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A Colonization Factor (Production of Lateral Flagella) of Mesophilic *Aeromonas* spp. Is Inactive in *Aeromonas salmonicida* Strains

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The nine *laf* (lateral flagellum) genes of mesophilic aeromonads are in the *Aeromonas salmonicida* genome. The *laf* genes are functional, except for *lafA* (flagellin gene), which was inactivated by transposase 8 (IS*3* family). A pathogenic characteristic of mesophilic aeromonads (lateral flagella) is abolished in this specialized pathogen with a narrow host range.

Aeromonas salmonicida is an important pathogen of salmonid fish, producing the systemic disease furunculosis (12). As in *Vibrio parahaemolyticus* (8, 9), two types of flagella are responsible for motility in aeromonads. A polar unsheathed flagellum is expressed constitutively that allows the bacteria to swim in liquid environments. In media where the polar flagellum is unable to propel the cell, aeromonads express peritrichous lateral flagella (21), a phenomenon associated with the colonization of surfaces, as such hyperflagellated cells demonstrate increased adherence. We have demonstrated the importance of the polar flagellum of *A. hydrophila* in the invasion of fish cell lines (11, 13). More recently, we have described a polar flagellar gene region in *A. caviae* that appears to be essential for adherence to human epithelial cells in vitro (19). Traditionally, the genus *Aeromonas* has been divided on the basis of motility, with *A. salmonicida* being the typical nonmotile species (17). However, a report by McIntosh and Austin (10) indicated that five *A. salmonicida* isolates were able to produce a pole-located sheathed flagellum when grown at supraoptimal incubation temperatures (30 to 37°C) and in the presence of 18% (wt/vol) Ficoll. However, incubation under these conditions never renders more than 1% of the population motile (twisting/tumbling movement) or flagellated. Genetic evidence demonstrated that *A. salmonicida* strains are capable of producing flagella, as two flagellin genes (*flA* and *-B*) were identified and characterized (24). Nine lateral flagellar genes, *lafA* to *-U*, for *A. hydrophila* and four *A. caviae* genes, *lafA1*, *lafA2*, *lafB*, and *fliU*, have been isolated (3). Mutant characterization and nucleotide and N-terminal sequencing demonstrated that the *A. hydrophila* and *A. caviae* lateral flagellins are almost identical but are distinct from their polar flagellum counterparts. Mutation of *Aeromonas lafB* or *lafS* or both *A. caviae* lateral flagellin genes caused the loss of lateral flagella and a reduction in adherence and biofilm formation. Mutation of *lafA1*, *lafA2*, *fliU*, or *lafT* resulted in strains that expressed lateral flagella but had reduced adherence levels. Mutation of the lateral flagellar loci did not affect polar flagellum synthesis, but the polarity of the transposon insertions on the *A. hydrophila lafT* and *-U* genes resulted in nonmotility (3). In a recent study of lateral flagella and swarming motility in different *Aeromonas* species, four different isolates of *A. salmonicida* reacted positively with a DNA probe for lateral flagella that was used to correlate lateral flagella and swarming motility in mesophilic *Aeromonas* spp. (7).

In this study, we provide the genetic basis to explain why *A. salmonicida* strains are able to hybridize with a DNA *laf* gene probe but are unable to produce lateral flagella.

Presence of *laf* genes and lateral flagella. The nucleotide sequences of the lateral flagellins from *A. hydrophila* AH-3 (*lafA*) and *A. caviae* Sch3N (*lafA1* and *lafA2*) were aligned by using ClustalW (22) in order to find common oligonucleotides able to amplify this DNA region. In the same alignment, the polar flagellins of *A. caviae* Sch3N (*flaA* and *flaB*) and *A. salmonicida* (*flaA* and *flaB*) were introduced in order to verify that the common oligonucleotides for lateral flagellins are unable to amplify the polar flagellins. From these data, we found two highly conserved domains (the C- and N-terminal domains) in which oligonucleotides could be designed to specifically amplify the lateral flagellins. The oligonucleotides designed were Laf1 (5′-GGTC TGCGCATCCAACTC-3′) and Laf2 (5′-GCTCCAGACGGTTGATG-3′). In a PCR with Laf1, Laf2, and genomic DNA from either *A. hydrophila* AH-3 or *A. caviae* Sch3N, we obtained a single band of approximately 550 bp that was confirmed to be the lateral flagellin gene by DNA sequencing (Fig. 1A).

