The Salmonella FlgA protein, a putative periplasmic chaperone essential for flagellar P ring formation

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INTRODUCTION

Many species of bacteria swim using flagella, which extend from the cytoplasmic face of the cell envelope into the external space. The individual flagellum consists of three contiguous substructures: a basal body, a hook and a filament. The basal body is embedded in the cell envelope and acts as a part of the rotary motor. Rotation generated in the basal body is transmitted via the flexible hook to the helical filament, which acts as a propeller to push a bacterial cell. The basal body of Gram-negative bacteria, such as Salmonella and Escherichia coli, consists of a central rod and three coaxial ring structures, named MS, P and L rings (DePamphilis & Adler, 1971a; Ueno et al., 1992). The MS and L rings lie within the cytoplasmic and outer membranes, respectively, while the rod fully traverses both membranes. The P ring lies between the MS and L rings and is believed to link to the peptidoglycan layer (DePamphilis & Adler, 1971b). The P ring forms together with the L ring a cylindrical architecture, which is believed to act as a molecular bushing of the rotary axial structure of the flagellum. This L–P ring complex shows remarkable stability against chemical and physical treatments (Akiba et al., 1991).

Flagellar structure is assembled through a highly ordered process, which begins with construction of the MS ring–proximal rod structure consisting of FliF (Ueno et al., 1992). The switch complex and flagellum-specific export apparatus are then mounted on the cytoplasmic face of the MS ring (Kubori et al., 1997). The remaining processes follow through two different export pathways of the component proteins: one is the flagellum-specific type III export pathway through a hollow channel in the nascent flagellar structure, and the other is the Sec secretory pathway involving cleavage of N-terminal signal sequences of respective pre-proteins. Among the component proteins of the periplasmic substructures, the rod proteins (FlgB, FlgC, FlgF and FlgG) are exported via the flagellum-specific pathway (Homma et al., 1990), whereas the P- and L-ring proteins (FlgI and FlgH, respectively) are exported via the Sec secretory pathway (Homma et al., 1987; Jones et al., 1987, 1989).
After being secreted, the four rod proteins assemble into a distal rod structure onto the proximal rod to construct the MS ring–rod complex (Kubori et al., 1992). After secretion and removal of the signal sequences, FlgI and FlgH assemble around the rod to form the P and L rings, respectively. The hook and filament then sequentially assemble onto the rod tip of the completed basal body structure.

In addition to the component proteins, several factors are known to be required for formation of the periplasmic substructures. Formation of the distal rod requires FlgJ, which is exported to the periplasmic space via the flagellum-specific pathway and hydrolyses the peptidoglycan just adjacent to the MS ring to allow the rod to penetrate the peptidoglycan layer (Nambu et al., 1999). Formation of the P ring requires the Dsb system, which is involved in intramolecular disulfide bond formation in the periplasm (Dailey & Berg, 1993). Furthermore, P ring formation also requires the function of one additional flagellar gene, flgA (Suzuki et al., 1978). We showed previously that the flgA gene can encode a protein of 219 amino acids possessing a potential signal sequence of 21 amino acids at its N-terminus (Kutsukake et al., 1994). This suggests that FlgA may function in the periplasm where P ring formation takes place. However, neither its molecular nature nor its function in P ring formation has been characterized yet. This study was carried out to address this issue. Here, we report several lines of evidence suggesting that FlgA is a periplasmic chaperone essential for P ring formation in Salmonella.

**METHODS**

**Bacterial strains, media and motility assay.** Bacterial strains used in the present study are listed in Table 1. L broth, L agar, minimal medium and motility agar plates were prepared as...
were detected as formation of spreading colonies (swarm) on Amersham-Pharmacia or Hokkaido System Science. Their oligonucleotides used for primers were purchased from Elmer (Perkin Elmer) according to the manufacturer’s instructions. Synthetic DNA ligase and the transformation technique were as described in vitro. Plasmids used are listed in Table 1. The Procedures for manipulation of DNA and DNA manipulation. Oligonucleotide primers used are listed in Table 2.

**Table 2. Oligonucleotide primers used**

<table>
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<th>Primer</th>
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<tr>
<td>PGA1</td>
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<td>PGA2</td>
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</tr>
<tr>
<td>PGA3</td>
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<td>PGI3</td>
<td>GCTGCCATGGCAGGATGTAAGC</td>
</tr>
<tr>
<td>P17b</td>
<td>CCGCTCAGAATTTCATTTTGTAAACTT</td>
</tr>
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described previously (Kutsukake, 1997). Unless otherwise specified, ampicillin and chloramphenicol were used at a final concentration of 50 µg ml⁻¹. Motility phenotypes of the cells were detected as formation of spreading colonies (swarm) on motility agar plates.

