Molecular Characterization of the *Salmonella typhimurium* flhB Operon and Its Protein Products

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The *flhB* and *flhA* genes constitute an operon called *flhB* operon on the *Salmonella typhimurium* chromosome. Their gene products are required for formation of the rod structure of flagellar apparatus. Furthermore, several lines of evidence suggest that they, together with *FliI* and *FlhB*, may constitute the export apparatus of flagellin, the component protein of flagellar filament. In this study, we determined the nucleotide sequence of the entire *flhB* operon from *S. typhimurium*. It was shown that the *flhB* and *flhA* genes encode highly hydrophilic polypeptides with calculated molecular masses of 42,322 and 74,848 Da, respectively. Both proteins have several potential membrane-spanning segments, suggesting that they may be integral membrane proteins. The *flhB* operon was found to contain an additional open reading frame capable of encoding a polypeptide with a calculated molecular mass of 14,973 Da. We designated this open reading frame *flhE*. The N-terminal 16 amino acids of *FlhE* displays a feature of a typical signal sequence. A maxicell labeling experiment enabled us to identify the precursor and mature forms of the *flhE* gene products. Insertion of a kanamycin-resistant gene cartridge into the chromosomal *flhE* gene did not affect the motility of the cells, indicating that the *flhE* gene is not essential for flagellar formation and function. We have overproduced and purified N-terminally truncated *FlhB* and *FlhA* proteins and raised antibodies against them. By use of these antibodies, localization of the *FlhB* and *FlhA* proteins was analyzed by Western blotting (immunoblotting) with the fractionated cell extracts. The results obtained indicated that both proteins are localized in the cytoplasmic membrane.

The flagellum of *Salmonella typhimurium* and *Escherichia coli* is composed of three distinct parts, the basal body, the hook, and the filament (27). The basal body is a complex structure with one inner ring (the MS ring), two outer rings (the L and P rings), and the rod. The basal body is embedded in the cell membrane, while the hook and filament portions extend into extracellular space. Flagellar assembly has been believed to proceed from cell-proximal to cell-distal structures; i.e., it begins with the basal body, proceeds with the hook, and is completed with the filament. Therefore, the flagellar proteins which are synthesized in the cytoplasm should be exported to the periplasmic space, the outer membrane, or the extracellular space where their assembly finally occurs (17, 36). The outer ring proteins, FlgH and FlgI, are synthesized as precursor forms with signal peptides and transported across the cytoplasmic membrane by the Sec-dependent pathway (13, 16). On the other hand, the component proteins of the axial structures, the rod, the hook, and the filament, do not have signal peptides at N termini and have been postulated to be exported through the flagellum-specific transport pathway (12, 14, 25, 27). Elongation of the filament proceeds in a remarkable fashion by adding the component protein, flagellin, at its distal end (15). Therefore, the most plausible candidate for the physical pathway of this transport is the central channel existing within the flagellar structure (29). The other axial proteins are also believed to be transported through the same pathway. However, very little is known about the molecular detail of the flagellum-specific transport pathway.

By using temperature-sensitive flagellation mutants of *S. typhimurium*, Vogler et al. (40) identified some candidate genes involved in this pathway. They include the *flhA* and *flil* genes. The facts that *Flil* shows similarity to the catalytic subunit of the F_{0}F_{1} ATPase (40) and that the purified *Flil* protein has an activity to bind ATP (7) suggested that *Flil* may act as an ATPase in the flagellum-specific export pathway. The *flhA* gene together with the *flhB* gene constitutes an operon called the *flhB* operon on the *S. typhimurium* chromosome (18, 23). In the accompanying paper, we present the genetic evidence suggesting that *FlhB* may also be involved in the flagellum-specific transport pathway (21). The DNA sequences of the *flhA* homolog of *Caullobacter crescentus* (*flbF*) and the *flhB* and *flhE* genes of *Bacillus subtilis* have been reported, and the similarity of their gene products to the virulence proteins from various pathogens has been demonstrated (4, 5, 32, 33). However, the molecular details of the *S. typhimurium* *flhB* operon and its protein products have not been described yet, and the exact role of the FlhA and FlhB proteins in the flagellum-specific transport pathway remains unknown.

