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Role of *flm* Locus in Mesophilic *Aeromonas* Species Adherence

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The adherence mechanism of *Aeromonas caviae* Sch3N to HEp-2 cells was initially investigated through four mini-Tn*5* mutants that showed a 10-fold decrease in adherence. These mutants lost motility, flagella, and their lipopolysaccharide (LPS) O antigen (O-Ag). Three genes, *flmB-neuA-flmD*, were found to be interrupted by the transposon insertions; additionally, two other genes, one lying upstream (*flmA*) and one downstream (*neuB*), were found to be clustered in the same operon. While the *flmA* and *flmB* genes were present in all mesophilic *Aeromonas* spp. (*A. hydrophila*, *A. caviae*, *A. veronii* bv. *veronii*, and *A. veronii* bv. *sobria*) tested, this was not the case for the *neuA-flmD-neuB* genes. Construction and characterization of *flmB* insertion mutants in five other mesophilic *Aeromonas* strains revealed the loss of motility, flagella, and adherence but did not alter the LPS composition of these strains. Taking the above findings into consideration, we conclude (i) that flagella and possibly the LPS O-Ag are involved in the adherence of the mesophilic *Aeromonas* to human epithelial cells; (ii) *flmA* and *flmB* are genes widely distributed in the mesophilic *Aeromonas* and are involved in flagella assembly, and thus adherence; and (iii) in *A. caviae* Sch3N the *flmA* and *flmB* genes are found in a putative operon together with *neuA*, *flmD*, and *neuB* and are involved in LPS O-Ag biosynthesis and probably have a role in flagellum assembly.

Mesophilic *Aeromonas* has been associated with gastrointestinal and wound infections of healthy humans, and less commonly with septicemias of immunocompromised patients (12). *A. caviae*, in particular, is reported as the most prevalent paediatric enteropathogenic species of the genus (26, 39).

A number of putative pathogenicity determinants have been reported for aeromonads; these include toxins, adhesins, and invasins (34). There is still little known about their adherence process, although long-wavy pili have been implicated as important colonization factors of *A. hydrophila* and *A. veronii* bv. *sobria* (14). However, many clinical isolates are poorly piliated or nonpiliated (15), and alternative adherence factors such as the lipopolysaccharide O antigen (O-Ag) and the polar flagellum have been suggested (35). Mesophilic aeromonads are usually motile by means of a single polar unsheathed flagellum; this has been proposed to aid adherence to and invasion of fish cell lines by *A. hydrophila* (24). The lipopolysaccharide (LPS) of the genus has been studied more extensively. *Aeromonas* LPS has been suggested to follow the characteristics of its counterparts in *Escherichia coli* and *Salmonella enterica* (21), with smooth ladder-like patterns predominating among clinical isolates (37). Approximately 100 serogroups have been described for the genus, with AX2, O:3, and O:17 being the commonest among *A. caviae* isolates (32). For *A. hydrophila* serogroup O:34, LPS O-Ag has been implicated in in vitro colonization and virulence in fish and mice (1, 22). Despite reports suggesting the involvement of O-Ag and the polar flagellum in *Aeromonas* colonization, none of the structural or the biosynthetic genes of either have been described to date.

In this study, we describe five genes of *A. caviae* that belong to a putative operon involved in the biosynthesis of LPS O-Ag and flagellum assembly. We have also investigated the distribution of these genes among the mesophilic *Aeromonas* species, which allowed us to draw conclusions about the roles of these two surface structures in the adherence of aeromonads to human epithelial cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. 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, while *Aeromonas* strains were grown either on tryptic soy broth or agar or in brain heart infusion broth (BHIB) (Oxoid). Ampicillin (50 μg/ml), nalidixic acid (50 μg/ml), kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), rifampin (100 μg/ml), and tetracycline (20 μg/ml) were added to the different media when needed.

HEp-2 cell culture and adherence assay. Tissue culture was maintained as described by Thornley et al. (34). The adherence assay was conducted as a slight modification of that described by Carrello et al. (4). Bacteria were grown statically in BHIB at 37°C, harvested by gentle centrifugation (1,600 × *g* for 5 min), and resuspended in phosphate-buffered saline (PBS), pH 7.2, at approximately 10^6 to 10^7 CFU/ml ($A_{600} \approx 0.07$). 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 with PBS, and the HEp-2 cells with the adherent bacteria were stained for 45 min in 10% (vol/vol) Giemsa stain (BDH, Poole, United Kingdom) prepared in Giemsa buffer. The coverslips were air dried, mounted, and viewed by oil immersion under a light microscope at ×1,000 magnification. Twenty HEp-2 cells/coverslip were randomly chosen, and the number of bacteria adhering per HEp-2 cell was recorded. Assays were carried out in duplicates or triplicates.

Whole-cell protein preparation, SDS-PAGE, and immunoblotting. Whole-cell proteins were obtained from *Aeromonas* strains grown statically overnight in BHIB at 37°C. Equivalent numbers of cells were harvested by centrifugation, and the cell pellet was resuspended in 50 to 200 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (29) and boiled

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or phenotype[a]	Reference(s) or source
Strains		
A. caviae		
Sch3	Clinical isolate	39
Sch3N	Sch3, spontaneous Nal^r	This work
IAG75	Sch3N*flmD*::mini-Tn*5*Cm, Nal^r Cm^r	This work
IAG570	Sch3N*flmB*::mini-Tn*5*Cm, Nal^r Cm^r	This work
IAG1419	Sch3N*neuA*::mini-Tn*5*Cm, Nal^r Cm^r	This work
IAG1639	Sch3N*flmB*::mini-Tn*5*Cm, Nal^r Cm^r	This work
A. hydrophila		
AH-3	O:34, wild type	20
AH-1726	AH-3*flmB*::Km^r	This work
O:3	O:3 wild type	1
AH-1881	O:3*flmB*::Km^r	This work
O:25	O:25 wild type	1
AH-1882	O:25*flmB*::Km^r	This work
A. veronii bv. *sobria*		
AH-1	O:11, wild type	1
AH-1883	AH-1*flmB*::Km^r	This work
A. veronii bv. *veronii*		
AS-28	O:11 wild type	1
AH-1884	AS-28*flmB*::Km^r	This work
E. coli		
BW19851	RP4-2*tet*::Mu-1*Kan*::Tn*7* integrant/Δ*uidA*::pir$^+$ *recA1 hsdR*17 *creB*510 *endA*1 *zbf-5 thi*	25
S17-1	*hsdR pro recA*, RP4-2 in chromosome Km::Tn*7* (Tc::Mu)	30
XL1-Blue	*endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1 lac*	Stratagene
DH5α	F$^-$ *endA hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyr-A96 80lacZ*	10
MC106(λ*pir*)	*thi thr1 leu6 proA2 his4 argE2 lacY1 galK2 ara14 xyl5 supE44 pir*	28
SM10(λ*pir*)	*thi thr leu tonA lacY supE recA*::*RP4-2-Tc*::*Mu Km^r pir*	28
Plasmids		
pUT mini-Tn*5*Cm	*bla ori* R6K *mob* RP4 *tnp* gene of Tn*5*-IS*50*$_R$ that lacks *Not*I site, MCS of M13tg131; 8.7 kb, Amp^r Cm^r	6, 11
pUC18	High-copy-number cloning vector, Amp^r	Gibco BRL
pUI2224	pUC18 with 2.2 kb of Sch3N DNA inserted in the *Hin*dIII site; 4.9 kb, Amp^r	This work
pUI2814	pUC18 with 2.8 kb of Sch3N DNA inserted in the *Hin*dIII site; 5.5 kb, Amp^r	This work
pDSK519	High-copy-number broad-host-range cloning vector; 8.1 kb, Km^r	13
pDI2211	pDSK519 with 2.2 kb of Sch3N DNA inserted in the *Hin*dIII site of the polylinker; 10.3 kb, Km^r	This work
pDI284	pDSK519 with 2.8 kb of Sch3N DNA inserted in the *Hin*dIII site of the polylinker; 10.9 kb, Km^r	This work
pDI54	pDI2211 with 2.8 kb of Sch3N DNA inserted in the *Hin*dIII site downstream of the 2.2-kb insert; 13.1 kb, Km^r	This work
pGEM-T	PCR cloning vector, Amp^r	Promega
pFS100	pGP704 suicide plasmid, *pir* dependent	28
pFS-Flm	pFS100 with an internal fragment (672 bp) of *flmB* gene	This work
pLA-Flm	pLA2917 cosmid with a complete *flmB* gene from strain AH-3	This work

