J. Biochem. **127**, 29–36 (2000)

Characterization of a Flagellar Sheath Component, PF60, and Its Structural Gene in Marine *Vibrio*[1]

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Received August 17, 1999; accepted October 5, 1999

The Polar flagella (Pof) of *Vibrio alginolyticus* are surrounded by a membrane structure called a sheath. Five major proteins, whose molecular masses are 60, 47, 45, 44, and 18 kDa (named PF60, PF47, PF45, PF44, and PF18, respectively), have been detected in polar flagella. PF47 and PF45 have been identified as flagellins while the other proteins are thought to be sheath-associated ones. In this study, we isolated and partially characterized a major sheath protein, PF60. We found that PF60 can be solubilized by Triton X-100 treatment, but not by heat or acid treatment. After digestion with a peptidase, the N-terminal sequences of several fragments were determined and the N-terminus of intact PF60 seemed to be blocked. Through PCR in conjunction with oligonucleotide primers deduced from the peptide sequences, a DNA fragment of PF60 was amplified. A 4.5 kb *Hin*dIII restriction fragment was cloned by colony hybridization using the PCR fragment. Subsequent sequence analysis revealed three complete and one partial open reading frame (ORFs). The three ORFs, which exhibit sequence homology, correspond to PF60 (named *pfsA*), an amino acid transport ATP-binding protein, and an amino acid binding periplasmic protein. The single *pfsA* gene constitutes an operon and encodes a protein of 491 amino acids containing a putative signal peptide sequence at the N-terminal. A sequence database search revealed no homologous protein. However, PfsA seems to resemble lipoproteins in the N-terminal signal sequence and the biochemical data obtained in this study are consistent with that PfsA is a lipoprotein. The expression of the *pfsA* gene may be coordinately regulated with flagellar formation and similarly regulated to PF47 flagellin.

Key words: flagella, flagellin, morphogenesis, sheath, *Vibrio*.

Vibrio alginolyticus has two types of flagellar systems for motility, polar flagella (Pof) and lateral flagella (Laf). The energy sources of the polar and lateral flagellar motors are Na^+ and H^+ motive forces, respectively (*1*, *2*). Polar flagella, whose rotation rate is very fast and stable at over 1,000 rps (*3*), are expressed constitutively in liquid media. Cells induce lateral flagella and elongate their bodies when they are transferred to the surface of a solid medium. As a result, they can move and spread on the surface of the medium (*4–7*). It has been shown that Pof function better for swimming in a low-viscosity environment than Laf (*8*), and the motor is characterized by its rotation speed and speed stability (*9*, *10*). The restriction of the Pof function on transfer to a solid surface is thought to initiate the expression of Laf (*11*, *12*).

Polar flagella are generated from the cell pole and the filament is sheathed in a membrane structure that is contiguous with the outer membrane (*13*, *14*). A polar flagellum has an apparent diameter of about 30 nm, and is easy to see under a dark-field microscope. On the other hand, lateral flagella are peritrichous and are not sheathed, similar to the flagella of *Escherichia coli* or *Salmonella typhimurium*. In the lateral flagella of *V. parahaemolyticus*, which is closely related to *V. alginolyticus*, the filament is composed of a flagellin, exhibiting an apparent molecular size of 27 kDa on SDS-PAGE gel, encoded by structural gene *lafA* (*12*, *15*). In contrast, the filament of polar flagella is composed of several kinds of flagellins that are encoded by *flaA*, *flaB*, *flaC*, and *flaD*, with apparent molecular sizes *ca.* 45 kDa. The four genes are arranged in two loci and two tandem flagellin genes are located in each locus.

Vibrio spp. may have a sheathed polar flagellum (*16*, *17*). The sheath of the *V. cholerae* flagellum is most likely not a simple extension of the outer membrane because some lipopolysaccharide (LPS) antigenic determinants differ between the outer membrane and the sheath (*17*, *18*). The sheath of the flagellum is essential for the virulence of *V. anguillarum*, which is a fish pathogen. It has been shown that the flagellum and a major surface antigen are expressed during fish infections (*19*). The genes, *virA* and *virB*, of *V. anguillarum* are involved in the biosynthesis of the major surface antigen of the sheath. Three proteins,

[1] This work was supported in part by Grants-in-Aid for Scientific Research (to I.K. and M.H.) from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: Laf, lateral flagella; Pof, polar flagella.

with apparent molecular sizes of 61.5, 60, and 56.5 kDa, were detected in the sheath as well as in the outer membrane of *V. cholerae* (*20*). In *Helicobacter pylori*, whose flagella are encased in a membrane-like sheath, a 29 kDa flagellar sheath protein was identified with a monoclonal antibody, which cross-reacted weakly with a 65 kDa protein of *V. parahaemolyticus* or *V. cholerae* (*21, 22*). The 29 kDa protein is similar to an *N*-acetylneuraminyllacetose-binding fibrillar hemagglutinin.