For the first step to elucidate the functions of the *flhA* and *flhB* gene products in the flagellum-specific export pathway, we decided to determine the structure of the *S. typhimurium* *flhB* operon and to characterize its protein products. The *flhB* and *flhA* genes encode polypeptides with the calculated molecular masses of 42,322 and 74,848 Da, respectively. Both FlhB and FlhA contain potential membrane-spanning regions, suggesting that they may be integral membrane proteins. This was confirmed by performing Western blotting (immunoblotting) with the fractionated cell extracts. Furthermore, we found that the *flhB* operon contains an additional open reading frame (ORF) capable of encoding a polypeptide of 14,973 Da whose N-terminal 16 amino acids show a feature of a typical signal
TABLE 1. Bacterial strains and plasmids used

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<td><strong>E. coli</strong></td>
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<tr>
<td>EKK22</td>
<td>hsd fbhD</td>
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<td>EKK24</td>
<td>hsd fbhA</td>
<td>This study</td>
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<tr>
<td>JM109</td>
<td>recA1 endA1 gyrA96 thi rpsL17 supE44 relA1 Δ(lac-proAB)F' [proAB+ lacF'] lacZΔM15 traD36]</td>
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<tr>
<td>MV1184</td>
<td>ana (lac-proAB) rpsL thi 80(alacZΔM15) Δ(srl-recA)306::Tn10/F' [proAB+ lacF'] lacZΔM15 traD36]</td>
<td>38</td>
</tr>
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<td><strong>S. typhimurium</strong></td>
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<tr>
<td>KK1004</td>
<td>Wild type</td>
<td>23</td>
</tr>
<tr>
<td>SJ12014</td>
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<td>SJ12033</td>
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<td>Plasmid for kanamycin-resistant gene cartridge</td>
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<td>The EcoRI site of pMK1 was converted into a HindIII site</td>
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<td>pMK2#1, -2, -3, -4, and -5</td>
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<td>The EcoRI site of pMK2#5 was converted into a BamHI site</td>
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<td>pMK2 and -4</td>
<td>A 2.1-kb Smal-PvuII fragment from pMK1 was cloned into the Smal site of pUC119</td>
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<td>pMK5</td>
<td>A 0.7-kb PvuII-EcoRI fragment from pKK1311EH was cloned into the Smal-EcoRI site of pUC118</td>
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<td>pMK6</td>
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<td>pMK7</td>
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<td>pMK8</td>
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<td>A 4.0-kb HindIII fragment of pMK1H was cloned into the HindIII site of pTrc99A</td>
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<td>pMK102</td>
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<td>pMK103</td>
<td>A 1.1-kb BglII-HindIII fragment from pMK1H was cloned into the BamHI-HindIII site of pTrc99C</td>
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<td>pMK104</td>
<td>A 0.6-kb BglII-BamHI fragment from pMK2#5B was cloned into the BamHI site of pTrc99B</td>
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sequence. Insertion of a kanamycin-resistant gene cartridge into this ORF on the chromosome did not affect motility of the cells, indicating that this ORF is not essential for flagellar formation and function.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and media.** The bacterial strains and plasmids used in this study are listed in Table 1. Ordinary culture media, including L broth, M9 minimal medium, L-agar plate, and motility agar plate, were prepared as described previously (23). Ampicillin and kanamycin were used at final concentrations of 50 and 25 μg/ml, respectively.

**DNA manipulation and DNA sequencing.** In vitro manipulation of DNA was performed by the methods described previously (24). Restriction endonucleases and DNA-modifying enzymes were purchased from Toyobo Co. Ltd. or Nippon Gene Co. Ltd.

Various restriction fragments from the 4.0-kb HindIII-EcoRI region of pKK1311EH were subcloned onto pUC118 or pUC119. Nested deletions of the cloned DNA fragments were obtained by the method of Henikoff (11).

Nucleotide sequences were determined for both strands by the dideoxy chain termination procedure with a Sequenase version 2.0 sequencing kit (U.S. Biochemicals). Sequence data were analyzed by use of the software GENETYX (Software Development Co., Tokyo, Japan).