**DNA manipulation.** Procedures for manipulation of DNA in vitro and the transformation technique were as described previously (Nambu et al., 1999). Restriction enzymes, T4 DNA ligase and Tag DNA polymerase were purchased from Toyobo, Nippon Gene and Promega, respectively. PCR was carried out using a DNA Thermal Cycler model 480 (Perkin Elmer) according to the manufacturer’s instructions. Synthetic oligonucleotides used for primers were purchased from Amersham-Pharmacia or Hokkaido System Science. Their sequences are summarized in Table 2.

**Plasmid construction.** Plasmids used are listed in Table 1. The flgA gene on pKK1417 was PCR-amplified with primers PGA1 and PGA2, digested with EcoRI and BamHI and inserted into the EcoRI–BamHI site of pTrc99B to obtain pTN601. The flgA gene on pKK1417 was PCR-amplified with primers PGA3 and PGA2, digested with Ndel and BamHI and inserted into the Ndel–BamHI site of pET19b and pET-FLAG-19b to obtain pTN603 and pTN607, respectively. A 0.6 kb Ndel–BamHI fragment containing the flgA gene was excised from pTN603 and inserted into the corresponding site of pET17b to obtain pTN603b. To obtain pTN609, the flgA gene with its upstream region on pTN603b was PCR-amplified with primers P17b and PGA2, digested with EcoRI and BamHI and inserted into the corresponding site of pTrc99A, whose Ndel and NcoI sites had been eliminated by filling-in reaction with Klenow enzyme following digestion with Ndel and NcoI, respectively. A 0.1 kb Ndel–BamHI fragment was excised from pET22b(+) and inserted into the corresponding site of pTN609 to obtain pTN609a. A 0.7 kb NcoI–BamHI fragment was excised from pTN603 and inserted into the corresponding site of pTN609c to obtain pTN610. The flgI gene on pKK1407 was PCR-amplified with primers PGI1 and PGI2, digested with Ndel and BamHI and inserted into the Ndel–BamHI site of pET19b and pET-FLAG-19b to obtain pTN701 and pTN705, respectively. The flgI gene on pKK1407 was PCR-amplified with primers PGI3 and PGI2, digested with EcoRI and BamHI and inserted into the EcoRI–BamHI site of pTrc99C to obtain pTN702. The flgA gene on pKK1417 was PCR-amplified using primers PGA4 and PGA5, digested with BamHI and HindIII and inserted into the corresponding site of pTN702 to obtain pTN703.

**Identification of gene products.** Maxicell labelling was carried out using E. coli JM109 by the method of Sancar et al. (1979) with slight modifications. An overnight L broth culture of JM109 harbouring an appropriate plasmid was diluted 100 times into 10 ml minimal-glucose medium containing 1% Casamino acids and 40 µg ampicillin ml⁻¹ and grown to mid-exponential phase at 37 °C. Cells were harvested by centrifugation and suspended in 10 ml minimal-glucose medium without Casamino acids. The cell suspension was placed in a Petri dish and irradiated with a UV light (254 nm) for 30 s with gentle shaking. After irradiation, the cell suspension was transferred into a 100 ml flask wrapped in aluminium foil and incubated for 1 h at 37 °C. β-Cyclodextrine (Wako), a β-alanine analogue, was then added to a final concentration of 100 µM and incubation was continued for a further 10 h. Maxicells were collected by centrifugation and suspended in 10 ml of a labelling medium [mixture of equal volumes of methionine assay medium (Difco) and minimal-glucose medium]. An aliquot (100 µl) of the maxicell sample was mixed with 1 mM IPTG for 20 min at 37 °C. The maxicells were then labelled with 1.85 MBq [³⁵S]methionine (Institute of Isotopes of the Hungarian Academy of Sciences) for 3 min, followed by chase for another 5 min after addition of 5 µl 100 mM non-labelled methionine. The chase reaction was stopped by adding an equal volume of ice-cold 10% trichloroacetic acid (TCA) and holding on ice for 30 min. The maxicells were collected, washed with 5% TCA and suspended in 50 µl of a Tris-base-saturated sample loading solution (100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol). The samples were heated for 3 min at 100 °C and separated by electrophoresis in SDS-15% polyacrylamide gels. After electrophoresis, the gels were placed in contact with an imaging plate (Fuji) for recording radioactive bands, which were then visualized by a BAS2000 system (Fuji).