At least five proteins (PF60, PF47, PF45, PF44, and PF18) are detectable in a Pof fraction of *V. alginolyticus* (*23*). It has been shown that PF47 and PF45 are flagellins from their N-terminal sequences, which are homologous to the flagellin sequences of *V. parahaemolyticus* (*24*). The remaining proteins, PF60, PF44, and PF18, are thought to be sheath components. The sheath structure is shed into the culture medium as vesicles by *V. alginolyticus* mutants that lack the Pof filament (*25*). The unassembled flagellin of the *V. alginolyticus* mutants seems to be shed with the vesicles, which can be solubilized with a detergent, Triton X-100. In addition, the amounts of PF60, PF47, and PF18 are increased in a long-Pof mutant of *V. alginolyticus*, the average length of the helical axis being 11.2 ± 3.6 μm, while that of the wild-type Pof is 5.5 ± 0.9 μm (*23*). The radius of the helical shape of long-Pof is smaller than that of wild-Pof, resulting in a slow swimming behavior. A DNA fragment of the *motX* promoter region suppresses the long-Pof phenotype, suggesting that this sequence may interact with a regulatory protein (*31*).

There have been few studies on the formation of the sheath structure. Furthermore, the role of flagellar sheaths in the interaction between a bacterium, while rotating rapidly with a flagellum, and a surface is unclear. In this study, to investigate the sheath structure and function, we characterize the sheath proteins in *V. alginolyticus* and clone a gene encoding one of the major sheath proteins.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions—The strains and plasmids mainly used in this study are shown in Table I. The Pof$^-$ mutant, YM44, may carry a defect in the σ^{54} gene (*rpoN*) (*26*). The other Pof$^-$ mutants, NMB126, NMB175, VIO560, and VIO587, were isolated from VIO5 as described previously (*28*). The plasmids, pSU21, pSU38, and pSU41, used have pACYC184-derived ori and are maintained in *V. alginolyticus* as well as *E. coli*.

To examine the influence of expression of the *pfsA* gene in *V. alginolyticus*, we subcloned the 3.5 kbp *SalI–XbaI* or 1.6 kbp *SalI–SacI* fragment of pMFS2 (see below) into the pSU41 vector. The resulting plasmids were named pMFS304 and pMFS306. A DNA fragment from pMFS2 was amplified by PCR using the primers of the upstream region (CGGGATCCGCTCAGTTTAGGAAAAAG) and the terminator (CCCAAGCTTAATAAATTTACAAAAGTG) region of the *pfsA* gene. The *Bam*HI and *Hin*dIII sites were added to the primers, respectively. Using these sites, the PCR product was cloned into pSU38 or pSU41, and the resulting plasmids were named pMFS301 and pMFS302, respectively.

V. alginolyticus cells were cultured at 30°C in VC medium (0.5% polypeptone, 0.5% yeast extract, 0.4% K_2HPO_4, 3% NaCl, 0.2% glucose) or VPG medium (1% polypeptone, 0.4% K_2HPO_4, 3% NaCl, 0.5% glycerol). For compact colony formation, VC 1.5% agar plates were used. For swarm assays, VPG 0.3% agar plates were used. When necessary, chloramphenicol, kanamycin, rifampicin, and tetracycline were added at final concentrations of 2.5, 100, 50, and 1.25 μg/ml, respectively. *E. coli* cells were cultured at 37°C in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl), and chloramphenicol, kanamycin, and tetracycline were added at final concentrations of 25, 25, and 12.5 μg/ml, respectively, when necessary.

TABLE I. **Bacterial strains and plasmids.**

Strains and plasmids	Genotype or description[a]	Reference or source
V. alginolyticus strains		
VIO5	VIK4 *laf* (Pof$^+$Laf$^-$)	(*27*)
YM4	138-2 *laf* (Pof$^+$Laf$^-$)	(*2*)
YM90	YM4 *laf* (Pof$^+$Laf$^-$)	(*23*)
NMB578	VIO5 *laf* (Pof$^+$Laf$^-$)	(*23*)
Plasmids		
pSU38	*kan* (Km^r) P_{lac} *lac* Zα	(*29*)
pSU41	*kan* (Km^r) P_{lac} *lac* Zα (MCS different from that in pSU38)	(*29*)
pSU21	*cat* (Cm^r) P_{lac} *lac* Zα (MCS same as that in pSU41)	(*29*)
pMFS301	pSU38 P_{lac} *pfsA*$^+$ (1.6 kb *Bam*HI–*Hin*dIII fragment)	This study
pMFS302	pSU41 *pfsA*$^+$ (1.6 kb *Bam*HI–*Hin*dIII fragment)	This study
pMFS304	pSU41 *pfsA*$^+$ (3.5 kb *SalI–Xba*I fragment)	This study
pMFS306	pSU41 *pfsA*$^-$ (1.6 kb *SalI–Sac*I fragment)	This study

[a]Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant; P_{lac}, *lac* promoter; Pof$^+$, long-polar flagella; MCS, multicloning site.

