with 2 mM glutamine and tetracycline. The complemented colonies which spread on the plates were isolated, and the plasmid pLA-FLR was recovered. Sequence analysis of pLA-FLR revealed three complete ORFs transcribed in the same direction, which encoded proteins related to the polar flagellum master regulatory proteins of different *Vibrio* species (35, 41, 52, 60). ORF2 (*flrB*) began 106 bp downstream of the ORF1 (*flrA*) stop codon, and ORF2 and ORF3 (*flrC*) are overlapping sequences. Moreover, 220 nucleotides upstream of the ORF1 (*flrA*) start codon is found a putative rho-independent terminator sequence and the stop codon (260 bp upstream) of a incomplete ORF whose encoded protein has a topoisomerase DNA binding C4 zinc finger domain.

Nucleotide analysis in silico shows putative Shine-Dalgarno sequences upstream of each ORF, two putative σ^{54} promoter sequences upstream of ORF1 and ORF2 (70 and 35 bp, respectively), and two rho-independent terminator sequences downstream of ORF1 and ORF3 (3 and 31 bp, respectively). RT-PCR using specific primers showed amplifications between ORFs 2 and 3 (*flrB* to *flrC*), and no amplifications were obtained with oligonucleotide pairs from ORFs 1 (*flrA*) and 2 (*flrB*) (Fig. 1). A search of the protein databases revealed that ORF1 (*flrA*) encoded a cytoplasmic protein of 475 amino acids which displayed more than 50% identity to the polar flagellum proteins FlrA of *V. cholerae* and FlaK of *V. parahaemolyticus*. ORF2 (*flrB*) encoded a transmembrane protein of 344 amino acids and ORF3 (*flrC*) a cytoplasmic protein of 447 amino acids, which are homologous to two-component flagellar regulatory proteins. ORF2 (*flrB*) encoded a protein which exhibited 38 to 40% identity to different two-component sensor kinases, such as FlrB of *V. cholerae* and FlaK of *V. parahaemolyticus*, and ORF3 (*flrC*) encoded a protein which shared high homology with the two-component response regulator proteins FlaM of *V. parahaemolyticus* (60% identity) and FlrC of *V. fischeri* (58% identity) (Table 2).

Construction of polar flagellum mutants and complementation studies. We constructed several mutants of *A. hydrophila* AH-3 to study the *Aeromonas* polar flagellum regions and to determine if they had any role in lateral flagellum formation. We obtained an in-frame *flaB* deletion mutant (AH-4425) and the following defined insertion mutants: *flaA*, *flaH*, and *maf-1*, with mutations in polar flagellum region 2; *fliM*, *flhA*, *fliA*, and *pomB*, with mutations in polar flagellum region 3; *motX*, with a mutation in polar flagellum region 4; and *flrC*, with a mutation in polar flagellum region 5. Amplified internal fragments of *flaA*, *maf-1*, *pomB*, *motX*, and *flrC* were ligated into pFS100 to construct plasmids pFS-FLAA (*flaA*), pFS-MAFP (*maf-1*), pFS-POMB (*pomB*), pFS-MOTX (*motX*), and pFS-FLRC (*flrC*), respectively. The Km cassette was inserted into *flaH*, *fliM*, *flhA*, and *fliA* and constructs ligated into pDM4, resulting in pDM-FLAH (*flaH*), pDM-FLIM (*fliM*), pDM-FLHA (*flhA*), and pDM-FLIA (*fliA*), respectively. Plasmids were independently introduced into *A. hydrophila* AH-405 by mating. Using this method, we obtained the following *A. hydrophila* mutant strains, with mutations in the genes in parentheses: AH-4426 (*flaA*), AH-4423 (*flaH*), AH-4424 (*maf-1*), AH-4441 (*fliM*), AH-4442 (*flhA*), AH-4443 (*fliA*), AH-4444 (*pomB*), AH-4461 (*motX*), AH-4462 (*flrC*), and AH-4427 (*flaA flaB* double mutant). The correct construction of all mutants was verified by Southern blot hybridization (data not shown). Swarming motility of *A. hydrophila* mutant strains was then assessed on swarm agar, swimming motility was assessed by light microscopy after growth in liquid media, and the presence/absence of both flagellum types (lateral and polar) was analyzed by TEM after growth in solid and liquid media. The motility phenotypes of the mutant strains are shown in Table 4. Mutations of either *flaA* (AH-4426) or *flaB* (AH-4425), located in *A. hydrophila* polar flagellum region 2, did not abolish motility and produced only a minor reduction in the swarm size on semisolid plates compared to the wild type, such as with *flaA* or *flaB A. caviae* mutant strains (53). Because single insertions in *flaA* or *flaB* do not abolish motility, we introduced the suicide plasmid pFS-FLAA into the *A. hydrophila* mutant AH-4425 in order to construct a double mutant strain (AH-4427), in which both genes were mutated in tandem. Motility assays showed that the double mutation abolished swimming motility and also caused a large decrease in swarming motility. TEM analysis and specific immunoblotting of the single mutant and double mutant whole cells, after growth in solid or liquid media, using lateral flagellin- or polar flagellin-specific antibodies, showed that *flaA* and *flaB* single mutant strains possess both flagellum types; however, the double mutant strain is unable to form polar flagella but is able to form lateral flagella (Fig. 3). This suggests

