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Flagellar Formation in C-Ring-Defective Mutants by Overproduction of FliI, the ATPase Specific for Flagellar Type III Secretion[∇]

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The flagellar cytoplasmic ring (C ring), which consists of three proteins, FliG, FliM, and FliN, is located on the cytoplasmic side of the flagellum. The C ring is a multifunctional structure necessary for flagellar protein secretion, torque generation, and switching of the rotational direction of the motor. The deletion of any one of the *fliG*, *fliM*, and *fliN* genes results in a Fla⁻ phenotype. Here, we show that the overproduction of the flagellum-specific ATPase FliI overcomes the inability of basal bodies with partial C-ring structures to produce complete flagella. Flagella made upon FliI overproduction were paralyzed, indicating that an intact C ring is essential for motor function. In FliN- or FliM-deficient mutants, flagellum production was about 10% of the wild-type level, while it was only a few percent in FliG-deficient mutants, suggesting that the size of partial C rings affects the extent of flagellation. For flagella made in C-ring mutants, the hook length varied considerably, with many being markedly shorter or longer than that of the wild type. The broad distribution of hook lengths suggests that defective C rings cannot control the hook length as tightly as the wild type even though FliK and FlhB are both intact.

The flagellum is the ultrastructure for motility in many bacterial species (1). Flagellar assembly requires about 50 genes, among which about 20 gene products are incorporated in the complete flagellum (12). Most structural proteins and others necessary for assembly are exported through a flagellum-specific type III secretion apparatus housed within the basal body. The apparatus consists of at least six integral membrane proteins: FlhA, FlhB, FliP, FliQ, FliR, and FliO (for salmonellae and other species) (1, 12). Other proteins are also involved. FliI is the only known ATPase among flagellar proteins (2). FliI interacts with FliJ, which is of unknown function, and with a dimer of FliH, an inhibitor of FliI. The apparatus can be visualized by quick-freeze electron microscopy and has been termed the C (cytoplasmic) rod by virtue of its appearance and membrane-proximal location inside the C ring (7). The C ring is composed of three component proteins: FliG, FliM, and FliN (3). Mutations or deletions of any of these proteins cause a nonflagellate (Fla⁻) phenotype, strongly suggesting that the C ring is necessary for flagellar protein export (6, 22, 26). The trimer FliH₂-FliI specifically binds FliN (4, 15), suggesting that FliI docks at the periphery of the C ring through interactions with FliN-bound FliH, standing ready to escort export substrates to the secretion gate that is probably composed by FlhA, FlhB, and others (15).

The C ring has long been studied with respect to motor function rather than export function. It has been proposed that FliG plays a major role in torque generation in concert with MotAB complexes, leaving the other two proteins, FliM and FliN, in minor and supporting roles (10, 11). However, as

mentioned above, all three components are required for flagellar protein export (6, 22, 26). Together with the C ring, FliI pushes export substrates into the gate using the energy of ATP hydrolysis. Just recently, it was shown that FliI ATPase activity is not absolutely necessary for protein export and that increasing proton motive force (PMF) or reversion mutations in FlhA and FlhB can compensate for its absence (17, 21).

In order to elucidate the roles that FliG, FliM, and FliN play in export, we employed C-ring-defective mutants. Here, we show that the overproduction of FliI allows flagellar formation in C-ring-defective mutants. We closely examined flagella formed in those mutants by electron microscopy, noting percentages of flagellation in each population, analyzing partially formed structures, and measuring hook length.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study originated from *Salmonella enterica* serovar Typhimurium strain SJW1103 and are listed in Table 1.

Media and standard genetic manipulations. Media, culturing, transductional methods, and motility assays were performed as described previously (18).

Measurement of cell motility. Cells were observed for motility by dark-field microscopy or by phase-contrast microscopy by our conventional method (14).

Electron microscopy. Cells and isolated flagellar structures were stained with 1% phosphotungstic acid (pH 7 or pH 5). The C-ring preparations were stained with 4% uranyl acetate (pH 4). Samples were observed with a JEOL 1200Ex electron microscope at 80 kV.

Measurement of hook length. Hook length is conventionally measured using hooks straightened by acidic pH (5). Because of concerns about low particle numbers resulting in loss during additional staining procedures, curved hooks were measured at neutral pH by using JW_cad, version 5.02a, software (<http://www.jwcad.net/>). Short straight lines running to the center of each hook were drawn and then summed to obtain a value for hook length.

