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Hook-Length Control of the Export-Switching Machinery Involves a Double-Locked Gate in *Salmonella typhimurium* Flagellar Morphogenesis

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During flagellar morphogenesis in *Salmonella typhimurium*, the genes involved in filament assembly are expressed fully only after completion of hook-basal body assembly. This coupling of gene expression to morphogenesis is achieved by exporting the flagellum-specific anti-sigma factor, FlgM, out of the cell through the mature hook-basal body structure. Therefore, the flagellum-specific export apparatus must be able to sense the assembly state of the flagellar structure and to turn on FlgM export at a specific stage of hook assembly. It has been suggested that FlhB may act as the molecular switch which mediates this ordered export. Here, I report genetic evidence that in addition to FlhB, the product of a newly identified gene, *rflH*, is involved in the negative regulation of FlgM export. FlgM is released through the basal body structure lacking the hook and the filament only when the *flhB* and *rflH* genes are both defective. Therefore, the export gate for FlgM should be double locked by FlhB and RflH. The *rflH* gene is located at around 52 min, where no flagellum-related gene has been found. I propose a revised model of the export-switching machinery which consists of two systems, the hook-length signal transduction pathway and the double-locked gate for FlgM export.

Salmonella typhimurium and *Escherichia coli* cells have 5 to 10 flagella, which are responsible for motility. An individual flagellum consists of three substructures: a basal body, a hook, and a filament. The filament extends into the extracellular space and is connected by the hook to the basal body. The basal body consists of an inner ring (MS ring), two outer rings (L and P rings), and a rod. The rod crosses the inner membrane, the periplasmic space, and the outer membrane. Flagellar assembly begins with the basal body, proceeds through the hook, and is completed by the filament. Assembly of extracellular structures such as the filament and the hook is believed to involve transport of the component proteins through a central channel residing within the growing flagellar structure. It has been postulated that a flagellum-specific export apparatus should exist at the cytoplasmic face of the basal body of each flagellum (24).

More than 50 genes are required for flagellar formation and function. These flagellar genes constitute at least 13 different operons, and their expression is highly organized within a regulon in which the flagellar operons are divided into three classes with respect to their relative positions in the expression hierarchy (10, 11, 19, 20). Class 2 operons are positively controlled by FlhD and FlhC, both of which are encoded by class 1 genes (22). Class 3 operons are controlled positively by FliA and negatively by FlgM (2, 16). FliA is a flagellum-specific sigma factor, whereas FlgM is an anti-sigma factor specific for FliA (5, 17, 27, 28).

The most remarkable feature of the flagellar regulon is the coupling of the sequential expression of the flagellar operons to the assembly process of the flagellar structure (19). All of the genes involved in formation of the hook-basal body structure belong to class 2, and those involved in filament formation and flagellar function belong to class 3. Mutations in any one of the hook-basal body genes not only inhibit normal assembly of the hook-basal body structure but also prevent the transcription of class 3 operons. This coupling is achieved by exporting FlgM out of the cell through the hook-basal body structure formed by the products of class 2 genes (4, 13). Strains containing a *flgM* null mutation express class 3 operons even in the absence of the hook-basal body structure (2, 16).

Wild-type cells have hooks with a relatively defined length of ca. 55 nm (3). Therefore, each flagellum should have a mechanism to monitor the state of its hook assembly (18, 23, 33). When the hook reaches its mature length, this system should signal to the flagellum-specific export apparatus to shut off export of hook protein and to initiate export of FlgM (Fig. 1). Removal of FlgM from the cytoplasm leads to expression of class 3 operons, and the proteins required for filament formation, FlgK, FlgL, FliD, and FliC, are then synthesized and exported through the hook-basal body structure. The proteins which are exported after completion of hook assembly are called late proteins.

Cells containing *fliK* mutations produce abnormally elongated hooks, called polyhooks, which lack a filament (30, 34). These mutants cannot export FlgM through the flagellar structures, and so inhibition of class 3 transcription is never lifted (13, 18). Therefore, FliK has the dual function of determining the length of the hook and of facilitating FlgM export. In a previous report (18), we showed that *fliK* mutants possessing specific alleles of the *flhB* gene can assemble filaments onto polyhooks (Fig. 1). We proposed that each flagellum may possess an export-switching machinery in which FlhB negatively regulates FlgM export before hook assembly is completed and FliK interferes with FlhB at a specific stage of hook assembly, resulting in cessation of the export of hook protein and in initiation of FlgM export. Williams et al. (38) recently proposed a similar model based on extensive analysis of *fliK* suppressor mutants.

