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Molecular Cloning and Expression of a Sodium-Driven Flagellar Motor Component Gene (*motX*) from *Vibrio fluvialis*

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Abstract The bacterial flagellar motor is a molecular machine that couples proton or sodium influx to force generation, mostly for driving rotation of the helical flagellar filament. In this study, we cloned a gene (*motX*) encoding a component of the sodium-driven flagellar motor from *Vibrio fluvialis*. The nucleotide sequence of the *motX* gene, composed of 633 bp and 211 amino acid residues, was determined. Overexpression of the *motX* gene in *Escherichia coli* using a strong promoter induced growth inhibition and cell lysis. The lethal effect of *E. coli* was suppressed by adding amiloride, as a potent inhibitor for the sodium channel. Electron microscopic observation of the expressed protein indicated that MotX protein induced by isopropyl β-D-thiogalactopyranoside caused the lysis of host cell.

Key words: *Vibrio fluvialis*, sodium-driven flagellar motor, MotX

For a successful growth, some microorganisms must use their flagella not only to transport the cells, but to act as a viscosity sensor in a typical aquatic environment. In *V. alginolyticus* and *V. parahaemolyticus*, two types of flagella, polar flagellum and lateral flagella, were identified in one cell [3, 15]. A single polar flagellum is suited for swimming in liquid, and numerous lateral flagella are suited for swimming over viscous surfaces [2]. The energy for the rotation of flagella is the electrochemical gradient of a specific ion, either a proton or a sodium ion. In some *Vibrio* sp., the energy source for the polar flagellum is known to be the sodium motive force, whereas the lateral flagella are known to be powered by the proton motive force [8]. The rotation of the polar flagellum is very fast, and the flagellar motor, which can stably rotate the flagellar filament, generates torques under the various sodium influxes [16].

Recently, the polar flagellar motor of *V. parahaemolyticus* was identified to consist of four components: *motA*, *motB*, *motX*, and *motY* gene products [7]. Among these, MotX protein is known to be a chemical component and MotY protein is a stator component [12, 13]. In *V. alginolyticus*, four proteins such as PomA, PomB, MotX, and MotY have also been reported as being essential elements for the sodium-driven flagellar motor [1, 20]. *MotY* genes of *V. alginolyticus* and *V. parahaemolyticus* have been cloned, and show 94.5% identity [17]. However, the *motX* gene of *V. parahaemolyticus* is the only available sequence for the channel component of the sodium-driven flagellar motor in *Vibrio* sp.

In the present study, the sodium-driven flagellar motor component gene was discovered from one recombinant clone, and the *motX* gene sequence and some characteristics of the recombinant protein on the host growth during overexpression are reported.

Materials and Methods

Bacterial Strains and Growth Condition

V. fluvialis (KCTC 2473) was obtained from Korean Collection for Type Cultures. This strain was cultivated aerobically in a brain heart infusion medium at 30°C for 2 days. The total chromosomal DNA was isolated by the procedure described previously [9].

Construction of Overexpression Plasmid, pVFMX6

The open reading frame (ORF) of the *motX* gene was amplified by PCR. A forward primer, 5'-GGAATTCCATATGAAGCTACGAACGGTAG-3' (*Nde*I site preceded the sense strand), and a reverse primer, 5'-CCCGGGAAGCTTCCAAAAGGTTCCGCGGCG-3' (*Hin*dIII site preceded

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the antisense strand) were used to amplify 10 ng of the template DNA. Plasmid pVFMX6 was obtained by ligation of the PCR product and pET22b (+) that had been double digested with *Nde*I and *Hin*dIII.

