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Flagellin A Is Essential for the Virulence of *Vibrio anguillarum*

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A flagellin gene from the fish pathogen *Vibrio anguillarum* was cloned, sequenced, and mutagenized. The DNA sequence suggests that the *flaA* gene encodes a 40.1-kDa protein and is a single transcriptional unit. A polar mutation and four in-frame deletion mutations (180 bp deleted from the 5′ end of the gene, 153 bp deleted from the 3′ end of the gene, a double deletion of both the 180- and 153-bp deletions, and 942 bp deleted from the entire gene) were made. Compared with the wild type, all mutants were partially motile, and a shortening of the flagellum was seen by electron microscopy. Wild-type phenotypes were regained when the mutations were transcomplemented with the *flaA* gene. Protein analysis indicated that the *flaA* gene corresponds to a 40-kDa protein and that the flagellum consists of three additional flagellin proteins with molecular masses of 41, 42, and 45 kDa. N-terminal sequence analysis confirmed that the additional proteins were flagellins with N termini that are 82 to 88% identical to the N terminus of FlaA. Virulence studies showed that the N terminal deletion, the double deletion, and the 942-bp deletion increased the 50% lethal dose between 70- and 700-fold via immersion infection, whereas infection via intraperitoneal injection showed no loss in virulence. In contrast, the polar mutant and the carboxy-terminal deletion mutant showed approximately a 10^4-fold increase in the 50% lethal dose by both immersion and intraperitoneal infection. In summary, FlaA is needed for crossing the fish integument and may play a role in virulence after invasion of the host.

Vibrio anguillarum, which causes vibriosis in marine fish, is a highly pathogenic bacterium and has become a severe problem for the fish farming industry. An earlier study (3) has correlated the virulence of *V. anguillarum* with the possession of more than one flagellum. The importance of the flagellum as a potential virulence factor has been demonstrated for other bacteria. In *Pseudomonas aeruginosa* (5), three wild-type strains and their respective isogenic motility mutants were tested in the burned mouse model. The nonmotile mutants proliferated in the wound but did not cause the characteristic systemic infection, indicating that motility contributes to the invasive capabilities of this organism. For *Campylobacter jejuni*, the flagellum is the best-characterized virulence factor. In vitro studies using epithelial cell lines have shown that the flagellum aids the bacterium either in adherence to (30) or in internalization within (11) the cultured cells. A more recent study (47) shows that motility and not flagellin A is required for the invasion of intestinal cells by *C. jejuni*. However, flagellin A can serve as a secondary adhesin for the adherence to intestinal cells, whereas other adhesins are present to aid the motility-dependent internalization in an intestinal cell line. Similarly, for *Vibrio cholerae*, studies using spontaneous mutants or chemically derived motility mutants indicate that either motility (12) or an adhesin, proposed to be associated with the flagellum (2, 17), is needed for colonization of intestinal tissues. Another *V. cholerae* study, which utilized transposon-derived and spontaneous flagellar mutants that were analyzed for their flagellar structures, suggested that motility but not the flagellar structure is essential in the colonization of rabbits (41). However, that report also noted a possible minor role in virulence for the sheath of the flagella of classical vibrios.

The flagellum has been implicated in pathogenicity as either a motility organelle or an organelle that carries an adhesive component. Although it is still unclear which characteristic of the flagellum is important for virulence, both lend an advantage to the bacterium for its invasive capabilities. The animal model for the fish pathogen *V. anguillarum* is ideal for studying the invasive mechanism of a bacterial pathogen, since the assay for host invasion uses a natural route of infection. In other words, the fish are allowed to swim in seawater containing bacteria. The virulence of a bacterial strain via this route of infection can be compared with the virulence via intraperitoneal infection to determine if there is a defect in the invasive capabilities of the bacterium. In this study, we have begun to identify the structural components of the flagellum and to determine if these components play any role in the virulence of *V. anguillarum* by using the fish animal model. The flagellin gene, *flaA*, was cloned, sequenced, and mutated either by allelic exchange or by insertional mutagenesis. All mutants were partially motile, and a shortened flagellum was visible by electron microscopy. Virulence analysis of the *flaA* mutants indicates that flagellin A, either as a part of the motility organelle or as some other component of the virulence mechanism, aids the invasion of the host by *V. anguillarum* but also plays a role in virulence after the fish integument is crossed.

MATERIALS AND METHODS

Strains, phages, plasmids, and media. *V. anguillarum* NB10 (serotype O1) is an isolate of our laboratory from the Gulf of Bothnia outside the Norrby Laboratory, Umeå, Sweden (34). The more competent strain *Escherichia coli* SY327 [Δ(*lac pro*) *argE*(Am) *rif malA recA56*] (31) was used for transformation after subcloning of fragments into either the pNQ705, pDM1, or pDM4 vector. All plasmids to be conjugated into *V. anguillarum* were transformed into either *E. coli* S17-1 (*thi pro hsdR hsdM*$^+$ *recA* RP4-2-Tc::Mu-Km::Tn7) (43) or *E. coli* SM10 (*thi thr leu tonA lacY supE recA* RP4-2-Tc::Mu::Km) (43), which were used as the donor strains. SY327, SM10, and S17-1 are bacteriophage lambda lysogens carrying the *pir* gene, which is needed for replication of the suicide vectors pNQ705, pDM1, and pDM4. *E. coli* XL1-Blue (42) (*recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1* [F′ *proAB lacI*^{q} *lacZ*ΔM15 Tn*10*]) was used for bacteriophage lambda infections and for most transformations.

Construction of the *V. anguillarum* genomic library by using the Lambda Zap

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II system from Stratagene and handling of this bacteriophage have been previously described (32).