According to our model (18), the *fliK*-suppressing FlhB pro-

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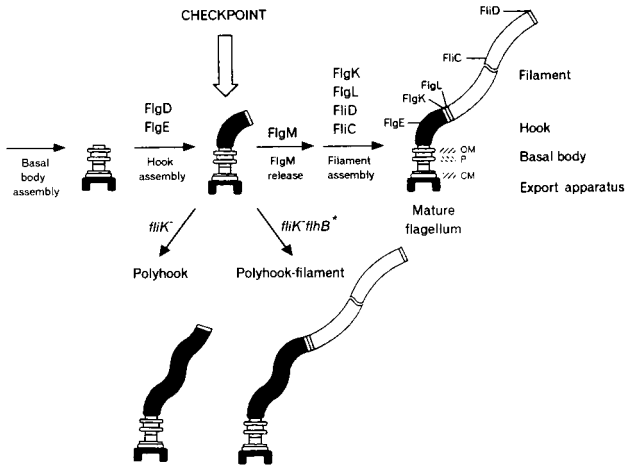

FIG. 1. Assembly pathway of hook and filament structures. The pathway in a wild-type strain is shown horizontally at the top. Proteins listed above the arrow are exported at that assembly step. Proteins exported after completion of hook assembly are called late proteins. The assembly pathways in *fliK* or *fliK flhB* mutants are also shown. OM, outer membrane; P, peptidoglycan layer; CM, cell membrane.

teins should lack the ability to inhibit FlgM export before completion of hook assembly. Therefore, *flhB* mutants might export FlgM through the mature basal body in the absence of the hook assembly. This work was initiated to test this possibility. I found that the basal body structure produced in the *flgE flhB* double mutant is not competent to export FlgM. This finding suggests that there may be an additional factor which responds to completion of hook assembly by turning on FlgM export. I isolated mutants defective in this factor and named its gene *rflH*. I propose a double-locked-gate model for FlgM export by the flagellum-specific export apparatus.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and media. *S. typhimurium* strains used in the present study were constructed by P22-mediated transduction from the strains listed in Table 1. All of the *rflH* mutants were isolated from strain KK1427. The *E. coli* strain used for complementation analysis of the *S. typhimurium rflH* mutations was JA200 (F^+ *trpE5 recA thr-1 leu-6 lacY thi gal xyl ara mtl*) carrying a recombinant ColE1 plasmid, pLC4-15 (26). P22 phages used for transduction and for Tn*10* mutagenesis were P22HT*int* and P22(*12*amN11 *13*amH101 *c2*ts29 *int3* Tn*10*), respectively (17).

L broth contained 10 g of Peptone S (Kyokuto, Tokyo, Japan), 5 g of yeast extract (Kyokuto), and 5 g of NaCl per liter of distilled water. L agar plates were prepared by adding 1.2% agar (Shoei, Tokyo, Japan) to L broth. Motility agar plates contained 15 g of Peptone S, 5 g of NaCl, and 2.5 g of agar per liter of distilled water. Other media, including P22 broth used for preparation of P22 transducing lysates, agar plates for selection of loss of tetracycline resistance, minimal medium, minimal agar plates, minimal lactose plates, and MacConkey lactose plates, were prepared as described previously (17, 18, 20, 21). Ampicillin, kanamycin, and tetracycline were used at final concentrations of 50, 25, and 20 μg/ml, respectively.

Genetic techniques. Transduction was performed with P22HT*int* as described previously (20). Mutant alleles of the *flg* genes were introduced into various strains by means of linked transduction with the *pyrC* gene. The *motA-lac* transcriptional fusion was introduced from KK1106 into other strains via P22-mediated transduction by selecting for ampicillin resistance (16). A strain carrying Tn*10* inserted near the *rflH* gene was constructed as described previously (17). The detailed procedure is described in Results. Complementation analysis of the *rflH* mutations was achieved by construction of merodiploids, using plasmid pLC4-15. This plasmid was introduced into the *rflH* mutant cells from JA200 by F-assisted conjugational transfer of a ColE1 plasmid.

