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Flk Couples *flgM* Translation to Flagellar Ring Assembly in *Salmonella typhimurium*

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The hook-basal body (HBB) is a key intermediate structure in the flagellar assembly pathway in *Salmonella typhimurium*. The FlgM protein inhibits the flagellum-specific transcription factor σ^{28} in the absence of the intact HBB structure and is secreted out of the cell following HBB completion. The *flk* gene encodes a positive regulator of the activity of FlgM at an assembly step just prior to HBB completion: at the point of assembly of the P- and L-rings. FlgM inhibition of σ^{28}-dependent class 3 flagellar gene transcription was relieved in P- and L-ring assembly mutants (*flgA*, *flgH*, and *flgI*) by introduction of a null mutation in the *flk* gene (J. E. Karlinsey et al., J. Bacteriol. 179:2389–2400, 1997). In P- and L-ring mutant strains, recessive mutations in *flk* resulted in a reduction in intracellular FlgM levels to those seen in wild-type (Fla$^+$) strains. The reduction in intracellular FlgM levels by mutations in the *flk* gene was concomitant with a 10-fold increase in transcription of the *flgMN* operon compared to that of the isogenic *flk*$^+$ strain, while transcription of the *flgAMN* operon was unaffected. This was true for both direct measurement of the *flgAMN* and *flgMN* mRNA transcripts by RNase T2 protection assays and for *lac* operon fusions to either the *flgAMN* or *flgMN* promoter. Loss of Flk did not allow secretion of FlgM through basal-body structures lacking the P- and L-rings. Intracellular FlgM was stable to proteolysis, and turnover occured primarily after export out of the cell. Loss of Flk did not result in increased FlgM turnover in either P- or L-ring mutant strains. With *lacZ* translational fusions to *flgM*, a null mutation in *flk* resulted in a significant reduction of *flgM-lacZ* mRNA translation, expressed from the class 3 *flgMN* promoter, in P- and L-ring mutant strains. No reduction in either *flgAMN* or *flgMN* mRNA stability was measured in the absence of Flk in Fla$^+$, ring mutant, or HBB deletion strains. We conclude that the reduction in the intracellular FlgM levels by mutation in the *flk* gene is only at the level of *flgM* mRNA translation.

Salmonella typhimurium can propel itself in a liquid environment by the rotation of 6 to 12 individual flagellar structures that are located peritrichously on the cell surface. The signal transduction pathway of the chemosensory system allows for the biased movement of an individual bacterium across a chemical gradient, a process called chemotaxis (reviewed in references 2 and 43). The flagella can be signaled to rotate in a clockwise manner, which causes the bacterium to tumble and change direction, or if this signal is suppressed, they will rotate in a counterclockwise manner, which propels the bacterium in a forward direction. The flagella are biased toward counterclockwise rotation when approaching an attractant or moving away from a repellent and are biased toward clockwise rotation when moving away from an attractant or toward a repellent.

The flagellum is generally broken down into three main structural components: (i) the basal body, (ii) the hook, and (iii) the long external filament (for recent reviews of flagellar structure and assembly, see references 1 and 33). Several stages of flagellar assembly are diagrammed in Fig. 1. The basal body traverses from the cytoplasm to the outside of the cell. Assembly begins with formation of the C- and MS-rings at the cytoplasmic base of the basal body. The C-ring extends into the cytoplasm and includes a type III secretion machinery and proteins that control the direction of flagellar rotation in response to the chemotactic signal transduction system. The C-ring is attached to the MS-ring, which is embedded within the cytoplasmic membrane. The next structure to be assembled is the rod that extends from the MS-ring to the lipopolysaccharide (LPS) layer. This is followed by P- and L-ring assembly in the peptidoglycan and LPS layers, respectively, which must occur before an external hook structure can polymerize. Assembly of extracytoplasmic components of the hook-basal body (HBB) requires the type III secretion system, whereas P- and L-ring subunits are exported out of the cytoplasm via the signal peptide-dependent general secretory pathway. Initiation of hook assembly can occur prior to P- and L-ring assembly, but elongation is blocked by the peptidoglycan and LPS layers (21). The hook is thought to act as a universal joint between the rotating part of the basal body and the long external filament. Hook completion is determined by the *fliK* gene product, which somehow signals the type III export apparatus to stop the export of hook proteins and initiate export of the flagellin subunits and associated proteins (13, 25, 47). The *flgK* and *flgL* genes encode the hook-associated proteins, which form small subunit rings at the hook-filament junction (17). The *fliD* gene encodes a flagellar cap protein located at the tip of the flagellum (18). Flagellin is encoded by alternatively expressed *fliC* and *fliB* genes encoding antigenically distinct flagellin subunits (44). Flagellin subunits polymerize first between the cap and the FlgL ring and then between the cap and the tip of the growing filament.

Regulation of the 50-plus genes in the flagellar and chemotaxis regulon occurs in coordination with flagellar assembly. The expression of these genes is organized into a regulatory hierarchy of three major classes: class 1, class 2, and class 3

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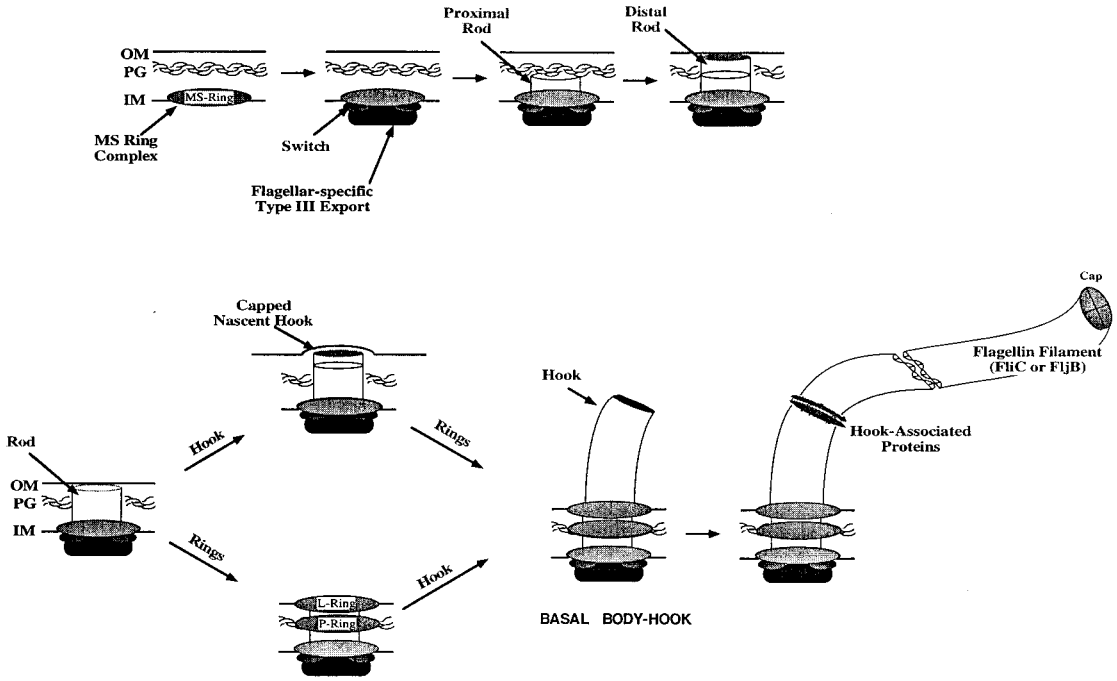

FIG. 1. Morphological pathway of flagellar assembly. MS-ring assembly in the inner membrane (IM) is followed by assembly of the switch complex and the flagellum-specific type III export apparatus. The rod components require the type III system to be exported across the inner membrane and into the periplasmic space, where they are assembled into a structure that penetrates the peptidoglycan layer (PG). The P- and L-rings (peptidoglycan and lipopolysaccharide, respectively) are assembled independently of hook initiation; however, hook elongation through the outer membrane (OM) requires the assembled P- and L-rings. After ring assembly, the hook is completed and the biosynthesis of the HBB intermediate structure is finished. At this point the class 3 flagellar proteins, including FlgM, are secreted. The hook-associated proteins and Cap are added to the end of the hook, followed by flagellin polymerization initially between the hook-associated proteins and Cap and then between the Cap and the elongating filament.

(22). Class 1 genes, *flhD* and *flhC*, are cotranscribed and represent the top of the flagellar transcriptional hierarchy. They are transcribed from a σ^{70}-dependent promoter, which is affected by a large number of global regulatory signals (28, 30, 31). The FlhD and FlhC proteins are required for expression of the rest of the genes in the regulon. These proteins form a heteromultimeric transcriptional activator complex which directs σ^{70}-dependent transcription of promoters for class 2 genes (30). Class 2 genes encode proteins required for the structure and assembly of the HBB intermediate flagellar structure and the FlgM and σ^{28} regulatory proteins. Class 3 genes include σ^{28}-dependent promoters and encode proteins required late in flagellar assembly, including the *flgK*, *flgL*, and *fliD* genes, and the filament genes *fliC* and *fliB*. In addition, genes required for chemotactic signal transduction are also class 3 genes.

This initial simplicity was complicated by the finding that multiple promoters transcribe most of the flagellar genes. The σ^{28} protein is an alternative transcription factor that interacts with RNA polymerase to direct transcription specifically from class 3 promoters but will also transcribe class 2 flagellar genes (23, 37). Several of the class 2 gene promoter regions have been shown to be transcribed by either σ^{28} holoenzyme or σ^{70} holoenzyme directed by the FlhDC complex (32). In vivo, all class 2 operons were shown to be dependent on either the *flhDC* operon or the σ^{28} structural gene, *fliA*, for expression (23). These results suggest that initiation of flagellar biosynthesis is completely dependent on FlhDC but that continued HBB gene expression results from FlhDC-dependent and/or σ^{28}-dependent class 2 transcription. The class 3 structural genes are of two types; the *flgK*, *flgL*, and *fliD* genes are transcribed from both a class 2 promoter and a σ^{28}-dependent class 3 promoter (26). The remaining class 3 structural genes are exclusively transcribed by σ^{28}-dependent promoters (23).

The class 3 genes were originally defined by their dependence on a functional HBB structure for their expression (22). This ability to couple class 3 gene expression to HBB completion is accomplished by the action of the FlgM protein on σ^{28}-dependent transcription (8, 9). FlgM is an anti-σ^{28} factor (38). When the HBB structure is incomplete or defective, FlgM interacts directly with σ^{28} to inhibit its activity as a transcription factor. FlgM can sense that the HBB structure is complete and export competent for external class 3 flagellar proteins by itself being a class 3 exported protein (16, 24). When the HBB structure is defective, FlgM inhibits σ^{28}-dependent transcription and is not found in the external growth medium. In a strain that makes the HBB structure, FlgM is found in the external growth medium and σ^{28}-dependent transcription occurs.