Isolation of Polar Flagella—Polar flagella were isolated as previously described (*23*). An overnight culture was plated on 1.5% VC plates and then incubated at 30°C overnight. Cells were scraped from the plates, suspended in V-buffer (25 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 mM $MgCl_2$), and then vortexed for 10 min to shear the flagella. The suspension was centrifuged at 10,000 ×*g* for 10 min, and the sedimented cells were resuspended in V-buffer and sheared again. The pooled supernatants were centrifuged at 80,000 ×*g* for 60 min. The pellet, Pof fraction, was suspended in V-buffer.

N-Terminal Sequences of the PF60 Peptides—The Pof fraction was treated with 1.0% Triton X-100 and then separated by ultracentrifugation (80,000 ×*g*, 1 h). The supernatant was separated by SDS-PAGE and the gel was stained with Coomassie Blue R250, and then the PF60 bands were cut out from the gel. After PF60 had been digested with lysylendopeptidase, the resultant peptide fragments were separated by reverse phase HPLC and then analyzed with a peptide sequencer (entrusted to APLO Science, Tokushima).

SDS-PAGE and Immunoblotting—SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting were performed as previously described (*30*).

Antibodies—The anti-PF45 and anti-PF60 antibodies were prepared previously (*25, 31*). The production of anti-peptide antibodies against PF60 was carried out by Sawaday (Tokyo). Peptide fragments were synthesized from the amino acid sequence predicted from the DNA sequence of the PF60 gene. The two fragments, PF60-96 (K96–D116)

and PF60-301 (E301–L320), were conjugated to KLH (keyhole limpet hemocyanin) through an additional C-terminal cysteine residue and used separately to immunize rabbits. The anti-peptide antibodies were affinity-purified using the corresponding peptide fragment coupled to agarose.

DNA Manipulations and Sequencing—Routine DNA manipulations were carried out according to standard procedures (*32*). Restriction endonucleases and other enzymes for DNA manipulations were purchased from Takara Shuzo (Kyoto) and New England Biolabs (Beverly, MA). The nucleotide sequence was determined by the dideoxy chain termination method using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI PRISM™ 377 DNA sequencer (Perkin-Elmer).

PCR Conditions—PCR amplification was performed using a Takara Ex *Taq* kit (Takara Shuzo, Kyoto) and a Thermal Cycler (Perkin-Elmer). PCR comprised 30 cycles of 30 s at 94°C, 2 min at 45°C, and 5 min at 72°C.

Screening of the Genomic Library—Plasmid pHSG398 (*33*) and chromosomal DNA from *V. alginolyticus* strain NMB90 were digested with *Hin*dIII and then ligated. The libraries were introduced into DH5α cells by electroporation. Colony hybridization was performed using the 231 bp fragment of pMFS1 which had been cloned by PCR-methods, using genomic *V. alginolyticus* DNA and synthesized mixed primers (49R and 58F; Fig. 4), into a TA cloning vector, pCR™2.1 (Invitrogen, CA). A positive clone was isolated, containing a plasmid (named pMFS2) with a 4.5 kb *Hin*dIII fragment insert.

Transformation in Vibrio—The transformation of *Vibrio* cells by electroporation was carried out as described previously (*34*). The cells were subjected to osmotic shock and then washed thoroughly with 20 mM $MgSO_4$. Electroporation was carried out using a Gene Pulser electroporation apparatus (Japan Bio-Rad Laboratories, Tokyo) at an electric field strength between 5.0 and 7.5 kV/cm.

Microscopy—Samples were negatively stained with 2% potassium phosphotungstate (pH 7.0) for electron microscopy and then observed under a JEM-1200 EXII electron microscope (JEOL, Tokyo).

The flagella were observed using a dark-field microscope (Olympus model BHT, Tokyo) equipped with a 100 W mercury lamp (Ushio USH-102, Tokyo).

RESULTS

Fractionation of Polar Flagella Components—Previously, we detected at least five major proteins (PF60, PF47, PF45, PF44, and PF18) in a Pof fraction of *V. alginolyticus* (*23*). In order to determine where these proteins are localized in the flagella structure of *V. alginolyticus*, polar flagella fractions of both a wild-type strain, YM4, and a long-Pof mutant, NMB90, were prepared by CsCl density gradient centrifugation. By means of this procedure, two major bands (upper and lower ones) were observed in the CsCl gradient for each fraction, and each band was analyzed by SDS-PAGE (Fig. 1). Two flagellins, PF47 and PF45, were detected in both the upper and lower bands, but were increased in the upper band of both fractions. In contrast, while PF60 and PF44 were also detectable of both the upper and lower bands, they were increased in the lower band of both fractions. In addition, on electron microscopic analysis, the flagellar filaments and the membrane structure were mainly found in the upper and lower bands, respectively (data not shown). These results suggest that CsCl centrifugation allows the enrichment of PF60 and PF44 in specific bands.