[a] Abbreviations: Amp, ampicillin; Ap, β-lactam antibiotics, including piperacillin; Cm, chloramphenicol; Km, kanamycin; Nal, nalidixic acid; MCS, multiple cloning site.

for 5 min, before adding an equal volume of distilled water and boiling for a further 5 min. The samples were centrifuged at 5,000 × *g* for 5 min at room temperature, and the supernatants were kept at −20°C until needed.

Protein samples were separated on SDS-polyacrylamide gels (12%) as described by Laemmli (17). For immunoblotting, proteins were transferred onto Hybond-C (Amersham) nitrocellulose membrane. Following transfer, membranes were blocked with 5% skim milk and probed with a polyclonal rabbit anti-polar flagellin antibody (1:500). The unbound antibody was removed by five washes in PBS, and a goat anti-rabbit peroxidase-conjugated secondary antibody (1:1,000) was added. The unbound secondary antibody was washed away with PBS as described for the primary antibody. The bound conjugate was then detected by the addition of 2 ml of 0.5% 4-chloro-1-naphthol (Sigma) prepared in methanol and diluted in 8 ml of PBS containing 50 μl of H_2O_2 (30% [wt/wt]).

Motility assay. Freshly grown bacterial colonies were transferred with a sterile toothpick into the center of motility agar (1% tryptone, 0.5% NaCl, 0.25% agar). The plates were incubated face up at 37°C for 16 to 24 h, and motility was assessed by examining the migration of bacteria through the agar from the center towards the periphery of the plate.

LPS extraction and PAGE analysis. LPS was purified by the method of Westphal and Jann (38). For screening purposes LPS was obtained after proteinase K digestion of whole cells according to the procedure of Darveau and Hancock (5). SDS-PAGE was performed, and LPS bands were detected by the silver staining method of Tsai and Frasch (36).

EM. Electron microscopy (EM) techniques for visualizing stained whole cells and flagella were previously described (20).

Mini-Tn*5*Cm mutagenesis. Conjugal transfer of pUT-mini-Tn*5*Cm from *E. coli* BW19851 to *A. caviae* Sch3N was performed using a filter mating technique. Bacterial conjugation was allowed to proceed for 6 to 8 h at 37°C on sterile nitrocellulose filters (0.45-μm pore size) placed onto an LB agar (LBA) plate. Serial dilutions of the mating mix were plated on LBA supplemented with

nalidixic acid and chloramphenicol, the latter added in order to select for mini-Tn*5*Cm.

General DNA methods. DNA manipulations were carried out essentially as previously described (29). DNA restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerase (Klenow fragment), and alkaline phosphatase were used as recommended by the suppliers.

Southern blot and dot blot hybridizations. Southern blotting was performed by capillary transfer (29). For dot blot hybridizations, the DNA was denatured by boiling for 5 min, chilled on ice for another 5 min, and spotted onto Hybond N^+ (Amersham) nylon membrane. Probe labeling, hybridization, and detection was carried out using the enhanced chemiluminescence labeling and detection system (Amersham) according to the manufacturer's instructions.

Cloning of DNA flanking mini-Tn*5*Cm insertions by inverse PCR. Chromosomal DNA of mini-Tn*5*Cm mutants IAG75, IAG570, IAG1419, and IAG1639 was digested with *Pst*I, purified, and then ligated overnight at 15°C. Samples of 100 to 200 ng of ligated DNA were then subjected to inverse PCR. The sequences flanking the transposon were amplified by using the primers 5′-AGAT CTGATCAAGAGACAG-3′ and 5′-ACTTGTGTATAAGAGTCAG-3′, which are specific to the 19-nucleotide (nt) I end and O end of miniTn*5*Cm, respectively. This was performed using *Pfu* DNA polymerase (Stratagene) at 2.5 mM $MgCl_2$ in a Hybaid *Omnigene* Thermal cycler. Initial DNA denaturation was carried out for 2 min, and amplification reactions were carried out for 25 cycles with denaturation at 95°C for 30 s, primer annealing at 48°C for 1 min, and elongation at 72°C for 8 min. A final elongation step of 10 min at 72°C was also performed. PCR products were ligated into the *Sma*I site of pUC18 and sequenced.

Nucleotide sequencing and sequence analysis. Double-stranded DNA sequencing was performed by using the Sanger dideoxy-chain termination method with the ABI Prism dye terminator cycle sequencing kit (Perkin-Elmer). DNA fragments were ligated into pUC18 and sequenced using an ABI PRISM 377 DNA sequencer (Perkin-Elmer Corporation). The 18-mer forward (5′-TGTAA AACGACGGCCAGT-3′) and the 22-mer reverse (5′-TCACACAGGAAACA GCTATGAC-3′) M13 primers were employed in sequencing the ends of the DNA inserts. Following the first sequencing reaction and whenever required, primers were designed until the inserts' sequences were complete. Primers used for DNA sequencing were purchased from Pharmacia LKB Biotechnology. 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 compared with those of DNA translated in all six frames from nonredundant GenBank and EMBL databases by using the BLAST network service at the National Center for Biotechnology Information (NCBI) (2). Multiple sequence alignments were carried out using the Clustal W program (33). Determination of possible terminator sequences was done by using the Terminator program from the Genetics Computer Group package (Madison, Wisconsin) in a VAX 4300. Hydropathy profiles were calculated according to the method of Kyte and Doolittle (16).