RESULTS AND DISCUSSION

Overproduction of FliI in C-ring-defective mutants. The overproduction of FliI can compensate for the deletion of *fliH*

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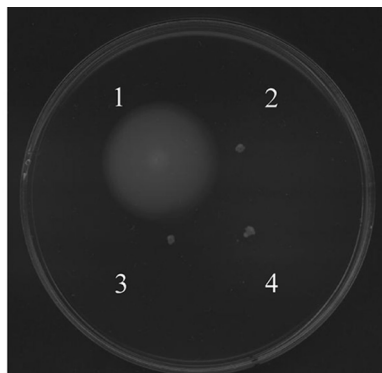
TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Reference
Strains		
SJW1103	Wild type	25
MKM001	$\Delta fliG$ ($\Delta 15-321$) ^a	8
MKM006	$\Delta fliM$ ($\Delta 16-325$)	8
MKM015	$\Delta fliN$ ($\Delta 15-133$)	4
Plasmids		
pTrc99AFF4	Modified pTrc expression vector	20
pMM1702 (<i>pfliI</i>)	pTrc99A His-FliI	16

^a The deletion numbers indicate positions of the amino acid residues deleted.

(17). González-Pedrajo et al. and others (4, 15) previously characterized a specific interaction between FliH and the C-ring protein FliN and demonstrated the formation of a complex between FliN, FliH, and FliI. Our rationale for the experiments described herein is the theory that FliI docks at the membrane-distal end of the C ring via interactions with FliH, which is in turn bound to FliN. FliI would thus stay near the export apparatus in preparation for arriving substrates. Without the C ring, FliI might diffuse away from the basal body, resulting in an inefficient export of proteins. If FliI is overproduced in mutants defective for C-ring proteins, the concentrations of FliI molecules may become high enough to export flagellar proteins without the use of C-ring docking.

We introduced the FliI overproduction plasmid into *fliG*, *fliM*, and *fliN* deletion mutants and observed the swimming ability of transductants on semisolid agar (Fig. 1). The wild-type strain harboring the vector only produced a large swim ring, while the C-ring-defective mutants did not swim but stayed at the inoculation points. As a routine process for the motility assay, we examined cells by dark-field microscopy to determine whether cells had deteriorated motility. There were no motile cells at all in the populations of any C-ring-defective mutants. Since this kind of microscopic observation is sensitive enough to detect one motile cell among thousands of nonmo-



1. SJW1103 + pTrc99AFF4 2. MKM001 + *pfliI*
3. MKM006 + *pfliI* 4. MKM015 + *pfliI*

FIG. 1. Motility assays of C-ring-defective mutants with overproduced FliI on semisolid agar plates. Semisolid agar contains 0.4% agar in LB medium, implemented with ampicillin and/or chloramphenicol. Plates were incubated at 37°C for 8 h. (1) SJW1103 harboring pTrc99AFF4; (2) MKM001 ($\Delta fliG$) harboring *pfliI*; (3) MKM006 ($\Delta fliM$) harboring *pfliI*; (4) MKM015 ($\Delta fliN$) harboring *pfliI*.

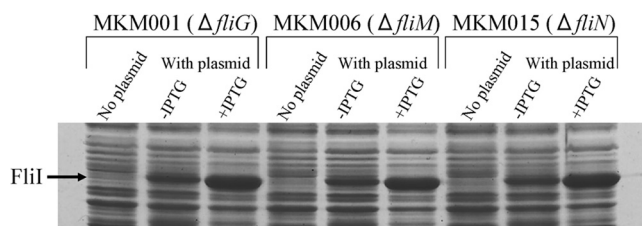


FIG. 2. Sodium dodecyl sulfate-polyacrylamide electrophoresis pattern of FliI in whole-cell extracts from MKM015 ($\Delta fliN$), MKM006 ($\Delta fliM$), and MKM001 ($\Delta fliG$). First lanes, without plasmid; second lanes, with plasmid and in the absence of IPTG; third lanes, with plasmid and in the presence of IPTG.

tile cells, we did not try further motility assays such as the tethered-cell method.