Recently, a novel flagellar regulatory gene, *flk*, was described as a gene whose product can sense the completion of the flagellar P- and L-rings of the basal body, which occurs just prior to hook assembly (20). The *flgA* and *flgI* genes are required for P-ring assembly, where the *flgI* gene encodes the structural component, and the *flgH* gene encodes the structural component for the L-ring (33). Like all genes involved in HBB assembly, mutations in any of the *flgA*, *flgH*, and *flgI* genes result in FlgM-dependent inhibition of σ^{28} activity (8). Unlike the other HBB genes, however, the negative regulatory effect of mutations in the *flgA*, *flgH*, or *flgI* gene can be suppressed by recessive insertion mutations in either *flgM* or *flk* (20). Insertions in *flgM*, but not those in *flk*, will suppress the negative regulatory effect caused by the loss of the remaining HBB genes (8, 20). In this paper, we provide evidence that the *flk*

TABLE 1. List of bacterial strains

S. typhimurium strain	Genotype	Source or reference[a]
SJW198	Δ*flgH2136 fliB*^{e,n,x} *hin*(*vh2*)	S. Yamaguchi
SJW200	Δ*flgI2453 fla fliB*^{e,n,x} *hin*(*vh2*)	S. Yamaguchi
SJW203	Δ*flgHI958 fliB*^{e,n,x} *hin*(*vh2*)	S. Yamaguchi
SJW204	Δ*flgE1204 fliB*^{e,n,x} *hin*(*vh2*)	S. Yamaguchi
SJW1376	Δ*flgI1376 fliB*^{e,n,x} *hin*(*vh2*)	S. Yamaguchi
SJW1518	Δ*fla-2157* (Δ*flgG-L*) fliB^{e,n,x} *hin*(*vh2*)	S. Yamaguchi
SJW1525	*flgB2164 fliB*^{e,n,x} *hin*(*vh2*)	S. Yamaguchi
SJW1529	Δ*flgA1529 fliB*^{e,n,x} *hin*(*vh2*)	S. Yamaguchi
TH1479	*fliA5059*::Tn*10d*Tc	
TH2157	*flgB2164 fljB5001*::Mu*d*J	8
TH2164	*flgI2002 fljB5001*::Mu*d*J	8
TH2413	*flk-5206*::Tn*10d*Cm	8
TH2512	*flgA5210*::Tn*10d*Tc	10
TH2541	*flk-5212*::Tn*10d*Tc	8
TH2575	*flgA5211*::Mu*d*A	10
TH2592	*fljB5001*::Mu*d*J	
TH2877	*flgM5207*::Mu*d*A	10
TH3282	*flk-5212*::Tn*10d*Tc *fljB5001*::Mu*d*J	
TH3303	*flgB2164 fljB5001*::Mu*d*J	
TH3441	Δ*flgA1529 fljB5001*::Mu*d*J	
TH3442	Δ*flgH2136 fljB5001*::Mu*d*J	
TH3443	Δ*flgI2453 fljB5001*::Mu*d*J	
TH3444	Δ*flgI1376 fljB5001*::Mu*d*J	
TH3445	Δ*flgHI958 fljB5001*::Mu*d*J	
TH3451	Δ*flgA1529 fljB5001*::Mu*d*J *flk-5212*::Tn*10d*Tc	
TH3452	Δ*flgH2136 fljB5001*::Mu*d*J *flk-5212*::Tn*10d*Tc	
TH3453	Δ*flgI2453 fljB5001*::Mu*d*J *flk-5212*::Tn*10d*Tc	
TH3454	Δ*flgI1376 fljB5001*::Mu*d*J *flk-5212*::Tn*10d*Tc	
TH3455	Δ*flgHI958 fljB5001*::Mu*d*J *flk-5212*::Tn*10d*Tc	
TH3504	*fljB5001*::Mu*d*Cm *flgB2164*	
TH3505	*fljB5001*::Mu*d*Cm	
TH3506	*fljB5001*::Mu*d*Cm *flk-5212*::Tn*10d*Tc	
TH3507	*flgB2164 fljB5001*::Mu*d*Cm *flk-5212*::Tn*10d*Tc	
TH3508	Δ*flgA1529 fljB5001*::Mu*d*Cm	
TH3513	Δ*flgA1529 fljB5001*::Mu*d*Cm *flk-5212*::Tn*10d*Tc	
TH3558	*ataA*::[P22 Δ(*mnt-arc*)::Km^r P_{fliA}-*lac-600*] *fljB5001*::Mu*d*Cm	
TH3559	*ataA*::[P22 Δ(*mnt-arc*)::Km^r P_{fliA}-*lac-600*] *fljB5001*::Mu*d*Cm *flgB2164*	
TH3560	*ataA*::[P22 Δ(*mnt-arc*)::Km^r P_{fliA}-*lac-600*] *fljB5001*::Mu*d*Cm Δ*flgA1529*	
TH3561	*ataA*::[P22 Δ(*mnt-arc*)::Km^r P_{fliA}-*lac-600*] *fljB5001*::Mu*d*Cm Δ*flgH2136*	
TH3562	*ataA*::[P22 Δ(*mnt-arc*)::Km^r P_{fliA}-*lac-600*] *fljB5001*::Mu*d*Cm Δ*flgI2453*	
TH3564	*ataA*::[P22 Δ(*mnt-arc*)::Km^r P_{fliA}-*lac-600*] *fljB5001*::Mu*d*Cm Δ*flgHI958*	
TH3565	*ataA*::[P22 Δ(*mnt-arc*)::Km^r P_{fliA}-*lac-600*] *fljB5001*::Mu*d*Cm *flk-5212*::Tn*10d*Tc	
TH3566	*ataA*::[P22 Δ(*mnt-arc*)::Km^r P_{fliA}-*lac-600*] *fljB5001*::Mu*d*Cm *flgB2164 flk-5212*::Tn*10d*Tc	
TH3567	*ataA*::[P22 Δ(*mnt-arc*)::Km^r P_{fliA}-*lac-600*] *fljB5001*::Mu*d*Cm Δ*flgA1529 flk-5212*::Tn*10d*Tc	
TH3568	*ataA*::[P22 Δ(*mnt-arc*)::Km^r P_{fliA}-*lac-600*] *fljB5001*::Mu*d*Cm Δ*flgH2136 flk-5212*::Tn*10d*Tc	
TH3569	*ataA*::[P22 Δ(*mnt-arc*)::Km^r P_{fliA}-*lac-600*] *fljB5001*::Mu*d*Cm Δ*flgI2453 flk-5212*::Tn*10d*Tc	
TH3571	*ataA*::[P22 Δ(*mnt-arc*)::Km^r P_{fliA}-*lac-600*] *fljB5001*::Mu*d*Cm Δ*flgHI958 flk-5212*::Tn*10d*Tc	
TH3590	DUP1127[(*purB1879*)*Mu*dA*(*flgA5211*)]	
TH3591	DUP1127[(Δ*flgA1529 purB1879*)*Mu*dA* (*flgA5211*)]	
TH3592	DUP1127[(Δ*flgG-L2157 purB1879*)*Mu*dA* (*flgA2511* Δ*flgG-L2157*)]	
TH3593	DUP1127[(*purB1879*)*Mu*dA*(*flgA5211*)]*flk-5206*::Tn*10d*Cm	
TH3594	DUP1127[(Δ*flgA1529 purB1879*)*Mu*dA* (*flgA5211*)]*flk-5206*::Tn*10d*Cm	
TH3595	DUP1127[(Δ*flgG-L2157 purB1879*)*Mu*dA* (*flgA5211* Δ*flgG-L2157*)]*flk-5206*::Tn*10d*Cm	
TH3596	DUP1113[(*purB1879*)*Mu*dA*(*flgM5207 flgA5210*::Tn*10d*Tc)]	
TH3597	DUP1113[(Δ*flgA1529 purB1879*)*Mu*dA* (*flgM5207 flgA5210*::Tn*10d*Tc)]	
TH3598	DUP1113[(Δ*flgG-L2157 purB1879*)*Mu*dA* (*flgM5207 flgA5210*::Tn*10d*Tc Δ*flgG-L2157*)]	
TH3599	DUP1113[(*purB1879*)*Mu*dA*(*flgM5207 flgA5210*::Tn*10d*Tc)]*flk-5206*::Tn*10d*Cm	
TH3600	DUP1113[(Δ*flgA1529 purB1879*)*Mu*dA* (*flgM5207 flgA5210*::Tn*10d*Tc)]*flk-5206*:: Tn*10d*Cn	
TH3601	DUP1113[(Δ*flgG-L2157 purB1879*)*Mu*dA* (*flgM5207 flgA5210*::Tn*10d*Tc Δ*flgG-L2157*)] *flk-5206*::Tn*10d*Cm	
TH3745	DEL[(*flgA5211*)*Mu*dJ*(*flgN5220*)] DEL1103(*tct-zfg-3516*::Tn*10d*Tc-*fljB5001*::Mu*d*J) *fla-2050* (Δ*fliA-D*)	
TH3907	*flgA5210*::Tn*10d*Tc *flgM5223 flgM5208*::Mu*d*K	
TH4023	DUP1142[(*purB1879*)*Mu*dB*(*flgM5208 flgM5223 flgA5210*::Tn*10d*Tc)]	
TH4024	DUP1142[(Δ*flgA1529 purB1879*)*Mu*dB* (*flgM5208 flgM5223 flgA5210*::Tn*10d*Tc)]	
TH4025	DUP1142[(Δ*flgG-L2157 purB1879*)*MudB* (*flgM5208 flgM5223 flgA5210*::Tn*10d*Tc Δ*flgG-L2157*)]	
TH4026	DUP1142[(*purB1879*)*Mu*dB*(*flgM5208 flgM5223 flgA5210*::Tn*10d*Tc)]*flk-5206*:: Tn*10d*Cm	
TH4027	DUP1142[(Δ*flgA1529 purB1879*)*Mu*dB* (*flgM5208 flgM5223 flgA5210*::Tn*10d*Tc)]*flk-5206*::Tn*10d*Cm	
TH4028	DUP1142[(Δ*flgG-L2157 purB1879*)*Mu*dB* (*flgM5208 flgM5223 flgA5210*::Tn*10d*Tc Δ*flgG-L2157*)]*flk-5206*::Tn*10d*Cm	
TH4029	DUP1142[(*purB1879*)*Mu*dB*(*flgM5208 flgM5223* Δ*flgA1529*)]	
TH4030	DUP1142[(Δ*flgA1529 purB1879*)*Mu*dB* (*flgM5208 flgM5223* Δ*flgA1529*)]	
TH4031	DUP1142[(Δ*flgG-L2157 purB1879*)*MudB* (*flgM5208 flgM5223* Δ*flgA1529* Δ*flgG-L2157*)]	
TH4032	DUP1142[(*purB1879*)*Mu*dB*(*flgM5208 flgM5223* Δ*flgA1529*)]*flk-5206*::Tn*10d*Cm	
TH4033	DUP1142[(Δ*flgA1529 purB1879*)*Mu*dB* (*flgM5208 flgM5223* Δ*flgA1529*)]*flk-5206*:: Tn*10d*Cm	
TH4034	DUP1142[(Δ*flgG-L2157 purB1879*)*Mu*dB* (*flgM5208 flgM5223* Δ*flgA1529* Δ*flgG-L2157*)] *flk-5206*::Tn*10d*Cm	
TH4041	Δ*flgA1529 flgM5223 flgM5208*::Mu*d*K	
TH4045	DUP1142[(*purB1879*)*Mu*dB*(*flgM5208 flgM5223* Δ*flgA1529*)]*fliA5050*::Tn*10d*Tc	
TH4046	DUP1142[(Δ*flgA1529 purB1879*)*Mu*dB* (*flgM5208 flgM5223* Δ*flgA1529*)] *fliA5050*:: Tn*10d*Tc	
TH4047	DUP1142[(Δ*flgG-L2157 purB1879*)*Mu*dB* (*flgM5208 flgM5223* Δ*flgA1529* Δ*flgG-L2157*)] *fliA5050*::Tn*10d*Tc	
TH4048	DUP1142[(*purB1879*)*Mu*dB*(*flgM5208 flgM5223* Δ*flgA1529*)]*fliA5050*::Tn*10d*Tc *flk-5206*::Tn*10d*Cm	
TH4049	DUP1142[(Δ*flgA1529 purB1879*)*Mu*dB* (*flgM5208 flgM5223* Δ*flgA1529*)] *fliA5050*:: Tn*10d*Tc *flk-5206*::Tn*10d*Cm	
TH4050	DUP1142[(Δ*flgG-L2157 purB1879*)*Mu*dB* (*flgM5208 flgM5223* Δ*flgA1529* Δ*flgG-L2157*)] *fliA5050*::Tn*10d*Tc *flk-5206*::Tn*10d*Cm	
TT10213	*purB1879*::Mu*d*A	John Roth

[a] Unless indicated otherwise, all strains were constructed during the course of this work.

gene plays a role in the translational control of *flgM* gene expression.

MATERIALS AND METHODS

Strains. The bacterial strains used in this study and their origins are listed in Table 1.

Media and standard genetic manipulations. Media, growth conditions, transductional methods, and motility assays were performed as described previously (7, 8).

Plasmid constructions. Plasmids p322KS and p322SK were constructed as described previously (20). The pGEM3Z vector was obtained from Promega Corp (Madison, Wis.). pJK282 served as a template for RNase T2 probes and was made as follows. A *Bsp*DI-*Hin*dIII 3,455-bp fragment from pMH71 (14) containing the *flg*AMN operon as well as the *flgB* and *flgC* genes was ligated into *Bsp*DI-*Hin*dIII-cut p322KS. pTX592 contained a 1.3-kbp insert obtained by PCR amplification with pJK286 (20) and primers 5′-GCCGGATCCTTCCCGTCC ACGCA and 5′-TTAAAGCTTCGACGCGTTCCATATTAA. This insert contained the first 182 bp of the *flgB* coding region, the entire *flgA* gene, and the first 198 bp of the *flgM* coding region. The amplified PCR product was digested with *Bam*HI and *Hin*dIII at sites that were engineered in the primers and were ligated to *Bam*HI- and *Hin*dIII-cut pGEM3Z to give plasmid pTX592. Plasmids pTX593 and pTX594 were obtained by digesting the 1.3-kbp PCR products with *Bam*HI and *Ava*I and *Hin*dIII and *Ava*I, respectively, and ligating the 777- and 511-bp fragments to the pGEM3Z vector cut with the same enzymes. pTX593 contained the first 182 bp of the *flgB* coding region, the intercistronic region of *flgB* and *flgA*, and the first 439 bp of the *flgA* coding region. pTX594 contained the last 221 bp of the *flgA* coding region, the intercistronic region of *flgA* and *flgM*, and the first 218 bp of the *flgM* coding region. pTX595 contained a 704-bp *Bam*HI-to-*Hpa*I fragment from pMS531 (42) cloned into the pGEM3Z vector. It contained 122 bp of the region upstream from *fliA* and the first 582 bp of the *fliA* coding region.