Effect of Overexpression of *motX* on Cell Growth

Plasmid pVFMX6 was transformed into *E. coli* BL21 (DE3) and grown at 37°C in modified Luria-Bertani (MLB) medium (1% tryptone, 0.5% yeast extract, 100 mM NaCl) supplemented with 50 μg/ml ampicillin. When the cultures reached an optical density (OD_{600}) of 0.6, the cells were harvested by centrifugation (15 min at 8,000 ×g, 4°C) and diluted to OD_{600} of 0.3 with MLB medium containing 50 μg/ml ampicillin, 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), and NaCl (100 mM or 200 mM). The cells were incubated at 37°C, and the optical density (OD_{600}) was checked. To examine the effect of amiloride, 0–1.5 mM concentration of amiloride was added to MLB medium.

Purification of Recombinant MotX and Preparation of anti-MotX Rabbit Serum

The cells were harvested after IPTG induction and resuspended in 20 mM Tris-HCl buffer (pH 8.0). The samples were disrupted by sonication and centrifuged to remove soluble components. Recovered insoluble fractions were solubilized with 6 M guanidine hydrochloride solution and applied to a Ni^{2+}-NTA agarose column (Novagen). Proteins were eluted with 30 mM imidazole solution [18]. The eluted fractions were analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To prepare the samples for immunization, the gel piece containing MotX was excised and purified from the gel slice at 4°C by electroelution. The antiserum against MotX was made by immunizing a rabbit intraperitoneally with approximately 100 μg *motX*. Immunization was done three times at 2 weeks intervals. At 2 weeks after the last injection, the blood was collected and the serum was obtained by centrifugation.

Western Blot Analysis

The membrane fractions were separated by the method of Doig and Trust [5]. Proteins separated by SDS-PAGE were visualized by staining with Coomassie brilliant blue R-250. For immunoblotting, the proteins were electrophoretically transferred to the nitrocellulose membrane. Goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase was used for a secondary antibody. Proteins were visualized after developing with 5-bromo-4-chloro-3-indolyphosphate [11].

Electron Microscopy

Bacteria were prepared for electron microscopy as described by Wagenaar *et al.* [19]. Ultrathin sections were examined with a Philips JEOL 1200 transmission electron microscope at an acceleration voltage of 80 kV. The TEM photographs were taken at Baek Hospital, Pusan, Korea [10].

Nucleotide Sequence Accession Number

The nucleotide sequence for *motX* has been deposited in the GenBank database under the accession no. AF134839.

RESULTS AND DISCUSSIONS

Sequence Analysis of the *motX* Gene

The nucleotide sequence of a 3.9 kb *Hin*dIII fragment (named pVFH192) was determined. Within the sequenced fragment, two complete ORFs were transcribed with opposite directions, and one partial ORF was truncated at the N-terminus (Fig. 1A). Homology search of the deduced amino acid sequence of ORFs revealed that the truncated sequence possessed as much as 90% identity to the adenylosuccinate synthetase (*purA*) of *V. parahaemolyticus* and the second ORF exhibited a high homology to *V. parahaemolyticus motX*, which is a protein associated with a channel component of the sodium-type flagellar motor. However, the third ORF existing downstream of *motX* was shown to have a low amino acid sequence homology of 24% to *E. coli* arylsulfatase.

The *motX* gene contained 633 bp with a predicted molecular mass of 24.1 kDa. As shown in Fig. 1B, a putative ribosome binding site (AAGAG) resembling those of *E. coli*, with a spacing 7 bp from an ATG initiation codon, was found. In the 5' nontranslated region of *motX*, two consensus promoter sequences known to be responsible for σ^{28} and σ^{54} were present. A 12 bp inverted repeat sequence containing the stop codon, which was assumed to be a rho-independent terminator, existed in the 3' end. The published sequences on the *motX* and *motY* genes from *V. parahaemolyticus* indicated that two consensus promoter sequences, which were very similar to the σ^{28} and σ^{54} promoter consensus sequences, could be identified together in the promoter region [12, 13]. Recently, a putative promoter region of *motX*, which was partially isolated, was identified in *V. alginolyticus* [6]. There were one σ^{54} promoter consensus sequence and one partial σ^{28} promoter consensus at only the −35 region. When the σ^{54} promoter consensus sequence of *V. alginolyticus motX* was introduced to a *V. alginolyticus* mutant containing a polar flagellum longer than the wild-type strain, the swimming speed and the length of the polar flagellum were recovered to the wild-type. The result obtained from transformation of the σ^{54} promoter sequence with elimination of the σ^{28} promoter sequence suggested that this sequence might interact with a regulatory protein for a normal control of the polar flagellum expression. The *motY* of *V. alginolyticus* has only a σ^{28} consensus in the promoter region [17]. Two consensus sequences were found in the 5' upstream region of *V. fluvialis motX*; $TAAAN_{15}GCCGATAC$ (at positions 1139 to 1165 in Fig. 1B) that is very similar to the proposed consensus sequence $TAAAN_{15}GCCGATAA$ for σ^{28}, and $TGGCAAN_4TTGCC$