β-Galactosidase assay. The Lac phenotype was tested by examining the colony color on MacConkey agar plates. β-Galactosidase activity was assayed by the method of Platt et al. (31), using cells grown in minimal medium containing 0.3% Casamino Acids (Nippon Pharmaceutical, Tokyo, Japan) and 0.2% glycerol, as described previously (19). The enzyme activities (in Miller units) reported here are the averages of at least three independent assays.

Export assay. Cells were grown at 37°C overnight in minimal medium containing 0.3% Casamino Acids and 0.2% glycerol. One milliliter of the culture was clarified by centrifugation, and the supernatant was passed through a 0.45-μm-pore-size membrane filter (Nippon Millipore, Tokyo, Japan) to remove contaminating cells (13). Proteins in the resulting cell-free supernatant were precipitated with 10% trichloroacetic acid and resuspended in sample loading buffer containing saturated Tris base as described previously (5, 39). Samples were heated at 100°C for 3 min and separated on sodium dodecyl sulfate (SDS)–18% polyacrylamide gels. For visualization of protein bands, gels were stained with 0.25% Coomassie brilliant blue R250. FlgM and FliC in the gel were detected by Western blotting using polyclonal antibodies as described previously (13).

Motility assay. Motility phenotypes of cells were detected as formation of spreading colonies (swarms) on motility agar plates at 30 or 37°C. Motility in liquid medium was observed with an Olympus BH-2 light microscope equipped with a dark-field condenser.

RESULTS

Expression of the *motA-lac* fusion in *flhB* mutants. FlhB contains 383 amino acids (25). The *fliK*-suppressing alleles used here were *flhB9001* and *flhB9002*, which cause an amino acid substitution (Ala to Thr) at position 298 and a premature termination at position 353 of the FlhB protein, respectively (18). I examined FlgM export in various hook-basal body mutants possessing these *flhB* mutations. I assessed FlgM export by monitoring the expression of one of the class 3 operons, *motA*, because the expression level of class 3 operons has been shown to be correlated with the efficiency of FlgM export (4, 13, 39). As expected, in the *fliK* mutant, the *flhB* mutations caused a three- or six-fold increase in *motA* transcription (Table 2).

The *fliF* gene encodes the component protein of the MS ring (37), and a strain with a mutation in this gene produces no detectable flagellar structure (12, 36). Therefore, this mutant lacks any flagellar structures that cross the bacterial membrane layers, and thus is possesses no export channel for late proteins. As expected, in this mutant the *flhB* mutations did not cause any increase in *motA* expression (Table 2).

The *flgE* gene encodes the hook protein (21), and a strain with a mutation in this gene produces the mature basal body lacking the hook and filament portions (36). I expected that this basal body structure might act as the export channel for FlgM. If the FlgM gate is always opened in the *flhB* mutant background, FlgM should be exported through the basal body, resulting in expression of the *motA* operon in the *flgE* mutant. However, the *flhB* mutations did not cause any increase in *motA* expression in this mutant (Table 2). This result suggests that the *flhB* mutations may cause FlgM release through the

TABLE 1. *S. typhimurium* strains used in this study

Strain	Relevant characteristics	Reference or source
KK1004	Wild type	20
KK1106	KK1004 *motA-lac* (Ap^r)	16
KK1140	KK1004 *flhB9001*	18
KK1143	KK1004 *flhB9002*	18
KK1326	KK1004 *flgD1155*	Laboratory stock
KK1327	KK1004 *flgE2004*	Laboratory stock
KK1427	KK1004 *flhB9002 flgE2004 motA-lac*	This study
KK2092	KK1004 *fliF*::Tn*10*	20
KK2143	KK1004 *fliK*::Tn*10*	20
KK2201	KK1004 *pyrC*::Tn*10*	Laboratory stock
KK2668	KK1004 *cheZ*::Tn*10*	20
PP1228	LT2 *pstI421*::Tn*10*	SGSC[a] (1)
TA3178	LT2 *hisJ8908*::Tn*10*	SGSC (1)
TT317	LT2 *purF1714*::Tn*10*	SGSC (1)
TT5866	LT2 *hisT290*::Tn*5*	SGSC (1)

[a] SGSC, *Salmonella* Genetic Stock Centre, University of Calgary, Calgary, Alberta, Canada.