Construction of pJK188 for the pulse-chase experiments was done as follows. A *Bsp*HI-*Hin*dIII 400-bp fragment filled in with Klenow from pMC64 (5) containing the *flgM* gene was ligated into pGEX-2T (Amersham Pharmacia Biotech, Piscataway, N.J.) cut with *Sma*I. The N terminus of FlgM was fused in frame to the C terminus of glutathione *S*-transferase (GST) to create a 39-kDa fusion protein under the inducible P_{tac} promoter.

Tandem chromosomal duplications of *flgM-lac* operon fusions. Duplications used to measure transcription separately from the *flgM* class 2 and class 3 promoters were constructed as described previously (15). Separately, a P22 lysate, grown on either the *flgA5211*::Mu*d*A or the *flgM5207*::Mu*d*A strain, was mixed with an equal titer of P22 lysate grown on the *purB1879*::Mu*d*A strain. The mixed lysates were used to transduce strain LT2 to Mu*d*A-encoded ampicillin resistance. Because of the large size of the Mu*d*A transposon (38 kbp) relative to the size of DNA packaged by P22 (42 kbp), inheritance of Mu*d*A by homologous recombination requires that two transduced fragments, each carrying part of the Mu*d* transposon, enter a recipient cell to inherit the Mu*d* insertion. Inheritance of Mu*d*A by homologous recombination involves three recombinational exchanges; one exchange joins the donor Mu*d*A fragments and two exchanges occur between the composite fragment and the recipient chromosome (15). Mixed lysates from either TH2575 (*flgA*::Mu*d*A) or TH2877 (*flgM*::Mu*d*A) and TT10213 (*purB*::Mu*d*A) were used to transduce LT2 to Mu*d*A-encoded ampicillin resistance. Duplication recombinants were distinguished from the parental recombinant types by the fact that they maintained wild-type copies of the *purB* and *flgA* genes and when cultures were grown in the absence of ampicillin, such that selection for Mu*d*A at the duplication join point was removed, they gave off Ap^s Lac^- (white in the presence of X-Gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside]) segregants. Loss of Mu*d*A is indicative of recombination between the duplicated homologies when selection for the Mu*d*A insertion at the join point is removed (15). Duplications were constructed in Δ*flgA* and Δ*flgG* to -*L* genetic backgrounds by transduction, selecting for Mu*d*A-encoded ampicillin resistance and screening for nonmotile transductants that kept the *flg* allele(s) of the recipient strain.

Tandem chromosomal duplications of *flgM-lacZ* gene fusions. Duplications used to measure translation of the *flgM5208-lacZ* gene fusion separately from either the *flgM* class 2 or class 3 promoters were constructed by first transducing strains TH3590, TH3591, TH3592, TH3593, TH3594, and TH3595 with a P22 transducing lysate grown on strain TH3907 (*flgA5210*::Tn*10d*Tc *flgM5223 flgM5208*::Mu*d*K) selecting for Tc^r. Recombination between the *lac* operon regions and the chromosomal regions clockwise to the site of the *flgA5210*::Tn*10d*Tc insertion in both the donor and recipient DNA segments yielded Tc^r, Ap^r, and Km^s transductants in which the *flgA5211*::Mu*d*A duplication join point was converted to an *flgM5208*::Mu*d*B join point to yield strains TH4023, TH4024, TH4025, TH4026, TH4027, and TH4028, respectively. All of these constructs carry the polar *flgA5210*::Tn*10d*Tc near the join point and the *flgM5223* (L66S) allele in the FlgM-LacZ fusions. This means that the FlgM-LacZ fusion containing the *flgM5223* (L66S) allele is transcribed and translated only from the class 3 *flgMN* promoter. Ampicillin was added to the growth medium to select for the duplication. When grown in the absence of ampicillin, the duplication strains segregated, yielding Ap^s, Lac^-, and Tc^s segregants, as expected.

To construct strains expressing the FlgM-LacZ fusion containing the *flgM5223* (L66S) allele from just the class 2 *flgAMN* promoter, the *flgA5210*::Tn*10d*Tc allele in strains TH4023 through TH4028, described above, was first removed by transducing these strains to tetracycline sensitivity (35) with a P22 transducing lysate grown on strain TH4041 (Δ*flgA1529 flgM5223 flgM5208*::Mu*d*K). A three-factor cross between the *flgM5208*::Mu*d*K, *flgA5210*::Tn*10d*Tc, and Δ*flgA1529* alleles revealed that the Δ*flgA1529* allele lies between the *flgM5208*::Mu*d*K and *flgA5210*::Tn*10d*Tc alleles (data not shown). This cross resulted in tetracycline-sensitive recombinants with a very high probability (>99%) of coinheriting the Δ*flgA1529* allele. To eliminate background spontaneous tetracycline-sensitive (Tc^s) revertants, phage lysates were mixed with cells at a high multiplicity of infection (i.e., 10) and were diluted 100- to 1,000-fold prior to being plated on Tc^s selection medium (35). In this way, the number of Tc^s transductants was 1,000-fold higher in frequency than that of spontaneous Tc^s revertants on cell-only control plates. These crosses yielded strains TH4029 through TH4034, which were Tc^s, Ap^r, and Km^s. Finally, TH4029 through TH4034 were transduced to Tc^r with a P22 transducing lysate on strain TH1479 (*fliA5059*::Tn*10d*Tc). Introduction of the *fliA*::Tn*10d*Tc allele prevents expression of the FlgM-LacZ fusion from the class 3 *flgMN* promoter (10). This yielded strains TH4045 through TH4050. In these strains, the FlgM-LacZ fusion carrying the *flgM5223* (L66S) allele is expressed only from the class 2 *flgAMN* promoter.

β-Galactosidase assays. β-Galactosidase assays were performed in triplicate on mid-log-phase cells as previously described (34). β-Galactosidase activities are expressed as nanomoles per minute per optical density unit at 650 nm.

Quantitative immunoblots. Intracellular levels of FlgM protein were determined by quantitative immunoblotting (3) with the following modifications. Cell lysates were prepared from mid-log-phase cells. Cells were pelleted from 3 ml of culture, resuspended in 50 μl of 1× sample buffer (29), and heated for 3 min. Protein concentrations from dilutions of the prepared lysates were determined with a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, Calif.). A total of 50 μg of each cell lysate was separated on 16.5% Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (40) and electrotransferred to Westran polyvinylidene difluoride membranes (Schleicher & Schuell, Keene, N.H.) in CAPS (3-[cyclohexylamino]-1-propane sulfonic acid) buffer (36). The blots were probed with anti-FlgM rabbit polyclonal antibodies and were immunostained by an alkaline phosphate-based reaction as described previously (16). Densitometric measurements of stained bands were accomplished by scanning the blots on a Agfa Arcus II Flatbed scanner by using Adobe Photoshop support software and then quantifying the image intensities with ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.). Standard protein concentration curves with purified FlgM protein were performed and showed the linearity intensity of immunostaining versus the protein concentration to be between 1 and 25 ng.

Pulse-chase experiments. Bacterial cells were grown in 1× E minimal medium supplemented with 0.2% glycerol and amino acid pools 6 to 11 without methionine (7) at 37°C to 40 Klett units. The cells were labeled for 1 min with [^{35}S] methionine-cysteine protein labeling mix (New England Nuclear, Boston, Mass.) at a final concentration of 50 μCi/ml. Cold methionine was added at a final concentration of 0.5%, and duplicate 1-ml samples were taken at various time points. Trichloroacetic acid (TCA) was added to a final concentration of 5%, and the mixture was incubated on ice for 20 min. The TCA precipitate was centrifuged in an Eppendorf microcentrifuge at 16,000 × *g* for 5 min at 4°C and was then washed two times in 80% cold acetone and dried. The pellet was resuspended in 50 μl of 1× SDS-sample buffer (29) and boiled for 3 min. Equal counts per minute from the time course samples were added to 900 μl of radioimmunoprecipitation buffer (RIPA) buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) (12) as well as a radiolabeled lysate from TH3746. Lysate from TH3746 has an N-terminal flgM fusion protein with glutathione *S*-transferase (39 kDa versus 10 kDa for native FlgM protein) and served as an internal control for the immunoprecipitation reaction. Anti-FlgM antibody (16) was added, and the mixture was incubated on ice for 1 h. A total of 50 μl of IgGSORB prepared as per the manufacturer's instructions (The Enzyme Center, Malden, Mass.) was added, and the mixture was incubated on ice for 30 min. The immunoprecipitated material was washed three times with cold RIPA buffer and then resuspended in 25 μl of 1× SDS-sample buffer. The samples were electrophoresed on 10% Tricine SDS-PAGE gels (40), and the gels were fixed in 10% methanol–10% acetic acid and dried. The gels were analyzed with a PhosphorImager (Molecular Dynamics), and the band intensities were quantified (phosphorimager units [PU]) by using ImageQuant software (Molecular Dynamics). Relative levels of FlgM were calculated as (PU of FlgM band/PU of GST-FlgM band) × 100.

FlgM export assay. Bacterial cells were grown in 25 ml of 1XE minimal medium supplemented with 0.4% glycerol and amino acid pools 6 to 11 (7) to 100 Klett units. The cells were pelleted at 17,000 × *g* for 30 min at 4°C, and the supernatant was saved and filtered through a 0.45-μm-pore-size cellulose acetate filter to remove any remaining bacterial cells. A 10-ml sample of filtered supernatant was filtered onto a prewetted BA85 0.2-μm-pore-size nitrocellulose filter (Schleicher & Schuell). The proteins were eluted by the addition of 50 μl of 1× SDS-sample buffer (29) and were heated at 65°C for 30 min. Samples were then analyzed by immunoblotting using anti-FlgM antibodies as described above.

RNA isolation and RNase T2 assays of chromosomal transcripts. RNA was purified from cells grown exponentially (50 to 65 Klett units at 660 nm) at 37°C by adding portions of bacterial cultures directly to lysis solutions without inter-

vening steps as described previously (46). RNase T2 protection assays of transcripts from the bacterial chromosomes were completed as described previously (46). RNA probes P1 to P4 covering parts of the *flgB*, *flgA*, and *flgM* regions are shown in Fig. 6 and were synthesized by using the following phage RNA polymerase and linearized plasmid templates: probe P1, SP6 and pTX592 with *Eco*RI; probe P2, SP6 and pTX594 with *Eco*RI; probe P3, T7 and pTX592 with *Hin*dIII; and probe P4, SP6 and pTX593 with *Eco*RI. An RNA probe for the detection of *fliA* transcript was synthesized by SP6 polymerase and pTX595 linearized with *Bam*HI. A series of labeled RNA molecules of known lengths were used as size standards to determine the lengths of the protected probes (46). Each hybridization reaction mixture contained 40 μg of total RNA or tRNA as a negative control. The opposite strand of probe P2 and the *fliA* probe were also synthesized and used to test for DNA contamination or antisense transcription. No bands were detected with these probes. The radioactivity in the bands on the gels was measured with the InstantImager (Packard, Meriden, Conn.).

Determination of mRNA stability. The stability of the *flgAMN* and *flgMN* mRNA was determined by previously described methods (11, 45). Rifampin was added to exponentially grown cells to a final concentration of 500 mg/ml, and the RNA was purified at different time points following addition of rifampin. Transcripts expressed from the *flgAMN* and *flgMN* promoters were analyzed as described above except that the radioactivity in the bands was measured with a PhosphorImager (Molecular Dynamics). The *flgAMN* and *flgMN* mRNA half-lives were calculated as described previously (45).

RESULTS

Mutations in the HBB structure result in an increase in the intracellular levels of FlgM, and loss of Flk restores wild-type levels of FlgM in ring mutant strains. Mutations in genes required for HBB formation result in the loss of class 3 gene expression (8). Because the negative regulator FlgM is believed to be exported in response to completion of the HBB structure, it was expected that a defective structure would lead to increased levels of FlgM protein in the cell. This model predicts that FlgM protein might accumulate in strains defective in HBB formation and result in the inhibition of σ^{28}-dependent class 3 gene expression. In order to test if a defective HBB structure leads to an increase in the intracellular levels of FlgM, quantitative immunoblot assays were used to measure the intracellular levels of FlgM in a variety of flagellar mutant strains.