Construction of *flmB*-defined insertion mutants. To obtain defined insertion mutants in *flmB* we used a method previously described (28) based on suicide plasmid pSF100. Briefly, an internal amplified fragment of this gene was ligated to vector pGEM-T (Promega) and transformed in *E. coli* DH5α. The fragment was recovered by restriction digestion; blunt ended with Klenow fragment; and finally ligated to *Eco*RV-digested, blunt-ended, and dephosphorylated pFS100 and transformed into *E. coli* MC1061(λ*pir*), selecting for Km^r to generate plasmid pFS-Flm. Plasmid pFS-Flm was isolated and transformed on *E. coli* SM10(λ*pir*). Plasmid pFS-Flm was transferred by conjugation to different mesophilic *Aeromonas* sp. rifampin-resistant (Rif^r) strains to obtain defined insertion mutants in *flmB* selecting for Rif^r and Km^r.

Nucleotide sequence accession number. The nucleotide sequence of the genes described here have been assigned the following GenBank accession number: AF126256.

RESULTS

Isolation of nonadherent mini-Tn*5*Cm mutants of *A. caviae* strain Sch3N. Conjugations between *A. caviae* Sch3N and *E. coli* BW19851 (pUT-mini-Tn*5*Cm) were carried out by filter mating. Transconjugants were grown for 48 to 72 h on LBA containing naladixic acid and chloramphenicol and subsequently subcultured and purified. Over 2,000 mutants generated by this method were qualitatively screened for reduced adherence to HEp-2 cells by adherence assay. Fourteen mutant strains consistently exhibiting an average of 15% of the wild-type adherence were isolated. All nonadherent mutants were then analyzed for the presence of the transposon by Southern hybridizations of *Pst*I chromosomal DNA digestions. As no *Pst*I restriction sites were present in the transposon, variable size bands larger than the transposon were observed for each mutant. A single band was detected in every mutant chromosome, indicating single transposon insertions. From the hybridizing *Pst*I bands, the size of *Aeromonas* DNA flanking the transposons could be estimated.

TABLE 2. Effect of centrifugation of *A. caviae* onto HEp-2 cells prior to infection

Strain	Mean no. of bacteria/HEp-2 cell ± SD[a]	
	Normal	Centrifuged
Sch3N (control)	23.5 ± 4.8	54.55 ± 15.55
IAG75	1.7 ± 1.5 (7.2)	26.8 ± 3.2 (49.1)
IAG570	1.25 ± 0.9 (5.3)	10.7 ± 1.9 (19.6)
IAG1419	3.5 ± 2.7 (14.9)	18.35 ± 8.35 (33.6)
IAG1639	3.2 ± 3.15 (13.6)	6.4 ± 1.25 (11.7)

[a] Bacteria were grown overnight statically in BHIB at 37°C, and adherence assays were carried out as normally (normal) or after centrifugation of bacteria onto the monolayers (centrifuged). Assays were carried out in duplicates on two separate occasions, and the mean number of adherent bacteria per HEp-2 cell ± standard deviation was recorded for every strain. Values in parentheses are percentages of control results.

Preliminary characterization of four nonadherent mutants. The four mutants IAG75, IAG570, IAG1419, and IAG1639 exhibited an average of 10% of Sch3N adherence (Table 2). The bands obtained on the *Pst*I Southern blots for these four mutants were of similar size (6 to 6.5 kb), and therefore the transposon insertions were thought to be in the same *Pst*I chromosomal DNA fragment, which was estimated around 2.5 to 3.0 kb. Since these mutants also exhibited identical phenotypes (see below) they were chosen for further study.

Loss of motility and flagellin expression by mutants. When the mutants were incubated statically in BHIB, they grew at the bottom of the culture as a "loose" pellet and not as a turbid suspension seen for Sch3N. Such a phenotype suggested the loss of motility by the mutants. This was confirmed by the inability of mutant cells to swim in semisolid motility agar and by immunoblotting of the mutant whole-cell protein preparations for the flagellin protein. In contrast to the parental strain, the polar flagellin was not detected in the mutant preparations, an observation suggesting the loss of flagellin protein expression and thus the absence of the polar flagellum filament (Fig. 1).

Effect of centrifugation of mutants onto HEp-2 cells. To determine whether motility per se was required for adherence, bacteria were centrifuged onto the HEp-2 cells prior to the 90-min infection period. In doing so, the motility defects of the mutants were bypassed. Centrifugation increased adherence of all the strains used (Table 2). Adherence of Sch3N went up from an average of 23.5 to 54.5 bacteria/HEp-2 cell. Centrifugation increased adherence of mutants IAG75 and IAG1419 up to 49.1 and 33.6% of the Sch3N adherence, respectively, whereas adherence of IAG570 and IAG1639 after centrifugation was much lower.

Sequence analysis of loci interrupted by mini-Tn*5*Cm in mutants IAG75, IAG570, IAG1419, and IAG1639. The DNA

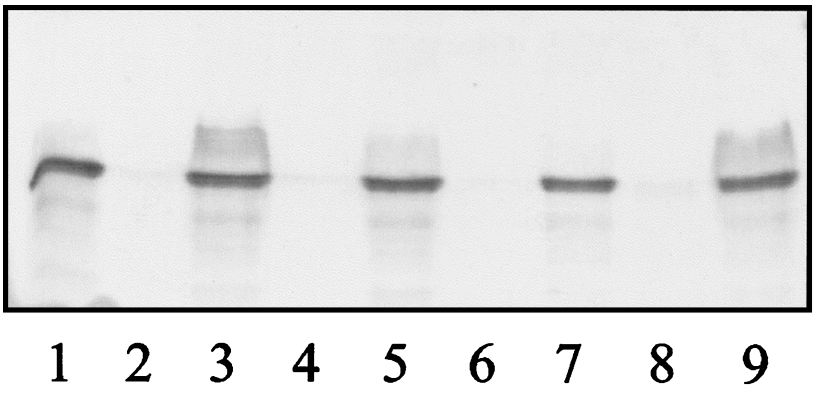

FIG. 1. Polar flagellin immunoblots of whole-cell proteins of the mutants IAG75 (lane 2), IAG570 (lane 4), IAG1419 (lane 6), IAG1639 (lane 8), and the respective complemented strains carrying plasmid pDI54 (lanes 3, 5, 7, and 9, respectively). A protein preparation of *A. caviae* Sch3N was also run as a positive control (lane 1). Proteins were obtained from bacteria grown at 37°C in BHIB and were analyzed by SDS–12% PAGE. They were transferred onto nitrocellulose membranes and immunoblotted with anti-polar flagellin antibodies (1:500).

flanking the transposon in all four mutants was isolated by inverse PCR and then cloned into pUC18 (see Materials and Methods). The PCR products obtained from all four mutants exhibited an identical size, 2.6 kb. Nucleotide sequencing of the four respective PCR products identified three mutated genes clustered on the same *Pst*I fragment. One of the PCR products was then used as a probe to screen a pUC18 *Hin*dIII chromosomal DNA library of Sch3N maintained in *E. coli* XL1-Blue. Subsequently two *Hin*dIII fragments of 2,237 and 2,830 bp carrying the wild-type genes were identified and sequenced. Sequence analysis of the two *Hin*dIII fragments of total length 5,067 bp identified five ORFs, ORF1 to ORF5, of which ORF1 was incomplete (Fig. 2). To decide on the stop and the start codons of the putative ORFs, the extent of the homology of the putative amino acid sequences to known proteins, the degree of overlap with preceding ORFs, and the presence or otherwise of Shine-Dalgarno sequences were taken into account. The genes appeared to be transcribed in the same direction, and no promoter sequences were identified between the putative ORFs. This suggested that the five genes were clustered into a single operon. Proteins homologous to the putative products of the five ORFs were identified using the BLASTX program (2) of the NCBI database (Table 3).