**Stability assay of the FlgA and FlgI proteins.** Maxicells were prepared from SJ10044A harbouring pTN601, pTN702 or pTN703 as described above and labelled for 1 min in the presence of [³⁵S]methionine. After 5, 15 or 30 min chase in the presence of excess amount of non-labelled methionine, the reaction was stopped and processed as described above.

**Purification of the His-tagged and His-FLAG-tagged proteins.** Procedures for overexpression and purification of His-tagged and His-FLAG-tagged proteins with a Ni²⁺-affinity resin were performed as described previously (Nambu et al., 1999). Purified proteins, which had been denatured with urea, were renatured according to the method described by Shimamoto et al. (1995).

**Western and Far-Western blot analyses.** Detection of the cellular FlgI protein was performed by Western blot analysis using a rabbit polyclonal antibody against FlgI according to the method described previously (Nambu et al., 1999). Interaction between two proteins was analysed by the Far-Western blotting procedure. The whole-cell lysates containing proteins to be analysed were separated by SDS-PAGE and subjected to electrotransfer onto a nitrocellulose membrane. Binding reaction was then performed in TBS-Tween (24 mg Tris base ml⁻³, 8.0 mg sodium chloride ml⁻³, 1% Tween 20, pH 7.6) containing one of the His-FLAG-tagged proteins (10 µg ml⁻³) for 10 h at room temperature. When required, an equal amount of the His-FlgA protein was also included in the binding reaction. The membranes were then washed three times with TBS-Tween. To detect the FLAG-tagged protein, the membrane was probed with anti-FLAG M2 monoclonal antibody (Eastman Kodak), which was then visualized with an ECL immunoblotting detection kit (Amersham-Pharmacia) according to the manufacturer’s instructions.
RESULTS

FlgA functions in the periplasmic space

In order to show that FlgA is synthesized as a precursor form possessing a cleavable signal sequence, we attempted to identify the flgA gene product. For this purpose, we used pTN601, which carries an entire coding region of the flgA gene transcriptionally fused to the IPTG-inducible trc promoter (Fig. 1a). The maxicells prepared from JM109 harbouring pTN601 synthesized a protein with an approximate molecular mass of 21 kDa upon induction with 1 mM IPTG (Fig. 1b, lane
4). When the maxicells were treated with 0–1 μM [35S]methionine and chased for 5, 15 or 30 min in the presence of an excess amount of non-labelled methionine. Arrows indicate the positions of the mature forms of FlgA and FlgI. X is a band of an unknown protein.

![Fig. 3. Affinity blots of the FlgA and FlgI proteins. A 20 μl volume of the mid-exponential culture of IPTG-induced E. coli HMS174(DE3)/pLysS harbouring pTN603 (lane 1) or pTN701 (lane 2) was separated by SDS-PAGE, and the gel was stained with 0.25% Coomassie brilliant blue (CBB). Proteins in the same gel were electro-transferred onto a nitrocellulose membrane, which was then soaked in the presence of purified His-FLAG–FlgA (lanes 3 and 4), His-FLAG–FlgI (lanes 5 and 6) or His-FLAG–FlgI and His-FlgA (lanes 7 and 8). The FLAG-tagged proteins were detected immunologically. Positions of the His-FlgI and His-FlgA proteins are indicated on the right. Positions of molecular mass markers are indicated on the left.](image)

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>flgA</th>
<th>flgL</th>
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<tbody>
<tr>
<td>pTN601</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>pTN703</td>
<td>+</td>
<td>+</td>
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<tr>
<td>pTN703</td>
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![Fig. 4. Stability of the mature forms of FlgA and FlgI. Maxicells prepared from SJ10044A harbouring pTN601, pTN702 or pTN703 were labelled for 1 min in the presence of [35S]methionine and chased for 5, 15 or 30 min in the presence of an excess amount of non-labelled methionine. Arrows indicate the positions of the mature forms of FlgA and FlgI. X is a band of an unknown protein.](image)

In order to examine whether FlgA functions in the periplasm, we constructed two plasmids, pTN609 and pTN610 (Fig. 1a). pTN609 encodes the mature form of FlgA without the signal sequence, while pTN610 encodes the mature form of FlgA fused to a heterologous signal sequence from PelB, a typical periplasmic protein (Lei et al., 1987). As in the case of pTN601, introduction of pTN610 could restore motility to the cells of a flgA mutant, KK1302, whereas pTN609 could not (Fig. 1c). This result indicates that the presence of a signal sequence, but not the amino acid sequence of the N-terminal region, is critical for FlgA to function in vivo. Therefore, we conclude that FlgA functions in the periplasmic space where P ring formation takes place.