Table 1 lists the strains used in this study. *A. salmonicida* strains were grown at 20°C, and mesophilic *Aeromonas* strains were grown at 30°C. The *A. salmonicida* strains were pathogenic isolates from moribund fish derived from a wide range of geographical locations (Canada, Japan, the United Kingdom, the United States, and Spain). All of the *A. salmonicida* strains used in this study were classified in accordance with *Bergey's Manual of Systematic Bacteriology*. All of the isolates were able to react with specific antibodies against the A layer and could be classified as typical pathogenic *A. salmonicida* strains (16).

All 50 (100%) of the *A. salmonicida* strains showed a positive PCR resulting in a single band of approximately 550 bp, which was confirmed by sequencing in a number of cases (Fig. 1A). The strains were also tested by colony blot hybridization against a *laf* probe and for swarming by methods previously

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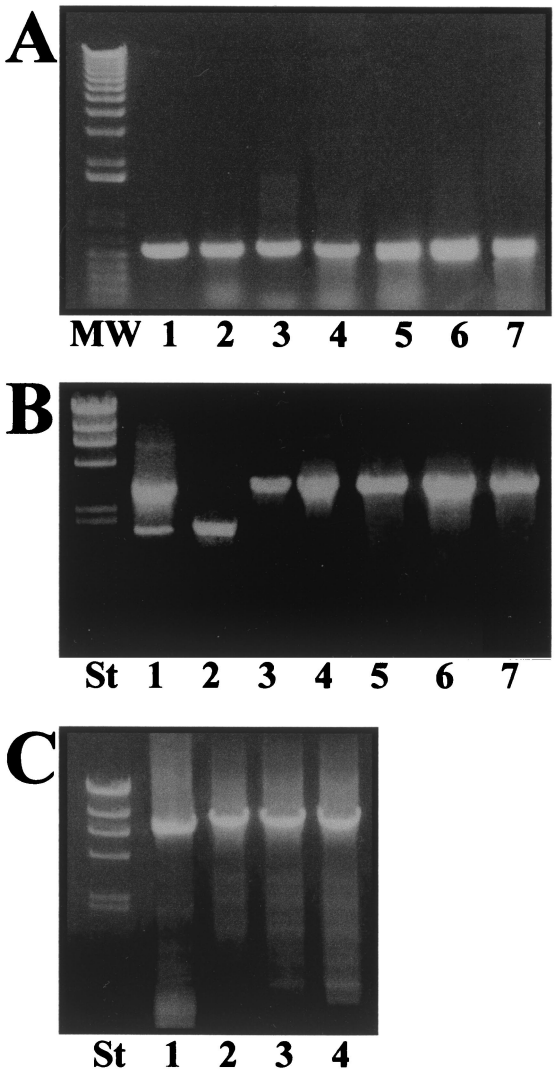

FIG. 1. PCR amplification with several *Aeromonas* sp. genomic DNAs. (A) DNA amplification fragments (550 bp) obtained with oligonucleotides Laf1 and -2 and *A. caviae* Sch3N (lane 1), *A. hydrophila* AH-3 (lane 2), *A. salmonicida* A450 (lane 3), and four different *A. salmonicida* isolates (lanes 4 to 7). Lane MW contained molecular weight markers (1 Kb PLUS DNA Ladder; Invitrogen). (B) DNA amplification fragments obtained with oligonucleotides Laf 1 and -5 and *A. caviae* Sch3N (lane 1, 2,751 and 1,737 bp), *A. hydrophila* AH-3 (lane 2, 1,715 bp), *A. salmonicida* A450 (lane 3, 2,952 bp), and the same *A. salmonicida* isolates previously used in panel A (lanes 4 to 7, 2,952 bp). Lane St contained λ DNA digested with *Hin*dIII. (C) DNA amplification fragments obtained with oligonucleotides Laf 1 and −9 and *A. hydrophila* AH-3 (lane 1, 7,120 bp), *A. salmonicida* A450 (lane 2, 8,360 bp), and two of the *A. salmonicida* isolates used previously (lanes 3 and 4, 8,360 bp). Lane St contained λ DNA digested with *Hin*dIII.