ORF1 (FlmA homologue). Only the 3′-end nucleotide sequence (nt 1 to 623) was obtained for ORF1, which was located upstream of the remaining four ORFs. Its deduced amino acid

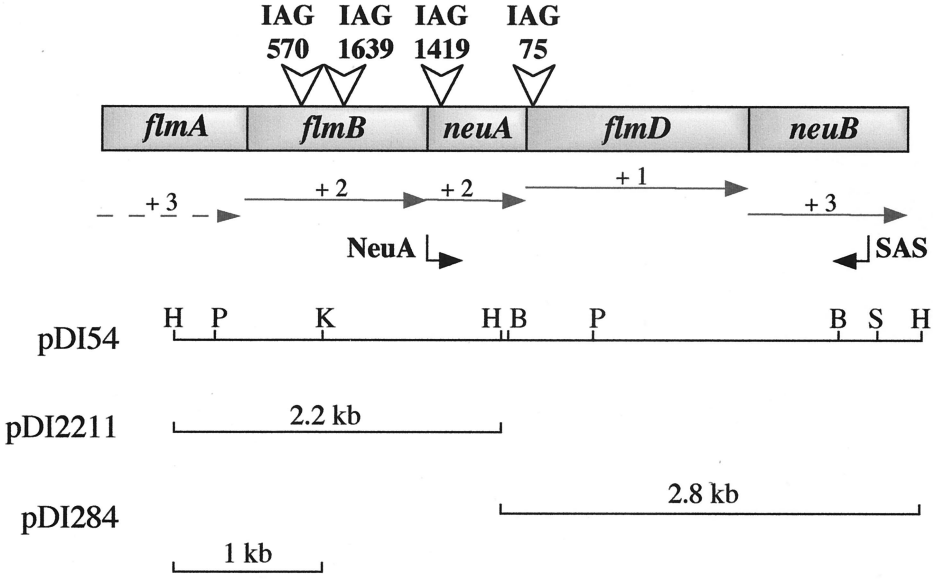

FIG. 2. Genetic organization of the *A. caviae* Sch3N cloned locus. The ~2.2-kb and the ~2.8-kb *Hin*dIII chromosomal DNA fragments were cloned separately or together into plasmid pDSK519 to give plasmids pDI2211, pDI284, and pDI54 respectively. Predicted ORFs were named after their homologues in other bacterial species. Open arrowheads indicate the site of mini-Tn*5*Cm insertion in every mutant's chromosome. Horizontal arrows indicating the direction of transcription and the reading frame (+1, +2, +3) are also drawn. The unknown *flmA* sequence is indicated by a dashed arrow. The approximate hybridization sites of the oligonucleotide primers NeuA and SAS are shown. *Bgl*II (B), *Hin*dIII (H), *Kpn*I (K), *Pst*I (P), and *Sac*II (S) restriction sites are indicated.

TABLE 3. Properties of five putative ORFs of *A. caviae* Sch3N

ORF (nt)[a]	Size (aa)[b]	Molecular mass (kDa)[a]	Homologous protein[c]	Homologue function	% Identity[c]	% Similarity[c]	GenBank accession no.
1 (621)[d]	206		FlaA1, *H. pylori*	Flagellar filament assembly	45	61	AE000595
			FlmA, *C. crescentus*	Flagellar filament assembly	42	57	U27301
			SpsD[e], *Methanococcus jannaschii*	Spore coat polysaccharide biosynthesis	39	60	U67549
2 (1,164)	387	43	FlmB, *C. crescentus*	Flagellar filament assembly	40	57	U27301
			SpsC[e] *Bacillus subtilis*	Spore coat polysaccharide biosynthesis	38	59	P39623
			SpsC[e] *M. jannaschii*	Spore coat polysaccharide biosynthesis	36	60	U67549
3 (687)	228	25.8	NeuA, *H. pylori*	CMP-NeuNAc synthetase	37	53	AE000550
			NeuA, *E. coli*	CMP-NeuNAc synthetase; capsule biosynthesis	31	50	P13266
			NeuA, *N meningitidis*	CMP-NeuAc synthetase; capsule biosynthesis	27	45	Q57385
			PtmB, *C. coli*	Flagellin glycosylation	27	45	U25992
4 (1,518)	505	56.8	FlmD, *C. crescentus*	Flagellar filament assembly	27	40	U27302
			SpsG[e], *M. jannaschii*	Spore coat polysaccharide biosynthesis	24	41	U67549
			SpsH[e], *B. subtilis*	Spore coat polysaccharide biosynthesis	23	40	Z99123
5 (1,059)	352	38.6	NeuB, *C. jejuni*	Sialic acid synthetase	40	55	AJ000855
			SpsE, *H. pylori*	Spore coat polysaccharide biosynthesis	39	59	AE000538
			SpsE[e], *M. jannaschii*	Spore coat polysaccharide biosynthesis	36	53	U67549
			SpsE[e] *B. subtilis*	Spore coat polysaccharide biosynthesis	31	50	P39625
			NeuB, *Streptococcus agalactiae*	Sialic acid synthetase; capsule biosynthesis	32	50	ABO17355
			NeuB, *E. coli*	Sialic acid synthetase; capsule biosynthesis	32	52	UO5248

[a] ORFs and molecular masses were determined by ORF finder of NCBI.
[b] aa, amino acids.
[c] Homologous proteins and identity and similarity scores over the homologous regions were determined by the BLASTX (2) program of NCBI.
[d] Incomplete ORF.
[e] Hypothetical protein.

sequence was found to be most similar to FlaA1 and FlmA of *Helicobacter pylori* and *Caulobacter crescentus* respectively. FlmA was recently proposed to be essential for flagellar filament assembly through flagellin or flagellar protein modification (18). These proteins have recently been included in the *Pseudomonas aeruginosa* WbpM subfamily 2, a large group of proteins with diverse functions involved in exopolysaccharide biosynthesis (3). Interestingly, homologues of FlmA have been included in the general protein glycosylation system of *Campylobacter jejuni* (31).