pBluescript (Stratagene) was used for the cloning and analysis of PCR fragments. The pBluescript plasmid derivative pBS6-1, which contained an approximately 7.7-kb chromosomal insert, was excised from the isolated Lambda Zap II recombinant bacteriophage 6-1 as previously described (32). To make handling of pBS6-1 simpler, approximately 3.7 kb upstream of the flagellin gene was removed by digestion with *Stu*I and *Xho*I. The *Xho*I sticky ends were then made blunt by using the Klenow fragment of *E. coli* DNA polymerase I (42). The *flaA*-containing fragment was purified from an agarose gel by using a DEAE-cellulose membrane (42) and then ligated to itself, resulting in pBS6-1A. The suicide vector pNQ705, which requires the *pir* gene for replication, is a chloramphenicol-resistant derivative of the pGP704 vector. Construction of pNQ705 has been described earlier (32). pDM1 and pDM4 are derivatives of pNQ705 that contain the *sacBR* genes from *Bacillus subtilis*; construction of these vectors was as follows. The *sacBR* genes were removed from the vector pKNG101 (19) (a gift from G. R. Cornelis) by digestion with *Pst*I. The 2.6-kb *sacBR*-containing fragment was purified from an agarose gel by using a DEAE-cellulose membrane (42) and then ligated into the unique *Pst*I site just upstream of the chloramphenicol resistance gene of pNQ705, resulting in the pDM1 vector. pDM4 was constructed from pDM1 by inserting a polylinker that contained restriction endonuclease sites absent in pDM1. A synthetic DNA fragment that contained the restriction sites *Xho*I, *Mlu*I, *Spe*I, *Apa*I, *Xba*I, *Bgl*II, *Sph*I, and *Bst*EII and that had *Sal*I and *Sac*I sticky ends was made. This DNA fragment was ligated to a *Sal*I-*Sac*I-digested pDM1 fragment, resulting in pDM4. pFlaA-2 is a pSup202P (32) derivative which contains the *flaA* gene. The *flaA* gene and its promoter region were removed from pBS6-1A by digestion with *Bgl*II and *Bam*HI. The 2.1-kb *flaA* gene-containing fragment was purified from an agarose gel by using a DEAE-cellulose membrane (42) and then ligated to the pSup202P vector, which had been digested with *Bgl*II. The resulting plasmid was pFlaA-2.

The medium routinely used for *E. coli* was TYS broth (10 g of Bacto Tryptone, 5 g of Bacto yeast extract, and 10 g of sodium chloride [all per liter]). For *V. anguillarum*, Trypticase soy medium (BBL) was used for routine growth. The vibrio selective medium TCBS (Difco) containing 5 μg of chloramphenicol per ml was used for the selection of *V. anguillarum* transconjugants when crosses were made with *E. coli*. Motility agar consisted of Trypticase soy broth plus 0.25% agar.

Antibiotics and enzymes. Antibiotic concentrations for all *E. coli* strains were as follows: ampicillin, 100 μg/ml; tetracycline, 10 μg/ml; and chloramphenicol, 25 μg/ml. Antibiotic concentrations for *V. anguillarum* were as follows: tetracycline, 5 μg/ml, and chloramphenicol, 5 μg/ml. Restriction enzymes were purchased from a variety of sources, and KGB buffer (42) was used for all digests. Reaction conditions for the DNA-modifying enzymes were as suggested by the manufacturers.

Bacterial matings. Plasmid transfers from *E. coli* to *V. anguillarum* were done by bacterial matings. Either *E. coli* S17-1 or SM10 was used as the donor strain for the transfer of all plasmid derivatives into *V. anguillarum*. These strains contain the *pir* gene, which is required for derivatives of the plasmids pNQ705, pDM1, and pDM4 to replicate. All strains were grown to the mid-logarithmic growth phase. At a ratio of approximately 10:1, the recipient and donor strains were mixed, pelleted together, and then spotted onto a Trypticase soy agar plate. The matings were allowed to progress at 26°C for 4 to 18 h. The selection for chloramphenicol-resistant transconjugants was done on the vibrio selective medium, TCBS (Difco), containing 5 μg chloramphenicol per ml.

DNA techniques and sequencing. Oligonucleotides for primers and for the pDM4 linker were synthesized with an Applied Biosystems DNA/RNA synthesizer model 392. Unless otherwise stated, all conditions for the various DNA techniques were as described by Sambrook et al. (42). Double-stranded DNA sequencing was performed by the dideoxy-chain termination method with T7 DNA polymerase (Pharmacia). The T3 and T7 primers were used for sequencing small DNA inserts within the pBluescript linker region. With the pBS6-1A plasmid, both strands of the *flaA* gene were sequenced by primer walking in two directions from the previously sequenced 119-bp PCR fragment from the 5′ end of the flagellin gene.

Screening of the genomic library. Bacteriophage plaques were plated with approximately 500 plaques per plate and screened by doing duplicate plaque lifts onto nitrocellulose filters (Schleicher & Schuell BA 85). The DNA was denatured as described by Sambrook et al. (42) and cross-linked to the nitrocellulose by UV irradiation. The prehybridization and hybridization solutions were the same (6× SSC [1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate], 5× Denhardt's solution [0.5 g of Ficoll, 0.5 g of polyvinylpyrrolidone, 0.5 g of bovine serum albumin, and water to 500 ml], 0.5% sodium dodecyl sulfate (SDS), and 100 μg of denatured, fragmented salmon sperm DNA per ml). Prehybridization was done for 1 to 2 h at 65°C, and hybridization was done for 16 to 20 h at the same temperature. After hybridization, the filters were washed at 65°C for 15 min with 0.1× SSC–0.5% SDS. The filters were then autoradiographed, and plaques that gave strong signals from both filters were picked and reprobed in the same manner for single-plaque isolation. The probe, a 119-bp fragment from the conserved 5′ end of the *V. anguillarum flaA* gene, was generated by PCR with primers complementary to a similar region of the *P. aeruginosa flaA* gene. The sequences of the primers are 5′-CTCATCGATCGCATCAACAGCGC-3′ and 5′-ATTGAGCTCGAGATACCGTCGTTGGCGTT-3′. The underlined sequences are complementary to the *P. aeruginosa* sequence (45), and restriction sites were included on the 5′ end for use in cloning of the product. For detection, the probe was labelled with [α-^{32}P]dCTP by random oligonucleotide priming with the Klenow fragment of *E. coli* DNA polymerase I (42).

Southern hybridization of chromosomal DNA. For the determination of the gene copy number, chromosomal DNA (2 μg) of *V. anguillarum* was digested with *Bgl*II, *Eco*RV, *Cla*I, *Eco*RI, and *Bam*HI and that of *E. coli* was digested with *Bgl*II. Enzymatic digests were incubated overnight at 37°C for complete digestion of the DNA. The DNA digests were electrophoresed through a 1% agarose gel in Tris-borate buffer (42). The DNA was denatured in the gel and transferred to a nitrocellulose filter as described by Sambrook et al. (42). The DNA was cross-linked to the filter by UV irradiation. A 2.1-kb *Bgl*II-*Bam*HI fragment from pBS6-1A, which contained the *flaA* gene and its promoter region, was labelled by random oligonucleotide priming with the Klenow fragment of *E. coli* DNA polymerase I (42) and hybridized to the filter. Prehybridization and hybridization conditions were the same as described above for the genomic library screening except that the temperature used throughout was 60°C.