described (3, 19). There was a complete correlation among the PCR-positive and colony blot-positive strains. However, while *laf*-positive mesophilic *Aeromonas* sp. strains were able to swarm, none of the *A. salmonicida* strains tested were able to. Furthermore, when several *A. salmonicida* strains were examined by electron microscopy (EM) and a Western immunoblot assay for the presence of lateral flagella and production of lateral flagellin (3, 19), they were always negative.

As can be observed from the DNA sequences of *A. hydrophila* AH-3 (accession no. AY028400) and *A. caviae* Sch3N (accession no. AF348135), *lafB* is the highly conserved gene immediately downstream of the lateral flagellin gene(s) in the *laf* cluster. An oligonucleotide was designed within the *lafB* gene, Laf5 (5′-ATCGCTGGAGGTCATCTTG-3′). In a PCR with Laf1, Laf5, and genomic DNA from *A. hydrophila* AH-3 or *A. caviae* Sch3N, we obtained a single band (1,715 bp) for *A. hydrophila* AH-3 and two bands (2,751 and 1,737 bp) for *A. caviae* Sch3N (depending upon whether they have one or two flagellin genes). When we tested the *A. salmonicida* strains, we amplified a single band of 2,952 bp (Fig. 1B). The complete DNA sequence of the *A. salmonicida* band showed the presence of a putative transposase 8 from the IS*3* family inserted within the *A. salmonicida lafA* gene. When other *A. salmonicida* strains were tested ($n = 10$) with the same oligonucleotides, the same *lafA* band (2,952 bp) containing the putative transposase was amplified. This putative transposase showed a high degree of identity and similarity (more than 60 and 75%, respectively) to TnpA from *Pseudomonas putida* or *Erwinia carotovora* and putative transposases from *Pantoea agglomerans*, *Xanthomonas campestris*, and *Agrobacterium tumefaciens*. Furthermore, it showed the typical transposase region at the beginning of the sequence and the integrase core domain at the end.

The complete *A. salmonicida* strain A450 *laf* gene cluster. With either genomic DNA from *A. hydrophila* AH-3 or the cosmid pCOS-LAF (3) in a PCR (annealing temperature of 56°C, extension time of 7 min 30 s, and PlatinumTaq polymerase high fidelity from Invitrogen) with oligonucleotides Laf1 and Laf9 (5′-CCAGATTCTTTTCCGCCTG-3′), a single DNA band of 7,120 bp was obtained (Fig. 1C). Oligonucleotide Laf9 is located in the last gene of the *laf* cluster, *lafU*; this allowed complete amplification of the nine *laf* genes. By applying the same amplification procedure to *A. caviae* Sch3N or *A. veronii* bv. *sobria* AH-1 genomic DNA, we obtained a single DNA band with a size similar to that of the AH-3 band. Sequence analysis of both edges confirmed the presence of the *lafA* and *lafU* genes in these strains. However, when *A. salmonicida* strain A450 was tested, we obtained a single band of 8,360 bp (Fig. 1C). Sequencing of the complete DNA band amplified from *A. salmonicida* strain A450 (accession no. AY1295578) revealed nine open reading frames (ORFs): *lafA*, *-B*, *-C*, *-X*, *-E*, *-F*, *-S*, *-T*, and *-U* (Fig. 2). All of the ORFs were transcribed in the same direction, and putative Shine-Dalgarno sequences were found upstream of all of the ORF start codons. The only putative transcriptional terminator sequence was found between *lafA* and *lafB*, suggesting that *lafB*- to *U* form a single transcriptional unit. The coding sequence was preceded by a putative σ^{54} promoter sequence. From *lafB* to *lafU*, the DNA sequence homology with the same region of *A. hydrophila* AH-3 was always greater than 90% (3).