ORF2 (FlmB homologue, nt 632 to 1795). ORF2 started 8 nt downstream of ORF1, with the alternative start codon GTG. It encoded a protein of 387 amino acids with a predicted molecular mass of 43 kDa. Transposon insertions in both mutants IAG570 and IAG1639 mapped within this ORF, between nt 1002 and 1003 and 1222 and 1223, respectively. The deduced amino acid sequence of ORF2 was most similar (40% identity) to the flagellar protein FlmB of *C. crescentus*. FlmB was also encoded by the operon that encoded FlmA, and again was proposed to be required for flagellar filament assembly (18). Moreover, FlmB homologues in *C. jejuni* (PglE) also belong to the general protein glycosylation system (31).

ORF3 (NeuA homologue, nt 1796 to 2482). ORF3 was the smallest of the complete ORFs identified. It encoded a putative protein of 228 amino acids with a predicted molecular mass of 25.8 kDa. Mutant IAG1419 carried the transposon insertion near the 5′ end of this ORF, between nt 1887 and 1888. ORF3 started immediately downstream of ORF2 and thus was transcribed in the same reading frame. Its predicted amino acid sequence matched a series of CMP-NeuNAc synthetases (NeuA) of *H. pylori*, *E. coli*, and *Neisseria meningitidis* required for the condensation of *N*-acetylneuraminic acid (NeuNAc) and CTP into CMP-NeuNAc. These enzymes were previously shown to be required for the biosynthesis of polysialic acid capsules of *E. coli* K1 (40) and *N. meningitidis* group B (7). The fourth-highest homology was to the recently identified PtmB of *Campylobacter coli* VC167, which was proposed to be required for the sialylation of the polar flagellins (9).

ORF4 (FlmD homologue, nt 2488 to 4005). ORF4 started 6 nt downstream of ORF3 and encoded a putative protein of 505 amino acids with a predicted molecular mass of 56.8 kDa. The transposon insertion of IAG75 mapped between nucleotides 2549 and 2550 and would have therefore inserted just inside the 5′ end of the putative gene. The deduced amino acid sequence of ORF4 was found mainly similar to the flagellar protein FlmD of *C. crescentus* and matched the deduced amino acid sequence of ORF4 throughout its length. Similar to FlmA and FlmB, FlmD was thought to be required for flagellar filament assembly, but the gene encoding this protein mapped with FlmC in a separate operon (18).

ORF5 (NeuB homologue, nt 3993 to 5051). ORF5 overlapped with ORF4 and started 13 nt within it. It encoded a putative protein of 352 amino acids with a predicted molecular mass of 38.6 kDa. Its deduced amino acid sequence was found to be homologous to a series of spore coat polysaccharide biosynthetic proteins and sialic acid synthetases (NeuB) from

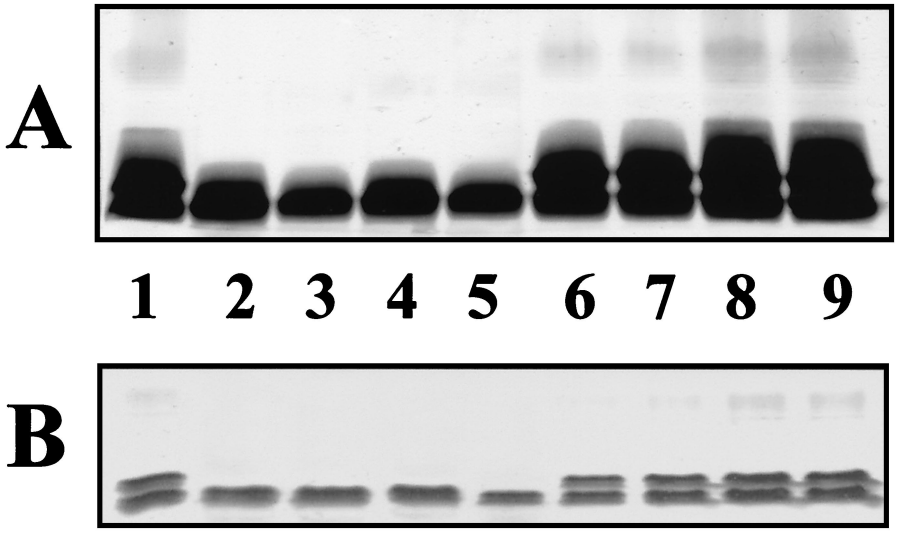

FIG. 3. Analysis of LPS isolated from *A. caviae* Sch3N (lane 1) and the four mutants IAG75, IAG570, IAG1419, and IAG1639 before (lanes 2, 3, 4, and 5, respectively) and after (lanes 6, 7, 8, and 9, respectively) complementation with plasmid pDI54. (A) LPS extracted and analysed by SDS-PAGE (12%) according to Darveau and Hancock (5) and silver stained (36). (B) The same LPS analyzed by Tricine-SDS-PAGE for LPS oligosaccharides (23).

different bacterial species. Three homologues of each protein were identified in the top six matches, and all matched the entire length of the *A. caviae* putative protein. Recently, one of the three *neuB* genes possessed by *C. jejuni* was shown to be required for motility and flagellum production (19).

LPS extraction and PAGE analysis. Following the database homology results, the possibility that the mutants carried defects in polysaccharide biosynthesis was investigated. As the *A. caviae* strain studied did not produce spores or a capsule, the LPS was examined. Strain Sch3N and the mutants IAG75, IAG570, IAG1419, and IAG1639 were grown overnight in BHIB at 37°C, and LPS was extracted and analyzed by PAGE (Fig. 3). As can be observed strain Sch3N was able to exhibit a smooth LPS (O-Ag$^+$) while the mutants were unable to do it (O-Ag$^-$) (Fig. 3A). Furthermore, when we studied the LPS core, we observed (Fig. 3B) that the LPS of the mutants lacks one of the bands always present in the LPS of the wild-type strain. The highest-migrating band, the one lost in the LPS of the mutants, could be part of the outer-core LPS.

Complementation analysis of mutants. The 2.2- and the 2.8-kb *Hin*dIII DNA fragments that carried wild-type copies of the mutated genes were expressed in the mutants in *trans*, in an attempt to complement the defects caused by the transposon insertions. The two fragments were ligated separately or together into the *Hin*dIII site of the broad-host-range plasmid pDSK519 (13) in an orientation that allowed expression from the *lac* promoter. The resulting pDSK519 derivatives carrying the 2.2 kb, the 2.8 kb, or the total of ~5 kb were designated pDI2211, pDI284, and pDI54, respectively. *E. coli* S17-1 transformants each carrying one of the three plasmids were subsequently obtained. The plasmids were then introduced separately into mutants IAG75, IAG570, IAG1419, and IAG1639 by bacterial conjugation (see Materials and Methods), and the wild-type genes were expressed from the *lac* promoter in *trans*. Transconjugant strains grown on both kanamycin and chloramphenicol were isolated from every conjugation experiment. Upon *Hin*dIII and *Pst*I digestion, all three plasmids isolated from the *Aeromonas* transconjugants gave the same restriction patterns as the original plasmids maintained in *E. coli* S17-1. Adherence, motility, and LPS phenotypes of the resulting 12 transconjugant strains were examined (Table 4).