The amounts of overexpressed FliI were similar among deletion mutants, as seen in sodium dodecyl sulfate gels (Fig. 2). There were no visible FliI bands in deletion mutants (Fig. 2, first lanes in three sets) in which a wild-type level of FliI was expressed. FliI bands were detected in deletion mutants with pTrc99A His-FliI (*pfliI*) even in the absence of IPTG (isopropyl- β -D-thiogalactopyranoside) (Fig. 2, second lanes in three sets). In the presence of IPTG, FliI bands were the major ones in the whole-cell extracts (Fig. 2, third lanes in three sets).

Electron microscopic observation of flagella. When observed by electron microscopy, a small number of flagella were found in all strains (Fig. 3A). Most cells do not have flagella (Fig. 3A, left), and some cells have two flagella (center), but the others have only one flagellum (right). Since populations of flagellated cells and the total number of flagella were so low, we had to repeat experiments several times to reach conclusions as follows. In one experiment, we found 7.9% (33 flagella altogether in 417 cells) for MKM015 ($\Delta fliN$) harboring *pfliI*, 12.6% (59/466) for MKM006 ($\Delta fliM$) harboring *pfliI*, and 0.4% (2/453) for MKM001 ($\Delta fliG$) harboring *pfliI*. Some variance was observed in different preparations of the same populations, but the yield of flagella was always about 10% for MKM015 ($\Delta fliN$) harboring *pfliI* and MKM006 ($\Delta fliM$) harboring *pfliI* and was less than 1% for MKM001 ($\Delta fliG$) harboring *pfliI*. Flagella formed in these strains appeared complete and long enough to swim but were likely paralyzed, as no motility was observed by dark-field microscopy, as mentioned above. Therefore, we conclude that the C ring is essential for motility but that its role in flagellar assembly can be replaced by the overproduction of FliI.

Since there were, at most, one or two flagella per cell, we scrutinized the cell surfaces, suspecting that there might be shorter flagella that were overlooked by routine survey. Osmotically shocked cells were produced from MKM015 ($\Delta fliN$) cells harboring *pfliI* as described previously (18). In flagellated cells, besides complete flagella, we observed hook-basal body structures sitting on the membranes (Fig. 3B, first three panels). However, there was no sign of filaments attached distally to hooks in those structures. The absence of filaments became obvious when we isolated flagella by lysing cells with lysozyme. Only complete flagella and hook-basal bodies were observed; there were no short-filament structures present (Fig. 3B, last panel). The hook-basal bodies outnumbered complete flagella, suggesting that there is a time gap between hook completion