TABLE 2. Effects of the *flhB* mutations on expression of the *motA-lac* fusion in various mutant backgrounds[a]

Hook-basal body mutation	Flagellar structure[b]	β-Galactosidase activity (Miller units) with indicated *flhB* allele		
		flhB$^+$	*flhB9001*	*flhB9002*
None	Mature flagellum	190	148	145
fliK::Tn*10*	Basal body-polyhook	10	30	63
flgE2004	Basal body	9	10	11
flgD1155	Basal body	10	9	12
fliF::Tn*10*	None	8	8	8

[a] All strains carry the *motA-lac* fusion introduced from strain KK1106 by P22-mediated transduction.
[b] The most complete structure observed is listed, based on the most recent morphological studies (12, 36).

basal body only in the presence of the hook assembly. Alternatively, the export apparatus may have a mechanism whereby it allows FlgM export only after export of the hook protein.

The *flgD* gene product is necessary for polymerization of newly exported hook subunits onto the tip of the rod (29). A *flgD* mutant produces a basal body lacking the hook and filament portions (36), and it excretes hook proteins into the culture media (29). As in the *flgE* mutant, the *flhB* mutations did not upregulate *motA* transcription in the *flgD* mutant (Table 2). This result indicates that prior export of the hook protein does not suffice for FlgM release through the basal body. I conclude that the FlgM gate is still shut in the absence of the hook assembly in *flhB* mutants.

This finding raises the possibility that there is another factor in addition to FlhB which inhibits FlgM export before completion of hook assembly. This inhibition may be relieved in response to the signal that the hook has reached its mature length. To test this possibility, I attempted to isolate mutants defective in this factor. We have used the symbol *rfl* followed by *A*, *B*, *C*, etc., to designate mutations which affect export or function of FlgM (17, 19). Because the last known *rfl* mutation is *rflG* (14), the mutation in this putative factor is called *rflH* in this report.

Isolation of the *rflH* mutants. I expected that the *rflH* mutations would relieve the inhibition of FlgM export only in the presence of the *fliK*-suppressing FlhB protein and the mature basal body structure. Therefore, I attempted to isolate *rflH* mutants in strain KK1427 (Table 1). This strain is a *flgE2004 flhB9002* double mutant carrying the *motA-lac* transcriptional fusion, and it displays a Lac$^-$ phenotype (Table 2). From this strain, I selected spontaneous Lac$^+$ revertants on minimal lactose plates. Next, the *fliF*::Tn*10* mutation was introduced into the revertants by P22-mediated transduction from strain KK2092.

The resulting transductants were examined for their Lac phenotypes on MacConkey lactose plates. More than 300 independent Lac$^+$ revertants were subjected to this test. All except three gave rise to Lac$^+$ transductants, suggesting that they might carry mutations which inactivate FlgM or render FliA insensitive to FlgM. According to our previous work (5, 17), at least some of them should carry mutations in either *flgM* or *fliA* gene. These revertants were not analyzed further.

The remaining three revertants gave rise only to Lac$^-$ transductants. This result suggests that they carry mutations which relieve the inhibition of FlgM export only in the presence of the mature basal body. These were considered to be potential *rflH* mutants. They were therefore named KK1451, KK1452, and KK1453, and their mutant alleles were designated *rflH115*, *rflH116*, and *rflH221*, respectively.

TABLE 3. Effects of the *flhB9002* and *rflH221* mutations on the *motA-lac* fusion expression in various mutant backgrounds[a]

Flagellar genotype				β-Galactosidase activity (Miller units) with indicated *rflH* allele[b]			
flgE	*flhB*	*fliF*	*fliK*	*rflH*$^+$	*rflH115*	*rflH116*	*rflH221*
2004	*9002*	+	+	11	103	105	104
2004	*9002*	Tn*10*	+	7	9	10	9
2004	+	+	+	9	18	19	13
+	+	+	+	190	ND[b]	ND	145
+	+	+	Tn*10*	10	ND	ND	8

[a] All strains carry the *motA-lac* fusion.
[b] ND, not done.