Adherence. The ability of the four mutant strains carrying either of the three pDSK519-derived vectors, in addition to Sch3N and the four original nonadherent transposon mutants, to adhere to HEp-2 cells was tested by adherence assay. All mutants harboring pDI2211 (*flmB*$^+$) remained nonadherent, with their mean adhesion values ranging between 0.5 to 3 adherent bacteria/HEp-2 cell. Similar low mean adhesion values of 0.6 to 0.9 adherent bacteria/HEp-2 cell were recorded for IAG570 (*flmB*), IAG1419 (*neuA*), and IAG1639 (*flmB*) carrying pDI284 (*flmD*$^+$ *neuB*$^+$). In contrast, IAG75 (*flmD*) harboring pDI284 (*flmD*$^+$ *neuB*$^+$) and all four mutants carrying pDI54 (*flmB*$^+$ *neuA*$^+$ *flmD*$^+$ *neuB*$^+$) exhibited dramatically increased adherence, with mean adhesion values of 15 and 12 to 17 adherent bacteria/HEp-2 cell, respectively.

Motility and flagellin expression. Motility of all four mutants expressing either of the three plasmids was tested in motility agar. Again only strain IAG75 expressing pDI284 and all four mutants expressing pDI54 regained their ability to swim in the semisolid motility agar, whereas the rest of the mutants remained nonmotile. To confirm the motility assays results, the whole-cell proteins of the 12 transconjugant mutant strains, in addition to the respective proteins of the four original nonadherent mutant strains and Sch3N, were immunoblotted for the polar flagellin protein. Polar flagellin proteins of an identical size to those of the parental strain were detected in the preparations of the adherent transconjugant strains, whereas no flagellin proteins were detected in the preparations of the nonadherent transconjugants and the original transposon mutant strains (Fig. 1).

TABLE 4. Phenotypic characteristics of original and complemented *A. caviae* Sch3N mutants

Strain or plasmid and mutant	Adherence[a]	Motility[b]	Polar flagellin immunoblot	LPS[c]
Sch3N	17.4 ± 11.3	+	+	S
IAG75 (*flmD*)	2.1 ± 1	−	−	R
IAG570 (*flmB*)	1.3 ± 0.5	−	−	R
IAG1419 (*neuA*)	1.1 ± 0.5	−	−	R
IAG1639 (*flmB*)	3.3 ± 1.3	−	−	R
pDI221 (*flmB*$^+$)				
IAG75 (*flmD*)	1.6 ± 0.6	−	−	R
IAG570 (*flmB*)	1.5 ± 0.6	−	−	R
IAG1419 (*neuA*)	0.5 ± 0.2	−	−	R
IAG1639 (*flmB*)	3 ± 1.3	−	−	R
pDI284 (*flmD*$^+$ *neuB*$^+$)				
IAG75 (*flmD*)	15 ± 10.4	+	+	S
IAG570 (*flmB*)	0.7 ± 0.3	−	−	R
IAG1419 (*neuA*)	0.9 ± 0.5	−	−	R
IAG1639 (*flmB*)	0.6 ± 0.4	−	−	R
pDI54 (*flmB*$^+$ *neuA*$^+$ *flmD*$^+$ *neuB*$^+$)				
IAG75 (*flmD*)	11.9 ± 5	+	+	S
IAG570 (*flmB*)	16.7 ± 7.8	+	+	S
IAG1419 (*neuA*)	17 ± 4.4	+	+	S
IAG1639 (*flmB*)	14.1 ± 3.6	+	+	S

[a] Mean number of adherent bacteria per HEp-2 cell ± standard deviation.
[b] Bacteria were inoculated in motility agar (1% tryptone, 0.05% NaCl, 0.25% agar) for 16 to 24 h. +, fully motile, bacteria cover most of the plate; −, nonmotile, no migration from the site of inoculation.
[c] S, smooth; R, rough.

LPS analysis. The profiles of all the mutants harboring pDI2211 and those of mutants IAG570, IAG1419, and IAG1639 harboring pDI284 remained unchanged (data not shown). The remaining mutants which were complemented for adherence, flagellin expression, and motility exhibited fully complemented LPS profiles (Fig. 3). Specifically, these mutants regained the O-Ag as well as the highest-migrating band of the outer-core LPS.

Distribution of *flmA*, *flmB*, neuA, *flmD*, and *neuB* in mesophilic aeromonads. Using five separate PCR-generated probes to each of the *A. caviae* Sch3N genes, we investigated the distribution of these genes among 20 mesophilic *Aeromonas* spp. (including *A. hydrophila*, *A. veronii* bv. *veronii*, and *A. veronii* bv. *sobria*), by dot blot hybridization. Both *flmA* and *flmB* probes hybridized to the chromosomal DNA of all the strains tested, whereas no hybridization was observed for the probes to *neuA*, *neuB*, or *flmD*. Additionally, the oligonucleotide primers NeuA, 5′-GACTCATATGAATATTGCCATCA TCCC-3′, and SAS, 5′-CTTTACATAACGCAGCAA-3′, were used to amplify the 2,998-bp *neuA-flmD-neuB* region from *A. caviae* Sch3N by PCR. This PCR product was used as a probe to screen 19 strains of *A. caviae* by Southern hybridization. However, only the chromosomal DNA of *A. caviae* Sch3N hybridized with the probe. Oligonucleotides flmB2, 5′-TCTG ATTTTCTAACTCAGGG-3′ (initial base 67), and flmB4, 5′-GTCATTCGGTAGTTAAAGCC-3′ (final base 739), were then used to amplify an internal fragment (672 bp) of both the *A. caviae* Sch3N and *A. hydrophila* AH-3 *flmB* genes; these were subsequently confirmed by sequencing. The amplified fragment from strain AH-3 was ligated into the suicide plasmid pFS100 to generate plasmid pFS-FlmB, in order to obtain defined insertion mutants as previously described (28). Using this plasmid we created defined *flmB* insertion mutants in *A. hydrophila* AH-3 as well as in four other mesophilic *Aeromonas* strains (*A. hydrophila* serogroup O:3 and O:25, *A. veronii* bv. *sobria* AH-1 [serogroup O:11], and *A. veronii* bv. *veronii* AS-28 [serogroup O:11]). All of these mutants were nonmotile when assayed on semisolid motility agar and lacked polar flagella, while their lateral flagella were not attached to the cell surface (Fig. 4 shows a comparison between AH-3 and a *flmB*-defined insertion mutant from the same strain). However, no changes could be observed in the LPS profiles of any of these strains.