**Gene product analysis.** Maxicells were labeled with [35S]methionine (HAS, Budapest, Hungary) as described by Ohnishi et al. (30). The labeled cells were suspended in 60 μl of sample loading buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue, 10% glycerol) and heated at 100°C for 3 min. After the samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the gels were soaked in Enlightning (DuPont, Wilmington, Del.) and fluorographed.

**Gene disruption experiments.** Gene disruption experiments with the *fbhE* gene were performed as described by Yamada et al. (41). pMK8 carried the *fbhE* gene within which the kanamycin-resistant gene cartridge from pUC4K was inserted.
From this plasmid, the 3.9-kb BamHI fragment carrying the flhE::Km gene was excised, ligated, and introduced into KK1004 by electroporation with a Gene Pulser (Bio-Rad). The treated cells were plated onto the L-agar plates containing kanamycin and incubated overnight at 37°C. The structure of the chromosomal flhE gene was examined with the resulting kanamycin-resistant clones by Southern blotting analysis with a DIG DNA labeling and detection kit (Boehringer Mannheim).

**Purification of proteins.** Plasmids pMK103 and pMK104 carried the 5' terminally truncated flhA and flhB genes inserted in frame into the high-expression vectors pTrc99C and pTrc 99B, respectively. EKK22 harboring pMK103 or pMK104 was grown overnight in 20 ml of L broth containing ampicillin at 37°C. The whole culture was inoculated into 2 liters of the same medium and incubated at 37°C with gentle shaking. When the cell density reached 60 Klett units, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at the final concentration of 1 mM, and incubation was continued for another 5 h. Cells were harvested, suspended in 80 ml of 50 mM Tris-HCl (pH 7.2)–0.1 M NaCl, and disrupted by sonication. Cell debris was removed by low-speed centrifugation, and the resulting supernatant was centrifuged at 35 krpm for 1 h in a Beckman no. 65 rotor. To the supernatant, ammonium sulfate was added to 60% saturation and mixed by stirring on ice. The precipitate was collected by centrifugation at 15 krpm for 30 min.

For purification of the N-terminally truncated FlhA protein, the precipitate was dissolved in 20 ml of 50 mM Tris-HCl (pH 7.2)–0.1 M NaCl and dialyzed three times against 1 liter of the same buffer. The sample was loaded on a TOYOPPEARL HW-50 column and eluted with the same buffer. Each fraction was monitored by SDS-PAGE, and fractions containing the protein with an apparent molecular mass of 20 kDa, which corresponded to the N-terminally truncated FlhA protein, were pooled. The pooled fraction was subjected again to the same gel filtration procedure. After dialysis three times against 1 liter of 100 mM sodium phosphate buffer (pH 6.8) containing 1 M ammonium sulfate, the sample was applied to an Econo-Pac HIC cartridge (Bio-Rad). The column was washed with 5 ml of the same buffer, and the adsorbed proteins were eluted with 50 ml of a 1 to 0 M ammonium sulfate linear gradient. Fractions containing the protein of 20 kDa were pooled, dialyzed three times against 1 liter of 10 mM sodium phosphate (pH 6.5), and applied to an Econo-Pac CM cartridge (Bio-Rad). The column was washed with 10 ml of the same buffer, and adsorbed proteins were eluted with 60 ml of a 0 to 1.5 M NaCl linear gradient. pMK103 also carried the flhE gene. We noticed that the cell extract from EKK22 harboring pMK103 also contained a large amount of 12-kDa protein which corresponded to the mature form of FlhE. This 12-kDa protein was purified by the same chromatographic procedure.

For purification of the N-terminally truncated FlhB protein, the ammonium sulfate precipitate of cell extract from EKK22 harboring pMK104 was dissolved in 20 ml of 10 mM sodium phosphate (pH 6.5) and dialyzed three times against 1 liter of the same buffer. The sample was loaded onto an Econo-Pac CM cartridge, washed, and eluted as described above. Each fraction was monitored by SDS-PAGE, and fractions containing the protein with an apparent molecular mass of 14 kDa which corresponded to the N-terminally truncated FlhB protein were pooled. To the pooled fraction, Triton X-100 was added to a final concentration of 1%. The sample was then subjected to the gel filtration procedure as described above.

**Amino acid sequencing.** N-terminal amino acid sequences of the purified proteins were analyzed with a protein sequencer, ABI 477A/120A.