TABLE 4. Phenotypes of in-frame and defined insertion mutants

Strain	Result for:				Gene defect
	Motility		Flagellation type[c]		
	Swarming (cm)[a]	Swimming[b]	Lateral	Polar	
AH-3	3.5	+	+	+	
AH-4423	0.8	−	+	−	*flaH*
AH-4424	0.7	−	+	−	*maf-1*
AH-4425	2.1	+	+	+	*flaB*
AH-4426	2.3	+	+	+	*flaA*
AH-4427	0.7	−	+	−	*flaA flaB*
AH-4441	0.9	−	+	−	*fliM*
AH-4442	0.8	−	+	−	*flhA*
AH-4443	0.7	−	+	−	*fliA*
AH-4444	3.3	+	+	+	*pomB*
AH-4461	0.9	−	+	+	*motX*
AH-4462	0.6	−	+	−	*flrC*

[a] Migration in centimeters of bacteria through the swarm agar plates from the center towards the periphery of the plate.
[b] −, no motility in swim plates and liquid media; +, motility equivalent to that of the wild-type strain in swim plates or liquid media.
[c] −, absence of flagella; +, flagella equivalent to amount in the wild type.

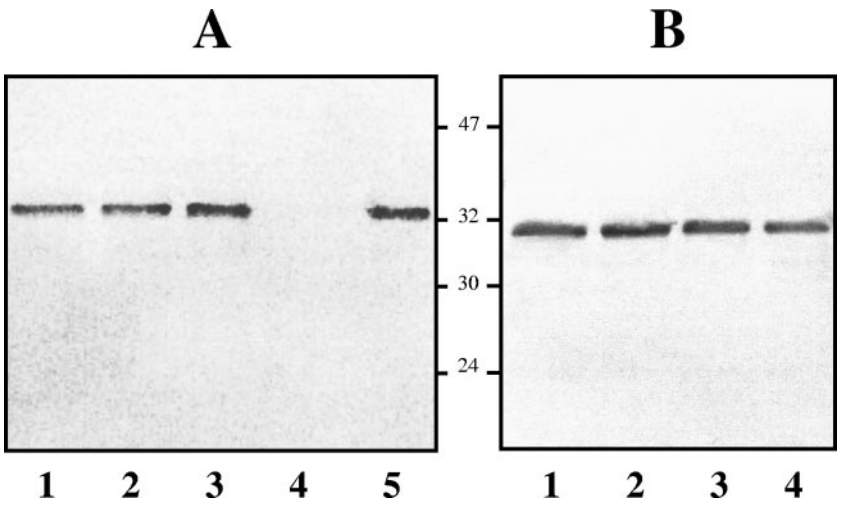

FIG. 3. (A) Western blot analysis with anti-polar flagellin (1:1,000) polyclonal antibodies of whole-cell preparations of *A. hydrophila* AH-3 (wild type) and mutants AH-4425 (*flaB*), AH-4426 (*flaA*), AH-4427 (*flaA flaB*), and AH-4427; bacteria were complemented with plasmid pLA-FLA (lanes 1, 2, 3, 4, and 5, respectively), grown at 30°C in TSB. (B) Western blot analysis with anti-lateral flagellin (1:1,000) polyclonal antibodies of whole-cell preparations of *A. hydrophila* AH-3 (wild type) and mutants AH-4425 (*flaB*), AH-4426 (*flaA*), and AH-4427 (*flaA flaB*) (lanes 1, 2, 3, and 4, respectively), grown at 30°C in TSA. Molecular mass markers (in kilodaltons) are noted between the blots.

that either of the flagellin proteins is able to substitute for the other to a certain extent, causing only a slight loss of motility, and that these flagellins are not involved in lateral flagellum formation. The *flaH* (AH-4423), *maf-1* (AH-4424), *fliM* (AH-4441), *flhA* (AH-4442), *fliA* (AH-4443), and *flrC* (AH-4462) defined insertion mutants were unable to swim (examined by microscopy) and exhibited a 60% decreased swarming motility, and under TEM, all of these mutant strains showed lateral flagella but no polar flagella, as seen previously for the miniTn*5* mutants AH-4422 and AH-4440 (Fig. 4). In contrast, the *pomB* (AH-4444) defined insertion mutant did not show any differences in swimming or swarming motility from the wild-type strain. Finally, the *motX* defined insertion mutant (AH-4461) was unable to swim and had a 60% decrease in swarming motility, and TEM assays showed both flagellum types.