When a Pof fraction derived from the NMB90 mutant was heated to 60 or 80°C and then centrifuged at high speed, the PF47 and PF45 flagellins were recovered in the supernatant, and PF60 and PF44 were recovered in the pellet (Fig. 2). This result suggests that the flagellar filaments are disassembled into component flagellin molecules as a result of the heat treatment. When the Pof fraction was treated with Triton X-100, PF60 was recovered in the supernatant on high-speed centrifugation, but PF44 was not solubilized with this treatment (Fig. 2). PF18 behaved similarly to PF60 (data not shown).

From the large amount of cells grown on the VC plates, the Pof fraction was treated with Triton X-100 and the

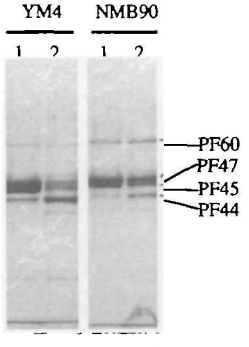

Fig. 1. **Pof fractionation by CsCl density-gradient centrifugation.** CsCl (1.3 g) was added to 3 ml of the Pof fractions isolated from YM4 and NMB90, followed by centrifugation (270,000 ×*g*, 5 h). The proteins in the two main bands, upper (lane 1) and lower (lane 2), on CsCl density-gradient centrifugation were separated by SDS-PAGE and stained with Coomassie Blue R250.

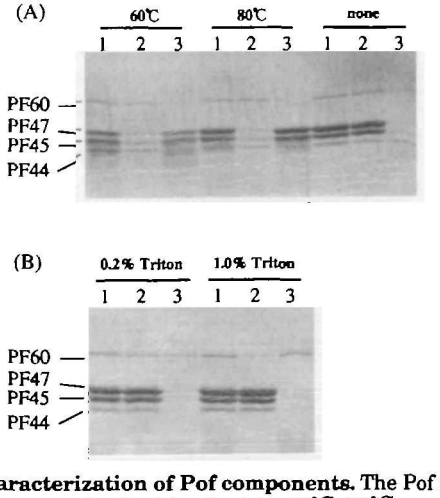

Fig. 2. **Characterization of Pof components.** The Pof fraction of NMB90 was treated with either heat (A; 60°C, 80°C, none) or a detergent (B; 0.2% Triton X-100, 1% Triton X-100) and then separated by ultracentrifugation (80,000 ×*g*, 1 h). The pre-separated sample (lane 1), precipitate (lane 2), and supernatant (lane 3) were analyzed by SDS-PAGE, followed by staining with Coomassie Blue R250.

sheath proteins were isolated (Fig. 3). We could separate the PF60 protein as the main protein in the sheath fraction of the long-Pof mutant. Using the sheath fraction derived on Triton X-100 treatment, we attempted to determine the N-terminal sequences of the PF60, PF44, and PF18 proteins. Our attempts were unsuccessful for PF60 and PF18, probably due to blocked N-terminals. However, we were successful in determining twenty amino acids of the N-terminal of PF44. A database search revealed that the identified sequence, AEVFKQEEGSVDFYGQLRPT, was similar to the N-terminal sequence of major outer membrane proteins of various bacteria.

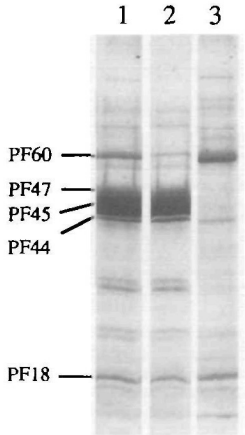

Fig. 3. **Separation of PF60.** The Pof fraction of NMB90 was treated with 1% Triton X-100 and then centrifuged at 80,000 ×*g* for 1 h (lane 1, Pof fraction; lane 2, precipitate; lane 3, supernatant). They were separated by SDS-PAGE and stained with Coomassie Blue R250.

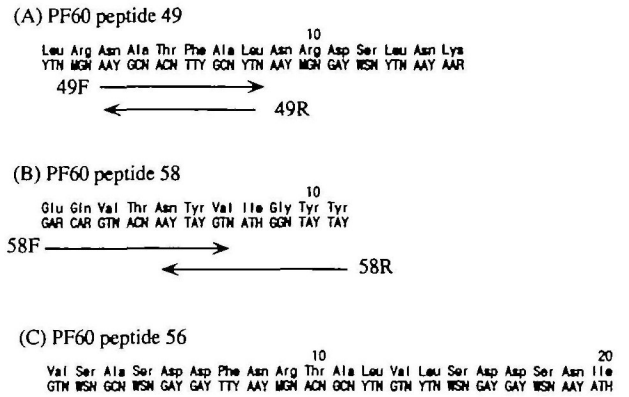

Fig. 4. **The sequence of the PF60 peptide fragments.** The peptide fragments were digested with an endoproteinase and separated by reversed-phase HPLC, and then the sequences of the N-termini of the three fragments were determined. The nucleotides corresponding to the amino acids are shown under the peptide sequences. The arrows indicate the primer regions for PCR.