**Preparation of antibodies against FlhA and FlhB.** The

N-terminally truncated FlhA and FlhB proteins prepared as described above were separated by SDS-PAGE, excised from the gels, and used to prepare antibodies against them. Approximately 1 mg of each protein was used to immunize a rabbit. The effectiveness of the antibodies was confirmed by Western blotting.

**Fractionation of cell extracts and Western blotting.** For the analysis of FlhA, KK1004 carrying pMK102 was grown overnight in 2 ml of L broth containing ampicillin at 37°C. Four hundred microliters of the overnight culture was inoculated into 40 ml of L broth containing ampicillin and incubated at 37°C with shaking. At the middle of the exponential growth, IPTG was added to a final concentration of 1 mM, and incubation was continued for another 3 h. For the analysis of FlhB, KK1004 harboring pMK2#5 was grown exponentially in L broth containing ampicillin.

Cells were harvested, resuspended in 10 mM sodium phosphate (pH 6.8) containing 0.2 mM diithiothreitol, andsonicated. After the cell debris was removed by low-speed centrifugation, the cell lysate was centrifuged at 35 krpm for 1 h in a Beckman no. 65 rotor. The supernatant and pellet fractions which contained the cytoplasmic and membrane proteins, respectively, were collected separately. Aliquots of the pellet fraction were treated with 1 M NaCl or 1.2% sodium lauryl sarcosinate (Sarkosyl) at 4°C overnight and centrifuged at 35 krpm for 2 h. The supernatant and the pellet fractions were collected separately. After the proteins in each fraction were separated by SDS-PAGE, Western blotting was performed as

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**FIG. 1.** Physical structure (left) and complementation ability (right) of the recombinant plasmids. The bold bars represent the *S. typhimurium* DNA fragments present in the plasmids. These plasmids were introduced into SJ12035 (flhB) and SJ12014 (flhA) by transformation, and the ability of the transformants to form swarms was examined on the motility agar plates. Complementation ability (+ and − [presence and absence, respectively]) was judged from the colony types (swarms or compact colonies) after incubation for 5 h at 37°C. +/−, swarms were produced, but only after prolonged incubation (more than 12 h; we suppose that these swarms might be raised by homologous recombination between genes on the chromosome and the plasmid). Restriction site abbreviations: E, EcoRI; H, HindIII; Hc, HincII; M, MluI; P, PstI; Pv, PvuII; Sa, SacI; Sm, SmaI.
FIG. 2. Nucleotide sequence of the entire flhB operon of *S. typhimurium* and deduced amino acid sequences of FlhB, FlhA, and FlhE. Numbers on the right refer to nucleotides from the HindIII site or to amino acids from the N terminus of each protein. Potential ribosome-binding sites are indicated by dotted lines. The stop codons are marked with asterisks. The signal sequence of FlhE is underlined. The convergent arrow indicates the inverted repeat sequence.
The accession number D32203.

DDBJ, EMBL, data

and fragment containing

nested deletion

termination potential

plasmids plausible

sites sequence

and A.

FIG. 3. sequence

of the

window

of FlhB (A), FlhA (B), and FlhE (C). The algorithm of Kyte and Doolittle (26) was used with a window span of 19. For each panel, hydropathy values are shown on the vertical axis, and the position of each amino acid is shown on the abscissa. The positive and negative values correspond to hydrophobic and hydrophilic segments, respectively.

described previously (30) with the antibodies against FlhA and FlhB.

Nucleotide sequence accession number. The nucleotide sequence data presented here have been submitted to the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases under the accession number D32203.

RESULTS AND DISCUSSION

Sequence analysis of the flhB operon. Although Stock and Stock (35) reported a partial sequence of the S. typhimurium flhB operon, the entire structure of the operon has not been determined. From pKK1311EH, which carried the entire flhB operon (19), various portions of the 4.0-kb HindIII-EcoRI fragment containing the flhB operon were recloned into pUC118 and pUC119. From each of these plasmids, a set of nested deletion mutants was constructed in vitro. These deletion plasmids were introduced into SJ12014 (flhA) and SJ12033 (flhB), and motility recovery was investigated. On the basis of complementation pattern, the approximate locations of flhB and flhA were determined as shown in Fig. 1.