Complementation studies were undertaken to determine if wild-type motility could be restored to the mutants by providing polar flagellum genes in *trans*. The plasmid pLA-FLA, containing *A. hydrophila* polar flagellum region 2, was introduced into the mutant strains AH-4423, AH-4424, AH-4425, AH-4426, and AH-4427. Swimming motility and polar flagellum production were restored in AH-4423, AH-4424, and AH-4427 (Fig. 3), whereas in AH-4425 and AH-4426, full swarming motility was recovered to the level of the wild type (data not shown). Furthermore, plasmid pACYC-MAF (see Materials and Methods) was able to fully complement mutant AH-4424 (Fig. 4) and the mini-Tn*5* mutant AH-4422. Plasmids pLA-FLIP1 and pLA-FLIP2, containing the *A. hydrophila fliE* to *cheY* and *fliQ* to *orf29* genes, respectively, of polar flagellum region 3, were introduced into AH-4441, AH-4442, and AH-4443 mutant strains. The *flhA* and *fliA* mutants (AH-4442 and AH-4443, respectively) were able to swim and to produce polar flagella when pLA-FLIP1 or pLA-FLIP2 was introduced independently. In contrast, AH-4441 (*fliM*) recovered the wild-type swimming phenotype and the ability to produce polar flagella only when plasmid pLA-FLIP1 was introduced (data not shown). When plasmid pLA-MOTX, containing the *A. hydrophila motX* gene of polar flagellum region 4, was introduced into AH-4461, the mutant strain was fully able to swim (data not shown). Introduction of plasmid pLA-FLR (containing *A. hydrophila* polar flagellum region 5) and plasmid pACYC-FLR1 (containing the *flrBC* genes [see Materials and Methods]) into the AH-4462 mutant strain rescued the swimming phenotype and the ability to synthesize polar flagella, as determined by TEM (Fig. 4). Moreover, *V. cholerae* KKV59 (Δ*flrA*) and KKV98 (Δ*flrC*) (35) mutant strains complemented with plasmid pLA-FLR spread on *Vibrio* motility plates as fast as the wild type, and the formation of polar flagella was confirmed by TEM (data not shown). No complementation was observed when only the vector pLA2917 or pACYC184 was introduced into the mutants.