PCR conditions. PCR cycle times for all primers were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s (on the last cycle, 72°C for 5 min). Before *Taq* DNA polymerase (Promega) was added, each reaction was preceded by a 5-min denaturation step at 95°C. Template DNA was obtained from either a chromosomal DNA preparation (100 ng was used [42]), bacterial cells, or PCR. Chromosomal DNA was obtained from a single bacterial colony by touching a sterile pipette tip to a colony and then mixing the cells into the PCR mixture. When PCR-generated fragments were used, the fragments were purified from a 12% nondenaturing polyacrylamide gel (acrylamide-bisacrylamide [29:1], Tris-borate buffer; 200 V) by using a DEAE cellulose membrane (42), and 10 to 100 ng was added to the PCR mixture. PCR-generated fragments were difficult to obtain from the terminator region of the *flaA* gene. To optimize these PCRs, 2 to 5 μg of a single-strand-binding protein, T4 gene 32 protein (Boehringer Mannheim), was added to the reaction mixture. For analysis, one-fifth of the PCR-generated fragments were electrophoresed through a 12% nondenaturing polyacrylamide gel.

Construction of *flaA* mutations. Two types of *flaA* mutations were made, i.e., chromosomal insertions and in-frame deletions. The chromosomal insertions were made by integrating a plasmid into the *flaA* gene, and the in-frame deletions were made by allelic exchange. For all constructions, suicide vectors (pNQ705 for the insertional mutations and pDM1 or pDM4 for the in-frame deletions) that contain fragments with or without in-frame deletions complementary to the *flaA* gene or regions flanking the *flaA* gene were used. To introduce these plasmids into *V. anguillarum*, conjugal matings were done. For the suicide plasmids to be maintained in *V. anguillarum* after conjugal transfer from *E. coli*, they must have integrated into the chromosome, most likely within the complementary *flaA* gene. This integration was required for generating the insertional mutations and for completing the first step of constructing the in-frame deletions. Transconjugants were selected by utilizing the chloramphenicol resistance gene located on the suicide plasmid. All insertional mutations were confirmed either by Southern analysis (described above) or by PCR analysis. PCR analysis utilized a primer complementary to the plasmid just outside the linker region of pNQ705 and another primer complementary to the chromosome just outside the *flaA* gene fragment. Restriction endonuclease analysis confirmed that the fragments derived this way were from the *flaA* gene or its flanking regions. Wild-type chromosomal DNA, used as a control, gave no PCR product when the same primers were used.

To complete the allelic exchange for the in-frame deletions, the integrated suicide plasmids were coerced to recombine out the chromosome by utilizing the *sacB* gene located on pDM1 and pDM4, and chloramphenicol-sensitive transconjugants were selected. The *sacB* gene product has been shown to be lethal for gram-negative bacteria (8). Strains carrying this gene can be induced to express the *sacB* gene product by the addition of 5% sucrose to the growth medium. Thus, only those strains lacking the *sacB* gene should grow on this medium. However, for *V. anguillarum*, the response to the induced *sacB* gene was not as simple as growth or no growth. The induction of the *sacB* gene product had two noticeable effects. First, the *sacB*-containing strains had a colony morphology on sucrose-containing plates that was different from that of strains without *sacB*, and second, the lethal effect was delayed by 36 to 48 h. After that time, the bacterial colonies containing the *sacB* gene began to lyse on sucrose-containing medium. Although the effect was delayed, the colony morphology difference made the selection for the loss of the plasmid simpler. Overnight cultures of the *sacB*-containing strains grown in the presence of 5% sucrose were streaked onto Trypticase soy agar containing 5% sucrose. Colonies that were wild type in appearance were patched onto Trypticase soy agar with and without chloramphenicol. The colonies that were sensitive to chloramphenicol were analyzed for the deletion. Fragments spanning the deleted regions were generated by PCR, and the sizes of the fragments were compared on a 12% nondenaturing polyacrylamide gel with that of a similar fragment derived from the wild-type chromosomal DNA. Fragments that migrated faster than the wild-type fragment were considered to have the deletion. Strains carrying the desired deletions were selected, and the same PCR fragments were cloned from these strains and sequenced to confirm that the correct deletions were made.

Chromosomal insertion mutant RO5 (*flaA4*) was constructed by using pNQ-FlaA4, a derivative of pNQ705 that contained a PCR fragment from the 5′

FIG. 1. Nucleotide sequence of the *flaA* gene. The deduced amino acid sequence of the open reading frame is indicated and corresponds to a protein with a molecular weight of 40,111. A putative ribosome-binding site (RBS) and the possible σ^{28} promoter consensus sequence are underlined. The deleted amino acids for each in-frame mutation are indicated by two labelled arrows for each deletion. The downward vertical arrow indicates the position of the plasmid insertion in strain RO5.

terminus of the *flaA* gene (residues 297 to 581) (Fig. 1). Chromosomal insertion mutant DM3 was constructed by using pNQFlaAend, a derivative of pNQ705 that contained a PCR fragment from the 3′-flanking region of the *flaA* gene (residues 1547 to 1754) (Fig. 1).

PCR fragments carried in either pDM1 or pDM4 that were utilized for the in-frame deletion mutations were generated by overlap PCR (16). This requires that two PCR fragments, which flank the region to be deleted, have complementary 3′ ends. The ends were made complementary through the construction of the PCR primers. These two PCR fragments were then used as templates in a second PCR with the two outermost primers from the first two PCRs to generate a recombinant fragment that contains the desired deletion. The in-frame deletion mutant RO2 (*flaA1*) was constructed by using pDMFlaA1, a derivative of pDM1 that contains a recombinant PCR fragment (residues 12 to 296 joined to residues 477 to 731) (Fig. 1) with a 180-bp deletion. Mutant DM16 (*flaA2*) was constructed by using pDMFlaA2, a derivative of pDM1 that contains a recombinant PCR fragment (residues 921 to 1226 joined to residues 1379 to 1681) (Fig. 1) with a 153-bp deletion. The in-frame deletion mutant RO8 (*flaA6*) was constructed by using pDMFlaA6, a derivative of pDM4 that contains a recombinant PCR fragment (residues 69 to 359 joined to residues 1302 to 1592) (Fig. 1) with a 942-bp deletion. For the double mutant DM17 (*flaA1 flaA2*), pDMFlaA2 was utilized to construct a second in-frame mutation in strain RO2.

SDS-PAGE. For separation of the flagellar proteins, SDS–11% polyacrylamide gel electrophoresis (PAGE), as described by Lugtenberg et al. (28) was used, except that 30 mM sodium chloride was added to the separating gel to enhance band separation. Protein concentrations were measured with a Micro BCA Protein Assay kit from Pierce. Twenty micrograms of crude flagellar protein was applied to a gel slot from each preparation. Gels were fixed and stained with 0.1% Coomassie brilliant blue in 40% methanol–10% acetic acid and then destained in 40% methanol–10% acetic acid.