By use of the same deletion mutants, the nucleotide sequence of the entire HindIII-EcoRI fragment was determined (Fig. 2). The sequence contains three ORFs of 1,149 bp (orf1), 2,076 bp (orf2), and 390 bp (orf3), oriented in the same direction. They are all preceded by well-placed potential ribosome-binding sites (Shine-Dalgarno sequences). Since there is no potential termination signal in the intergenic regions, it is most plausible that these three ORFs constitute an operon. From the complementation data shown in Fig. 1, we concluded that orf1 and orf2 correspond to the flhB and flhA genes, respectively. In this paper, orf3 is tentatively called flhE.

Like many other flagellar genes (for examples, see references 16, 28, and 40), the coding regions for these three genes overlap with one another. The flhB gene terminates in an 8-bp overlap (ATGGCTAA) with the start of flhA. The flhA gene terminates in a 1-bp overlap (TAATG) with the start of flhE. These features may reflect translational coupling. As Stock and Stock (35) pointed out, the upstream region of flhB contains an inverted repeat sequence that would permit the formation of a stem-loop structure with 11 paired bases in the stem and 10 bases in the loop. This might be the terminator of transcription of the tar operon which exists upstream of the flhB operon. As described previously (19, 22), the promoter structure of the flhB gene is not evident.

Characteristics of the primary sequence of the FlhB, FlhA, and FlhE proteins. The flhB and flhA genes encode 383- and 692-amino-acid proteins with calculated molecular masses of 42,322 and 74,848 Da, respectively. They are both extremely hydrophobic proteins. The hydropathy profiles indicated that both FlhA and FlhB have several potential membrane-spanning segments at the N-terminal half (Fig. 3), suggesting that they may be integral membrane proteins. Their C-terminal half is quite hydrophilic and is likely to represent a water-soluble domain. As expected, FlhA shows strong homology to B. subtilis FlhA and C. crescentus FlhF (43 and 40% identities, respectively) (4, 32, 33). As reported previously (2, 9, 31, 39), these proteins also show homology to the virulence proteins such as Yersinia pestis LcrD, Shigella flexneri MixA, and S. typhimurium InvA (data not shown). Similarly, FlhB shows strong homology to B. subtilis FlhB (38% identity) (5), and these proteins share homology with another family of virulence proteins such as S. flexneri Spa4O (34) and S. typhimurium SpaS (10). An increasing amount of evidence suggested that many of the flagellar proteins are highly homologous to various virulence proteins required for the surface presentation of invasion antigens. Although the significance of this sequence homology has not been understood well, it has been postulated that this superfamily of proteins may be dedicated to the export of the component proteins of the extracellular suprastructures by a mechanism that does not involve signal sequence cleavage (3, 10, 28).

The flhE gene encodes a 130-amino-acid protein with a calculated molecular mass of 14,073. FlhE has the characteristics of a cleavable signal sequence in the N-terminal 16 amino acids. The sequence contains a predominantly hydrophobic region (WLALLLF), which is preceded by a short positively charged N-terminal sequence (MRK) and followed by a potential recognition site for signal peptidase cleavage (VQA). A helix-breaking Pro residue is also present in this region (Pro-11). The cleavage should take place between Ala-16 and Ala-17. This feature suggests that, after synthesis within the cytoplasm as a precursor form, FlhE may be transported across the cytoplasmic membrane through the Sec-dependent pathway and the N-terminal signal peptide may be cleaved during the export process. The protein sequence of FlhE was compared with sequences of other proteins, but no significant homology was found.

Identification of the flhE gene product. To confirm that the flhE gene product might be subjected to processing, we analyzed the protein products from pMK103 which carried the 5' terminally truncated flhA gene and the intact flhE gene under the tac promoter. This plasmid was introduced into JM109 by transformation, and protein labeling was performed in maxicells prepared from the resulting transformant (Fig. 4).
The maxicells were found to synthesize three species of proteins with approximate molecular masses of 20, 14, and 12 kDa only when induced with 1 mM IPTG. When the maxicells were treated with 0.5 mM carbonyl cyanide m-chlorophenyl-hydrazone (CCCP), which is known to inhibit the cleavage of the signal sequence (6), the amount of the 12-kDa protein decreased and that of the 14-kDa protein increased. This result indicated that the 12- and 14-kDa proteins correspond to mature and precursor forms of FlhE, respectively. Consistent with this, the 12-kDa protein purified as described in Materials and Methods has an N-terminal amino acid sequence, AGE GAWDDS, which is exactly identical with that of the putative mature FlhE protein. The 20-kDa protein corresponded to the N-terminally truncated FlhA protein (see Materials and Methods).