Distribution of polar flagellum genes in mesophilic *Aeromonas* strains. The distribution of polar flagellum genes in mesophilic *Aeromonas* strains ($n = 50$) was analyzed by dot blot hybridization experiments against total genomic DNA, using independent PCR probes. The distribution of polar flagellum region 2 was analyzed using three PCR probes (*flaB* to *flaH*, *flaA*, and *maf-1*); region 3 was analyzed using three PCR probes (*fliK* to *fliM*, *flhA* to *fliA*, and *pomAB*); region 4 was determined by an intragenic *motX* probe; and master regulatory genes on region 5 were analyzed using two PCR probes (*flrA* and *flrBC*).

Polar flagellum probes hybridized to the chromosomal DNA of all mesophilic *Aeromonas* strains tested, whether or not the strains were able to produce lateral flagella.

Adhesion to HEp-2 cells and biofilm formation. In order to correlate polar flagellum presence and motility with adherence to mammalian cells, we examined the interaction of polar flagellum mutants with cultured monolayers of HEp-2 cells. Differences in adherence were calculated by determining the average numbers of bacteria adhering to HEp-2 cells (Fig. 5A).

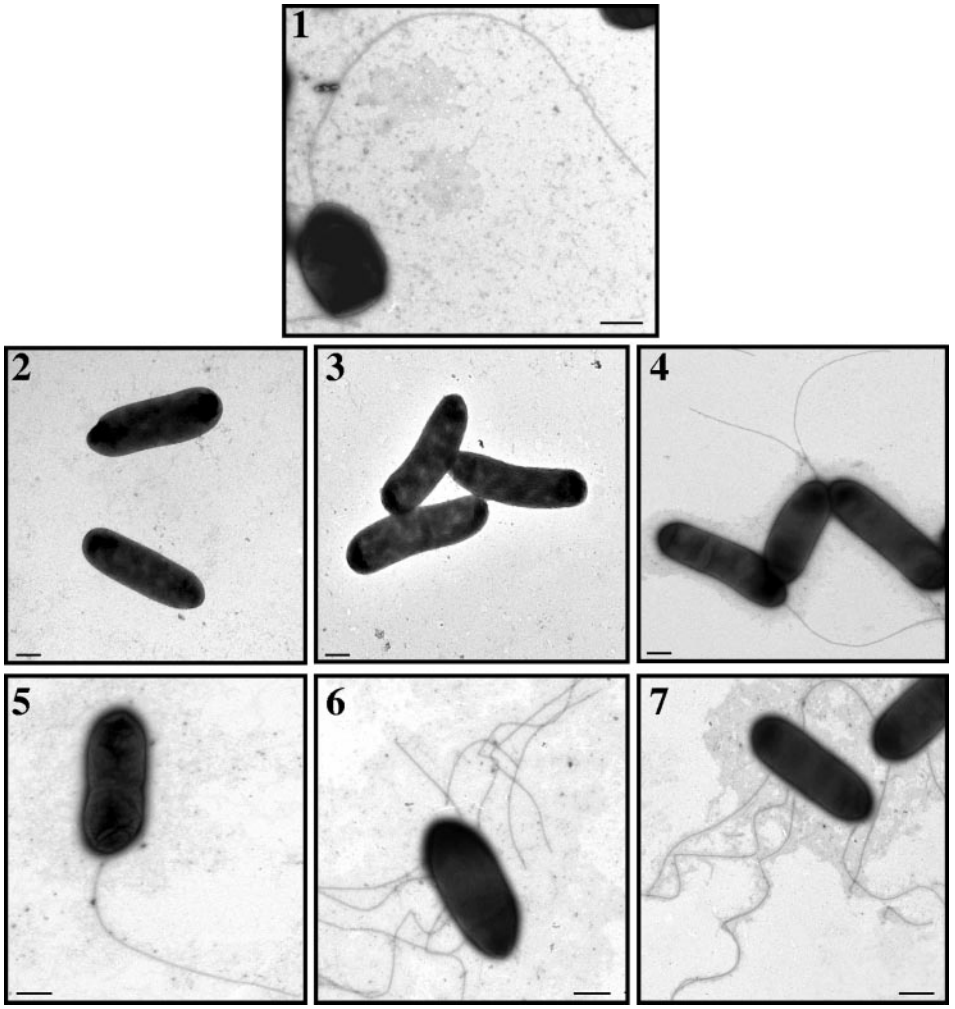

FIG. 4. TEM of *A. hydrophila* AH-3 (wild type) and mutants AH-4424 (*maf-1*), AH-4462 (*flrC*), and AH-4424 complemented with plasmid pACYC-MAF and AH-4462 complemented with plasmid pACYC-FLR1 (panels 1, 2, 3, 4, and 5, respectively), grown at 30°C in TSB. Lanes 6 and 7 show AH-4424 and AH-4462 mutants, respectively, grown at 30°C on TSA. Bacteria were gently placed onto Formvar-coated copper grids and negatively stained using 2% uranyl acetate. Bar = 0.5 μm.