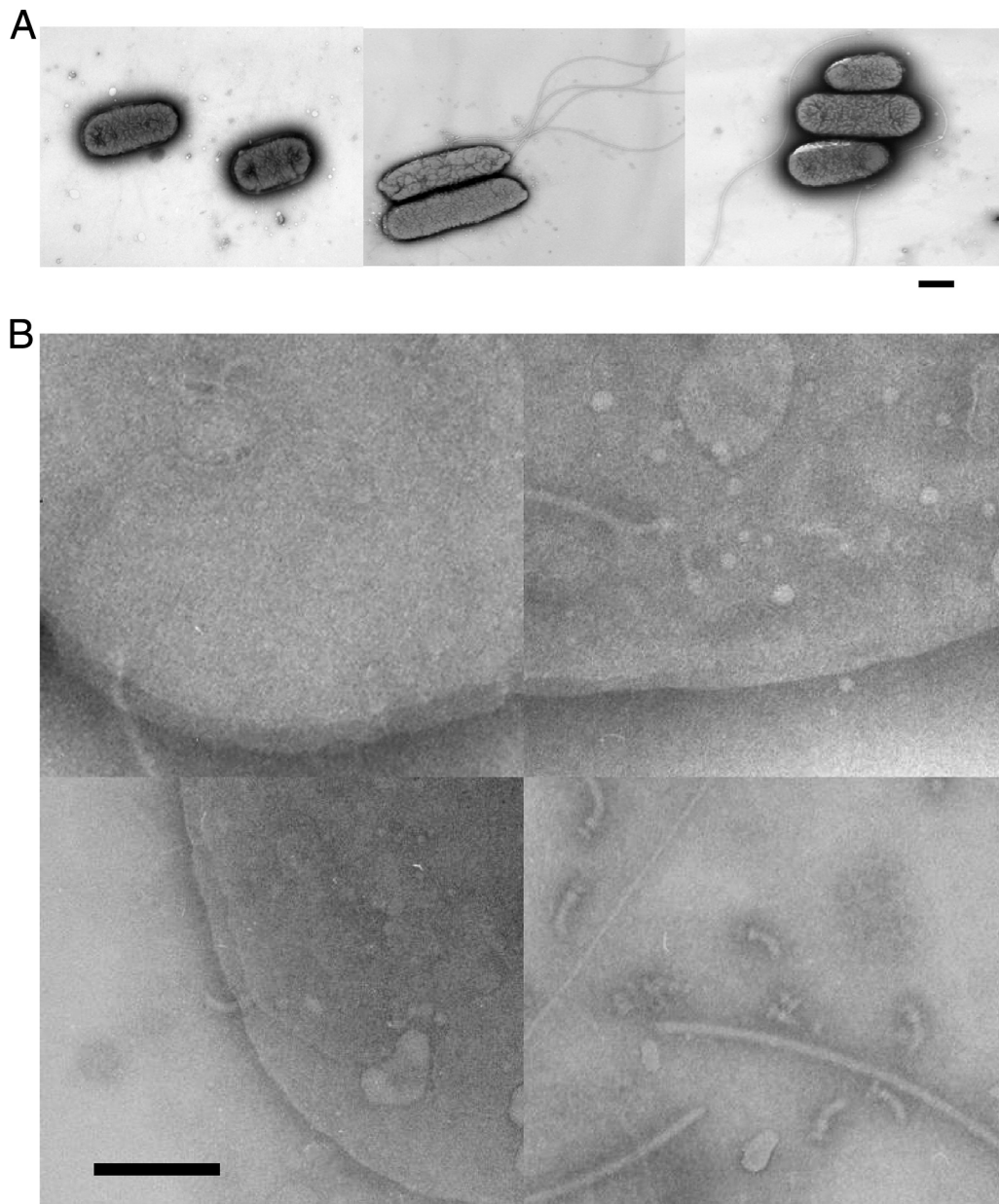


FIG. 3. (A) Electron microscopic images of flagellated cells. (Left) MKM015 ($\Delta fliN$); (center) MKM006 ($\Delta fliM$) harboring *pflil*; (right) MKM015 ($\Delta fliN$) harboring *pflil*. Percentages of flagellation were very low. Flagellated cells had a few flagella, while nonflagellated cells had none including the hook-basal bodies. (B) Osmotically shocked cells show the hook-basal bodies on the membranes (upper and lower left panels). Flagella and hook-basal bodies were isolated from cells of MKM015 ($\Delta fliN$) harboring *pflil* (lower right panel). Bars indicate 200 nm.

and filament initiation in the construction process. This might be caused by deteriorated export rates of the hook proteins, resulting in a slow growth of hooks. An alternative possibility is that the deteriorated function of FliK in the absence of the C ring may result in a failure to switch substrate specificity from the hook-rod mode to the flagellin mode. It should be noted that flagellated cells and nonflagellated cells are clearly separated; there were no hook-basal bodies in nonflagellated cells. It remains unclear why some cells are capable of growing flagella but others are not at all. From these data, we estimate that more than 20% of the population in the C-ring-defective mutants (MKM006 and MKM015)

produced several flagellar basal bodies, and about one-half of those exhibited complete filament formation.

Sizes of defective C-ring structures. When planning the experiments, it was expected that the deletions in the *fliG* (MKM001), *fliM* (MKM006), and *fliN* (MKM015) genes would be large enough to prevent the formation of complete C-ring structures (Table 1). To confirm if this genetic evidence was true, C-ring structures were isolated and observed by electron microscopy (3). In isolation procedures, we grew mutant cells in the absence of IPTG, because cells grown in the presence of IPTG were hard to lyse by conventional methods using ly-