We performed quantitative immunoblot assays with anti-FlgM antibody on eight isogenic strains (Fig. 2) to measure the effects of rod (*flgB*), P-ring (*flgA*, *flgI*), and L-ring (*flgH*) mutations on the levels of FlgM in the cell. Each HBB mutation resulted in about a twofold increase in the levels of FlgM in the cell compared to that for the isogenic Fla$^+$ strain. This result supports the original hypothesis that mutations in the HBB structure prevent the export of the FlgM negative regulator, which then accumulates in the cell to inhibit σ^{28}-dependent class 3 gene transcription.

The effects of a null mutation in the *flk* gene on the levels of FlgM protein were determined in Fla$^+$, ring, and rod mutant strains (Fig. 2). As mentioned above, intracellular levels of FlgM increased approximately twofold when export of FlgM was blocked by the presence of either a ring or a rod mutation compared to the levels of FlgM present in wild-type cells. Insertional inactivation of the *flk* gene had no effect on intracellular FlgM levels in either the wild-type or rod mutant strains, but intracellular levels of FlgM were reduced in the ring mutant strains in the presence of the *flk* null allele. This result demonstrates that inactivation of the *flk* locus in ring mutant strains leads to a reduction in the intracellular concentration of the FlgM anti-sigma factor. This could account for the observed expression of the *fliB-lac* fusion in P- and L-ring mutant strains when the *flk* gene is also disrupted (20).

FlgM turnover is unaffected by mutations in *flk*. The reduction in the intracellular levels of FlgM protein by loss of Flk in the ring mutant strains would account for the increase in class 3 gene transcription. There are several mechanisms by which the loss of Flk could lead to reduced FlgM levels. Flk could affect FlgM stability, FlgM export, transcription of the *flgM* gene, *flgM* mRNA translation, *flgM* mRNA stability, FlgM anti-σ^{28} activity, or some other mechanism that affects FlgM protein levels.

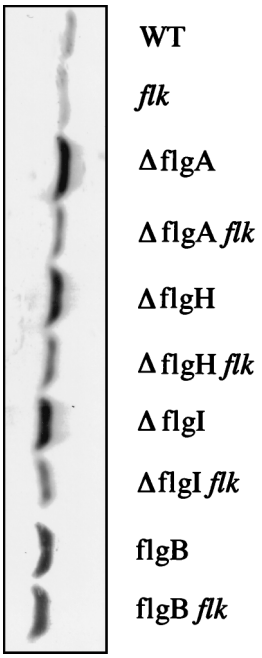

FIG. 2. Effects of HBB mutations and Flk on intracellular levels of FlgM protein. A quantitative immunoblot was performed with anti-FlgM antibody on cell extracts prepared from exponentially growing cells of isogenic wild-type and flagellar mutant strains. WT, TH2592; *flk*, TH3282; *flgB*, TH2157; *flgB flk*, TH3303; *flgA*, TH3441; *flgA flk*, TH3451; *flgH*, TH3442; *flgH flk*, TH3452; *flgI*, TH3444; *flgI flk*, TH3454.

One mechanism to reduce FlgM protein levels in the cell by loss of Flk in a ring mutant strain would be to decrease FlgM protein stability. The possibility that the reduction of intracellular FlgM levels by inactivation of *flk* in ring mutant strains was due to an increase in FlgM protein degradation was tested by measuring the effect of inactivation of *flk* on FlgM half-life. FlgM turnover was measured by adding ^{35}S-labeled methionine and cysteine to growing cells followed by a chase with unlabeled methionine and cysteine. At various times after introduction of label, both intracellular and secreted labeled FlgM were measured (see Materials and Methods) to determine if loss of Flk results in an increase in FlgM turnover in P- and L-ring mutant strains. We found that FlgM turnover occurred only in export-competent (Fla$^+$) cells. (Note that we measured the combined precipitated FlgM from both inside and outside the cells.) In Fla$^+$ cells, the measured half-life of FlgM was 7.3 min (Fig. 3A). This was increased to 11 min in the presence of a *flk* null allele. If FlgM export was prevented by a mutation in either an *flgB* or a Δ*flgHI* double mutant strain, then FlgM had no detectable turnover (Fig. 3B and C). (The *flgB* mutation is defective in the formation of the proximal rod portion of the basal body, while the Δ*flgHI* mutant strain is deleted for the P- and L-ring structural genes.) Loss of Flk did not result in increased FlgM turnover in the Δ*flgHI* strain even though loss of Flk did allow class 3 gene expression in the same Δ*flgHI* mutant background. These results suggest that the reduction in intracellular FlgM seen by loss of Flk in ring mutant strains does not result from an increase in FlgM

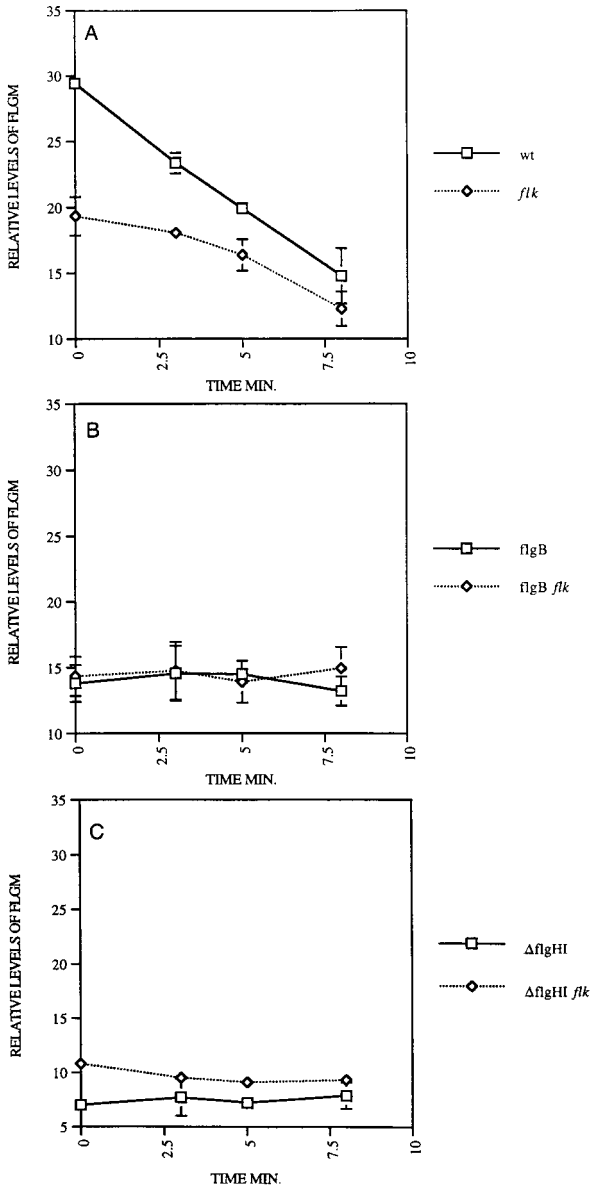

FIG. 3. Turnover of intracellular FlgM protein as measured by pulse-chase experiments with [^{35}S]Met-Cys on exponentially growing cells of isogenic strains. (A) Intracellular turnover of labeled FlgM in a wild-type (wt) strain (half-life = 7.3 min) (TH2592) and an isogenic *flk* mutant strain (half-life = 11 min) (TH3282). (B) Intracellular turnover of labeled FlgM protein in isogenic *flgB* (rod-defective, TH2157) and *flgB flk* (TH3303) mutant strains. (C) Intracellular turnover of labeled FlgM protein in isogenic Δ*flgHI* (ring-defective, TH3445) and Δ*flgHI flk* (TH3455) mutant strains.

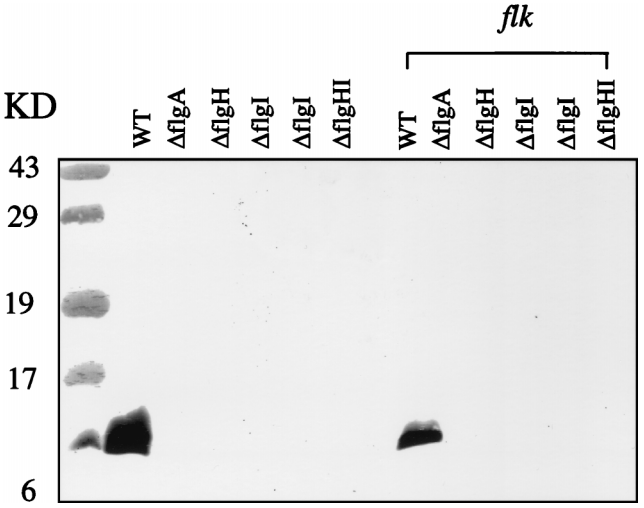

FIG. 4. Effect of Flk on the secretion of FlgM into the external growth medium. Immunoblot analysis was performed with anti-FlgM antibody on protein found in the spent growth medium of exponentially growing isogenic strains. WT, TH2592; *flgA*, TH3441; *flgH*, TH3442; *flgI* [left], TH3443; *flgI* [right], TH3444; *flgHI*, TH3445; WT *flk*, TH3282; *flgA flk*, TH3451; *flgH flk*, TH3452; *flgI flk* [left], TH3453; *flgI flk* [right], TH3454; *flgHI flk*, TH3455.

turnover. They also suggest that FlgM is stable to proteolysis inside the cell but not in the spent growth medium.

FlgM export is unaffected by mutations in *flk*. The possibility that the reduction of intracellular FlgM levels by inactivation of *flk* in ring mutant strains was due to export of FlgM was assayed by measuring levels of exported FlgM in the external medium in a variety of flagellar mutant backgrounds. The results presented in Fig. 4 show that FlgM is present in the spent growth medium of a wild-type flagellar strain but not in that of isogenic ring mutant strains. Introduction of an insertion mutation in *flk* had no effect on FlgM export. In a wild-type Fla$^+$ strain, FlgM is present in the external growth medium with or without a mutation in *flk*. In ring mutant strains no FlgM was detected in the spent growth medium even in the absence of a functional *flk* gene. These results do not support a model in which loss of Flk allows FlgM export in ring mutant strains.

Effects of mutations in *flk* on expression of *lac* operon fusions to the *flgM* class 2 and class 3 promoters in wild-type and flagellar mutant strains. To measure the effect of Flk on expression from the *flgAMN* class 2 promoter and the *flgMN* class 3 promoter in response to flagellar structural mutations, tandem chromosomal duplications were constructed between Mu*d*A insertions in the *purB* and either the *flgA* or *flgM* gene by a previously described method (15). DNA sequence analysis revealed that the *flgA5211*::Mu*d*A and the *flgM5207*::Mu*d*A insertions used in these assays resulted from Mu*d* transposition into the *flgA* gene just after codon 180 and into the *flgM* gene at codon 86 (CTC leu, 5′CT-Mu*d*; however, insertion recreates a Leu codon at the fusion join point [10]). The duplication between Mu*d*A insertions in *flgA* and *purB* resulted in the *lac* operon being expressed from the class 2 promoter of the *flgAMN* operon (Fig. 5A). The duplication between Mu*d*A insertions in the *flgM* and *purB* genes resulted in the *lac* operon being expressed from the class 3 promoter of the *flgMN* operon (Fig. 5B). In this *purB-flgM* duplication, transcription from the upstream class 2 promoter is prevented by a polar *flgA*::Tn*10d*Tc insertion mutation characterized previously (10).

Expression of *lac* from the *flgAMN* class 2 promoter was increased about 40% in the presence of an HBB deletion compared to the level of expression in the wild-type strain. In a ring mutant strain, transcription from the *flgAMN* class 2 promoter was unaffected (Fig. 5A). Introduction of an insertion mutation in the *flk* locus had no significant effect on the expression of the *flgAMN* class 2 promoter in any of the three backgrounds tested (Fig. 5A).