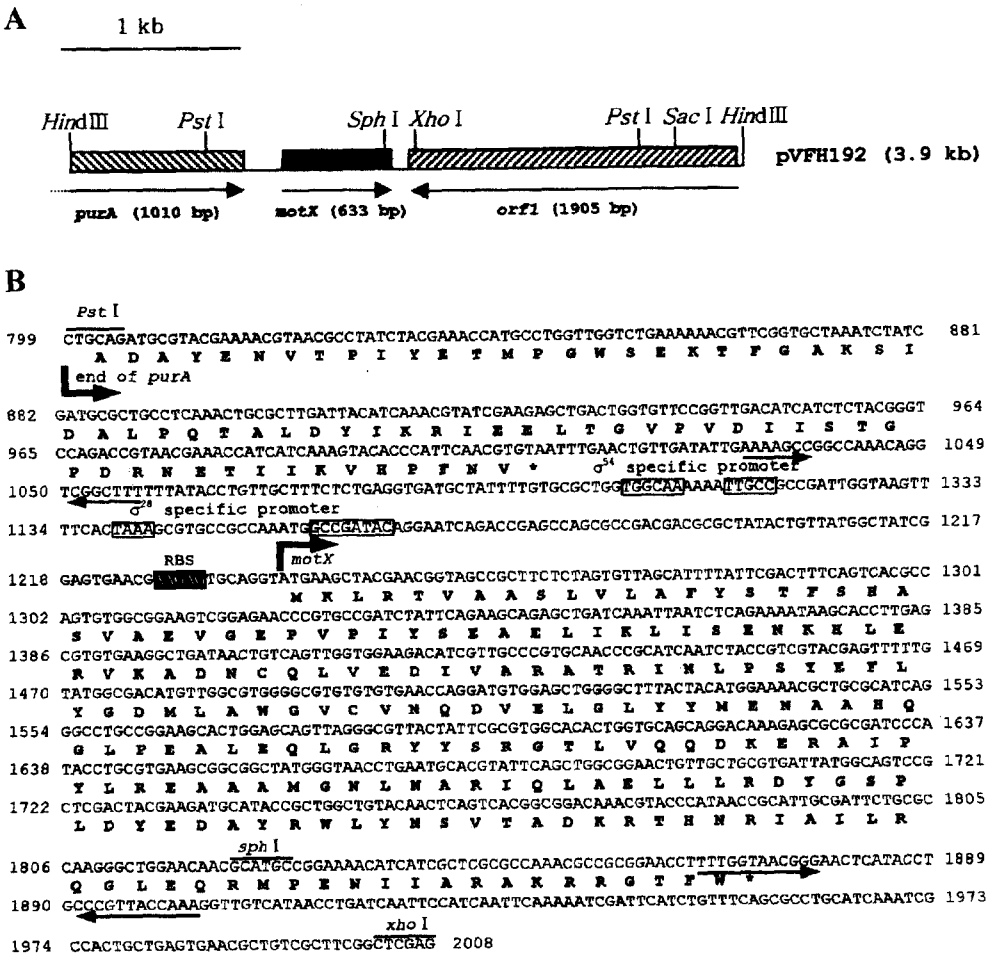

Fig. 1. Restriction map of pVFH192 (A) and nucleotide sequence of the *motX* gene region of pVFH192 (B).
The features of a potential σ^{54} and σ^{28} promoter are indicated by a square.