The expression level of the *motA-lac* fusion was examined with these three strains in the absence and presence of the *fliF*::Tn*10* mutation (Table 3). In the *fliF*$^+$ background, these *rflH* mutations caused nearly 10-fold increases in *motA* expression. However, in the *fliF* mutant background, *motA* expression remained low even in the presence of the *rflH* mutations.

Next, the *flhB9002* allele in the revertants was replaced with the wild-type allele by means of linked transduction with *cheZ*::Tn*10*. The resulting *flhB*$^+$ transductants showed only slightly higher levels of *motA* expression than the *rflH*$^+$ parent (Table 3). These results indicate that both an *flhB* mutation and an *rflH* mutation are required to enable FlgM export in the *flgE* mutant.

FlgM export from the *rflH* mutants. To confirm that FlgM is actually exported from the *flgE* mutant cells in the *flhB rflH* double mutant, proteins in culture supernatants were concentrated by trichloroacetic acid precipitation and separated by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. On the Coomassie blue-stained gel, no major protein band was detected in the culture supernatants of the *flhB*$^+$ *rflH*$^+$, *flhB9002 rflH*$^+$, and *flhB*$^+$ *rflH221* strains (Fig. 2A, lanes 1 to 3). In contrast, at least two protein bands were observed in the culture supernatant from the *flhB9002 rflH221* double mutant (Fig. 2A, lane 4). Their electrophoretic mobil-

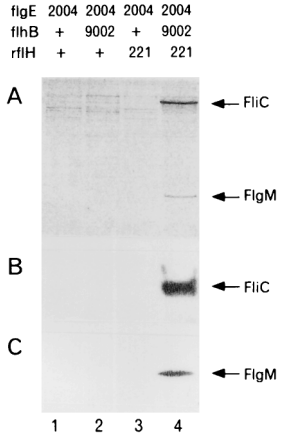

FIG. 2. Excretion of FlgM and FliC into the culture medium. The genotypes of the strains used are indicated at the top. Proteins in the culture supernatant were separated by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. (A) The gel was stained with Coomassie brilliant blue. The positions of FliC and FlgM are indicated on the right. (B and C) The proteins on the gel were electrophoretically transferred onto a nitrocellulose membrane and visualized with FliC (B) and FlgM (C) antibodies.

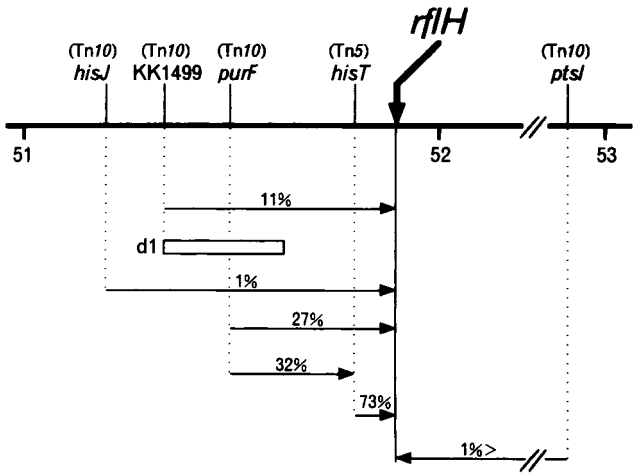

FIG. 3. Chromosomal location of the *rflH* gene relative to known markers. Cotransduction frequencies between two genes are indicated above the arrow. The gene at the base of the arrow was used as the donor marker, and the gene at the arrowhead was the recipient marker. The open box labeled dl is the area which is deleted in strain KK1499dl.

ities suggest that the fast-migrating, faint band may correspond to FlgM and that the slowly migrating, heavy one may correspond to FliC, the major component protein of the filament. This prediction was confirmed by Western blotting using antibodies specific for FlgM and FliC (Fig. 2B and C). Similar results were obtained with the *rflH115* and *rflH116* mutants (data not shown). Therefore, it appears that in the presence of the *flhB* and *rflH* mutations, both FlgM and FliC can be exported through the basal body lacking the hook and the filament.