Expression of *lac* from the *flgMN* class 3 promoter was reduced 10-fold in the presence of either an HBB deletion mutation or a ring mutation (Fig. 5B). Introduction of an insertion mutation in the *flk* locus had no effect on expression of the *flgMN* class 3 promoter in the wild-type strain and increased its

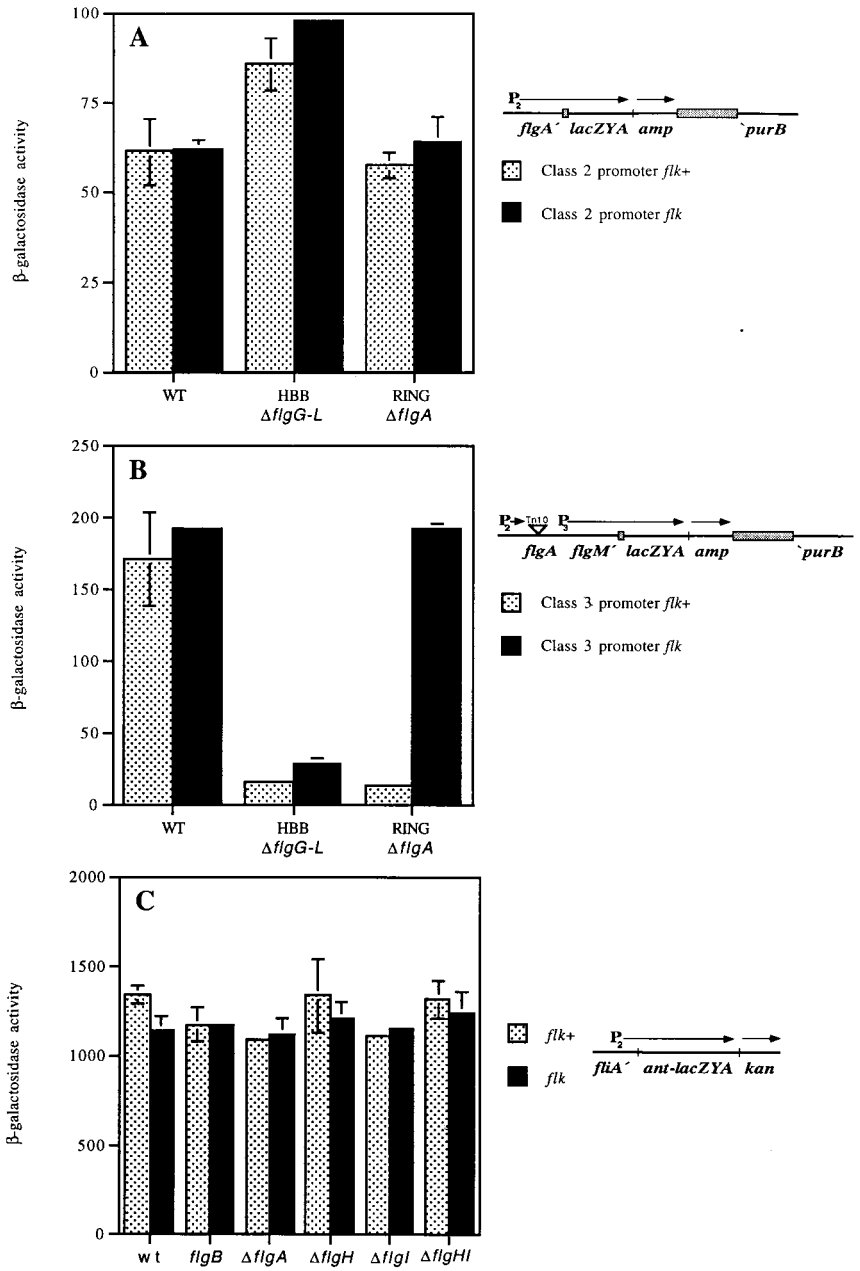

FIG. 5. Effects of Flk on *flgM* gene expression from both the class 2 *flgA* promoter and class 3 *flgM* promoter and the effect of Flk on expression of the *fliA* gene. (A) Expression of an *flgA-lac* operon fusion from the class 2 *flgA* promoter in isogenic wild-type and flagellar mutant strains. WT, TH3590, a duplication strain carrying an *flgA-lac* fusion and an intact *flgAMN* operon; WT *flk*, TH3593 (TH3590 *flk-5206*::Tn*10d*Cm), the identical strain defective in *flk*; Δ*flgG-L flk*$^+$, TH3592 (same as TH3590 except both duplicated regions carry the Δ*flgG-L2157* allele), a deletion of basal-body genes; Δ*flgG-L flk*, TH3595 (TH3592 *flk-5206*::Tn*10d*Cm); Δ*flgA flk*$^+$, TH3591 (same as TH3590 except the *flgAMN* operon carries a nonpolar internal deletion of the *flgA* gene); Δ*flgA flk*, TH3594 (TH3591 *flk-5206*::Tn*10d*Cm). (B) Expression of an *flgM-lac* operon fusion from the class 3 *flgM* promoter in isogenic wild-type and flagellar mutant strains. For the results from all the strains presented in this panel, expression of the intact *flgMN* operon from the *flgA* promoter is prevented by the introduction of a polar upstream *flgA*::Tn*10d*Tc insertion. WT, TH3596, a duplication strain carrying an *flgM-lac* fusion and an intact *flgMN* operon; WT *flk*, TH3599 (TH3596 *flk-5206*::Tn*10d*Cm), the identical strain defective in *flk*; Δ*flgG-L flk*$^+$, TH3598 (same as TH3596 except both duplicated regions carry the Δ*flgG-L2157* allele), a deletion of basal-body genes; Δ*flgG-L flk*, TH3601 (TH3598 *flk-5206*::Tn*10d*Cm); Δ*flgA flk*$^+$, TH3597 (same as TH3590 except the *flgAMN* operon carries a nonpolar internal deletion of the *flgA* gene); Δ*flgA flk*, TH3600 (TH3597 *flk-5206*::Tn*10d*Cm). (C) Expression of the *lac* operon from an isolated *fliA* promoter which includes DNA from −588 to +19 relative to the transcriptional start site from the *fliA* gene in the isogenic wild-type, the flagellar rod (*flgB*), and the different flagellar ring (*flgA*, *flgH*, and *flgI*) mutant strains with and without a mutation in the *flk* locus. WT, TH3558; WT *flk*, TH3565; *flgB flk*$^+$, TH3559; *flgB flk*, TH3566; *flgA flk*$^+$, TH3560; *flgA flk*, TH3567; *flgH flk*$^+$, TH3561; *flgH flk*, TH3568; *flgI flk*$^+$, TH3562; *flgI flk*, TH3569; *flgHI flk*$^+$, TH3564; *flgHI flk*, TH3571.

expression only slightly (less than twofold) in the HBB deletion mutant strain; however, in the ring mutant background, loss of *flk* by insertional inactivation restored expression from the class 3 *flgMN* promoter to levels seen in the isogenic wild-type strain (Fig. 5B). This result does not support the model that the reduction in the levels of FlgM protein seen in a ring *flk* double mutant compared to the levels in the isogenic ring mutant *flk*$^+$ strain is due to a reduction in transcription of the *flgM* gene. In fact, expression of *flgM* was increased in the ring mutant strain by introduction of the *flk* null allele, and the class

TABLE 2. Effects of *flk* mutation on the amounts of transcripts from class 2 and class 3 promoters in parent, *flgB2164*, and Δ*flgA1529* strains[a]

Transcript	Transcript amount (cpm)[b]					
	TH3505 (parent)	TH3506 (*flk*)	TH3504 (*flgB*)	TH3507 (*flgB flk*)	TH3508 (Δ*flgA*)	TH3513 (Δ*flgA flk*)
flgA-flgM[c]	274	197	256	266	251	261
P_{flgM}	818	413	36	34	33	477
P_{flgB}[d]	912	551	162	153	760	719
P_{fliA}	840	499	413	440	416	563

[a] Class 2 promoters include P_{flgA}, which produces *flgA-flgM* cotranscript, and P_{flgB} and P_{fliA} (22). Class 3 promoters include P_{flgM} (10).

[b] Total RNA was isolated from cells grown exponentially in Luria-Bertani medium at 37°C and was hybridized with RNA probes in RNase T2 protection assays. *flgA-flgM* and P_{flgM}, P_{flgB}, and P_{fliA} transcripts were quantitated from hybridized transcripts with probes P2 and P4 (Fig. 5) and the probe for P_{fliA} (Materials and Methods), respectively. Hybridized RNA fragments were analyzed and quantitated as described in Materials and Methods. cpm, counts per minute.

[c] The protected *flgA-flgM* cotranscripts in Δ*flgA1529* mutants were 410 nt in length compared to 504 nt in the $flgA^+$ parent.

[d] Quantitation of P_{flgB} included the sum of the 197- and 187-nt transcripts in the $flgB^+$ parent and the 85- and 92-nt transcripts in the *flgB2164* mutant.

3 *flgMN* promoter is regulated like any other class 3 promoter in response to effects of HBB mutations and *flk*.

Effect of mutations in *flk* on expression of *lac* operon fusions to the *fliA* promoter region in wild-type and flagellar mutant strains. We examined the effect of Flk on expression of the *fliA* promoter in a rod mutant and the different ring mutant strains (Fig. 5C). It was possible that the increased expression from class 3 promoters in ring mutant strains by introduction of an *flk* null allele could be due to a reduction of FlgM levels or an increase in FliA (σ^{28}) levels by increasing transcription from the *fliA* promoter. Introduction of the *fliA* gene on a multicopy plasmid overcomes FlgM inhibition of class 3 gene expression (16). A defective bacteriophage P22 vector carrying the *lac* operon transcribed from the *fliA* promoter region from −588 to +19 relative to the translational start site was constructed (5a). This phage was lysogenized into a variety of flagellar mutant strains with and without a functional *flk* gene, and the effect of Flk on *fliA* transcription was determined. Expression of *lac* from the *fliA* promoter was unaffected by a mutation in the rod structural gene, *flgB*, or any of the genes needed for P- or L-ring formation. Introduction of an insertion in the *flk* gene had no effect on expression of the *fliA* promoter in any of the strains tested (Fig. 5C). Thus, the increased expression from class 3 promoters in ring mutant strains by introduction of a *flk* null allele does not coincide with a reduction in transcription of the *flgM* gene or an increase in transcription of the *fliA* gene.

Effects of Flk on *flgM* mRNA synthesis. The effects of the *flk* mutation on class 2 and class 3 transcription of *flgM* were also examined at the level of mRNA synthesis by using RNase T2 protection assays. Levels of mRNA transcripts from the class 2 and class 3 *flgM* promoters were measured directly in wild-type, rod (*flgB*), and ring (*flgA*) mutant strains with and without a functional flk^+ gene (Table 2; Fig. 6). The ring mutant used in these experiments was the Δ*flgA* allele (Δ*flgA1529*) previously shown to have no effect on transcription or translation of the downstream *flgM* gene (10). Protected transcripts were detected with RNA probes P1 to P4 shown in Fig. 6A. As controls, the levels of class 2 transcripts from the *flgB* to *-L* operon and the *fliAZY* operon (probe not shown) were also measured.

We performed RNase T2 assays in six isogenic strains (TH3505, parent; TH3506, *flk*; TH3504, *flgB2164*; TH3507, *flgB2164 flk*; TH3508, Δ*flgA1529*; TH3513, Δ*flgA1529 flk*) (Table 2) to measure the effects of *flk* mutation on the amounts of transcripts from class 2 (P_{flgA}, P_{flgB}, and P_{fliA}) and class 3 promoters (P_{flgM}) in parent, non-ring (rod) mutant (*flgB*), and ring mutant (Δ*flgA*) strains. The transcription start site from the class 3 P_{flgM} promoter has been previously mapped (10) and a predicted 234-nucleotide (nt) protected transcript (Fig.