(at positions 1104 to 1118 in Fig. 1B) that is similar to the proposed consensus sequence TGGCACN$_5$TTGCA for σ^{54}, respectively.

When the deduced amino acid sequence of *V. parahaemolyticus* MotX was aligned with *V. fluvialis* MotX, 85% of identity was found (Fig. 2). It is important to point out that a high portion of hydrophobic residues existed at the N-terminus. In the *V. parahaemolyticus* MotX, the hydrophobic segment of the N-terminus was proposed to be a transmembrane spanning region [14]. The deduced amino acid sequence of a 27 transmembrane spanning region (from Val[6] to Ile[32]) was also observed in *V. fluvialis* MotX (Fig. 2). Eleven residues among 27 amino acids in a putative transmembrane domain near the N-terminus were different from those of *V. parahaemolyticus* MotX; nevertheless, the hydrophobicity of the transmembrane domain was found to be largely hydrophobic.

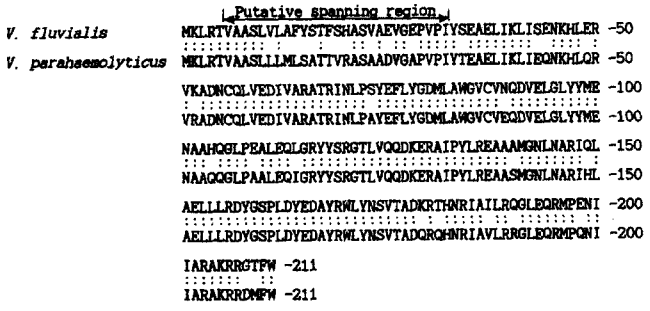

Fig. 2. Alignment of the amino acid sequences between the *V. fluvialis* and *V. parahaemolyticus motX* genes.

Expression and Purification of Recombinant MotX

High levels of overexpression of *motX* were achieved by subcloning the *motX* coding region, which was obtained by PCR, and by inserting into pET22b (+) to construct a plasmid pVFMX6. pVFMX6 was then transformed to *E. coli* BL21 (DE3). The *E. coli* BL21 (DE3) transformant was cultivated in MLB medium and IPTG was added when the absorbance level reached 0.6 at 600 nm. As shown in Fig.

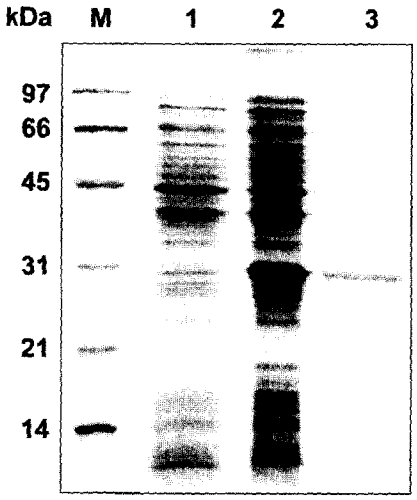

Fig. 3. The expression and purification of MotX.
Cells (pVFMX6) were harvested and purified after 3 h induction with IPTG. Lanes: M, low range marker; 1, pET22b(+) BL21 (DE3); 2, pVFMX6/BL21 (DE3); 3, purified MotX.

3 (lane 2), an accumulation of a 30-kDa protein was observed in *E. coli* carrying pVFMX6, but not in *E. coli* carrying pET22b(+). After the host cell lysis, purification was performed as described in Materials and Methods. The purified recombinant protein contained a molecular mass of 30 kDa, obtained by SDS-PAGE (Fig. 3, lane 3).