Also, we compared the abilities of the wild-type and polar mutant strains to form biofilms in microtiter plates (Fig. 5B). The *A. hydrophila* wild-type strain, AH-3, exhibited an adhesion value of 18.3 (±1.7) bacteria adhered per HEp-2 cell and a biofilm formation ability with an optical density at 570 nm of 1.3 (±0.1). Polar flagellum mutant strains AH-4423 (*flaH*), AH-4424 (*maf-1*), AH-4427 (*flaA flaB*), AH-4441 (*fliM*), AH-4442 (*flhA*), AH-4443 (*fliA*), and AH-4462 (*flrC*), which produce lateral flagella but are unable to produce polar flagella, showed approximately an 86% reduction in adhesion to HEp-2 cells and a 55% reduction in biofilm formation. These results were similar to those for *A. hydrophila flg* polar flagellum mutants described previously (2). Mutant strains AH-4425 (*flaB*) and AH-4426 (*flaA*), which possess both flagellum types but show a small reduction in motility phenotype, exhibit a slight reduction in adhesion to HEp-2 cells, as well as in ability to form biofilms (approximately 35% and 10%, respectively). Finally, AH-4461 (*motX*), which has both flagellum types but is unable to swim, exhibited approximately half of the adhesion capacity to HEp-2 cells compared to the wild-type strain and had a 37% reduction in its ability to form biofilms. No changes in adhesion or biofilm formation were observed for the AH-4444 (*pomB*) mutant in comparison to the wild-type strain. Complementation studies using polar flagellum mutants AH-4423, AH-4425, AH-4426, and AH-4427 with plasmid pLA-FLA; AH-4424 with plasmid pLA-FLA or pACYC-MAF; AH-4441 with plasmid pLA-FLIP1; AH-4442 and AH-4443 with plasmid pLA-FLIP1 or pLA-FLIP2; AH-4461 with plasmid pLA-MOTX; and AH-4462 with plasmid pLA-FLR or pACYC-FLR1 showed values similar to those for the wild-type strain regarding adhesion to HEp-2 cells and biofilm formation. These adhesion and biofilm formation assays showed that polar flagella have an important role in cellular adhesion and biofilm formation, as was previously reported (2).

DISCUSSION

Mesophilic *Aeromonas* spp. produce a single polar flagellum that is expressed in both liquid and solid media; in addition, 50 to 60% of strains also have an inducible lateral flagellum sys-