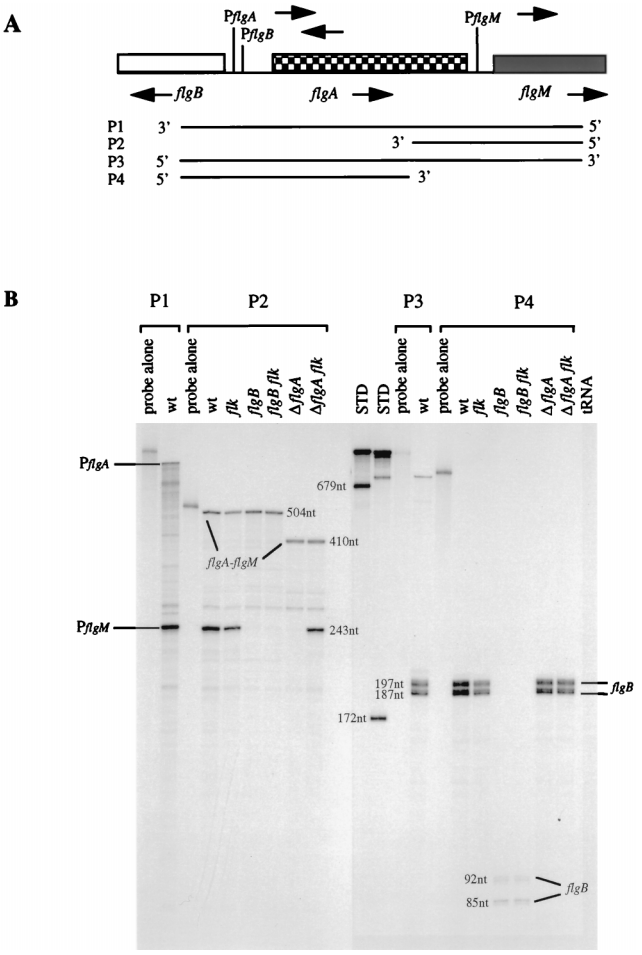

FIG. 6. RNase T2 assay showing the effects of *flk* mutation on the amounts of transcripts in the *flgB*, *flgA* and *flgM* regions. (A) Structure and transcription of the *flgB*, *flgA*, and *flgM* regions in *S. typhimurium*. The figure, which is drawn to scale, is based on sequences in the GenBank database (accession no. D00498 for *flgA* and *flgB* and accession no. M74222 for *flgM*). The start sites of the promoters P_{flgB} and P_{flgA} were based on consensus sequences for promoters in class 2 flagellar operons (22). The start site of P_{flgM} was mapped previously (9). Also indicated are the RNA probes P1 to P4 used in the RNase T2 protection assay. (B) Protected transcripts detected with RNA probes P1 to P4. Total RNA isolated from exponentially growing cells was hybridized with RNA probes P1 to P4 as described in Materials and Methods. The bands indicated correspond to transcription initiation from P_{flgA} and P_{flgM}, the *flgA-flgM* cotranscript, and transcripts in the *flgB* region. Lane 1, RNA probe P1; lane 2, protected transcripts obtained from hybridization of probe P1 with total RNA from TH3505 (wild type [wt]); lane 3, RNA probe P2; lanes 4 to 9, protected transcripts obtained from hybridization of probe P2 with total RNA obtained from TH3505 (wt), TH3506 (*flk*), TH3504 (*flgB2164*), TH3507 (*flgB2164 flk*), TH3508 (Δ*flgA1529*), and TH3515 (Δ*flgA1529 flk*), respectively; lanes 10 and 11, molecular weight standards [STD]; lane 12, RNA probe P3; lane 13, protected transcripts obtained from hybridization of probe P3 with total RNA from TH3505 (wt); lane 14, RNA probe P4; lanes 15 to 20, protected transcripts obtained from hybridization of probe P4 with total RNA obtained from TH3505 (wt), TH3506 (*flk*), TH3504 (*flgB2164*), TH3507 (*flgB2164 flk*), TH3508 (Δ*flgA1529*), and TH3513 (Δ*flgA1529 flk*), respectively. A total of 40 μg of total RNA was used in each hybridization. tRNA was used as a negative control (lane 21).

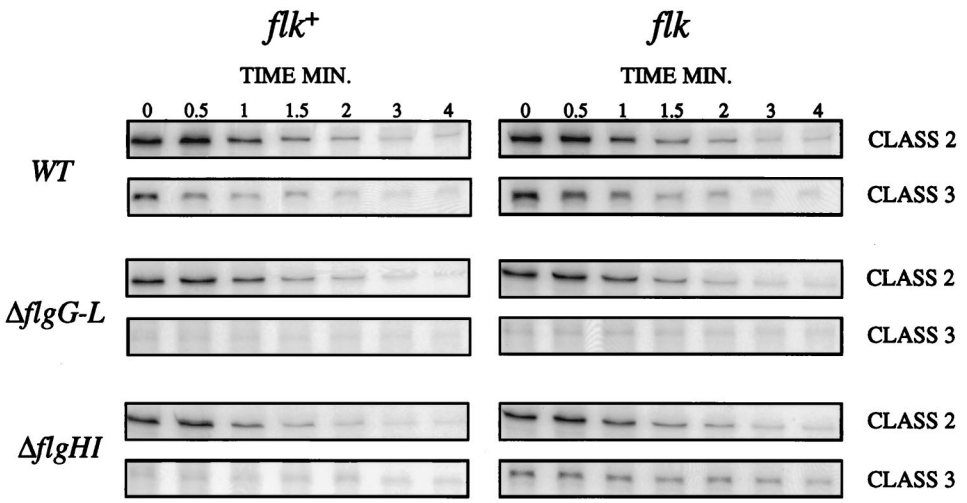

FIG. 7. Stability of *flgM* mRNA transcripts. Class 2 flgAMN and class 3 flgMN mRNA stability assays were performed on flagellar wild-type (WT: *flk*$^+$, LT2; *flk*, TH2413), HBB deletion (Δ*flgG-L flk*$^+$, TH3586; Δ*flgG-L flk*, TH3886), and ring mutant (Δ*flgHI flk*$^+$, TH3852; Δ*flgHI flk*, TH3888) strains in the presence and absence of a functional *flk* gene. The results of RNAase T2 protection assays on RNA samples, taken at the different time points as indicated following addition of rifampin, are shown (see Materials and Methods). The probe used in these assays was the P2 probe depicted in Fig. 6A, which hybridizes to both class 2 *flgAMN* and class 3 *flgMN* transcripts.

6B) from this promoter was detected by the overlapping P1 (lane 1) and P2 (lane 2) probes. The amount of P_{flgM} transcript decreased twofold in the *flk* mutant compared to that in the parent, TH3505 (Table 2). Mutation in *flgB* or *flgA* in the *flk*$^+$ strain caused a dramatic (23- or 25-fold, respectively) decrease in P_{flgM} transcript amounts (Table 2), consistent with the β-galactosidase activities of operon fusion strains. However, *flk* mutation reversed this decrease in the amount of P_{flgM} transcript in the *flgA* mutant but not in the *flgB* mutant. The P_{flgM} transcript amount was 14-fold higher in the *flgA flk* double mutant than the amount in the *flgA* mutant but remained low in the *flgB flk* double mutant, as in the *flgB* mutant.

In contrast to their effects on class 3 promoters, *flgB*, *flgA*, and *flk* mutations did not cause large changes in the amounts of transcript from class 2 promoters (Table 2). Transcripts initiated from P_{flgA} gave rise to an *flgA-flgM* cotranscript detected as the predicted 504-nt full-length protected transcripts with probe 2 and as 410-nt transcripts in Δ*flgA1529* mutants (Fig. 6B). Amounts of *flgA-flgM* transcript decreased slightly in the *flk* mutant and were similar in *flgB*, *flgB flk*, *flgA*, and *flgA flk* mutant strains compared to that in the parent strain. Transcripts initiated from P_{flgB} were detected as 197- and 187-nt protected fragments with probes P3 and P4 in *flgB*$^+$ strains. The 5′ ends of the *flgB* transcript were mapped to 27 and 37 nt downstream of the predicted P_{flgB} transcript start site based on consensus sequences for class 2 promoters (22). It is possible that the detected transcripts were processed transcripts from the predicted promoter. The amounts of *flgB* transcript quantitated as the sum of the 197- and 187-nt species were slightly lower in the *flk* mutant than in the parent but were similar in *flgA* and *flgA flk* strains. The protected *flgB* transcripts in *flgB2164* mutants were detected as shorter (92 and 85 nt) and less abundant species than the native *flgB* transcripts. The *flk* mutation had no effect on the amount of the mutant *flgB* transcript. Protected transcripts initiated from P_{fliA} were detected by the *fliA* probe as a predicted 630-nt species (data not shown). The *flk* mutation caused a slight decrease of P_{fliA} transcript in the wild-type flagellar background but did not change the transcript amounts in *flgB* and *flgA* mutant strains. These results are consistent with the genetic results obtained for the *lac* operon fusions presented above.

Effects of Flk on *flgM* mRNA stability. Another mechanism by which Flk could reduce FlgM levels in P- and L-ring mutant strains could be through an effect on *flgM* mRNA stability. Given that the class 3 *flgMN* mRNA transcript increases 10-fold in an *flk* ring mutant compared to that in the ring mutant strain alone, any reduction in *flgM* mRNA stability might be expected to occur on this transcript, although it is possible that both the *flgAMN* and the *flgMN* transcripts are affected. The stabilities of the class 2 *flgAMN* and class 3 *flgMN* transcripts were determined in wild-type (Fla$^+$), HBB deletion (Δ*flgG* to *-L*), and ring mutant backgrounds in the presence and absence of an *flk* null allele (Fig. 7 and Table 3). There was a slight increase in the mRNA stability (50 to 60%) of the class 2 transcript in the HBB deletion (Δ*flgG* to *-L*) strain and no effect of the ring mutation on the stability of the class 2 transcript. There were 1.5- and 2-fold increases in the half-lives of the class 3 transcript in the HBB deletion mutant and ring mutant backgrounds, respectively, compared to that of the Fla$^+$ background. Finally, there was no effect of *flk* on the class 2 transcript in the different backgrounds and a less than two-

TABLE 3. Effects of *flk* mutation on the half-lives of mRNA transcripts from class 2 *flgAMN* and class 3 *flgMN* promoters in wild-type, ring mutant (Δ*flgHI*), and HBB deletion (Δ*flgG* to *-L*) strains

Flagellar genotype	mRNA half-life[a] (min)			
	Class 2 transcript		Class 3 transcript	
	flk$^+$	*flk*	*flk*$^+$	*flk*
Fla$^+$	0.45 ± 0.05	0.53 ± 0.2	0.98 ± 0.2	0.63 ± 0.3
Δ*flgHI*	0.43 ± 0.01	0.54 ± 0.1	2.2 ± 0.8	3.7 ± 0.2
Δ*flgG* to *-L*	0.74 ± 0.2	0.8 ± 0.2	1.5 ± 0.2	1.8 ± 0.1

[a] Half-lives were determined by linear regression analysis, as described in Materials and Methods, for two independent experimental trials, the results from one of which are shown in Fig. 7.

fold increase in stability in the ring mutant background. There was a further slight increase (less than twofold) in the stability of the class 3 transcript in the ring mutant background when Flk was removed, but removal of Flk did not significantly affect the stability of the class 3 transcript in the HBB deletion background. Thus, there is no evidence suggesting that the reduction of intracellular FlgM levels in ring mutant backgrounds is due to a decrease in either class 2 or class 3 *flgM* mRNA stability. If anything, there was a three- to fourfold increase in the stability of the class 3 transcript in the ring *flk* double mutant strain compared to that of the Fla^+ ($flk^{+/-}$) strains.

Characterization of a LacZ translational fusion to FlgM. We have shown that the loss of *flk* results in a reduction in intracellular FlgM levels in P- and L-ring mutant strains (Fig. 2). Reduction in FlgM levels leads to increased transcription of the class 3 promoter, including the class 3 promoter for the *flgM* gene itself (20) (Table 2 and Fig. 5 and 6). FlgM turnover, FlgM secretion, *flgM* transcription, and *flgM* mRNA stability were not found to account for the observed reduction in FlgM levels (Fig. 3, 4, and 7 and Table 3). Another possibility is that translation of the class 2 and/or the class 3 *flgM* mRNA transcripts might be reduced by loss of *flk* in the ring mutant strains. We tested this possibility with a *lacZ* translational fusion to *flgM* and used β-galactosidase assays to examine the effects of *flk* on *flgM* translation. We previously reported the isolation of a Mu*d*K insertion in the *flgM* gene, *flgM5208*::Mu*d*K, that resulted in a translational fusion of *lacZ* to *flgM* (10). DNA sequence analysis revealed that the Mu*d* transposed into the *flgM* gene at codon 86 (CTC Leu, 5′CT-MudK) (10). This is the exact same site of insertion for the *flgM5207*::Mu*d*A insertion used to characterize the effect of *flk* on *flgM* transcription from the class 3 promoter presented above (Fig. 5).