Taking advantage of a previously constructed genomic library of strain AH-3 (1) we complemented the FlmB mutant of this strain (AH-1726, Km^r) selecting for Km^r Tc^r clones that were motile on semisolid motility agar. Plasmid pLA-Flm, which complemented the mutation, was sequenced, and two incomplete ORFs (ORF1 and ORF3) and one complete ORF (ORF2) were identified. The deduced amino acid sequence of ORF1 was clearly homologus to that of FlmA, while ORF2 was a homologue of FlmB. ORF3 is transcribed in the opposite direction, and its deduced amino acid sequence was found to be similar to a putative sugar nucleotidyl transferase (Snt) from *C. jejuni* (Fig. 5). Oligonucleotide primers were designed that hybridized to the edges of ORF1 and ORF2, flmC 5′-G AGAAGTTGCATGAAGTGATG-3′ and flmB5 5′-GTCATG CGGTAGTTGAAACC-3′. These amplified a single fragment of 976 bp from *A. hydrophila* AH-3 in a PCR. Single bands were also obtained in PCRs using chromosomal DNA from the *Aeromonas* strains: *A. caviae* Sch3N, *A. hydrophila* serogroups O:3 and O:25, *A. veronii* bv. *sobria* AH-1 (serogroup O:11), and *A. veronii* bv. *veronii* AS-28 (serogroup O:11). When oligonucleotide primers were designed that hybridized to the edges of ORF2 and ORF3, flmB3 5′-TCCGATTTTCTGACTCAGG G-3′ and flmB7 5′-GGGGAAATTGATTCACC-3′ (Fig. 5), again a single band was amplified by PCR in all strains tested with the notable exception of *A. caviae* Sch3N. Both the PCR and Southern hybridization results suggest that for the mesophilic aeromonads, *flmA* and *flmB* are usually found together upstream of *snt* and not in a putative operon along with *neuA-flmD-neuB* as is the case for *A. caviae* Sch3N.

All of the FlmB-defined insertion mutants exhibited reduced adherence to HEp-2 cells that could either be fully restored by complementation with pLA-Flm or by centrifugation onto the monolayer (Table 5). After complementation with pLA-Flm, all the mutants were motile and expressed polar or lateral flagella depending on the medium fluidity, similar to their respective wild types.

DISCUSSION

In this study we used the HEp-2 cell model to initially identify the genes involved in the adherence mechanism of *A. caviae* Sch3, a strain able to exhibit similar patterns of diffuse adherence to the human epithelial cell lines HEp-2 and Caco-2 (34). Preliminary data generated in our laboratory during this and previous studies (34) suggested the involvement of the polar flagellum in the adherence of *A. caviae* Sch3. Moreover,

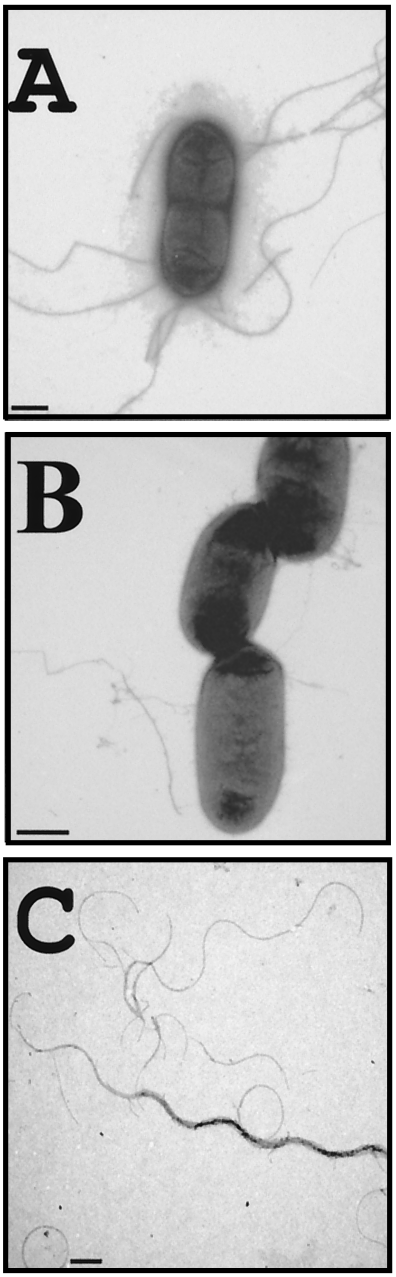

FIG. 4. EM of *A. hydrophila* motile strain AH-3 (wild type) (A), and nonmotile mutant AH-1726 (*flmB*) derived from AH-3(B) grown on motility agar for 12 h at 30°C. Samples were picked and gently resuspended before being adsorbed on the grid. As can be observed, loose flagella are frequently observed (C) on preparations of mutant strain AH-1726, while nothing similar could be observed on wild-type strain AH-3. Bar, 0.5 μm.

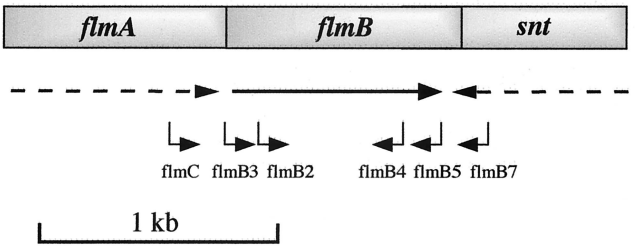

FIG. 5. Genetic organization of the *A. hydrophila flm* locus. Predicted ORFs were named after their homologues in other bacterial species. Horizontal arrows indicate the direction of transcription, and partial ORFs are indicated by dashed arrows. The approximate hybridization sites of the oligonucleotide primers flmC, flmB3, flmB2, flmB4, flmB5, and flmB7 are shown.

phenotypic characterization of the nonadherent transposon mutants of *A. caviae* Sch3N IAG75, IAG570, IAG1419, and IAG1639 strengthened this view. The wild-type copies of the genes mutated in these transposon mutants were cloned and sequenced and were shown to be clustered in a putative operon involved in LPS O-Ag biosynthesis and possibly flagellum assembly. The products of the five putative *A. caviae* genes (ORF1 to ORF5) were similar to a series of bacterial polysaccharide biosynthesis proteins, although the products of ORF1, ORF2, and ORF4 were most similar to the flagellar proteins FlmA, FlmB, and FlmD of *C. crescentus*, respectively. Flm proteins were reported to be involved in flagellar filament assembly of *Caulobacter*, possibly through glycosylation of the flagellin or another flagellar protein(s) (18). However, the *C. crescentus flm* mutants do not exhibit any LPS defects (18), in contrast to those of *A. caviae*. As the *A. caviae* Sch3N transposon mutations affect the LPS O-Ag, an alternative explanation for the loss of flagella could be the rough phenotype which has been reported for LPS core mutants (*rfa*) of *E. coli* (27). Such mutations occur in the LPS core and are thought to

TABLE 5. Adhesion and effect of centrifugation of different *Aeromonas* strains onto HEp-2 cells prior to infection