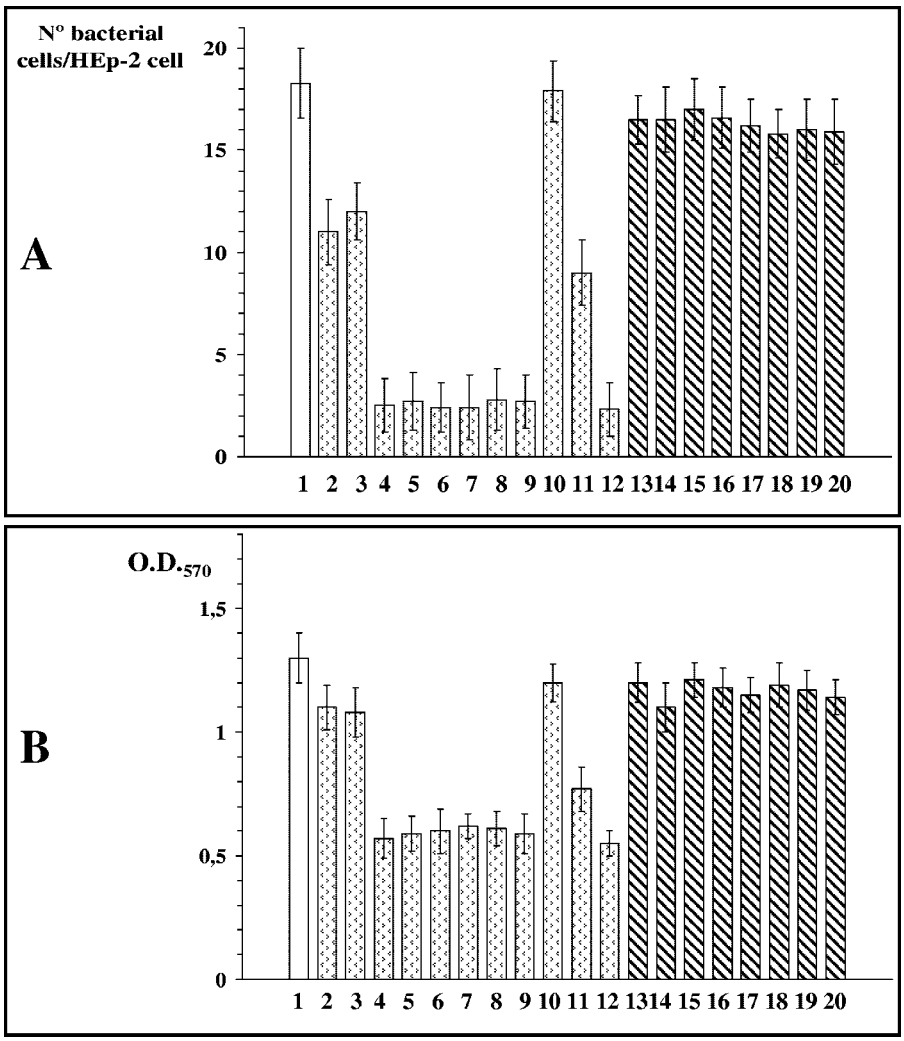

FIG. 5. (A) Adhesion of *A. hydrophila* AH-3 and its lateral flagellum mutants to HEp-2 cells. Mean numbers of adherent bacteria per HEp-2 cell are shown. (B) Biofilm formation ability of *A. hydrophila* AH-3 and its lateral flagellum mutants. The optical density at 570 nm ($O.D._{570}$) quantifies the amount of crystal violet retained by the biofilm on the microtiter plates after staining. Columns: 1, AH-3 (wild type); 2, AH-4425 (*flaB*); 3, AH-4426 (*flaA*); 4, AH-4427 (*flaA flaB*); 5, AH-4423 (*flaH*); 6, AH-4424 (*maf-1*); 7, AH-4441 (*fliM*); 8, AH-4442 (*flhA*); 9, AH-4443 (*fliA*); 10, AH-4444 (*pomB*); 11, AH-4461 (*motX*); 12, AH-4462 (*flrC*); 13, AH-4427 (*flaA flaB*) with plasmid pLA-FLA; 14, AH-4423 (*flaH*) with plasmid pLA-FLA; 15, AH-4424 (*maf-1*) with plasmid pACYC-MAF; 16, AH-4441 (*fliM*) with plasmid pLA-FLIP1; 17, AH-4442 (*flhA*) with plasmid pLA-FLIP2; 18, AH-4443 (*fliA*) with plasmid pLA-FLIP2; 19, AH-4461 (*motX*) with plasmid pLA-MOTX; and 20, AH-4462 (*flrC*) with plasmid pACYC-FLR1. The averages of three independent experiments (each experiment performed in duplicate) are shown.