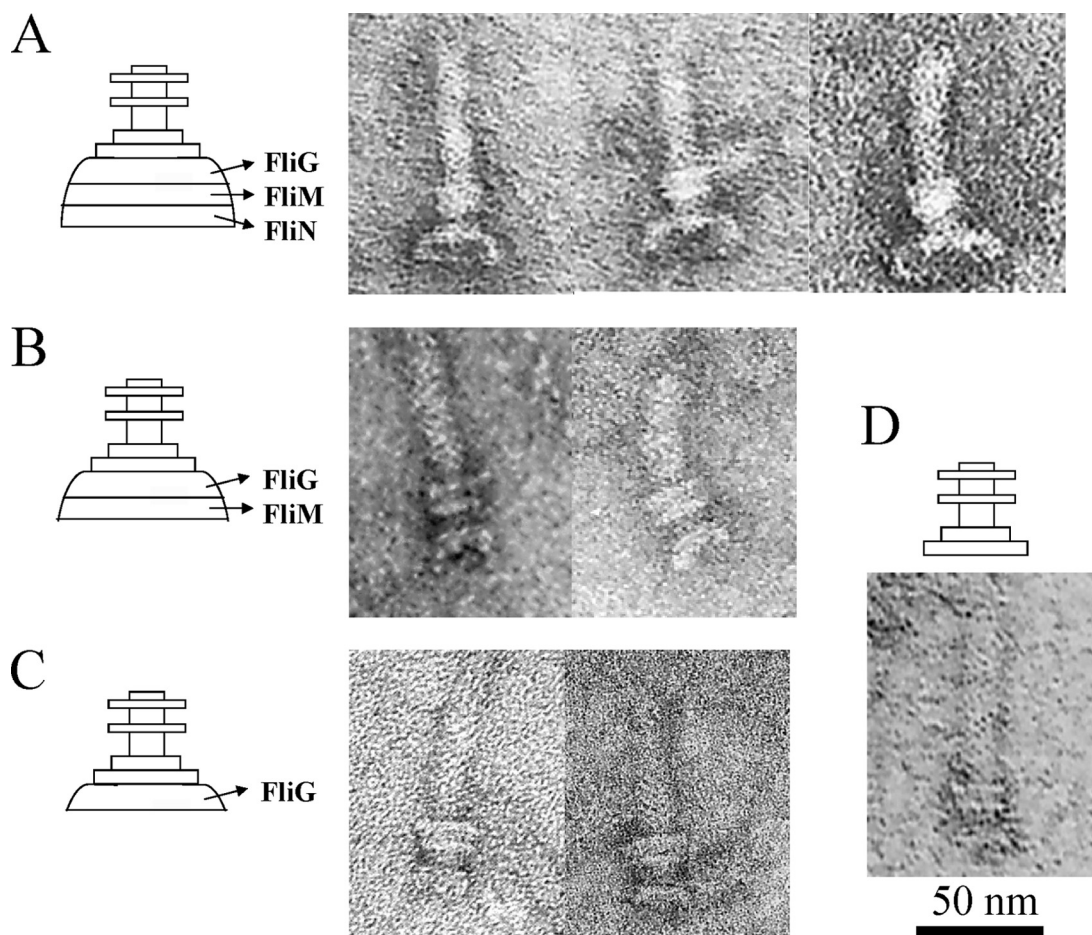


FIG. 4. Electron microscopic images of C rings isolated from C-ring-defective mutants. (A) SJW1103 (wild type) harboring pTrc99AFF4; (B) MKM015 ($\Delta fliN$) harboring *pfliI*; (C) MKM006 ($\Delta fliM$) harboring *pfliI*; (D) MKM001 ($\Delta fliG$) harboring *pfliI*. The bar indicates 50 nm.

sozyme. As described above, FliI was overexpressed from the plasmid even in the absence of IPTG (Fig. 2).

C rings were visualized by staining with 4% uranyl acetate. Since the C ring is a fragile component, we measured the height and width of the largest C rings. There was no clear boundary between the M ring and the C ring. Therefore, we measured the distance from the gap between the S and M rings to the lowest bottom of the C ring as a height of the C ring. The width was measured as the largest distance across the C ring. In the wild-type C-ring control, the average height was 15.4 nm and the average width was 45.8 nm (Fig. 4A) (24). In the C-ring mutants, the height was 9.07 nm and the width was 24.5 nm for MKM015 ($\Delta fliN$) harboring *pfliI* (Fig. 4B), the height and width were 4.30 nm and 22.4 nm, respectively, for MKM006 ($\Delta fliM$) harboring *pfliI* (Fig. 4C), and there were none for MKM001 ($\Delta fliG$) harboring *pfliI* (Fig. 4D). Although the number of particles measured was only a few, the numbers obtained were reasonable, judging from numbers of the wild-type C ring that showed good agreement with the numbers obtained by more sophisticated image analyses of the C ring: 16.5 nm in height and 46.5 to 49.0 nm in width (23). In conclusion, the results strongly suggest that MKM015 ($\Delta fliN$) lost the lowest (FliN) ring and that MKM006 ($\Delta fliM$) lost the lowest (FliN) and middle (FliM) rings, leaving the top (FliG) ring directly

attached the M ring. Thus, the order of the sizes of the incomplete C rings was wild-type > MKM015 ($\Delta fliN$) > MKM006 ($\Delta fliM$) > MKM001 ($\Delta fliG$), as originally expected.