Strain	Mean no. of bacteria/HEp-2 cell ± SD[a]	
	Normal	Centrifuged
A. hydrophila AH-3 (wild type)	18.3 ± 2.2	31.6 ± 3.0
FlmB insertion mutant from AH-3 (AH-1726)	9.4 ± 1.2 (51)	29.6 ± 3.2 (93)
AH-1726 complemented with pLA-Flm	18.0 ± 2.5	32.5 ± 2.3
A. hydrophila O:3 (wild type)	21.6 ± 2.4	42.7 ± 4.3
FlmB insertion mutant from O:3 (AH-1881)	10.1 ± 1.9 (46)	40.8 ± 5.1 (95)
AH-1881 complemented with pLA-Flm	21.9 ± 3.2	42.1 ± 5.2
A. hydrophila O:25 (wild type)	16.0 ± 2.4	29.3 ± 2.8
FlmB insertion mutant from O:25 (AH-1882)	7.7 ± 1.3 (48)	28.6 ± 2.9 (96)
AH-1882 complemented with pLA-Flm	15.7 ± 2.6	29.9 ± 3.1
A. veronii bv. *sobria* AH-1 (wild type)	32.3 ± 3.9	71.4 ± 10.7
FlmB insertion mutant from AH-1 (AH-1883)	18.1 ± 2.2 (56)	67.3 ± 9.9 (94)
AH-1883 complemented with pLA-Flm	31.6 ± 4.4	70.3 ± 9.2
A. veronii bv. *veronii* AS-28 (wild type)	31.7 ± 4.6	66.8 ± 10.2
FlmB insertion mutant from AS-28 (AH-1884)	15.5 ± 3.4 (48)	63.3 ± 9.1 (94)
AH-1884 complemented with pLA-Flm	32.4 ± 3.8	65.9 ± 10.0

[a] See footnote *a* to Table 2.

destabilize in a pleiotropic way the outer membrane and affect outer membrane protein insertion and O-Ag attachment. Mutations in the *flm* locus of *A. caviae* could possibly be affecting the insertion of another adhesin into the outer membrane. However, this putative adhesin did not appear to be an outer membrane protein as the outer membrane protein profiles of the four mutations did not differ from the wild types when analyzed on polyacrylamide gels (data not shown).

In order to try to explain this phenomenon we investigated the presence of these genes (*flmA*, *flmB*, *neuA*, *flmD*, and *neuB*) in different mesophilic *Aeromonas* spp., including the strain *A. hydrophila* AH-3 (serogroup O:34). We used this strain in particular because the genes responsible for the O:34 LPS antigen biosynthesis have been cloned and sequenced and the LPS composition is known (unpublished results). The *flmA*- and *flmB*-like genes were found in all mesophilic *Aeromonas* strains (*A. hydrophila*, *A. caviae*, *A. veronii* bv. *veronii*, and *A. veronii* bv. *sobria*) when tested by dot blot hybridization, while only *A. caviae* Sch3N contained the *neuA*-, *flmD*-, and *neuB*-like genes. This suggests that *A. caviae* Sch3N is different from most of the other mesophilic aeromonads (including *A. caviae*) and that a genetic rearrangement has probably occurred in this strain to incorporate *flmA* and *flmB* in a putative operon along with *neuA*, *flmD*, and *neuB*.

Using PCR we were able to amplify an internal fragment of the *flmB*-like gene from *A. hydrophila* AH-3; this allowed us to generate a defined insertion mutant in this strain. The *flmB* mutant of strain AH-3 lacked flagella (polar and lateral flagella) and therefore was nonmotile. It was clear by EM that these mutants were able to produce flagella but unable to assemble them on the cell. However, no changes were seen in the LPS of this mutant, a situation similar to that of *Caulobacter* and the other mesophilic aeromonad *flmB* mutants created in this study but different from that observed for *A. caviae* Sch3N.

After complementation of the *flmB* mutant of strain AH-3, it was clear that *flmA* and *flmB* are transcribed in the same direction, and no *neuA*-like gene could be found downstream of *flmB*. Instead, a gene similar to a putative sugar nucleotidyl transferase from *C. jejuni*, one that is transcribed in the opposite direction, was present. This could be a possible reason that no changes in the LPS profile were observed in the *flmB* mutant of AH-3. A similar situation was observed for the *flmB* mutants of the four other mesophilic *Aeromonas* strains.

In the case of *A. caviae* Sch3N the phenotype of the original and the complemented mutants did not allow us to assess the separate roles of the polar flagellum and the LPS O-Ag in adherence. Either of the two or both could be responsible for the in vitro adherence of *A. caviae* Sch3N. The ability of the complemented motile mutants to adhere to HEp-2 cells and the increased adherence seen after the centrifugation of bacteria onto the monolayers suggested the involvement of both the polar flagellum and motility in adherence. Furthermore, the approximate 50% inhibition of adherence produced by the polar flagellin antibodies (data not shown) also supported the importance of motility and flagella in the process. We previously indicated that the polar flagellum and motility are required for adherence to, and invasion of, fish cell lines by *A. hydrophila* serogroup O:34, as nonflagellate Tn*5* mutants lost adherence and invasiveness; however, the mutated genes were not identified (24). The LPS O-Ag has also been described as an adhesin in *Aeromonas*. Ourselves and other workers have associated LPS O-Ag expression with adherence of *A. hydrophila* and *A. veronii* bv. *sobria* clinical isolates to HEp-2 cells (1, 8, 23). Furthermore, LPS O-Ag transposon mutants of *A. hydrophila* O:34 were shown to be unable to colonize the germfree chicken gut model (22). Nevertheless, the *flmB* mutations in *A. hydrophila* AH-3 and the other mesophilic aeromonads (excluding *A. caviae* Sch3N), allowed us to conclude that the flagellum is essential for HEp-2 cell colonization, because these strains were nonadherent and nonmotile and lacked flagella but retained complete LPS profiles. However, there was only a 60% decrease in adherence for the *A. hydrophila* AH-3 *flmB* mutant, compared to the 80 to 90% decrease in adherence observed for *A. caviae* Sch3N *flm* locus transposon mutants. Additionally, the *A. hydrophila* AH-3 *flmB* mutation could be partially rescued, and adherence to wild-type levels could be recovered by centrifugation of the bacteria onto the monolayer. This was not the case for the *A. caviae* mutants, for which centrifugation alone only restored the adherence to 12 to 50% of the wild-type levels. This is most probably due to the *A. caviae* mutants' lacking two adhesins: flagellar and LPS O-Ag. After centrifugation the mutations in *flmB* (IAG570 and IAG1639) caused the greatest loss of HEp-2 cell colonization. This could be possibly due to FlmB being required for the expression of another adhesin or acting as an adhesin itself, although the outer membrane protein profiles of the *flmB* mutants did not differ from those of the other mutants or that of the wild types (data not shown). From all the results we can conclude (i) that flagella and possibly the LPS O antigen are surface structures of mesophilic *Aeromonas* involved in adherence to human epithelial cells; (ii) *flmA* and *flmB* are genes widely distributed in mesophilic *Aeromonas* and are involved in flagellum assembly, and thus adherence; and (iii) in *A. caviae* Sch3N the *flmA* and *flmB* genes are located in a putative operon together with *neuA*, *flmD*, and *neuB*. These genes perform a role in LPS O-Ag biosynthesis and are likely to be involved flagellum assembly.

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