tem that is expressed in high viscosity. Previously, only a few genes required for polar flagellum formation have been published (2, 53). The isolation of *A. hydrophila* AH-3 transposon mutants unable to swim allowed us to genetically characterize three of five *A. hydrophila* AH-3 chromosomal regions (regions 2, 3, and 4) containing polar flagellum genes (Fig. 1). Region 2 is composed of *fla* and *maf* genes; *fli*, *flh*, chemotaxis, and motor genes are in region 3; and the motor gene *motX* is alone in region 4. Polar flagellum loci that have been sequenced in other bacteria, such as *Vibrio* or *Pseudomonas*, indicated that the gene organization seems highly conserved, although their distributions in chromosomal regions are different between organisms (Fig. 2). Genes coding for flagellins and capping protein (*fla*), as well as genes coding for the basal body, hook length regulator, switch, export apparatus, flagellum placement determinant, flagellum number regulator, σ^{28} factor, chemotaxis, and motor proteins (*fli* and *flh*) of *A. hydrophila* AH-3, are distributed in two different chromosomal regions (2 and 3). In contrast, *V. parahaemolyticus* (Fig. 2) showed all of these genes grouped in a single chromosomal region (34, 41).

Organization of the *A. hydrophila fla* genes in region 2 is identical to that of *A. caviae* (53). A similar gene organization is also observed with *V. parahaemolyticus* polar flagellum region 2 (Fig. 2); however, this organism possesses one more flagellin gene (*flaF*) and a gene encoding a putative chaperone (*flaI*) between *flaH* and *flaJ* (30, 36). The *A. hydrophila* insertional *flaH* mutant and *flaA flaB* double mutant showed lateral flagella, absence of polar flagella, and a dramatic reduction in adhesion to HEp-2 cells and ability to form biofilms. All of these effects were rescued by the introduction of pLA-FLA in

the mutant strains, suggesting that the *fla* genes distributed in this chromosomal region are involved only in polar flagellum biosynthesis or assembly, and both flagellin genes (*flaA* and *flaB*) are required for optimal polar flagellum function. Recent unpublished work performed with *A. caviae* has shown that nonpolar mutants (*flaA flaB* and *flaH*) do in fact express lateral flagella, in contrast to the previously reported results (53). The *A. hydrophila* AH-3 strains with mutations in *flaH*, *flaA*, *flaB*, and *flaA flaB* demonstrated the same phenotypes as their *A. caviae* counterparts (53). In addition we found, downstream of the *A. hydrophila* polar flagellum *fla* loci and preceded by a σ^{28} promoter sequence, an independently transcribed gene not described before for *Aeromonas* spp. or *Vibrio* spp. This gene encoded a homologue of the Maf proteins reported for *Helicobacter pylori*, *Clostridium acetobutylicum*, and *Campylobacter jejuni*. In all of these bacteria, the genes encoding Maf proteins are linked to either flagellum biosynthesis genes and/or genes involved in sugar biosynthesis and transport (26, 33). The inactivation of the *A. hydrophila maf-1* gene abolished only polar flagellum formation and did not affect lateral flagella; the wild-type phenotype was restored by introduction of pACYC-MAF (Fig. 4). This fact together with the knowledge that *A. hydrophila* AH-3 polar flagellins are glycosylated (unpublished observation), similarly to the *A. caviae* polar flagellins (25, 53), may suggest that the encoded protein (Maf-1) is involved in posttranslational polar flagellum glycosylation, but their exact role in flagellar biosynthesis remains unknown.

The *A. hydrophila* polar flagellum region 3 (Table 3) showed an organization similar to that of the genes downstream of *flaM* in *V. parahaemolyticus* polar flagellum region 2, with the absence of the motor genes (34, 41) (Fig. 2). No master regulatory genes encoding homologues of *V. parahaemolyticus* FlaK, FlaL, and FlaM, *V. cholerae* FlrA, FlrB, and FlrC (52), or *P. aeruginosa* FleQ, FleS, and FleR (4, 54) were found upstream of *A. hydrophila fliE*. In contrast to the *Vibrio* polar flagellum systems, we found two genes (*pomA* and *pomB*) which encode orthologues of the MotA and MotB motor proteins of *Pseudomonas* (15). The *A. hydrophila pomA* and *pomB* genes are transcribed independently, according to the RT-PCR results, while in *P. aeruginosa*, these genes are cotranscribed with some chemotaxis genes (15).