Isolation of flagella. For the isolation of crude flagella, *V. anguillarum* was grown overnight at room temperature in Trypticase soy broth with gentle shaking. For isolation of flagella from the *flaA* mutants, 1,000-ml cultures were used, and for all other strains, 500-ml cultures were used. The following protocol is a modification of that described by Geis et al. (9). The bacterial cells were pelleted (12,400 × *g*, 15 min, 4°C), washed in 0.9% sodium chloride, and resuspended in 40 ml of Tris hydrochloride buffer (pH 7.2). Flagella were sheared from the bacteria at 4°C with a Sorvall Omnimixer (setting 4 for 3 min). The bacterial cells were pelleted (5,500 × *g*, 15 min, 4°C) twice to minimize protein contamination from the bacterial cell. The supernatant was collected, and the flagella were pelleted by ultracentrifugation (270,000 × *g*, 1 h, 10°C). The flagella were resuspended overnight in 500 μl of water. To prevent distortion and smearing of the proteins during SDS-PAGE, lipid extraction with chloroform and methanol was done as described by Thomashow and Rittenberg (44). After lipid extraction, the pellet was resuspended in 200 μl of Tris hydrochloride (pH 7.2) containing 1% SDS to solubilize all proteins.

Motility agar assay. Motility was measured by the movement of bacterial cells through Trypticase soy broth containing 0.25% agar. The optical density at 600 nm (OD_{600}) was determined for overnight cultures of all of the strains used. Equal amounts of cells in 10 μl were spotted in the centers of the plates, and movement away from the center was measured after 24 h of growth at room temperature.

Growth rate determination. For all strains, an overnight culture was diluted

100-fold into prewarmed (30°C) Trypticase soy broth and growth with shaking was begun. The OD_{600} was measured every hour. When the culture reached an OD_{600} of 0.8 to 1.0, it was diluted 10-fold into prewarmed Trypticase soy broth, and OD_{600} readings were continued every 30 min. The doubling time was determined from readings of the final dilution.

Electron microscopy. Immunogold electron microscopy was performed via a modification of the method of Fuerst and Perry (7). Bacteria were grown overnight at room temperature in Trypticase soy broth. Cells were pelleted and resuspended in the same volume of 0.01% gluteraldehyde in 0.2 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], pH 7. The remainder of the protocol was the same as described previously (7) except that PIPES buffer instead of phosphate-buffered saline was used throughout. The primary antiserum was raised against formalin-killed whole cells of *V. anguillarum* 775.17B and was previously shown to be highly antigenic for the lipopolysaccharide (LPS) in the sheath of the flagellum (35). To increase the specificity for the LPS, the antiserum was immunoadsorbed to a 775.17B LPS mutant, VAN70 (35). VAN70 was washed in phosphate-buffered saline, and 100 μl of the cells was mixed with 1 ml of antiserum and allowed to absorb for 1 h. The cells were removed by centrifugation, and the immunoadsorption was repeated one more time. The goat anti-rabbit immunoglobulin G–gold particles (10-nm diameter) were purchased from British BioCell International. Specimens were examined with a Zeiss EM 109 transmission electron microscope operated at an accelerating voltage of 50 kV.

N-terminal amino acid sequencing. The FlaA mutant strain RO2 was used for the isolation of four of the five major protein bands found in a crude wild-type flagellum preparation. Flagellar proteins from RO2 were separated by SDS–11% PAGE and transferred to an Immobilon-P membrane filter (Millipore). The membrane was stained with Coomassie brilliant blue and then destained as described for SDS-PAGE. The four protein bands were cut out of the filter and washed first in methanol and then in water. The proteins were then sent to Uppsala, Sweden, for protein sequence analysis.

Computer analysis. Database searches were done by using the Genetics Computer Group Sequence Analysis software (4) of the Genetics Computer Group, Inc. (University of Wisconsin).

Fish infections. Rainbow trout (*Oncorhynchus mykiss*) with an approximate weight of 10 to 15 g were infected with *V. anguillarum* either by intraperitoneal injections or by immersion of the fish in seawater containing *V. anguillarum* as previously described (34). The immersion and intraperitoneal infections were done at least two times. Five fish were infected for each bacterial dilution used. The 50% lethal doses were calculated as described by Reed and Muench (40). The 50% lethal doses recorded are averages of results for all infections for each strain.

Nucleotide sequence accession number. The DNA sequence reported here has been submitted to GenBank, and its accession number is L47122.

RESULTS

Cloning and sequencing of the *flaA* gene. A comparison of flagellin proteins from a variety of bacterial species showed that the N and C termini were highly conserved. Since there was such a high conservation of the amino acid sequence between species, PCR primers were constructed such that the 3′ end of each primer was complementary to a conserved region of the 3′ end of the *P. aeruginosa flaA* gene (45) (see Materials and Methods). Chromosomal DNA from *V. anguillarum* NB10 was used, and a PCR product approximately the same size as that expected for *P. aeruginosa* (128 bp) was obtained (data not shown). The PCR product was then cloned and sequenced. The deduced amino acid sequence was 72% identical to the deduced *P. aeruginosa* amino acid sequence. Thus, the PCR product was assumed to be from the *V. anguillarum* flagellin gene, now designated *flaA*.

Previously, a *V. anguillarum* genomic library was constructed by using the Lambda Zap II bacteriophage (32). By plaque hybridization, this library was screened for the flagellin gene with the PCR product described above as the probe. Approximately 2,000 bacteriophage plaques were screened, and of these, 5 hybridized to the probe. These five positive plaques were picked and reprobed for single-plaque isolation. Each bacteriophage was considered to contain all or a portion of the flagellin gene, and one, 6-1, was chosen for further analyses.

The pBluescript plasmid, pBS6-1, containing the *V. anguillarum* chromosomal DNA was excised from bacteriophage 6-1. Restriction endonuclease analysis revealed that pBS6-1 contained an approximately 7.7-kb fragment. To enhance the quality of the sequencing data, approximately 3.7 kb of DNA upstream of the *flaA* gene was deleted from the insert, resulting in plasmid pBS6-1A. Since a part of the DNA sequence was known from the cloned PCR product, sequencing was begun by walking along the DNA in two directions with oligonucleotide primers. The complete nucleotide sequence is shown in Fig. 1.

The open reading frame begins at nucleotide 261, just downstream of a putative ribosome-binding site, and ends at nucleotide 1400, thus potentially encoding a polypeptide of 379 amino acids with a calculated molecular weight of approximately 40,111. A possible σ^{28} promoter sequence (the *Bacillus subtilis* consensus sequence $TAAAN_{15}GCCGATAT$) (14) is located 35 bp upstream of the start of the open reading frame (Fig. 1). No strong termination site was obvious from the sequence, and no other putative open reading frame was observed at either the 3′ or 5′ end of the gene. The deduced amino acid sequence was compared with those for other bacterial flagellin genes and showed strong similarities to those for many of these genes, especially at the highly conserved amino and carboxy termini. The highest degree of similarity was to the *flaC* gene product of *V. parahaemolyticus* (29), which gave 80% identity throughout the entire protein.