Growth Inhibition by MotX Induction

A thorough study was carried out to examine whether a lethal effect from overproduction of *V. fluvialis* MotX could indeed occur in *E. coli*. At different time intervals, growths were measured by absorbance determination. The cells were growing exponentially for 30 min after IPTG induction at different NaCl concentration levels. However, the cessation of growth appeared thereafter, and the growth decreased to a constant level (Fig. 4A). Simultaneously, an accumulation of MotX was apparent for 30 min of induction and the amount of expressed protein did not change (Fig. 4A, inset). In many cases, *E. coli* failed to grow when the recombinant proteins were overexpressed, since the overproduced proteins were found to be toxic to the host cells. To prove whether the cell growth was harmed by the overexpressed protein itself, that was shown in ordinary cases of pET systems or by inhibiting sodium channel by MotX, amiloride, a specific inhibitor of the sodium channel, was used. As shown in Fig. 4B, the deleterious effect of MotX induction was diminished by increasing concentrations of amiloride. In contrast, the constant expression levels were maintained (data not shown), although the growth inhibition was not completely prevented at 1.5 mM concentration of amiloride. The helical flagellar filaments of many bacteria rotate by the flagellar motor. Energy to rotate the motor is not supplied by ATP

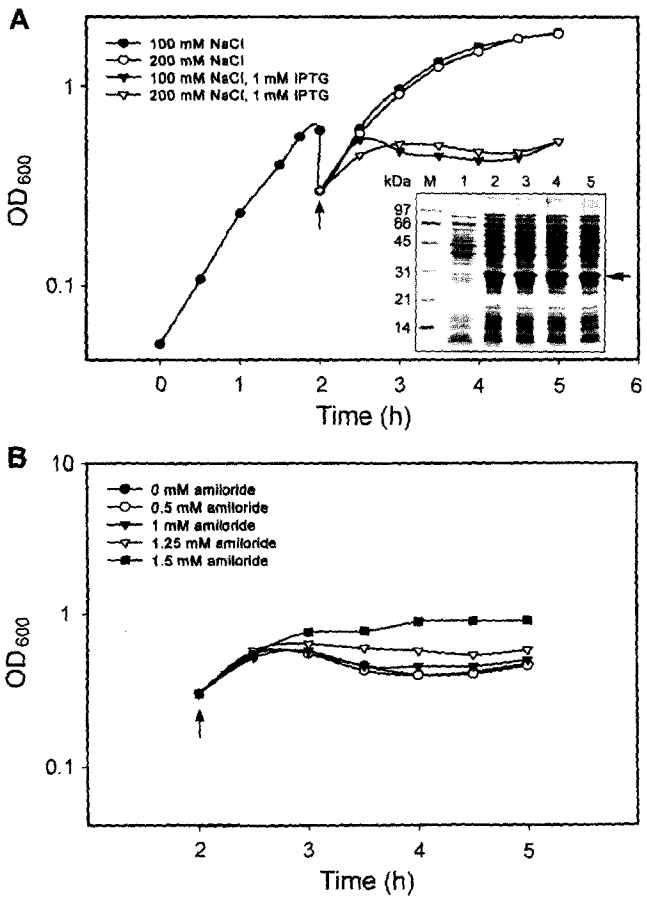

Fig. 4. Effect of NaCl and amiloride concentrations on MotX induction and the growth of *E. coli*.
Cells (pVFMX6) were grown in MLB plus IPTG and various concentrations of NaCl (A) and amiloride (B). Inset; Time course of MotX expression. Lanes: M, low range marker; 1, no induction; 2, 0.5 h after induction; 3, 1.5 h after induction; 4, 2.5 h after induction; 5, 5.5 h after induction. ↑; the point for the dilution and substitution of the medium. ←; the position of the expressed MotX protein.

hydrolysis but derived from the electrochemical gradient of specific ions across the cytoplasmic membrane. For some alkalophilic *Bacillus* sp. and a marine *Vibrio* sp., the sodium motive force is an essential energy source [4, 12]. Sodium flux couples to generate forces. In *V. alginolyticus*, lithium ion could be used for the flagellar motor. As the ion was substituted from sodium to lithium, the maximal swimming speed was reduced due to the lowered permeability [12].