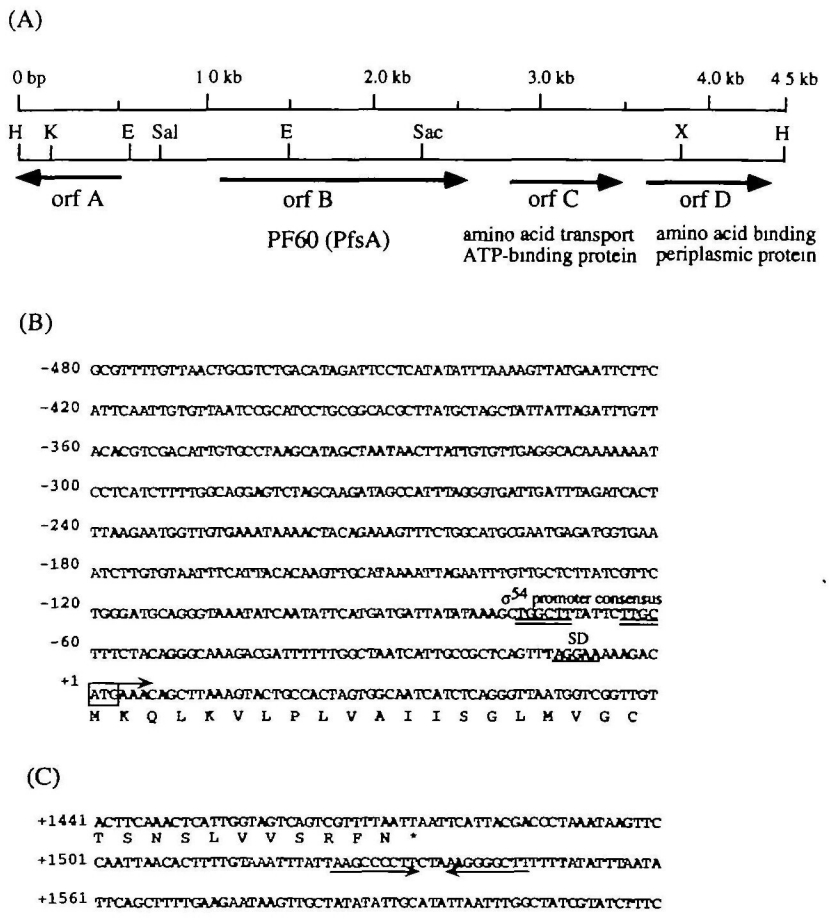

Fig. 5. **4.5 kb *Hin*dIII fragment.** (A) Physical map around the PF60 gene. The PF60 gene and ORFs are indicated by arrows. The restriction sites are denoted as follows: E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Sac, *Sac*I; Sal, *Sal*I; X, *Xba*I. (B) The promoter region of the PF60 gene. The consensus sequence of a potential σ^{54} promoter region is double-underlined. A proposed Shine-Dalgarno (SD) sequence is underlined. The start codon is boxed. (C) The terminator region of the PF60 gene. The potential transcriptional terminator is indicated by arrows. The sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number (AB032934).

Cloning of the Gene Encoding PF60—The PF60 protein, isolated by SDS-PAGE, was digested with an endoproteinase, and the resultant peptides were separated by HPLC. The N-terminal sequences of three of the peptides were determined (Fig. 4). DNA fragments were amplified by PCR-methods using genomic *V. alginolyticus* DNA and synthesized mixed primers deduced from the amino acid sequence of these peptides. A 0.2 kb fragment was amplified with primers 49R and 58F (Fig. 4). The resultant fragment was cloned into a TA cloning vector, and the resultant plasmid was named pMFS1. The insert was sequenced. The fragment is 231 bp in length and the amino acid sequence deduced from the fragment corresponds to the N-terminal sequences of the peptides derived from PF60. From these results, we concluded that the fragment is derived from the PF60 gene.

The DNA probe generated from pMFS1 hybridized to chromosomal DNA digested with various restriction enzymes (data not shown). All the enzymes used gave a single detectable band. In order to clone the entire region of the PF60 gene, we used a genomic library derived from chromosomal DNA of NMB90 digested with *Hin*dIII. *Hin*dIII-digestion of chromosomal DNA results in a 4.5 kb fragment detected using the PF60 fragment DNA probe. Colonies generated from the *Hin*dIII library were hybridized with the 231 bp fragment probe. A positive clone was isolated and found to contain a plasmid, named pMSF2, that possesses the 4.5 kb *Hin*dIII fragment.