Construction of chromosomal flagellin A mutants. A flagellin A-deficient mutant, RO5 (*flaA4*), was generated by integrating into the *V. anguillarum* NB10 chromosome a mobilizable suicide plasmid, pNQFlaA4, which contained a 292-bp PCR fragment from the N terminus-coding region of the *flaA* gene (for details, see Materials and Methods) (Fig. 1). The plasmid was conjugated into *V. anguillarum* NB10, and one chloramphenicol-resistant transconjugant was chosen. Chromosomal integration was confirmed by PCR and Southern analysis (data not shown). To determine the stability of the *flaA4* mutation, RO5 was grown for 30 generations in the absence of antibiotic selection and 100 colonies were tested for resistance to chloramphenicol. All colonies tested showed resistance to the antibiotic, indicating that the plasmid was stably maintained (data not shown).

The *flaA4* mutation is a polar mutation which is useful for creating a flagellin A-deficient mutant (RO5). However, many bacterial species contain more than one flagellin gene that are often tandemly oriented and may be affected by the polar mutation. If this is also true for *V. anguillarum*, then the use of a polar mutation may not adequately assess the importance of this gene for virulence. Thus, in-frame mutations were made in the highly conserved amino and carboxy termini, which are believed to be important in the transport and polymerization of the flagellin into the filament (15, 25). The *flaA1* mutation (mutant RO2) deleted 60 amino acids from the amino terminus of the flagellin protein (Fig. 1). The *flaA2* mutation (mutant DM16) deleted 51 amino acids from the carboxy terminus of the flagellin protein (Fig. 1). In addition, a double mutation, *flaA1 flaA2* (mutant DM17), that deleted both of the above-described regions was made. A final mutation, *flaA6* (mutant RO8), deleted 314 of 379 amino acids from the flagellin protein (Fig. 1). All in-frame mutations were generated by allelic exchange (see Materials and Methods for specific details). Each deletion was confirmed by sequencing cloned PCR products derived from the deleted regions of the *flaA* gene. All deletion mutations were free from PCR-generated base substitutions except that in mutant DM16, which had a substitution downstream of the *flaA* gene at position 1572 (Fig. 1). In addition, the growth rate, which influences both virulence and motility analyses, was tested for all mutant strains. A growth rate comparable to that of the wild type was seen.

Functional analysis of the mutant FlaA proteins. To determine loss of flagellar function, each of the mutants was ana-

TABLE 1. Results of motility and virulence assays

Strain	Mutation(s)	Motility (Diam [cm] of growth)[a]	Virulence determined by:	
			Intraperitoneal injection (LD_{50})[b]	Immersion (cells/ml)
NB10	None (wild type)	5.5	22	9×10^2
NB10(pFlaA-2)		5.2		
RO2	60-aa[c] N-terminal deletion	3.1	6	1×10^5
RO2(pFlaA-2)		4.8		
RO5	Plasmid insertion	3.2	2×10^5	$>1 \times 10^5$[d]
RO5(pFlaA-2)		4.6		
RO8	314-aa central deletion	2.9	41	6×10^5
RO8(pFlaA-2)		4.8		
DM16	51-aa C-terminal deletion	3.7	8×10^5	$>1 \times 10^5$[d]
DM16(pFlaA-2)		4.9		
DM17	N-terminal and C-terminal deletions	3.5	7	6×10^4
DM17(pFlaA-2)		4.8		
DM3	Plasmid insertion downstream	6.0	44	1×10^4

[a] All cultures were grown overnight with antibiotic when appropriate. The OD_{600} was determined, and equal numbers of cells were spotted in 10 μl in the center of each plate of motility agar. Diameters of growth were measured after 24 h of growth at room temperature. The numbers presented represent averages from three experiments.
[b] LD_{50}, 50% lethal dose.
[c] aa, amino acid.
[d] The infection was done only once.

lyzed for motility by light microscopy and by swimming through soft agar. For all mutants light microscopy revealed partial motility compared with the wild type. Approximately 50% of the cells appeared to have the wild-type motility, while the remaining 50% appeared to be nonmotile. No observable differences between the mutants could be seen. Furthermore, Table 1 shows that after 24 h of growth, all mutants displayed a decrease in motility within soft agar compared with the wild type. Thus, the loss of the FlaA protein reduced the motility but did not eliminate it, suggesting the presence of more than one flagellin gene.

Structural analysis of the flagella of the *flaA* mutants. A previous study (35) has shown that *V. anguillarum* NB10 has a single polar flagellum. To determine if the mutants still possessed this flagellum, which could explain the partial motility, structural analysis of the mutant flagella was done by electron microscopy. All mutants had a truncated flagellum, which would account for the partial motility seen with the light microscope and with the motility agar. The wild-type flagellum and a typical example representing all of the mutants are shown in Fig. 2. In addition, for all mutants, a flagellum, with approximately the same length as wild type, could occasionally be seen.

Identification of more than one flagellin protein. Multiple flagellin genes have been found in other bacteria (10, 13, 24, 27, 29, 33, 36, 39). To determine the chromosomal copy number of the *flaA* gene and to identify a possible second DNA locus containing other flagellin genes, Southern blot analysis was done with the *flaA* gene and its promoter region as the probe (Fig. 3). Stringent hybridization and wash conditions were used, and the filter was exposed to film for a week to visualize weakly hybridizing bands. Restriction enzymes that have one site within *flaA* (*Eco*RV and *Eco*RI) gave two bands that hybridized to the probe, and enzymes that do not cut within *flaA* (*Bgl*II, *Cla*I, and *Bam*HI) gave only one band. In addition, all digests except the *Bam*HI digest contained a second, weakly hybridizing band (Fig. 3). These results indicate that there is only one copy of the *flaA* gene and that another flagellin gene(s) located at another DNA locus with some homology to the *flaA* gene may also be present in *V. anguillarum*. In an attempt to identify a second flagellin gene, additional PCR clones obtained by using the original primers complementary to the *P. aeruginosa* flagellin gene were sequenced. However, all clones gave a sequence identical to that of *flaA*.