Genetic analysis of the *rflH* mutations. Linkage analysis of the *rflH* mutations revealed that these mutations are not in the known flagellar gene clusters. To determine the map locations of the *rflH* mutations, Tn*10* was inserted near the *rflH* gene by the following procedure. First, a pool of mutants in which Tn*10* is randomly inserted into the chromosome of wild-type strain KK1004 was established (17). A P22HT*int* phage lysate propagated on this pool was used to infect one of the *rflH* mutants, KK1453 (*flgE2004 flhB9002 rflH221 motA-lac*), and tetracycline-resistant, Lac$^-$ transductants were selected. One such transductant was designated KK1499. The Tn*10* insertion in this strain was shown by P22-mediated transduction to be 11% linked to the *rflH221* mutation. The remaining two *rflH* mutations were also cotransducible with this Tn*10* insertion. This result suggests that all three *rflH* mutations occurred within a single gene.

By using a method described previously (15), tetracycline-sensitive derivatives were isolated from KK1499 to obtain mutants carrying Tn*10* excision-associated deletions (9). Among them, one (KK1499dl) was found to be an adenine-requiring mutant. Transductional analysis with various *pur* mutant recipients revealed that a P22 lysate made with strain KK1499dl lacks the ability to produce Pur$^+$ recombinants when crossed with a *purF* mutant strain, TT317. Therefore, I conclude that the deleted region in KK1499dl includes the *purF* gene, which is at 51.7 min on the newest genetic map of *S. typhimurium* (32). I then examined the linkage of the *rflH221* mutation with various markers located near the *purF* gene (Fig. 3). I found that the *rflH* gene is closely linked to *hisT* and conclude that the *rflH* gene is located at around 52 min. No flagellar gene has been previously identified in this region.

Next, I carried out complementation tests to ascertain whether the *rflH* mutations are dominant or recessive. For this purpose, I used plasmid pLC4-15, which carries the 52-min region (*hisJ-purF-aroC*) from *E. coli* (26). A *purF*::Tn*10* derivative of strain KK1453 (*flgE2004 flhB9002 rflH221 motA-lac purF*::Tn*10*) was conjugated with strain JA200 carrying pLC4-15, and tetracycline-resistant, Pur$^+$ transconjugants were selected. The resulting merodiploid was Lac$^-$, indicating that *E. coli* has a gene which is functionally homologous to the *S. typhimurium rflH* gene and dominant over the *rflH221* mutation. The same result was obtained with the *rflH115* and *rflH116* mutations. Therefore, I conclude that the *rflH* mutations obtained in this study are all recessive.

Characterization of the *rflH* mutants. By use of linked transduction with the *purF* gene, I introduced the *rflH221* mutation into an otherwise wild-type strain. The resulting *rflH* mutant (KK1498) was found to form swarms on motility agar plates. The swarm size was comparable to that of swarms formed by an isogenic *rflH*$^+$ strain (KK1004). When observed by dark-field light microscopy, cells were actively motile in liquid medium. Therefore, the *rflH* mutation alone does not have a noticeable effect on flagellar formation or function.

The *fliK*::Tn*10* mutation was introduced into strain KK1498 by transduction from strain KK2143. The resulting transductant (*rflH221 fliK*::Tn*10*) did not form swarms on motility agar plate, indicating that, unlike the *flhB* mutations, the *rflH* mutation can not suppress the *fliK* defect. Consistent with this conclusion, expression of the *motA-lac* fusion was severely inhibited in this strain (Table 3).

DISCUSSION

The ordered expression of flagellar genes is regulated by a mechanism which allows the flagellum-specific export apparatus to export FlgM only after completion of hook assembly (4, 13, 23, 33). We previously proposed a model for switching specificity of the export machinery which requires two distinct systems; one is a signal transduction pathway which relays information that the hook has reached its mature length, and the other is a molecular switch which changes the substrate specificity of the flagellum-specific export apparatus from the hook protein to FlgM when it receives the signal (18). On the basis of the observation that *flhB* mutations allow *fliK* mutants to assemble filaments onto polyhooks, we proposed that FliK is involved in the signal transduction pathway and FlhB acts as the switch (18). FlgM export is postulated to be inhibited until FlhB receives the hook-completion signal via FliK.