Complementation analysis of mesophilic *Aeromonas* Laf⁻ mutants with *A. salmonicida laf* genes. The AH-1982 (*lafB*) and AH-1983 (*lafS*) mutant strains of *A. hydrophila* and mutant strain AAR9 (*lafB*) or tandem flagellin mutant strain AAR6 (*lafA1 lafA2*) of *A. caviae* produced polar flagella but not lateral flagella (3). Mutant AH-1984 (*lafT*) of *A. hydrophila* pro-

TABLE 1. Bacterial strains and plasmid used in this study

Strain or plasmid	Genotype and/or phenotype	Source or reference
Strains		
A. salmonicida A450		14
A. hydrophila AH-3	O:34 wild type	11
A. hydrophila AH-1982	Defined *lafB* insertion mutant from AH-3; Km^r	3
A. hydrophila AH-1983	Defined *lafS* insertion mutant from AH-3; Km^r	3
A. hydrophila AH-1984	Defined *lafT* insertion mutant from AH-3; Km^r	3
A. caviae Sch3N	Wild type	5
A. caviae AAR6	Defined *lafA1-lafA2 double*-insertion mutant from Sch3N; Km^r Cm^r	3
A. caviae AAR9	Defined *lafB* insertion mutant from Sch3N; Cm^r	3
E. coli DH5α	F^- *endA hdsR17* (r_K^- m_k^+) *supE44 thi-1 recA1 gyrA96 80lacZ*	6
E. coli XL1-Blue	*endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1 lac*	Stratagene
Plasmids		
pGEMT	Cloning vector; Ap^r	Promega
pRK2073	Helper plasmid; Sp^r	20
pLA2917	Cosmid vector; Tc^r Km^r	1
pCOS-LAF	pLA2917 cosmid with complete *laf* gene cluster; Tc^r	3
pACYC184	Cloning vector	15
pINA1	pACYC184 containing *A. salmonicida lafA*	This work
pINA2	pACYC184 containing *A. salmonicida lafB*	This work
pINA3	pACYC184 containing *A. salmonicida lafS*	This work
pINA4	pACYC184 containing *A. salmonicida lafT*	This work

duced polar and lateral flagella but was nonmotile (3). Mutant AAR6 was unable to be complemented by plasmid pINA1 (*lafA* of *A. salmonicida* with the putative transposase inserted), as lateral flagella were not detected by EM.

However, mutants AAR9 and AH-1982 were fully complemented when plasmid pINA2 (*lafB* of *A. salmonicida*) was introduced into them, as lateral flagella were detected on the complemented strains by EM. A similar situation was observed for mutant AH-1983 and plasmid pINA3 (*lafS* of *A. salmonicida*). Furthermore, plasmid pINA4 (*lafT* of *A. salmonicida*) was fully able to complement the nonmotile phenotype when introduced into mutant AH-1984. None of these strains were complemented when we introduced the plasmid vector alone (pACYC184) into the mutants.

Lateral flagella, as well as the polar flagellum, in mesophilic *Aeromonas* strains seem to be involved in bacterial adhesion to eukaryotic cells and the ability to form biofilms (3, 5, 19). In order to see if the *A. salmonicida laf* genes were fully functional, we tested them for the ability to complement the mesophilic *Aeromonas laf* mutants for adhesion to HEp-2 cells and biofilm formation in vitro by using previously described assays (18, 23). As shown in Table 2, *A. hydrophila* mutants AH-1982 (*lafB*) and AH-1983 (*lafS*) and *A. caviae* mutants AAR6 (*lafA1 lafA2*) and AAR9 (*lafB*) showed an approximately 85% reduction in adherence to HEp-2 cells in comparison with the respective wild-type strains whereas mutant AH-1984 showed only a 50% reduction ($P = <0.0005$). Furthermore, the same mutants showed a drastic reduction in the ability to form biofilms (Table 2). The results obtained with the complemented strains (Table 2) are in agreement with the