To identify the FlaA protein and other potential flagellin proteins, protein analysis was done. The flagellum and its sheath were isolated from the wild-type and mutant strains, the lipids were extracted, and the crude proteins were electrophoresed through an SDS-polyacrylamide gel. As shown for the wild type in Fig. 4, five major protein bands with approximate molecular masses of 38, 40, 41, 42, and 45 kDa were present. The *flaA* mutant strains are missing the 40-kDa protein, suggesting that this protein is flagellin A. In addition, the size of this protein is consistent with the DNA sequencing data. To determine if any of the remaining four proteins are flagellin proteins, N-terminal protein sequencing was done. Flagellar proteins from one of the mutants, RO2, were chosen for the isolation of the four remaining proteins, since the FlaA protein migrates close to the 41-kDa protein. Of the four proteins sequenced, three proteins had an amino acid sequence 82% identical to that of FlaA (Fig. 5). Of these three proteins, two had identical amino acid sequences. Thus, the flagellum appears to consist of three (possibly four) flagellin proteins. Until further conformational studies are done, these proteins will be referred to as FlaA (40 kDa), FlaB (41 kDa), FlaC (42 kDa), and FlaD (45 kDa). The presence of four flagellin proteins explains the partial motility and the shortened flagellum observed for the FlaA mutants, as well as the weakly hybridizing band in the Southern data. The sequence of the 38-kDa protein was not good because of the presence of contaminating proteins. The amino acid sequence that was obtained showed no homology to that of the FlaA protein but did show partial homology to those of numerous outer membrane proteins from a variety of genera.

Transcomplementation of the *flaA* mutations. Transcomplementation studies were done to determine if the wild-type motility and flagellum length could be restored by providing the *flaA* gene in *trans*. A mobilizable vector, pFlaA-2, carrying the *flaA* gene and its promoter was conjugated into the mutant strains. The transconjugants were analyzed in a fashion similar to that used for the mutant strains. Electron microscopy showed that the length of the flagellum returned to wild-type

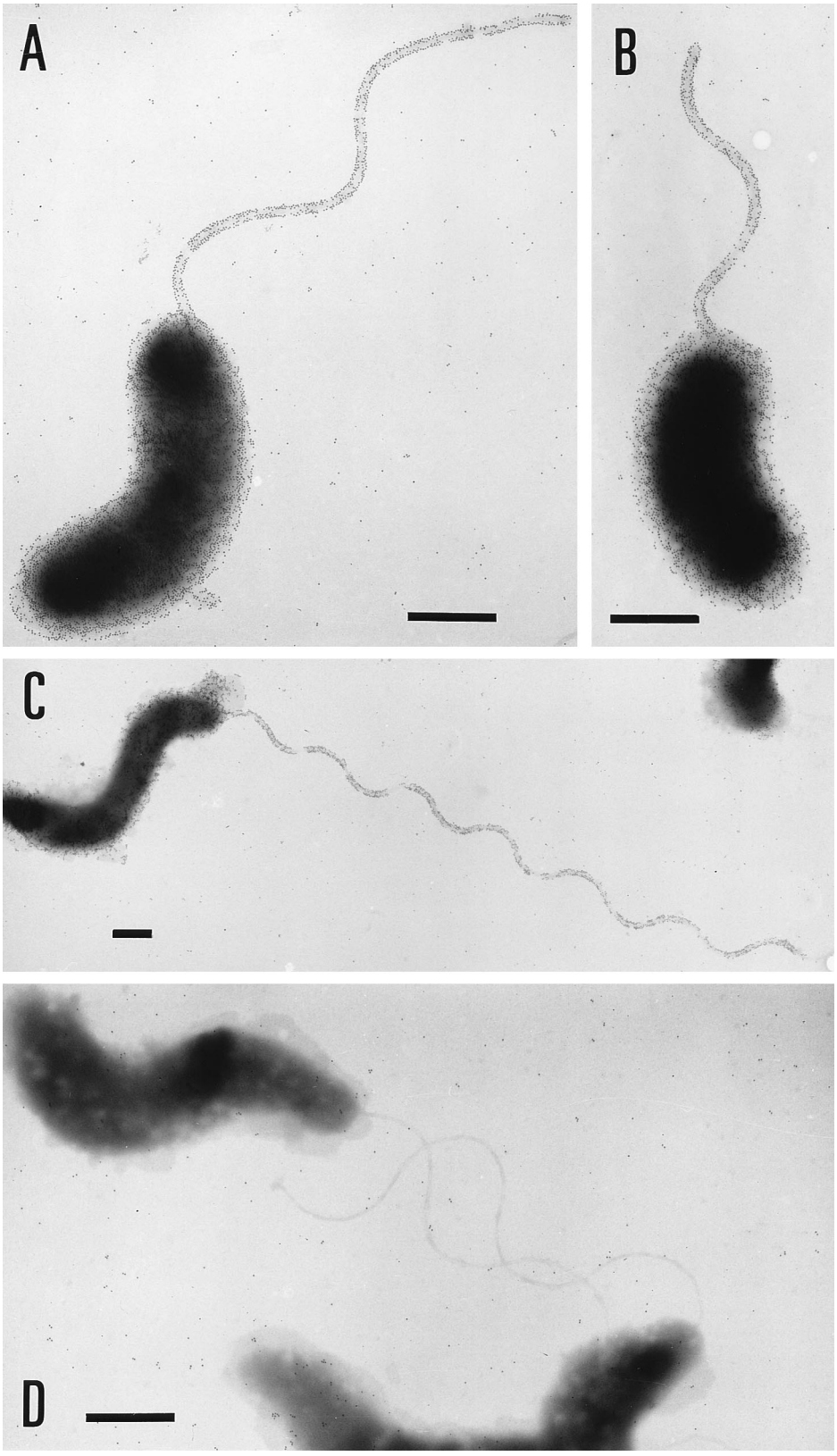

FIG. 2. Immunogold electron microscopy. Primary antiserum was raised against formalin-killed whole cells of *V. anguillarum* 775.17B and has been shown to be highly antigenic for the LPS in the sheath. (A) Wild-type strain NB10. (B) *flaA* mutant strain. (C) *flaA* mutant strain which carries the pFlaA-2 plasmid. (D) Transposon mutant VAN70 (negative control), which was previously characterized as lacking the LPS. Bars, 0.5 μm.