This hypothesis predicts that the *fliK*-suppressing *flhB* mutations would allow mature basal bodies to export FlgM even in the absence of the hook assembly. However, this prediction was invalidated by the observation that the *flgE* or *flgD* mutant which produces a basal body lacking the hook and the filament cannot export FlgM even in the presence of the *flhB* mutations (Table 2; Fig. 3). Here, I report the isolation and characterization of *rflH* mutants which are defective in inhibition of FlgM export before completion of hook assembly. Based on the results obtained in this present study, I propose a revised scheme of the export switching machinery (Fig. 4).

Double-locked gate of the FlgM export. The *rflH* mutations obtained in this study allow *flgE* mutants to release FlgM into the medium only in strains having the *flhB* mutations (Table 3; Fig. 3). This result indicates that the *flhB* and *rflH* mutations are both required for FlgM release through the basal body in the absence of the hook assembly. In a previous report (18), we showed that the *flhB* mutations used in this study are both recessive. Here, I present evidence that the *rflH* mutations

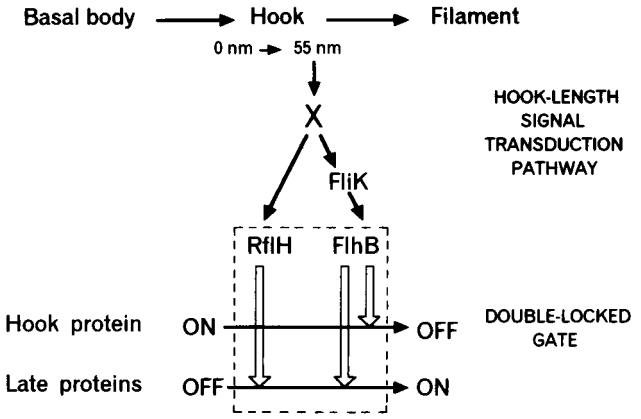

FIG. 4. Model of the export-switching machinery. It consists of the signal transduction pathway for hook length and the double-locked gate. In the former, X is a putative sensor that detects the state of hook assembly and transmits the information that the mature length (55 nm) has been attained. This signal is received by FlhB via FliK and by RflH directly. FlhB and RflH independently inhibit the export of late proteins before hook assembly is complete. Therefore, the export gate for late proteins is double locked. When these proteins receive the signal that the mature hook length has been reached, they open the gate for late proteins. At the same time, FlhB shuts the gate for hook protein.

obtained in this study are also recessive. Therefore, the results shown in Table 3 and Fig. 3 can be explained as follows. Both FlhB and RflH may independently inhibit the FlgM export through the basal body in the absence of the hook assembly. The *flhB* and *rflH* mutants used in this study may lack this ability to inhibit. Therefore, I propose that the gate for FlgM export in the flagellum-specific export apparatus is double locked by FlhB and RflH. Each lock is opened independently in response to the signal that the mature hook length has been attained (Fig. 4).

The *flhB* mutations allow the *fliK* mutants to assemble filaments attached to polyhooks (3, 18, 38). This observation indicates that *flhB* polyhook mutants can export not only FlgM but also all of the other late proteins, FlgK, FlgL, FliD, and FliC, required for filament assembly. I show here that the *flhB rflH* double mutants export at least the major filament protein, FliC, and FlgM through the basal body formed in the *flgE* mutant.

These results do not necessarily mean that in the absence of normal hook assembly, FlhB and RflH inhibit the export of the filament proteins in addition to FlgM. The failure of the *fliK* and *flgE* mutants to export the filament proteins in a wild-type *flhB* or *rflH* background can be attributed to their low level of expression of the filament proteins, because FlgM is not exported from these mutants and therefore inhibits expression of class 3 genes (4, 13). However, the observation that the *flgM fliK* and *flgM flgE* double mutants express the genes for the filament proteins but cannot release them into the medium (14) suggests that export of the filament proteins may be regulated in the same manner as that of FlgM. Therefore, I conclude that the FlhB RflH double-locked gate acts as the export switch for all late proteins. This interpretation suggests that the export-switching machinery accomplishes two missions: it allows an individual flagellum to undergo ordered export of its component proteins to ensure the ordered assembly of the flagellum, and it allows the flagellar regulon to respond directly to the assembly state of the flagellum.