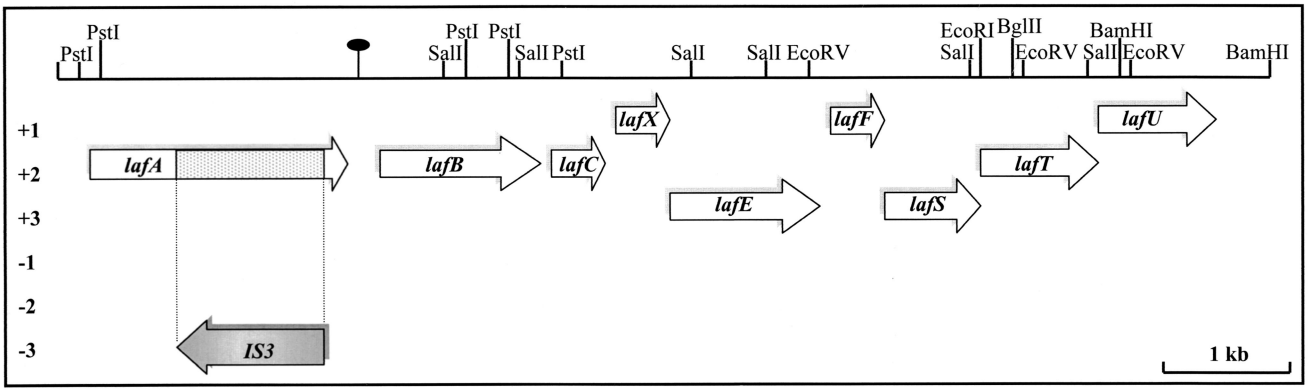

FIG. 2. Schematic representation of the *A. salmonicida* A450 lateral flagellin loci. Flagellar genes and ORFs are indicated by arrows, as is the direction of transcription. ORFs are named after their homologues in the *A. hydrophila* AH-3 *laf* gene cluster. The putative transposase 8 of the IS*3* family is indicated by a shaded arrow along with its direction of transcription. RF (+1, +2, +3, −1, −2, and −3) indicates the reading frame. The lollipop structure depicts the approximate position of the putative transcriptional terminator. *Pst*I, *Sal*I, *Eco*RV, *Bam*HI, and *Bgl*II restriction sites are shown.

TABLE 2. Adhesion to HEp-2 cells and biofilm formation of different mesophilic *Aeromonas* wild-type and mutant strains[a]

Strain	Mean no. of adherent bacteria/HEp-2 cell ± SD	Biofilm formation ability (OD_{570} ± SD)
AH-3 (wild type)	18.3 ± 1.3	1.1 ± 0.2
AH-1982 (*lafB*)	2.7 ± 0.8 (15)	0.4 ± 0.1 (36)
AH-1983 (*lafS*)	2.8 ± 0.7 (16)	0.4 ± 0.1 (36)
AH-1984 (*lafT*)	9.2 ± 1.3 (51)	0.7 ± 0.1 (63)
AH-1982 (pINA2)	18.0 ± 1.1	1.1 ± 0.1
AH-1983 (pINA3)	17.4 ± 2.0	1.1 ± 0.2
AH-1984 (pINA4)	18.7 ± 1.4	1.1 ± 0.1
Sch3N (wild type)	39.1 ± 4.8	1.5 ± 0.2
AAR6 (*lafA1 lafA2*)	5.5 ± 2.7 (14)	0.6 ± 0.1 (40)
AAR9 (*lafB*)	6.3 ± 1.7 (16)	0.6 ± 0.1 (40)
AAR6 (pINA1)	5.6 ± 2.2 (14)	0.6 ± 0.1 (40)
AAR9 (pINA2)	37.9 ± 3.6	1.4 ± 0.2