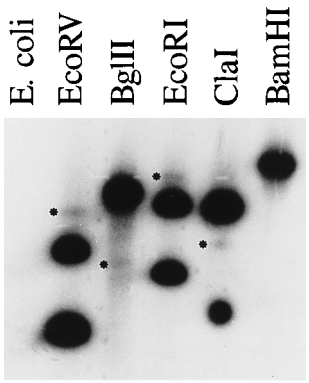

FIG. 3. Southern analysis of chromosomal DNA of *V. anguillarum* NB10. Approximate fragment sizes were calculated from the agarose gel. Lanes: 1, *Bgl*II digest of *E. coli* XL1-Blue (negative control); 2, *Eco*RV digest (3.1- and 1.7-kb fragments); 3, *Bgl*II digest (6.5-kb fragment); 4, *Eco*RV digest (5.5- and 2.7-kb fragments); 5, *Cla*I digest (5-kb fragment); and 6, *Bam*HI digest (13.5-kb fragment). The complete *flaA* gene was used as the probe, and the filter was exposed to film for 1 week. Weakly hybridizing bands are marked with an asterisk.

length or longer (Fig. 2). Motility agar analysis (Table 1) and wet mount observation with the light microscope indicated that the motility was approximately that of the wild type, and protein analysis showed the return of the 40-kDa protein band (Fig. 4). However, the amount of FlaA protein found in the transcomplemented strains was larger than that in the wild type. This probably reflects the increase in copy number, since the same increase was seen if the *flaA* gene was provided in *trans* for the wild-type strain (Fig. 4). This may suggest that the amount of FlaA polymerized into flagellar filaments is dependent on the amount expressed within the cell, since the amount of FlaA seen in the flagellum increases when the copy number of the gene increases.

Fish infection studies. To determine if loss of the FlaA protein caused a decrease or loss in virulence, fish were infected with the mutant strains via either intraperitoneal injection or bathing of the fish in infected seawater (Table 1). Interestingly, two phenotypes were seen. RO2, DM17, and RO8 showed a loss in virulence only with infection via immersion, whereas RO5 and DM16 showed a loss in virulence via both routes of infection. Since several flagellin proteins have been identified and recombination may occur to obtain a functional *flaA* gene, the presence of each *flaA* mutation was confirmed by PCR analysis of these strains isolated from infected fish. When a fish died from vibriosis (usually 4 to 7 days after infection), each strain was isolated from the kidney of the fish, and a mixture of *V. anguillarum* colonies from the kidney was utilized in PCR analysis and in soft agar motility tests. PCR confirmed that all mutations were still present and that motility, as measured in soft agar, was comparable to the results shown in Table 1. Thus, these results indicate that FlaA is important for virulence at two different levels. The protein is needed for crossing the initial fish barriers, and it plays a role once the bacterium has entered the host.

Although transcomplementation of the *flaA* gene was accomplished when the strains were analyzed in vitro, transcomplementation of the *flaA* gene did not work for the fish infections (data not shown). This could be due to instability of the plasmid that contains the wild-type copy of the *flaA* gene, since proliferation of the bacterium during infection of the fish was in the absence of antibiotic. When plasmid stability was tested for these strains, 75% of the cells lost the plasmid after 30 generations of growth in the absence of antibiotic (data not shown). Thus, to circumvent the transcomplementation problem and to confirm that the loss of virulence for the polar mutant RO5 was due to the loss of the FlaA protein and not due to a loss of some downstream product, the suicide plasmid was inserted into the chromosome just downstream of the *flaA* gene, creating strain DM3. This chromosomal insertion should mimic the polar effect of the RO5 mutant while maintaining the wild-type *flaA* gene. If there is a polar effect on downstream virulence determinants, then this strain should also be avirulent. Instead, strain DM3 showed no loss in virulence. Structural, functional, and protein analyses of DM3 (Fig. 4 and Table 1) indicated a wild-type flagellum, suggesting no polar effects on potential downstream flagellar genes. As for the *flaA* mutant strains described above, strain DM3 was isolated after a challenge in the fish model, and the insertion mutation and the motility were shown to be unchanged after the challenge. Thus, some property of the FlaA protein or motility plays a role in the virulence of *V. anguillarum*.

Detection of the major surface antigen. Previously (35) in our laboratory, a surface antigen (LPS) associated with the sheath of the flagellum has been shown to be involved in the virulence of *V. anguillarum*. Consequently, the role of the FlaA protein may be complicated, since the sheath is closely associated with the flagellum. To separate the role of the sheath from that of the flagellum requires analyses beyond what could be done in this study. However, confirmation that the loss of the

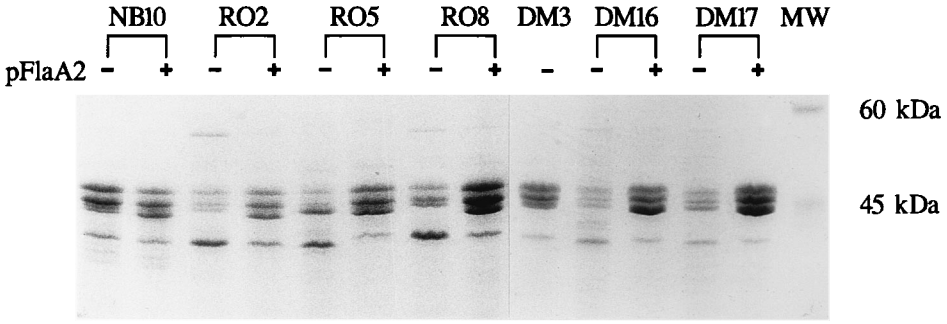

FIG. 4. Protein analysis of crude flagellum preparations from the wild type, the mutants, and the transcomplemented strains. Flagellar proteins (20 μg) were analyzed by electrophoresis through an SDS–11% polyacrylamide gel. Lane MW contains molecular mass markers, and the remaining lanes contain the indicated strains. NB10 is the wild-type strain, RO2 contains an in-frame deletion at the 5′ end of the gene, RO5 carries the plasmid insertion, RO8 has an in-frame deletion of most of the gene, DM16 contains an in-frame deletion at the 3′ end of the gene, DM17 contains both the 3′ and the 5′ end deletions, and DM3 has a plasmid insertion downstream of the *flaA* gene. A plus sign indicates the presence of the pFlaA-2 plasmid, which contains a copy of the *flaA* gene and its promoter region; a minus sign indicates the absence of pFlaA-2.

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FlaA   ThrIleThrValAsnThrAsnValSerAlaMetThrAlaGlnArgTyrLeu
FlaB   AlaIleAsnValSerThrAsnValSerAlaMetThrAlaGlnArgTyrLeu
FlaC   AlaValAsnValAsnThrAsnValSerAlaMetThrAlaGlnArgTyrLeu
FlaD   AlaValAsnValAsnThrAsnValSerAlaMetThrAlaGlnArgTyrLeu
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FIG. 5. Comparison of the N-terminal amino acid sequences of the FlaA, FlaB, FlaC, and FlaD flagellin proteins. The FlaA amino acid sequence was deduced from the nucleotide sequence in Fig. 1. Boldface lettering indicates the nonconserved amino acids.