Effects of mutations in *flk* on expression of *flgM-lacZ* gene fusions expressed from the *flgM* class 2 and class 3 promoters in wild-type and flagellar mutant strains. We decided to compare expression of β-galactosidase with the *flgM-lacZ* gene fusion (where β-galactosidase levels are dependent on both transcription and translation of *flgM*) to expression of β-galactosidase with the *flgM-lac* operon fusion (where β-galactosidase levels are dependent only on transcription of *flgM*). This is feasible only if the putative translational regulatory signals are 5′ to the site of insertion of the Mu*d* transposon at amino acid 86 of FlgM. To measure the effect of Flk on translation of FlgM-LacZ expressed from the class 2 and class 3 promoters in response to flagellar structural mutations, tandem chromosomal duplications were constructed as described in Materials and Methods. The duplications resulted in two tandem chromosomal copies of the *flgM* region. One copy contains a wild-type *flgM* gene. The second copy of the *flgM* chromosomal region contains the *flgM-lacZ* gene fusion expressed from either the class 2 promoter of the *flgAMN* operon or the class 3 promoter of the *flgMN* operon. In all cases the fusion protein carried the L66S substitution mutation. This is a base substitution mutation at codon 66 in FlgM that results in substitution of leucine by serine at this position (6). The L66S mutation renders FlgM defective in binding to σ^{28} (6). This mutation was used in these assays so that changes in the level of the FlgM-LacZ protein fusion would not have the capacity to feedback-regulate its own expression at the class 3 promoter. In the cases where the FlgM-LacZ fusion was expressed from the class 2 promoter, an internal, nonpolar deletion of *flgA*, Δ*flgA1529*, was included which when duplicated for the *flgA1529* allele renders the cell defective in P-ring assembly.

Effects of mutations in *flk* on expression of *lac* operon fusions to the *flgM* class 2 and class 3 promoters in wild-type and flagellar mutant strains. Expression of *flgM-lacZ* from the *flgAMN* class 2 promoter increased less than twofold in the presence of an HBB deletion and increased about 2.5-fold in the ring mutant background compared to the level in the isogenic wild-type strain (Fig. 8A). Introduction of an insertion mutation in the *flk* locus showed only a slight reduction of about 20% in the expression of the FlgM-LacZ fusion from the *flgAMN* class 2 promoter in all of the three backgrounds tested (Fig. 8A).

Expression of FlgM-LacZ from the *flgMN* class 3 promoter was reduced 25-fold in the presence of either an HBB deletion mutation or a ring mutation (Fig. 8B). This is presumably due to the FlgM-mediated inhibition of transcription from the class 3 promoter in an HBB mutant strain (Fig. 5B). Introduction of an insertion mutation in the *flk* locus had no effect on *flgM* expression in the wild-type strain and had a slight increase (less than twofold) in the HBB deletion mutant strain. There was a fivefold increase in expression in the ring mutant background (Fig. 8B). In these assays with the *flgM-lacZ* gene fusion, we did not observe restoration of β-galactosidase activity to wild-type levels by loss of *flk* as seen in the *flgM-lac* operon fusion constructs expressed from the class 3 promoter in the ring mutant background (Fig. 5B). Thus, by comparing the effect of Flk on expression of the *flgM-lacZ* translational fusion (Fig. 8) to the effect of Flk on expression of the *flgM-lac* transcriptional fusion (Fig. 5), we conclude that loss of Flk leads to a reduction in the translation of *flgM* expressed from the class 3 transcript in the ring mutant background but not in either the wild-type or the HBB deletion background.

DISCUSSION

Loss of Flk reduces FlgM levels in ring mutant strains. The flagellar regulon is a regulatory network of over 50 genes in which expression of these genes is tightly coupled to the assembly of the flagellar organelle. The FlgM negative regulator is responsible for keeping expression of σ^{28}-dependent promoters off until the HBB structure is complete. A novel genetic locus, *flk*, was discovered which allowed class 3 gene expression in ring mutant strains. Results presented in this report demonstrate that restoration of class 3 gene expression in ring mutant strains by loss of Flk is the result of reduced FlgM levels in the cell. This work examined many possible mechanisms in which loss of Flk could result in a reduction in FlgM levels in the cell. This work demonstrates that the Flk effect on class 3 transcription in ring mutant strains is not due to any of the following: (i) a reduction in transcription of the *flgM* gene, (ii) a reduction in *flgM* mRNA levels, (iii) a reduction in *flgM* mRNA stability, (iv) a decrease in FlgM protein stability, or (v) the ability to export FlgM through a "ringless" HBB structure. Transcription of *flgM* from the class 3 promoter increased about 15-fold when *flk* was mutated, suggesting that the effect of Flk on FlgM levels is posttranscriptional. Indeed *flgM* translation was reduced when a null mutation in *flk* was introduced into a ring mutant background (discussed below).

FlgM accumulates in export-deficient cells despite a reduction in *flgM* transcription. The hypothesis that FlgM could sense the completion of the HBB structure by being a substrate for secretion through the completed structure was supported by the finding that intracellular levels of FlgM increase by about 2-fold in HBB mutant strains despite a 10-fold decrease in FlgM transcription from the class 3 promoter. We had shown previously that about 80% of *flgM* transcription during exponential growth comes from the class 3 promoter. We have determined that in HBB mutant strains, FlgM was stable and its turnover was dependent on the presence of a functional

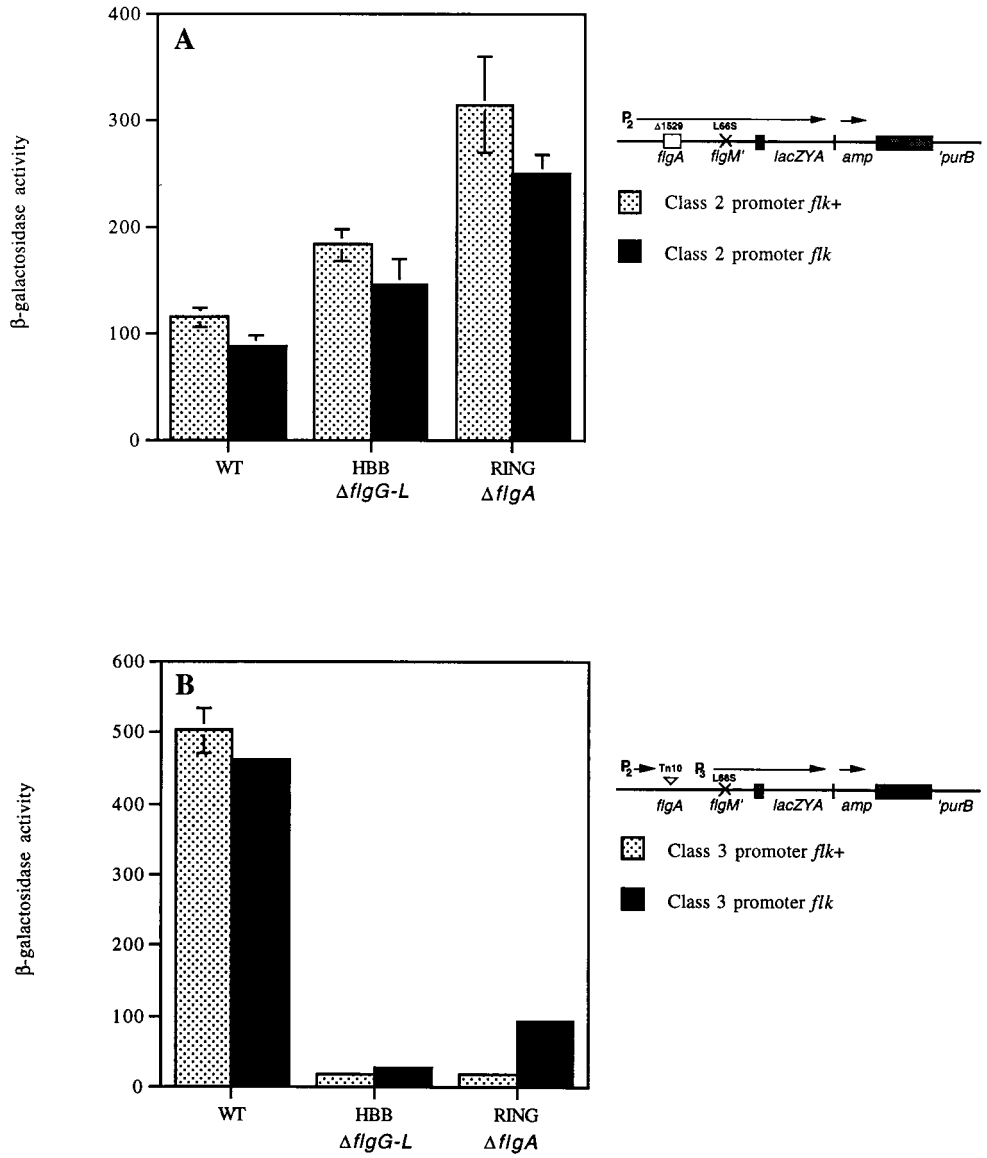

FIG. 8. Effects of Flk on *flgM-lacZ* expression from the class 2 *flgAMN* and class 3 *flgMN* promoters. (A) Expression of the *flgM5208-lacZ* gene fusion from the class 2 *flgA* promoter in isogenic wild-type and flagellar mutant strains. All strains harbor tandem, chromosomal duplications of the *flgM* through *purB* genes with the *flgM5208-lacZ* gene fusion located at the join point of the duplicated region. WT, TH4045; WT *flk*, TH4048; HBB Δ*flgG-L flk*$^+$, TH4047 (same as TH4045 except both duplicated regions carry the Δ*flgG-L2157* allele), HBB Δ*flgG-L flk*, TH4050 (TH4047 *flk-5206*::Tn*10d*Cm); RING Δ*flgA flk*$^+$, TH4046 (same as TH4045 except both the *flgAM-lacZYA* and *flgAMN* operons carry a nonpolar internal deletion of the *flgA* gene); RING Δ*flgA flk*, TH4049 (TH4046 *flk-5206*::Tn*10d*Cm). All six strains carry the *fliA5059*::Tn*10d*Tc insertion, which prevents expression of *flgM* and *flgM-lacZ* from the class 3 *flgMN* promoter (10). (B) Expression of the *flgM5208-lac* operon fusion from the class 3 *flgM* promoter in isogenic wild-type and flagellar mutant strains. All strains harbor tandem, chromosomal duplications of the *flgM* through *purB* genes with the *flgM5208-lacZ* gene fusion located at the join point of the duplicated region. WT, TH4023; WT *flk*, TH4026; HBB Δ*flgG-L flk*$^+$, TH4025 (same as TH4023 except both duplicated regions carry the Δ*flgG-L2157* allele); HBB Δ*flgG-L flk*, TH4028 (TH4025 *flk-5206*::Tn*10d*Cm); RING Δ*flgA flk*$^+$, TH4024 (same as TH4023 except that the *flgAMN* operon carries a nonpolar internal deletion of the *flgA* gene); RING Δ*flgA flk*, TH4027 (TH4024 *flk-5206*::Tn*10d*Cm). All six strains carry the *flgA5210*::Tn*10d*Tc insertion, which prevents expression of *flgM-lacZ* from the class 2 *flgAMN* promoter (10).

HBB structure, suggesting that turnover of FlgM in wild-type strains during exponential growth is dependent on a gene product produced or activated following HBB completion or that FlgM turnover occurs only on extracellular FlgM.

In examining the effect of Flk on *flgM* gene transcription, we saw that loss of Flk in ring mutant strains resulted in the simultaneous 10-fold increase in transcription from the *flgM* class 3 promoter and a return of intracellular FlgM down to wild-type levels. How does increased *flgM* transcription correlate with decreased FlgM protein levels? The answer has to do with the feedback regulation that FlgM has on its own structural gene expressed from the class 3 promoter in response to completion of the HBB structure. FlgM responds to the completed HBB structure by itself being a substrate for export through it into the external medium. Because FlgM is autoregulatory, the rate at which FlgM protein is produced (transcription-translation) and the rate at which FlgM is exported through the structure will both determine and depend on how much FlgM accumulates in the cell. FlgM inhibits σ^{28}-dependent transcription, and 80% of *flgM* gene transcription is from a σ^{28}-dependent, class 3 promoter. When the HBB is defective, FlgM protein accumulates and, as was shown in this work, it is

a very stable intracellular protein. Accumulation of FlgM inhibits σ^{28}-dependent transcription, including that directed by the class 3 promoter for the *flgM* gene. We have presented both genetic data, by individually assaying both the *flgM* class 2 (P_{flgA}) and class 3 (P_{flgM}) promoters fused to the *lac* operon (Fig. 5), and molecular data, by measuring the individual transcripts expressed from the P_{flgA} and P_{flgM} promoters (Fig. 6 and Table 2), to support this model.

Flk couples *flgM* translation to ring assembly. The observation that the large amount of *flgM*-containing transcript from the class 3 promoter in a ring *flk* double mutant strain does not result in an increase in intracellular FlgM protein levels (Fig. 2 and 3) suggested that FlgM translation was reduced when *flk* was defective.