Overproduction of FlgI suppresses flgA mutations

Though the component proteins of flagellum have been intensively investigated (Aizawa et al., 1985; Jones et al., 1987; Jones & Macnab, 1990; Kubori et al., 1992), FlgA has not been detected in the purified flagellar structures. This suggests that FlgA is not a component of the P ring but has a logistic role in P ring assembly. If so, there is a possibility that overproduction of FlgI may suppress defects in the flgA gene. In order to test this possibility, we constructed a plasmid, pTN702, which carries the entire flgI gene under the trc promoter on pTrc99C, and examined the ability of this plasmid to recover motility to three flgA mutants (Fig. 2a). All the flgA mutants harbouring pTrc99C formed compact colonies on motility agar plates. However, though prolonged incubation was required, those harbouring pTN702 formed swarms even in the absence of IPTG, and the size of these swarms increased upon induction with increasing amounts of IPTG. Because the cellular levels of the FlgI protein also increased under these conditions (Fig. 2b), this result indicates that, when an excess amount of the FlgI protein is supplied, Salmonella cells can produce functional flagella in the absence of FlgA. Flagellar structures were prepared from one of the flgA mutants, KK1302, harbouring pTN702 by the method of Suzuki et al. (1978) and inspected by electron microscopy. They possessed P rings and their overall structure was indistinguishable from that of the wild-type flagella (data not shown). Therefore, we conclude that FlgA is not a component of the P ring but exerts an auxiliary role in P ring assembly.
FlgA interacts with FlgI and FlgA

Next, we attempted to show the physical interaction between the FlgA and FlgI proteins by the Far-Western blotting method. For this purpose, we constructed two plasmids, pTN607 and pTN705, which specify the mature forms of FlgA and FlgI, respectively, fused to the His$_{16}$ and FLAG tags at their N-termini. In this paper, these hybrid proteins are called His-FLAG–FlgA and His-FLAG–FlgI, respectively. These proteins were affinity-purified from $E$. coli cells of HMS174(DE3)/pLysS harbouring these plasmids. We also constructed another two plasmids, pTN603 and pTN701, which specify the mature forms of FlgA and FlgI, respectively, N-terminally fused to the His$_{16}$ tag lacking the FLAG tag. These hybrid proteins are called His–FlgA and His–FlgI, respectively. Whole-cell proteins synthesized by HMS174(DE3)/pLysS harbouring pTN603 or pTN701 were separated by SDS-PAGE. The proteins in the gel were transferred onto a nitrocellulose membrane and probed with either purified His-FLAG–FlgA or His-FLAG–FlgI protein, which was then visualized with anti-FLAG antibody. It was found that His-FLAG–FlgA specifically bound to both His–FlgA and His–FlgI (Fig. 3, lanes 3 and 4), while His-FLAG–FlgI specifically bound to His–FlgA but not to His–FlgI (Fig. 3, lanes 5 and 6).

Though the FlgI proteins must interact with each other in the P ring structure, we could not detect any interaction in vitro between His-FLAG–FlgI and His–FlgI in the Far-Western blotting method. This suggests that FlgA is required for establishment of a stable interaction between the FlgI proteins. Therefore, next we examined the interaction between His-FLAG–FlgI and His–FlgI in the presence of His–FlgA (Fig. 3, lane 8). The anti-FLAG antibody detected a positive band in the position corresponding to His–FlgI, suggesting a direct or indirect FlgI–FlgI interaction in the presence of FlgA.