FlaA protein did not result in the loss of the LPS was obtained by immunogold electron microscopy (Fig. 2). An antiserum that was raised against formalin-killed whole cells of *V. anguillarum* 775.17B and that was then immunoabsorbed against the 775.17B LPS mutant VAN70 to increase the specificity of the antiserum for the LPS was used to detect the LPS associated with the sheath. This is the same antiserum used in the previous study (35) that identified the major surface antigen. The LPS was present for all mutants, confirming that it is the loss of the FlaA protein and not a lack of the surface antigen that is altering the virulence. The LPS mutant VAN70 was used as a negative control and showed no binding of the gold particles. Surprisingly, the surface antigen was located over the whole surface of the bacterium. This result is in contradiction to the previous report (35) from our laboratory, which showed that the surface antigen and the sheath are located only on the flagellum. Since the *V. anguillarum* parent strains were different in the two studies, the parent strain 775.17B from the previous study was also analyzed, and the results were the same as those for strain NB10 (data not shown). The major difference between the two studies is the methods used for preparing the specimens for analysis.

DISCUSSION

The flagellum has been suggested to influence the virulence of *V. anguillarum* (3, 35). In one study, Chart (3) showed a correlation between virulence and the possession of more than one flagellum. He used seven different strains of *V. anguillarum*, and of these strains only those that possessed more than one flagellum were virulent for eels. This correlation cannot be a general rule for all *V. anguillarum* strains, since our wild-type strain NB10 expresses only one flagellum during infection of the fish (35) and is highly virulent for rainbow trout.

In another study (35), a major surface antigen, characterized as LPS and located specifically within the sheath of the flagellum, was suggested to aid in proliferation of the bacterium after entry into the fish. The loss of this surface antigen resulted in the loss of virulence. Our results (Fig. 2) do not contradict the importance of the LPS in virulence but they do contradict the specific localization of this major surface antigen to the sheath of the flagellum. This contradiction of results can be explained by the two different methods used for electron microscopy. In the present study, a 5-min 0.01% gluteraldehyde treatment was utilized, whereas in our previous studies, the bacterium was used without pretreatment with gluteraldehyde and sloughing of an outer layer from the bacterial cell but not from the flagellum was seen, indicating that the outer layer on the cell is fragile. Moreover, a negative stain was used in the previous study but not in the present study, which makes the bacterial cell too electron dense to visualize gold particles that may be bound to bits of the sheath that may have remained after handling of the grids for the binding of the gold particles. The localization of the major surface antigen both on the sheath of the flagellum and on the cell surface has been shown for the Inaba and Ogawa strains of *V. cholerae* (7). In addition, preliminary results from our laboratory indicate that this LPS is involved in serum resistance of *V. anguillarum* (38), suggesting that the LPS is located on the entire surface of the cell instead of only on the flagellum. Thus, *V. anguillarum* contains a sheath-like structure surrounding both the cell and the flagellum, in which at least the antigenic portion of the LPS is embedded. In addition, the presence of the LPS on the surface of the bacterium thus far appears to have no correlation with the presence of the flagellum. A spontaneous flagellum-minus, motility-negative mutant also binds the LPS antibody on the cell surface (data not shown). Thus, loss of virulence due to alterations in the structure of the flagellum is not influenced by the LPS.

How FlaA is involved in virulence cannot be determined from the data presented here. However, it is clear that FlaA plays some role in crossing the integument of the fish. Chemotactic motility has been shown to be required for *V. anguillarum* to invade the fish (37). Clearly, mutations in the *flaA* gene decreased motility, which would account for the loss in virulence when the fish were infected via immersion. However, the two mutants for which a portion or all of the C terminus was removed, DM16 and RO5, were also avirulent via intraperitoneal infection. Homma et al. (15) have suggested that export of a protein by the flagellum-specific export pathway involves the recognition of a structural conformation at the secondary level or higher. In agreement with this idea, Kuwajima et al. (25) have shown that 183 residues of the N terminus of the *E. coli* flagellin are required for secretion. Likewise, a 21-amino-acid sequence near the N terminus of a *Caulobacter* flagellar hook protein is essential for secretion of this protein via the flagellum-specific pathway (23). Possibly the C terminus contains the information needed to secrete FlaA from *V. anguillarum*. Consequently, the FlaA proteins that are lacking a portion of the C terminus would not be secreted from the cytoplasm and may then suppress virulence determinants, leading to a decreased virulence via intraperitoneal injection as seen for the mutants DM16 and RO5. The coordinate regulation of flagellin genes and virulence genes has been shown for several pathogens (1, 6, 20). Some virulence determinants require the expression of flagellar genes for induction, whereas others require the suppression of flagellar genes.

Multiple flagellin genes have been found in a number of bacterial species. Many species studied have two flagellin proteins (13, 24, 27, 36, 39). However, *Caulobacter crescentus* (33) has been shown to have three flagellin proteins; *Vibrio parahaemolyticus* (29) has been shown to have four flagellin proteins, for which the genes are located at two different DNA loci; and *Halobacterium halobium* (10) has as many as five flagellin proteins, with the genes also separated onto two different DNA loci. In this study, *V. anguillarum* was shown to have three, and possibly four, flagellin proteins. The N-terminal sequencing data clearly identified three flagellin proteins that differed from the FlaA protein. However, two of the three proteins had similar N-terminal amino acid sequences. This presents two possibilities. First, there could be four different

flagellin proteins, of which two of the proteins have identical N-terminal sequences. Alternatively, it could be that FlaC and FlaD originated from the same gene but FlaD is a posttranslationally modified FlaC. Posttranslational modification has been reported to occur in several other bacterial species (18, 21, 22, 26, 46). However, support for the likelihood of four flagellin genes may come from a *V. parahaemolyticus* study (29). In this related *Vibrio* species, four flagellin genes have been cloned and mapped to two distinct loci of the chromosome. One locus encodes FlaA and FlaB and another encodes FlaC and FlaD. Interestingly, FlaA from *V. anguillarum* is 80% identical to FlaC, and Southern analysis of the *V. anguillarum* chromosomal DNA showed a weakly hybridizing band after a lengthy exposure time (Fig. 3), suggesting that the four possible *V. anguillarum* genes may also be located on two distinct loci. Although no open reading frame was detected on either the 5′ or 3′ flanking region of the *flaA* gene, these regions could be analyzed further. This would require analysis of another clone, since the chromosomal insert of pBS6-1 ends 450 bp downstream of the *flaA* gene.

In conclusion, we have shown that flagellin A is one of four flagellin subunits that constitute the flagellar filament of *V. anguillarum* and that it is needed for full motility. Loss of this protein from the flagellar filament affects the ability of this bacterium to invade fish. Interestingly, the removal of only the conserved C terminus from this protein leads to a decreased virulence by both routes of infection, i.e., immersion and intraperitoneal injection, suggesting a possible role in virulence after the bacterium has crossed the fish integument. The characterization of the specific role(s) of flagellin A in the virulence of *V. anguillarum*, either as a part of the motility organelle or as some other component of the virulence mechanism, and the identification of the remaining flagellin genes are in progress.

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