Hook length in C-ring-defective mutant flagella. During observation of the C rings by electron microscopy, an unusually large number of shorter- and longer-than-normal hooks were found (Fig. 5). First, we overexpressed FliI in wild-type strain SJW1103 and measured the hook length. The distribution of the hook length was similar with that of the wild type without plasmid, at 56.1 ± 5.0 nm (Fig. 6A). We then measured hook lengths in complete flagella isolated from all three mutant strains: 63.0 ± 22.5 nm for MKM015 ($\Delta fliN$) harboring *pfliI* (Fig. 6B), 66.1 ± 16.0 nm for MKM006 ($\Delta fliM$) harboring *pfliI* (Fig. 6C), and 61.5 ± 15.2 nm for MKM001 ($\Delta fliG$) harboring *pfliI* (Fig. 6D). We also measured hook length in hook-basal bodies only from MKM015 ($\Delta fliN$) harboring *pfliI*, which averaged 47.0 ± 17.7 nm (Fig. 6E), which was distributed in a shorter region than those of complete flagella (Fig. 6B).

As seen in the length distributions, all the peaks were between 50 and 60 nm, indicating that there might be other mechanisms to measure the amounts of the hook subunits than the measuring-cup model, which we proposed in 2001 (13). This peak is unlikely to be controlled exclusively by FliK, because even in FliK-deficient mutants, the peak of the length

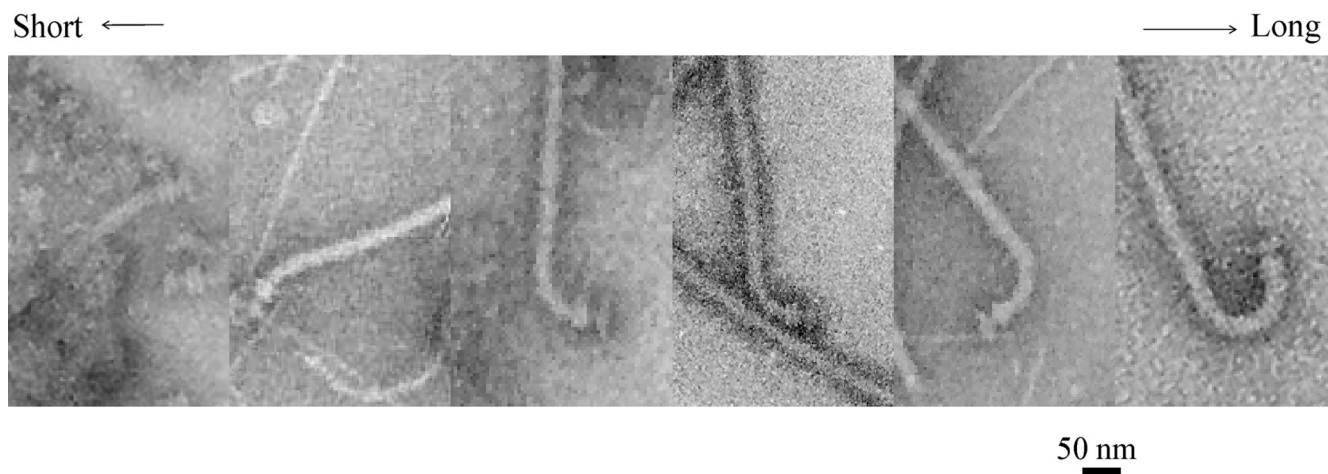


FIG. 5. Electron microscopic images of the hooks of flagella isolated from C-ring-defective mutants. Particles were arbitrarily chosen to show long and short hooks. The bar indicates 50 nm.

distribution is around 50 to 60 nm (9). On the other hand, the distributions of hook length in C-ring mutants with overproduced FliI were widely spread from 10 nm to more than 100 nm in the presence of intact FliK (Fig. 6B), which cannot be explained by the ruler model alone (19). These data suggest that the C ring is necessary for the tight control of the hook length but not for determinations of the hook length itself. Since it is likely that the disruption of the FliN-FliH interaction affects the efficiency of export, one tantalizing possibility is that the rate of hook export affects the ultimate length of the hook in concert with the role of the FliK-FliB interaction.