FlgI affects the stability of FlgA

If FlgA would interact also in vivo with FlgI, FlgA should affect the stability of FlgI or vice versa. In order to assess the stability of FlgI and FlgA, pulse–chase experiments were performed using maxicells harbouring pTN601 or pTN702. In these experiments, a $flhD$ mutant, SJ10044A, was used for maxicell preparation. Because the $flhD$ gene encodes the flagellar master regulator (Kutsukake et al., 1990), the $flhD$ mutant cannot assemble flagella at all. Therefore, using this strain enabled us to assess the stability of flagellar proteins in the absence of flagellar assembly. It was found that the mature form of FlgI was fairly stable during a 30 min chase, whereas that of FlgA was rapidly degraded (Fig. 4). When both proteins were expressed simultaneously from pTN703 in the same cells, FlgA as well as FlgI stably existed during the chase period (Fig. 4). These results indicate that FlgA is relatively labile in the periplasmic space and stabilized in the presence of FlgI. This suggests that FlgA and FlgI interact with each other in the periplasmic space.

DISCUSSION

The P ring is a homopolymeric structure consisting of 26 FlgI monomers assembled around the rod (Jones et al., 1990). The FlgI protein is synthesized as a precursor form and exported into the periplasmic space via the Sec secretory pathway (Homma et al., 1987; Jones et al., 1989), and forms an intramolecular disulfide bond with the aid of the Dsb system (Dailey & Berg, 1993). Because the flagA mutation blocks flagellar assembly at the step of P ring formation (Suzuki et al., 1978), FlgA must be required for P ring formation. In this study, we showed that FlgA functions in the periplasmic space where P ring formation takes place and that the flagA defects can be overcome by overproduction of FlgI. These results indicate that FlgA has an auxiliary role in P ring assembly in the periplasmic space. We showed further that FlgA interacts in vitro with FlgI and promotes direct or indirect FlgI–FlgI interaction. Evidence was also presented suggesting the presence of the FlgA–FlgI interaction in vivo. Because FlgA is not detected in the complete flagellar structure, we would like to propose here a hypothesis that FlgA is a periplasmic chaperone for P ring assembly.

Molecular chaperones are proteins that help other proteins to fold and assemble (Horwich et al., 1999; Eisenberg, 1999). So far, at least two flagellar proteins have been suggested to act as chaperones for
flagellar assembly. One is FliS, which facilitates the export of FlIC, the filament subunit protein (Yokoseki et al., 1995). The other is FlgN, which facilitates the export of hook-associated proteins, FlgK and FlgL, needed for filament assembly (Kutsukake et al., 1994; Fraser et al., 1999). These two putative chaperones are both believed to be cytoplasmic proteins. Therefore, as far as we know, this paper is the first report describing a putative periplasmic chaperone for flagellar assembly. The most intensively studied periplasmic chaperone is PapD, which assists the assembly process of type P pili in uropathogenic strains of E. coli (Hultgren et al., 1996). Proteins belonging to the PapD family have an overall topology of immunoglobulin fold consisting of two globular domains with a deep cleft between them and bind to pilus subunit proteins in the periplasmic space to prevent their premature associations by capping their interactive surfaces. This capping process also facilitates their release from a membrane-tethered state as soluble chaperone–subunit complexes, which are in turn targeted to the outer-membrane usher, PapC, to assemble pili. Because FlgA and the PapD family proteins share no significant homology in primary or predicted secondary structure (data not shown), it is reasonable to imagine that these two types of periplasmic chaperones may employ different molecular mechanisms to assist in supramolecular assembly.

The P ring is believed to be a mechanically very tight structure, because it must act as molecular bushing in the rotary motor (Akiba et al., 1991). Therefore, the FlgI–FlgL interaction in the P ring is expected to be very strong. However, only a very weak interaction was observed between the FlgI proteins in the yeast two-hybrid reporter analysis (Marykwas et al., 1996). Consistent with this, we could not demonstrate FlgI–FlgL binding in the Far-Western blotting experiment (Fig. 3). These results suggest that, unlike pilus subunit proteins, the FlgI proteins do not assemble efficiently by themselves. However, overproduction of FlgI resulted in P ring assembly in the absence of FlgA (Fig. 2), indicating that, when supplied in excess, FlgI can assemble spontaneously in vivo. Because direct or indirect FlgI–FlgL interaction was observed in vitro in the presence of FlgA (Fig. 3), we anticipate that FlgA may facilitate the polymerization reaction of the FlgI proteins through FlgA–FlgL interaction. Our current model for the process of P ring assembly is summarized in Fig. 5. Kubori et al. (1992) showed that P ring formation is not observed in the absence of rod formation. This indicates that P ring assembly occurs only around the pre-existing rod structure. Experiments are now in progress to establish an in vitro system to reconstitute the P ring around the rod structure.

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REFERENCES


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