DNA Nucleotide Sequence of the 4.5 kb Fragment—The 4.5 kb fragment was sequenced and found to contain one partial (orfA) and three complete open reading frames (orfB, orfC, and orfD) (Fig. 5A). The DNA sequence of orfB corresponded in part to the 231 bp fragment of PF60, and the amino acid sequence of the third peptide fragment, peptide 56, matched part of the sequence of orfB. So, we concluded the ORF encodes PF60. This PF60 gene was named *pfsA* (polar flagellar sheath protein A). Translation of the *pfsA* gene may start with an ATG codon due to a sequence resembling the ribosome binding consensus which is located 6 bp upstream from the codon (Fig. 5B). Furthermore, a potential promoter sequence of TGGCTT (N5) TTGC, which is very similar to the σ^{54} promoter consensus was found about 70 bp upstream of the start codon. The transcriptional unit seems to consist of only the *pfsA* gene because a sequence resembling a rho-independent transcriptional terminator is present about 50 bp downstream from the termination codon (Fig. 5C). The orfC and orfD proteins are homologous to amino acid transport ATP-binding proteins and amino acid binding periplasmic proteins, respectively. No significant sequence homology was found with orfA.

Amino Acid Sequence of PF60—The amino acid sequence

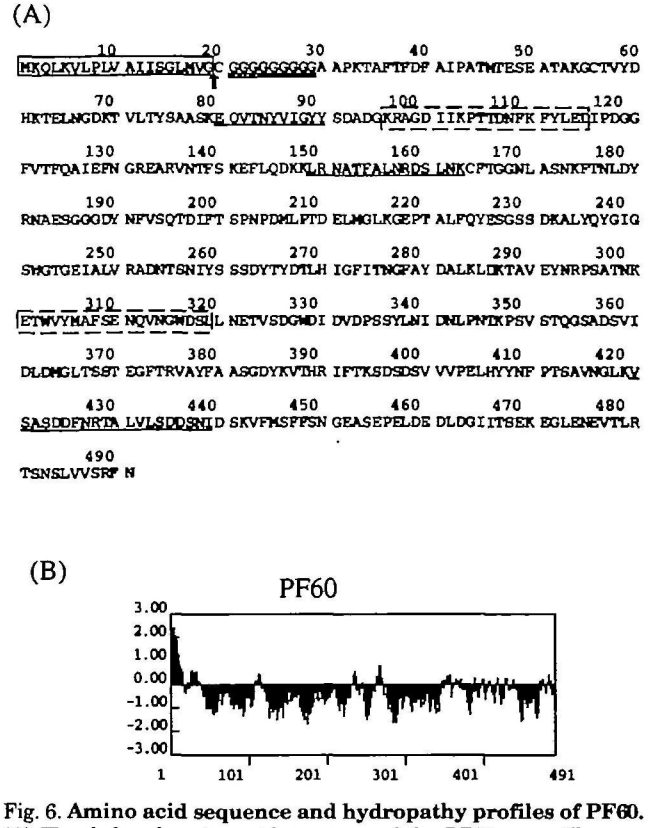

Fig. 6. **Amino acid sequence and hydropathy profiles of PF60.** (A) The deduced amino acid sequence of the PF60 gene. The predicted signal sequence is boxed. An arrow indicates the putative cleavage site for signal peptidase. The underlined peptide sequences indicate the N-terminus of the peptides digested with the endoproteinase. The peptides synthesized to prepare the PF60 antibodies are boxed by dashed lines. (B) The hydrophobic profiles. The hydropathy profile of PF60 was predicted by the method of Kyte and Doolittle.

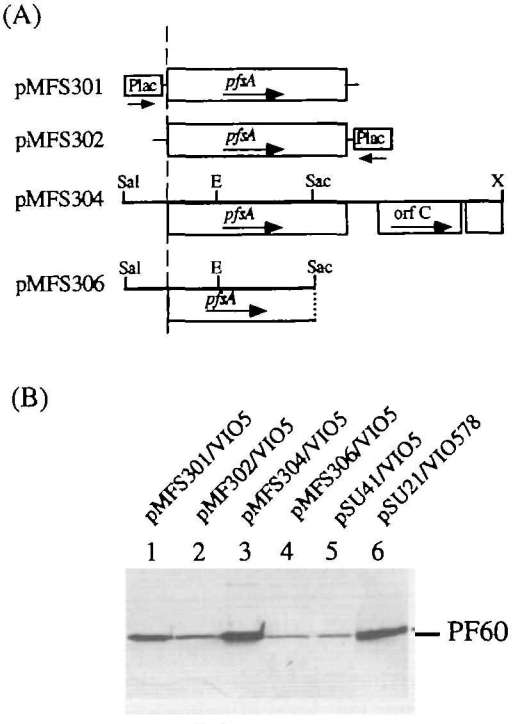

Fig. 7. **Detection of the *pfsA* product.** (A) Various plasmids carrying the *pfsA* gene were constructed. The arrows in the boxes, which represent the coding regions, show the direction of the translation. The arrows under the boxes show the direction of the *lac* promoter. (B) Cells transformed with the plasmids were harvested at the mid-log phase, and the proteins in the cells were separated by SDS-PAGE and immunoblotting with the affinity-purified antipeptide-PF60 antibody (PF60-96).