**Disruption of the chromosomal flhE gene.** To analyze the function of flhE in flagellar formation and function, we constructed a mutant in which the chromosomal flhE gene was specifically disrupted by insertion of a kanamycin-resistant gene cartridge. The procedure was described in Materials and Methods. On the motility agar plates, the flhE disruptant formed swarms of the same size as those formed by the wild-type strain (data not shown). Therefore, we concluded that the flhE gene is not essential for flagellar formation and function. At present, we have no idea as to the function of FlhE.

**Subcellular localization of the FlhB and FlhA proteins.** Plasmids pMK103 and pMK104 carried the 5'-terminally truncated flhA and flhB genes inserted in frame into the high-expression vectors pTrc99C and pTrc99B, respectively (Fig. 5). N-terminally truncated FlhB and FlhA proteins were purified from IPTG-induced cells of EKK22 harboring pMK104 and pMK103, respectively, as described in Materials and Methods. The purified proteins gave single bands upon SDS-PAGE (data not shown). They were analyzed with a protein sequencer, and N-terminal sequences were read as GNSSXXVP GDLVPGVVTLLTL for FlhA and GIRARXP for FlhB, respectively. Except for the N-terminal methionine residues, these were identical to the predicted N-terminal sequences of the truncated FlhA and FlhB proteins, respectively (Fig. 5). Antibodies against FlhB and FlhA were prepared by using these truncated proteins as immunogens. By use of these antibodies, we carried out Western blotting analysis of the fractionated cell extracts from KK1004 harboring pMK102 or pMK2#5, which carried the intact flhA or flhB genes, respectively.

After the cells of KK1004 harboring pMK102 were disrupted by sonication, cell lysates were fractionated into cytoplasmic and membrane fractions by ultracentrifugation. In the Western blotting analysis of these fractions with anti-FlhA antibody, FlhA was detected in the membrane fraction as well as the cytoplasmic fraction (Fig. 6A, lanes 2 and 3). Then, the membrane fraction was washed with 1 M NaCl and separated by ultracentrifugation into the pellet and supernatant fractions.
FlhA protein was detected exclusively in the pellet (Fig. 6A, lanes 4 and 5). Aliquots of the membrane fraction were treated with 1.2% Sarkosyl and separated by ultracentrifugation into the pellet and supernatant fractions. FlhA protein was detected exclusively in the supernatant (Fig. 6A, lanes 6 and 7). Because the integral membrane proteins are known to be solubilized by Sarkosyl treatment but not by salt treatment (8, 37), these results clearly indicated that FlhA is an integral cytoplasmic membrane protein. This is consistent with the feature of the predicted primary sequence of the FlhA protein.

In KK1004 harboring pMK2#5, FlhB was also detected in the membrane fraction (Fig. 6B, lanes 2 and 3). A salt wash of the membrane fraction did not solubilize FlhB (Fig. 6B, lanes 4 and 5), suggesting that FlhB may be integral to the cytoplasmic membrane. Sarkosyl treatment solubilized only a small amount of FlhB (Fig. 6B, lanes 6 and 7). This suggests that FlhB may form some supramolecular structure within the membrane.

The studies on the assembly process of flagellar structure have shown that FlhA and FlhB may participate in this process after the MS ring assembly which occurs within the cytoplasmic membrane (17, 36). Because it is reasonable to assume that the flagellum-specific transport apparatus may associate with the flagellar basal structures, we suspect that FlhA and FlhB may integrate into the cytoplasmic membrane and constitute the flagellum-specific transport apparatus in contact with the MS ring. However, because neither FlhA nor FlhB has been detected in the purified flagellar structures (17), they may be in a labile association with the MS ring.

ACKNOWLEDGMENTS

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