FlhB is postulated to be an integral membrane protein having its C-terminal domain extending into the cytoplasm (25). The *fliK*-suppressing mutations are all located in the cytoplasmic domain (18, 38). The *flhB* mutants produce slightly elongated hooks attached to filaments (3). This increased hook length indicates that the C-terminal portion of FlhB is also involved in shutting off the export of hook protein. Furthermore, the hydrophobic N-terminal portion of FlhB has been shown to be needed for formation of the rod and hook structures (12, 18, 36). Therefore, FlhB is involved in assembly of the hook-basal body structure and the filament. Because *fliK* mutants produce polyhooks in the $rflH^+$ background, RflH is unlikely to be involved in shutting off the export of hook protein.

The *rflH* gene is located at around 52 min of the *S. typhimurium* chromosome, where no flagellar gene has been identified. Because I have not characterized the *rflH* gene at a molecular level, no information is available on the structure or cellular localization of the *rflH* gene product. I did not recognize any phenotype conferred by the *rflH* mutations to a wild-type strain. If the *rflH* mutations obtained here manifest the null phenotype of the gene, RflH has no direct role in flagellar formation and function. Molecular characterization of the wild-type and mutant alleles of the *rflH* gene and careful electron microscopic observation of flagellar structures produced by *rflH* mutants will be required to address these issues.

Recently, we showed that FlgM export is negatively regulated by FliD, FliS, and FliT after completion of hook assembly (39). Therefore, during flagellar morphogenesis, FlgM export is still inhibited to some extent even after the FlhB RflH double-locked gate has been opened. This negative regulation may avoid unnecessary overproduction of flagellar proteins after completion of hook assembly.

Hook-length signal transduction pathway. Next, I consider the signal transduction pathway for hook length based on the results obtained in the present study. The *flhB* mutations can suppress the defects of *fliK* mutants in export of late proteins in the $rflH^+$ background. This observation indicates that FliK is needed to open the FlhB lock but not the RflH lock. The signal that directs the export apparatus to release FlgM is transmitted to RflH at a certain stage of the polyhook formation, probably at the stage when the hook length reaches ca. 55 nm. Therefore, I conclude that (i) the signal transduction pathway for hook length diverges into two branches, one leading to FlhB and the other leading to RflH; and (ii) FliK is involved in the signal transduction pathway leading to FlhB but not in the pathway leading to RflH. The finding that *rflH* mutations cannot suppress the *fliK* defects is consistent with this conclusion. However, for the FliK-FlhB signal transduction pathway, an alternative explanation is that the signal is received directly by FlhB and that only an FlhB molecule which has received the signal can interact with FliK.

Some *fliK* mutants with small internal in-frame deletions or substitutions of the gene produce filaments attached to the polyhooks (35, 38). Williams et al. (38) explained this phenomenon by assuming that FliK consists of two functional domains, one at the N terminus for measuring hook length and the other at the C terminus for determining export specificity. They hypothesized that the *fliK* mutants mentioned above should be defective in the N-terminal domain. However, as discussed below, FliK is unlikely to measure the hook length.

I propose a simpler explanation for the phenotype of these *fliK* mutants according to the model in Fig. 4. I assume that the mutant FliK proteins are partially defective in interacting with FlhB. When the hook-length signal was transmitted to these proteins, they could not interact effectively with FlhB, and consequently the export specificity failed to switch at the proper time. However, during the subsequent elongation of the polyhook, the mutant FliK proteins which had received the

signal should finally have sufficient time to interact with FlhB to switch the export specificity, resulting in formation of the polyhook-filament structures.

Most enigmatic is the question of what senses the state of hook assembly and transmits the signal. FliK has been predicted to be this sensor, because *fliK* mutants lack the hook length control (3, 8, 38). However, this prediction is invalidated by the observation that the RflH lock can be opened in response to the hook assembly even in the absence of FliK. In Fig. 4, "X" denotes this putative sensor. During morphogenesis of phage lambda, the H protein acts as a molecular ruler which measures and determines the tail length (6, 7). If sensor X acts as a molecular ruler like the H protein, it should extend from the tip of the rod to the growing end of the hook. Experiments are in progress to identify a molecule to have such a function.

Because the hook is an extracellular structure, the signal transduction pathway for hook length should cross three compartments: the outer membrane, the periplasmic space, and the cytoplasmic membrane. Because the basal body spans three compartments, the basal body itself may act as the pathway for the hook-length signal transduction.

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