Strains with mutations in the *fliM*, *flhA*, and *fliA* genes were able to produce lateral flagella but were unable to produce polar flagella, and they also showed a large reduction in adhesion to HEp-2 cells and ability to form biofilms. The data obtained from these mutants suggest that they are required only for the production of polar flagella but not for production of lateral flagella. The MotA-MotB complex constitutes the stator of the flagellum motor and is involved in the formation of a proton or sodium-conducting channel to generate the force necessary to drive the flagella (31, 61). The *pomB* insertion mutant had both flagellum types and was fully able to swim, adhere, and form biofilms as well as the wild-type strain. This situation was similar for *P. aeruginosa motB* mutants (18, 64) but not for *V. cholerae* or *V. parahaemolyticus motB* mutants, which were able to produce polar flagella but were nonmotile (8, 24). In *Pseudomonas*, there are two sets of *motAB*-like genes, *motAB* and *motCD*, distributed in different chromosomal regions, as well as another gene, *motY*, which contributes to proton-driven flagellar motility (Fig. 2). Loss of either *motAB*-like gene still resulted in motile bacteria in aqueous environments, and only mutations of both sets of genes encoding the MotA or MotB homologue were sufficient to abolish motility (18, 64). The data obtained from the *pomB* mutant suggest that PomB is not essential for swimming motility, leading to two different possible explanations: the stator of lateral flagella can supply its function, or another *pomAB*-like locus is present in *A. hydrophila*. Further studies are required in order to completely understand the motility process in *A. hydrophila* AH-3.

Region 4 of *A. hydrophila* AH-3 polar flagella includes a gene that encodes a homologue of the sodium-driven motor MotX of *V. alginolyticus* and *V. parahaemolyticus*, which is involved with MotA, MotB, and MotY in torque generation of polar flagella (8, 40), although its exact function is unknown. This *Aeromonas* gene has both a putative σ^{28} promoter sequence and a putative terminator sequence, suggesting that it is transcribed independently, as in *Vibrio* spp. Inactivation of the *A. hydrophila motX* gene did not affect either lateral or polar flagellum formation, but mutants were unable to swim in liquid media, as described for *V. parahaemolyticus*, and showed a reduction (50%) in adhesion to HEp-2 cells and ability to form biofilms (37% reduction) in comparison with the wild-type strain.

In contrast to master regulatory genes for other polar flagellated bacteria, such as *Vibrio* and *Pseudomonas* (Fig. 2), the *Aeromonas flrA* to *flrC* genes are located in an independent chromosomal region (region 5). The central domain of *Aeromonas* FlrB and FlrC homologues contains a σ^{54} interaction domain, which is present in σ^{54} activators, and carboxyl- and amino-terminal domains of FlrB and FlrC homologues, respectively, which define these two proteins as members of the two-component family of bacterial signal transducers. A characteristic histidine kinase domain was found in the FlrB homologue protein, and their N termini contain a PAS domain, usually associated with proteins that play a role in detection and adaptation to environmental change (62). A PAS domain was also found in *V. cholerae* FlrB and *P. aeruginosa* FleS N termini, in contrast to FlaL of *V. parahaemolyticus*. The *Aeromonas flrC* mutant was able to produce lateral flagella but unable to produce polar flagella and had a large reduction in adhesion to HEp-2 cells and ability to form biofilms. The data obtained from this mutant suggest that it is required only for the production of polar flagella but not for production of lateral flagella. Plasmids pLA-FLR and pACYC-FLR1 were able to fully complement the *flrC* defects when introduced independently into this mutant strain. With the possible exception of the motor genes, which require further investigation, the rest of the structural genes for polar and lateral flagellum formation in *A. hydrophila* AH-3 are clearly independent, and further studies are required in order to completely understand the motility process in *A. hydrophila* AH-3.

By comparing previous results with results described in this work for mutants unable to produce polar and lateral flagella (25), mutants able to produce polar but not lateral flagella (23), and mutants able to produce lateral but not polar flagella, we can conclude that both types of flagella contribute to HEp-2 cell adhesion and biofilm formation in *A. hydrophila* AH-3. In *V. parahaemolyticus*, only the polar flagella seem to be involved in these pathogenic features (20).

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