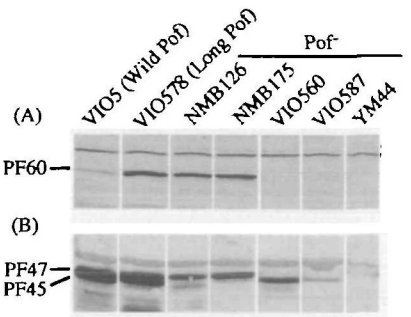

Fig. 8. **Expression levels of PF60 and flagellin.** Cells were harvested at the mid-log phase, and then suspended in D.W. These suspensions were subjected to SDS-PAGE and immunoblotting with the antipeptide-PF60 antibody (PF60-96) (A) and the anti-PF45 antibody (B).

deduced from the *pfsA* gene is shown in Fig. 6A. The gene encodes a 54 kDa protein comprising 491 amino acids. The protein has a putative signal peptide sequence at the N-terminus and a glycine-rich sequence just following the signal peptide. PfsA exhibits similarity to the N-terminal region of about 50 amino acids of *Pseudomonas fluorescens* XynF, which is a component of xylanases, is similar to that of PfsA (*35*). However, no genes exhibiting significant or meaningful similarity to PfsA could be found on database searches. PfsA is not hydrophobic except for the region of the putative signal sequence (Fig. 6B). The putative cleavage sequence is similar to the lipoprotein cleavage consensus (*36*). PF60 may be a lipoprotein.

Detection of the pfsA Product—Plasmid pMFS2, whose vector region is pHSG398 and whose replication origin comes from ColE1, can not be maintained in *Vibrio* cells. Therefore, the *pfsA* gene and derivative constructs were transferred to the pSU41 vector that has a P15A replicon, and the resultant plasmids were introduced into wild-Pof strain VIO5 (Fig. 7A). pMFS304 contains the native promoter region and the whole structural gene of *pfsA*, while pMFS306 lacks the C-terminal region of the *pfsA* gene. The *pfsA* gene without the promoter region, which originally was amplified by PCR, was cloned into the *lac* promoter region of the pSU41 vector and named pMFS301. A plasmid, pMFS302, that contains the *pfsA* gene in the opposite orientation was also constructed. Using an anti-peptide antibody (PF60-96), immunoblotting was performed on the wild-Pof cells (VIO5) containing vector plasmid pSU41 (pSU41/VIO5). The PfsA levels found in these cells were equivalent to that of pMFS306/VIO5. When the whole *pfsA* gene was present in the plasmid, overproduction of PfsA was observed. The highest expression level of PfsA, about 40-fold greater than the wild-type level, was found for pMFS304. This level of PsfA expression was equivalent to that of the long-Pof mutant (VIO578), but was greater than that found for pMFS301, which lacks the native promoter for the *pfsA* gene. The level of expression from pMFS304 may be due to the additional effect of the native promoter and the *lac* promoter. Even in cells containing pMFS302, in which the *lac* promoter is in the opposite orientation to the *pfsA* gene, PfsA appeared to be overproduced. This overexpression may be due to a copy number effect of the gene and starting of transcription from the vector region. Similar expression levels were detected using the another anti-peptide antibody (PF60-301) or an anti-PF60 antibody (data not shown).

The swarm ability of VIO5 cells containing the plasmids in a 0.3% agar VC plate was not different from that of the original VIO5 cells. In addition, neither the flagellar shape nor the length was affected by the plasmids (data not shown). These results suggest that the excess amount of PfsA, produced by the plasmids, does not affect the motility or the flagellar structure.

Expression of PF60 and Flagellin in pof Mutants—In the *pof* mutants isolated, the level of PF60 expression was increased or decreased, although the mutations have not been mapped to the *psfA* region (Fig. 8). In NMB126 and NMB175, PF60 expression was increased compared with in the wild-Pof strain (VIO5), and the level was comparable to in the long-Pof strain (VIO578). A trace amount of PF60 was detected in VIO560 and VIO587, but no PF60 was detected in YM44, which seems to be an *rpoN* mutant (*26*). The levels of flagellin expression and PF60 expression may be related. In NMB126 and NMB175, PF47 flagellin increased but PF45 flagellin was not detected. In VIO560 and VIO587, PF47 flagellin was not detected but PF45 flagellin was. Neither PF47 nor PF45 flagellin was detected in YM44. The amount of PF47 flagellin was increased in the long-Pof strain. These data suggest that the expression of PF60 is similarly regulated to that of PF47 flagellin.