[a] Values in parentheses are percentages of control results. All values are averages of three independent experiments ($P < 0.0005$). OD_{570}, optical density at 570 nm.

phenotypic complementation mentioned above. Briefly, *lafB*, *-S*, and *-T* mutants recovered values of adhesion to HEp-2 cells and biofilm formation similar to those of the corresponding wild-type strains. However, again, the AAR6 mutant of *A. caviae* (*lafA1 lafA2*) was not complemented by plasmid pINA1 (*lafA* of *A. salmonicida* with the putative transposase inserted). Again, no changes in adhesion to HEp-2 cells or biofilm formation were found in the mutants when the plasmid vector alone (pACYC184) was introduced.

We clearly demonstrated by mutant complementation of mesophilic *Aeromonas* spp. that *A. salmonicida* genes *lafB* to *-U* are fully functional either by their phenotypic traits (presence or absence of lateral flagella or swarming motility) or by their ability to adhere to HEp-2 cells and form biofilms in vitro. The only *A. salmonicida laf* gene that is nonfunctional is *lafA*, as assessed by mutant complementation of mesophilic *Aeromonas* spp. It was shown that in all of the *A. salmonicida* isolates tested, there was a putative transposase 8 from the IS*3* family inserted in the middle of the *lafA* gene. This gene is responsible for lateral flagellin production, which in some cases is present as a single gene, as in *A. hydrophila* AH-3 (3), or as two genes, as in *A. caviae* Sch3N (3). *A. salmonicida* appears to have only a single *lafA* gene, which was inactivated in all of the strains tested.

We tried repeatedly to introduce plasmid pCOS-LAF (3) into different *A. salmonicida* strains. Although cosmid vector pLA2917 alone (1) is able to enter and replicate in *A. salmonicida* strains, no transconjugants were obtained when pCOS-LAF was introduced. Moreover, if a plasmid construct (pACYC184) with the *A. hydrophila* AH-3 *lafA* gene alone was introduced into *A. salmonicida* strains, we obtained the same negative results.

The lack of transconjugants of *A. salmonicida* strains with pCOS-LAF or a plasmid construct with the *A. hydrophila* AH-3 *lafA* gene alone does not allow us to state that *A. salmonicida* strains just require a intact copy of the *lafA* gene for production of lateral flagella. Inactivation of *lafA* was enough to abolish production of lateral flagella in *A. salmonicida*, but maybe other genes affecting global (polar and lateral) flagellar synthesis are lacking in *A. salmonicida* strains, thus explaining the lack of production of polar and lateral flagella. For instance, *Vibrio parahaemolyticus* strains use more than 60 genes to produce polar flagella (8). It is tempting to speculate that supraoptimal incubation temperatures plus high-osmolarity (Ficoll) conditions may enhance excision of the transposase from the *A. salmonicida lafA* gene, rendering a small percentage of the population able to produce lateral flagella. In this respect, *A. salmonicida* appear to be similar to *Shigella* spp., which are also classified as nonmotile, as IS elements have been detected in the flagellar genes of various strains (2). However, flagellate and motile strains of *Shigella* spp. have been reported, although at a low frequency (4). As with *Shigella* spp., *A. salmonicida* does not show any degeneracy in its flagellar genes. This suggests that the insertion of the IS element was a recent event or that keeping these genes and having a small amount the *A. salmonicida* population able to become motile are somehow important for the survival and biology of the organism.

Production of lateral flagella by mesophilic *Aeromonas* spp. is a pathogenic factor, as it enhances adhesion to eukaryotic cells and the ability to form biofilms (3). *A. salmonicida* strains are pathogens with a narrow host range, salmonid fish. In this bacterium, this pathogenic factor is not expressed even though the genes appear to be present in its genome. This is, to our knowledge, the first genetically well-documented case in which a pathogenic character is abolished in a pathogen with a high level of specialization because of its narrow host range.

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