Lysis by MotX overproduction was studied by measuring the absorbance level, and the accumulation of MotX and the cell lysis were observed by an electron microscope. With transmission electron microscopy, we observed the lysis of *E. coli* from 2 h to 5 h after induction. When the cell membranes were fractionated and Western blot analysis was performed with an anti-rabbit serum against MotX, a positive signal could be detected only in the inner

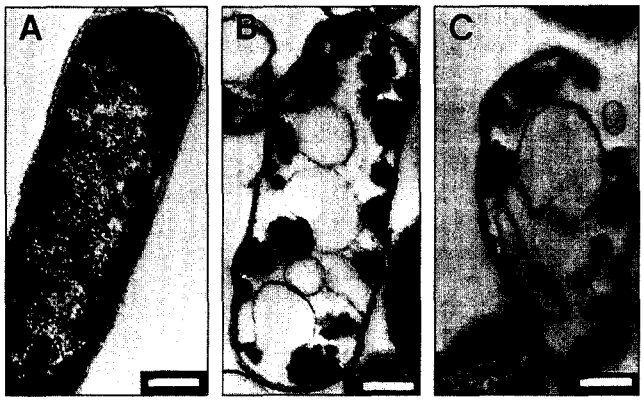

Fig. 5. Transmission electron micrographs of *E. coli* (pVFMX6). A, no induction; B, 2 h after IPTG induction; C, 5 h after IPTG induction. Bar, 200 nm.

membrane fraction of *E. coli*. Figure 6 shows that a single band against the antibody could be observed and the corresponding protein was clearly visualized on SDS-PAGE stained with Coomassie brilliant blue.

Overexpression of the *V. parahaemolyticus motX* gene by using a strong promoter in *E. coli* induced growth inhibition and cell death via an unknown mechanism. Moreover, the rate of growth inhibition by overexpression of the *motX* gene was promoted with the presence of an ion, such as potassium, lithium, and sodium ions. The lethal effect of *E. coli* by overexpression of the *motX* gene was suppressed by adding amiloride, which is a potent inhibitor of the sodium-ion channel in animal cells and specifically inhibits the rotation of the sodium-driven flagellar motors by competing with sodium ion in the medium. Comparative characterization with ions and a specific channel inhibitor implied that the growth inhibition by overexpression of the *motX* gene could be caused by the expressed protein affecting the ion channel, rather than other general detrimental effects such as formation of

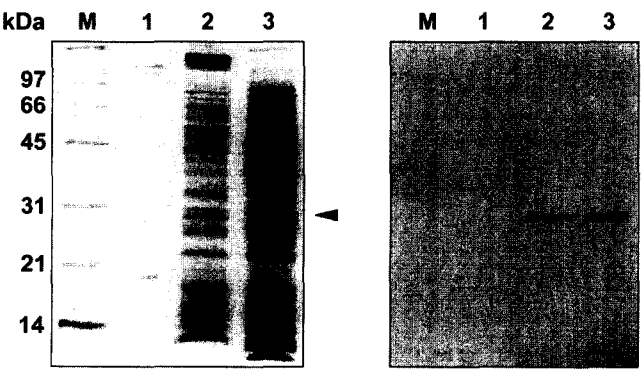

Fig. 6. The localization of MotX at *E. coli* (pVFMX6) was analyzed by Western blotting.
Lanes: M, low range marker; 1, outer membrane fraction; 2, inner membrane fraction; 3, total membrane fraction.

inclusion body [14]. From the overexpression of the *V. fluvialis motX* gene in *E. coli*, MotX protein was shown to inhibit the host cell growth in a same manner as with the *V. parahaemolyticus motX* gene.

This finding raises a possibility that the attached MotX proteins are involved in control of the ion flux. However, clarification of the mechanism of how the attached MotX protein reacts through the membrane and how the MotX protein plays a role in fluxing ions should be carried out in the near future.

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