DISCUSSION

In polar flagella, the three proteins PF60, PF44 and PF18 are detected as well as filament proteins, flagellins PF47 and PF45, suggesting that the three proteins comprise the filament sheath. PF60 and PF18 are detergent-soluble, while PF44 is not solubilized with a detergent and is recovered in the pellet fraction on high-speed centrifugation. These results are consistent with the evidence that the outer membrane proteins of *E. coli* are not solubilized by mild detergents, such as Triton X-100. The N-terminal sequence of PF44 exhibits similarity to the N-terminal sequence of outer membrane proteins. The hydropathy profile demonstrated that PF60 (PfsA) is not hydrophobic except for the signal peptide region. These data are consistent with other evidence that the sheath is contiguous with the outer membrane. The structural gene for PF60 was cloned using peptide sequence data and called *pfsA*. The *pfsA* gene encodes a 491 amino acid polypeptide with a putative N-terminal signal peptide sequence (19 amino acids) and a 9 glycine repeat sequence just following the signal sequence. XynF of *Pseudomonas fluorescens*, which is a component of xylanases, also has this signal peptide and glycine repeat (*35*). The function of the repeat sequence is unknown. The putative cleavage sequence is similar to the lipoprotein cleavage consensus (*36*). The N-terminal cysteine may be modified by lipids. This could explain the blocking on N-terminal sequencing of PF60, the characterization as a membrane protein despite the hydrophilic profile of PF60, and the easy solubility with Triton X-100. PF60 may be a lipoprotein. When PF60 was overproduced in a wild-Pof strain, the flagellar formation and shape were not affected. However, PF60 has not been shown to be necessary for flagellar formation. In *V. cholerae*, approximately 60 kDa proteins, similar in size to PF60, have been detected in its filament sheath (*20*). The polar flagella of *V.*

cholerae and *V. alginolyticus* are structurally and functionally similar to each other. The flagellar basal body of *V. alginolyticus* greatly resembles that of *V. cholerae* (*37*, *38*). When grown in a liquid medium, they mainly possess a single polar flagellum and swim rapidly. The partial sequences of the genes homologous to *pomA*, *pomB*, *motX*, and *motY*, essential for the rotation of the Na^+-motor of *V. alginolyticus*, are found in the database of the genome sequencing project on *V. cholerae*. It has been shown that the polar flagellar motor of *V. cholerae* is driven by the Na^+-motive force (*39*, *40*).

In the cloned fragment in this study, there are one partial (orfA) and three complete open reading frames (orfB, orfC, and orfD). orfB corresponds to PfsA (PF60). The *pfsA* gene probably forms a single transcription unit because a coding sequence resembling a rho-independent transcriptional terminator is present at about 50 bp downstream from the termination codon. The products of orfC and orfD were found to be homologous to amino acid transport ATP-binding proteins and amino acid binding periplasmic proteins, respectively.

In an *rpoN* mutant, which is defective in Pof formation, flagellins and PF60 are not expressed at all (*26*, this study). On the other hand, their expression levels vary in various *pof* mutants. This suggests that the expression of the *pfsA* gene is regulated negatively and positively by the polar-flagellar regulatory system. The σ^{54} factor is essential in this regulatory system. In the promoter regions of the polar flagellin genes for *V. cholerae* and *V. parahaemolyticus*, a σ^{54} consensus sequence has been found (*24*, *41*). It has been shown that *rpoN* mutants do not produce flagella in various bacteria which possess polar flagella (*41–45*).

In Pof length mutants, we found a long-Pof mutant with a Pof of about twice the mean length of the wild-Pof and an altered filament shape (*23*). The relative amounts of the sheath and the flagellar components of the long Pof are different from those of the wild Pof, with an increase in the amounts of PF60 and PF47 flagellin. The mutant phenotypes reverted almost to the wild type on the introduction of a 143 bp fragment whose sequence comprises the *motX* promoter region and contains a sequence similar to σ^{54} consensus (*31*). This shows that the gene affected in the long-Pof mutation may be a regulatory gene for sheath and flagellar genes. The *motX* fragment may reduce the levels of available σ^{54}, and alter the expression of the sheath and flagellar genes, resulting in suppression of the long-Pof phenotype. Among *pof* mutants, the amount of PF60 was increased or decreased, and PF60 was not detected at all in an *rpoN* mutant. The σ^{54} factor and the activator proteins for σ^{54} may also regulate the genes for cell division and colonization in addition to flagellar formation in *V. cholerae* (*41*).

We wish to thank Noriko Nishioka for the assistance with some of the biochemical experiments.

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