The present results show that C-ring mutants with overproduced FliI allow flagellar export as evidenced by hook-basal body and filament structures present at fractions of wild-type levels in the *fliN* deletion mutant. The flagella produced under

these conditions are paralyzed, as indicated by a lack of motile cells when viewed by dark-field microscopy. Thus, it can be concluded that the entire C ring is necessary for torque generation sufficient for motility and that it plays an integral but not insurmountable role in flagellar export, likely by supporting the localization of FliH-FliI complexes to the basal body. FliN, which resides at the bottom of the C ring and binds FliH, seems to be necessary for export. *fliM* and *fliG* mutants would likely not allow for the integration of FliN into the C ring, which explains the export defects of those mutants even though FliG and FliM exhibit no affinity for FliH (4). Much like FliH, the absence of FliN from the C rings of mutants described herein can be overcome by increased levels of FliI, effectively bypassing the proposed FliN-FliH-FliI complex (15). Flagella resulting from FliI overproduction exhibit broad distributions

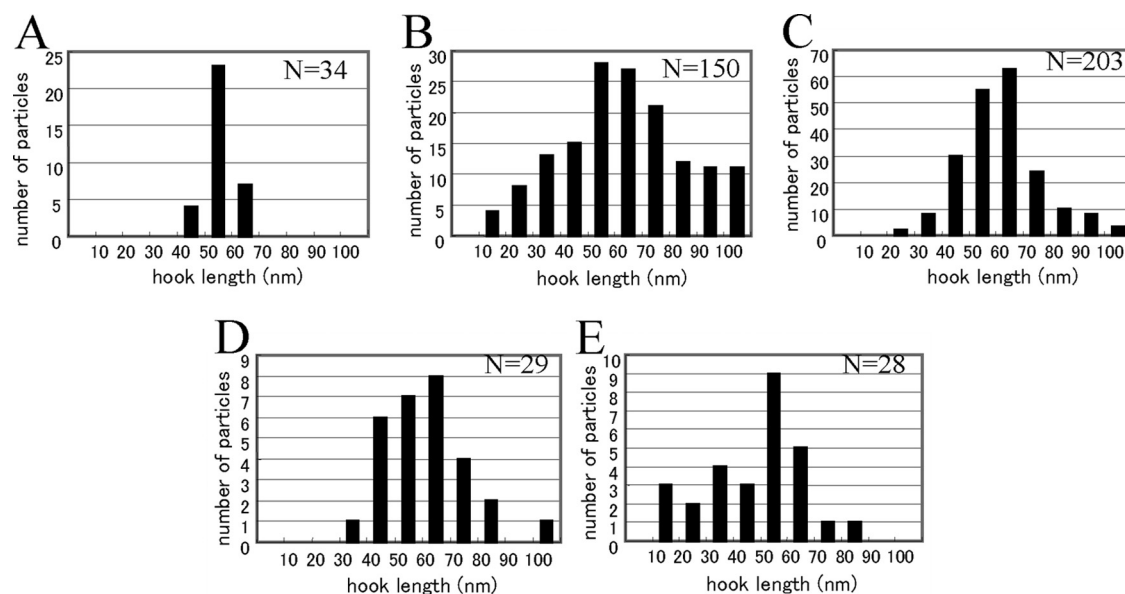


FIG. 6. Histograms of hook lengths of C-ring-defective mutants. (A) SJW1103 harboring pTrc99AFF4; (B) MKM015 ($\Delta fliN$) harboring *pfliI*; (C) MKM006 ($\Delta fliM$) harboring *pfliI*; (D) MKM001 ($\Delta fliG$) harboring *pfliI*; (E) hook-basal bodies only from cells of MKM015 ($\Delta fliN$) harboring *pfliI*.

of hook length, suggesting that the C ring is required for the tight control of hook length, perhaps by allowing the efficient export of proteins via FliN acting as a docking station for the soluble export proteins. The dynamic relationships among C ring, export apparatus, and substrate proteins indicate that export, substrate specificity, and hook length are governed by multiple factors. A complete understanding of hook length control and substrate specificity switching is sure to be more complex than current models (11, 21).

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