The effect of *flk* on *flgM* translation was tested with an FlgM-LacZ protein fusion in which the anti-σ^{28} activity was rendered defective by mutation (L66S). As a result of comparing the transcription of an *flgM-lac* operon fusion to the translation of an *flgM-lacZ* gene fusion in wild-type, HBB deletion, and ring mutant strains with and without a mutation in *flk* (Fig. 5 and 8), we can make the following conclusions. The effect of the HBB deletion resulted in a 50% increase in transcription of *lacZ* from the *flgAMN* class 2 promoter, while no increase in transcription was observed in the ring mutant background (Fig. 5A). The expression of the *flgM-lacZ* gene fusion also showed a 50% increase in the HBB deletion background (Fig. 7A), which can be attributed to the 50% increase in the HBB deletion background (Fig. 7A), which can be attributed to the 50% increase in transcription from the class 2 promoter (compare Fig. 5A to 8A). In the ring mutant background there was about a threefold increase in translation (Fig. 8A), and since there was no increase in transcription in the ring mutant strain (Fig. 5A), we conclude that there is a threefold increase in *flgM* translation in a ring mutant strain. We also conclude that there is differential transcriptional and translational regulation in different basal-body mutant strains. This implies that the coupling of flagellar gene expression to flagellar assembly occurs at assembly stages prior to HBB completion.

If we now compare the transcription and translation of *flgM* from the class 3 promoter, the effects are striking. As expected, the level of class 3 transcription is lower in either the HBB deletion or the ring mutant background (Fig. 5B). Loss of *flk* restores transcription of *flgM* class 3 transcription in the ring mutant background, but not in the HBB deletion background, to levels seen for the wild-type strain (Fig. 5B). This result is similar to what we observed previously for transcription from the class 3 *fliB* flagellin gene promoter (20). Finally, if we look at expression of the *flgM-lacZ* gene (translational) fusion, it is up 50% in the HBB mutant background (Fig. 8B), which can be accounted for by a 50% increase in transcription (compare Fig. 5B and 8B). In the ring mutant background, expression of the *flgM-lacZ* gene fusion is up when *flk* is mutated, but the level of β-galactosidase activity is only 20% of that measured in the Fla^+ strains, whereas the amount of β-galactosidase activity from the *flgM-lac* operon fusion in the ring mutant background was at 100% of that measured in the Fla^+ strains when *flk* was mutated (compare Fig. 5B and 8B). This result suggests that the translation of the *flgM-lacZ* gene fusion is reduced by the loss of Flk in the ring mutant strain. This accounts for the regulatory effect seen on flagellar class 3 gene expression in ring mutant strains. The results are in agreement with a model, presented in Fig. 9, that the Flk effects on FlgM protein levels are due, at least partly, to a reduction in the translation of the class 3 *flgM* mRNA. However, because the class 3 *flgM* mRNA is not transcribed in the ring mutant strains, there must be some effect of Flk on *flgM* expressed from the class 2 transcript.

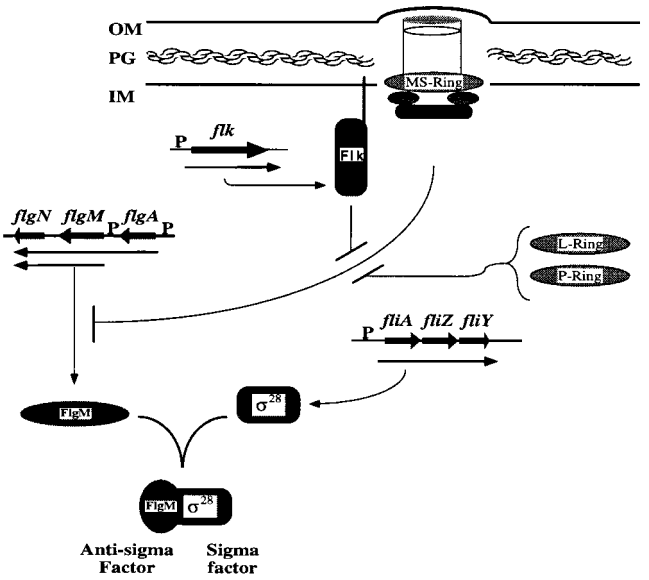

FIG. 9. Model diagramming the role of the Flk protein in the regulation of *flgM* mRNA translation. The ringless basal body is depicted transversing the inner membrane (IM) and peptidoglycan (PG) layers but is unable to penetrate the outer membrane-LPS (OM) layer. Flk is depicted to interact with the ringless basal body through its C-terminal hydrophobic 18-amino-acid tail in the inner membrane. Prior to P- and L-ring assembly, the ringless basal-body structure either directly or indirectly inhibits *flgM* mRNA translation. This effect requires a functional hook gene (*flgE*), so either the ringless basal body contains a nascent hook structure or hook subunits act separately in the cytoplasm. A reduction in *flgM* mRNA translation and subsequent FlgM protein levels in the cell would relieve inhibition of σ^{28} and allow class 3 flagellar gene expression. The negative regulatory effect of the ringless basal-body structure on *flgM* mRNA translation is inhibited either by the incorporation of the P- and L-rings into the basal body structure or by the action of the Flk protein. The genes are named above the chromosomal open reading frames (thickest arrows). mRNA transcripts are depicted below the genes, and the arrows indicate the direction of transcription. The arrow below each transcript indicates translation to the indicated protein product.

We observed a 20% reduction in *flgM* translation from the class 2 transcript in the ring mutant background when *flk* was mutated, but we saw the same effect in the Fla^+ and HBB deletion backgrounds as well. It may be that our error limits prevent us from detecting the small, but significant, reduction in *flgM* translation from the class 2 transcript when *flk* is mutated compared to the levels of *flgM* translation in the Fla^+ and HBB deletion backgrounds, and it is only translation of *flgM* from the class 3 transcript that is easily distinguished in the different backgrounds.

Homologies between Flk and RNA binding proteins of *E. coli*. An enhanced search of the protein database revealed homology between the so-called "S1 domain" of RNA binding proteins of *E. coli* (4) and a region of the deduced Flk protein sequence (amino acids 190 through 212 of *S. typhimurium* Flk and amino acids 192 through 214 of *E. coli* Flk). This could imply a direct interaction between Flk and the *flgM* mRNA.

FlgM, whose native structure is unfolded, is protected from intracellular degradation. While it is not unusual for proteins to be stable to turnover during exponential growth, the stability of intracellular FlgM seemed surprising given the recent discovery that the FlgM protein exists as an unfolded polypeptide (6). How does an unfolded protein stay resistant to protein degradation within the cell? One possible answer to this question is that intracellular FlgM stays bound by other proteins. The C-terminal half of FlgM contains the anti-σ^{28} domain (6, 19), while the N-terminal portion is essential for FlgM export

through the HBB structure (19). If binding of other proteins is necessary for FlgM stability, then two predictions can be made. First, in the absence of FlgM export, something may bind the N terminus of FlgM in the cell to prevent its degradation, while the σ^{28} protein binds the C terminus. If the level of FlgM exceeds that of σ^{28}, something may bind the C terminus of FlgM to protect it from degradation. Furthermore, only insertions in the *flgM* gene relieve class 3 gene expression in strains defective in HBB genes, except in the case of ring mutants, which can be suppressed by insertions in *flk* as well as in *flgM*. This suggests that if binding proteins essential for intracellular FlgM stability exist, they may be either essential to cell viability or essential for class 3 gene expression. Otherwise, selections that yielded *flgM* mutants would have yielded mutations in other genes as well. A second prediction is that the σ^{28} protein may be required for FlgM stability unless the anti-σ^{28} domain of FlgM binds other proteins as well as σ^{28}.

The ringless basal body is necessary for translational inhibition of *flgM* expression. What is the mechanism by which ring completion is coupled to translation of *flgM*, and what is the role of Flk in this mechanism? All of the HBB genes except the ring genes, *flgA*, *flgH*, and *flgI*, are required to be functional for the Flk effect on class 3 gene expression to be seen. This suggests that it is the ringless HBB structure that is directly or indirectly responsible for reduction in FlgM protein levels in the absence of Flk (Fig. 9). If any one of the other 23 known HBB genes is mutated, then loss of Flk does not exhibit the large effect on class 3 gene expression. We propose that Flk is involved in detecting completion of the L- and P-rings for the purpose of initiating some class 3 gene derepression prior to completion of the L- and P-rings for the purpose of initiating some class 3 gene dererpression prior to completion of the HBB structure. Otherwise, class 3 gene derepression must wait until the hook is complete and FlgM is exported out of the cell. This would result in a lag time between hook completion and the transcription, translation, and assembly of flagellin filament protein. The following scenario is hypothesized for when flagellin is expressed only after FlgM export occurs: the hook is completed, FlgM is exported, transcription of the flagellin filament structural genes (*fliC* or *fliB*) is initiated, and flagellin mRNA is transcribed and translated into protein subunits ready for export and polymerization at the hook-filament junction. Thus, there would be a time loss between hook completion and filament polymerization, potentially conferring a competitive disadvantage. In the presence of Flk, the following scenario is hypothesized. The rings are completed, and at this moment two things happen simultaneously: (i) hook elongation begins and (ii) Flk, sensing ring completion and/or hook elongation, directly or indirectly inhibits translation of *flgM* mRNA while production of σ^{28} protein continues. The σ^{28}/FlgM ratio increases and allows some transcription of flagellin subunits while the hook is elongating. Once hook biosynthesis is complete, FlgM is exported and flagellin transcription is fully derepressed, but enough flagellin has already been synthesized to initiate its export and polymerization without a lapse in time for flagellar assembly. Flk is predicted to eliminate the potential time loss from hook completion and FlgM export to flagellin expression, leading to more efficient biosynthesis of the flagellum and a greater survival advantage over a cell less efficient in flagellar assembly.

The above model also allows a role for Flk in detecting ringless HBB structures in wild-type cells. There are two distinct secretion pathways utilized in the assembly of the HBB. The type III flagellum-specific secretion system is required to direct components assembled beyond the cytoplasmic membrane into the hollow core of the growing structure to the site of assembly for each component. The rod components are assembled first, followed by the hook scaffolding protein FlgD and then the hook (21, 39). The P- and L-ring subunits are secreted into the periplasm by the general signal-sequence-dependent secretory pathway (14, 41). Thus, it is possible that ringless HBB structures could form while awaiting secretion of ring components into the periplasm. It is the presence of this structure that results in a reduction in FlgM levels in ring *flk* double mutant strains. Flk may be there to prevent this structure from reducing *flgM* translation until the rings form and HBB completion is imminent. This suggests that there may be conditions where ring assembly is not efficient or does not occur, leading to accumulation of the ringless HBB structures. Flk may be present to prevent any premature flagellin expression under such conditions.

The *flk* and *rflH* mutants may represent alleles of the same gene. A new regulatory gene of the flagellar regulon, *rflH*, was recently described as having a negative regulatory role in FlgM export (27). Because the chromosomal location of the *rflH* locus is placed at a location similar to the *flk* locus, it is possible that the *rflH* mutations are alleles of the *flk* gene. The FliK protein determines hook length. (Note that FliK and Flk are not the same protein.) Null mutations in the *fliK* gene result in what is known as the polyhook phenotype, in which the transition from export of hook subunits to export of class 3-exported proteins (FlgM, FlgK, FlgL, FliD, FliC, and FljB) does not occur. As a result, null alleles of the *fliK* gene are nonmotile. Motile revertants of *fliK* null alleles map to the *flhB* locus, which is part of the type III flagellum-specific export apparatus (13, 25). This realization led to the model that the FliK protein measured hook length and that, when hook length reached about 55 nm, the FliK protein was able to signal the export machinery, at FlhB, to stop export of hook subunits and begin export of class 3 exported proteins. Thus, in a strain carrying a *flhB* suppressor of the *fliK* null allele, FlgM is exported and class 3 genes are derepressed in the absence of the *fliK* gene. However, if the hook gene is mutated in the same background, FlgM is not exported and class 3 transcription is repressed (27). Selection for class 3 gene expression in an *flgE flhB* (*fliK* bypass mutant) strain yielded mutations in *rflH* that allowed FlgM export in this strain. A model was proposed that export is controlled by a double-locked gate where RflH and FlhB act as locks to independently inhibit export of class 3 proteins and that these locks are released by a hook completion signal (sensed directly by RflH and via FliK to FlhB), resulting in class 3 protien export. If *flk* and *rflH* are the same locus, then this protein has a dual role in flagellar assembly at the level of ring completion (and FlgM translation) and at the level of preventing class 3 protein export in the absence of the hook structure. We suggest that Flk does not sense hook completion as proposed but that it senses the translation and/or export and/or polymerization of hook following ring completion. This model is consistent with the phenotypes of both the *rflH* and *flk* alleles reported.

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