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The *flk* Gene of *Salmonella typhimurium* Couples Flagellar P- and L-Ring Assembly to Flagellar Morphogenesis

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The flagellum of *Salmonella typhimurium* is assembled in stages, and the negative regulatory protein, FlgM, is able to sense the completion of an intermediate stage of assembly, the basal body-hook (BBH) structure. Mutations in steps leading to the formation of the BBH structure do not express the flagellar filament structural genes, *fliC* and *fljB*, due to negative regulation by FlgM (K. L. Gillen and K. T. Hughes, J. Bacteriol. 173:6453–6459, 1991). We have discovered another novel regulatory gene, *flk*, which appears to sense the completion of another assembly stage in the flagellar morphogenic pathway just prior to BBH formation: the completion of the P- and L-rings. Cells that are unable to assemble the L- or P-rings do not express the flagellin structural genes. Mutations by insertional inactivation in either the *flk* or *flgM* locus allow expression of the *fljB* flagellin structural gene in strains defective in flagellar P- and L-ring assembly. Mutations in the *flgM* gene, but not mutations in the *flk* gene, allow expression of the *fljB* gene in strains defective in all of the steps leading to BBH formation. The *flk* gene was mapped to min 52 of the *S. typhimurium* linkage map between the *pdxB* and *fabB* loci. A null allele of *flk* was complemented in *trans* by a *flk*$^+$ allele present in a multicopy pBR-based plasmid. DNA sequence analysis of the *flk* gene has revealed it to be identical to a gene of *Escherichia coli* of unknown function which has an overlapping, divergent promoter with the *pdxB* gene promoter (P. A. Schoenlein, B. B. Roa, and M. E. Winkler, J. Bacteriol. 174:6256–6263, 1992). An open reading frame of 333 amino acids corresponding to the *flk* gene product of *S. typhimurium* and 331 amino acids from the *E. coli* sequence was identified. The transcriptional start site of the *S. typhimurium flk* gene was determined and transcription of the *flk* gene was independent of the FlhDC and σ^{28} flagellar transcription factors. The Flk protein observed in a T7 RNA polymerase-mediated expression system showed an apparent molecular mass of 35 kDa, slightly smaller than the predicted size of 37 kDa. The predicted structure of Flk is a mostly hydrophilic protein with a very C-terminal membrane-spanning segment preceded by positively charged amino acids. This finding predicts Flk to be inserted into the cytoplasmic membrane facing inside the cytoplasm.

Flagellar biosynthesis in *Salmonella typhimurium* and *Escherichia coli* represents the best-understood system in which a large regulatory network of over 50 genes is coupled to the assembly of a large, complex structure, the flagellar organelle. The flagellum is required for motility in response to chemical attractants and repellents in a liquid environment (5, 35, 54). The flagellum is commonly divided into three parts: the basal body, the hook, and the filament. The basal body is contained within the cell envelope, and over 30 genes are required for its synthesis (reviewed in references 2 and 35). The flagellar structure is diagrammed in Fig. 1. The basal body is composed of the following components: (i) a reversible rotary motor driven by a proton pump; (ii) a rod structure that transverses the cell wall, cell membranes, and periplasm; (iii) three ring proteins that stabilize the rod structure in the inner membrane, peptidoglycan layer, and lipopolysaccharide; (iv) a switch complex that determines clockwise or counterclockwise rotation in response to the chemosensory system; and (v) a flagellum-specific type III secretion pathway. Attached to the basal body on the outside of the cell is a flexible hook structure, from which the long filament structure extends. The flagellum is hollow, and a flagellum-specific export pathway sends subunits through the hollow passage to be assembled at the tip of the growing structure (35).

The flagellar genes are organized into a transcriptional hierarchy of three classes that are coupled to morphogenesis of the flagellar organelle (Fig. 1) (25). Each class must be functional for the subsequent class to be expressed.

Class 1 genes, *flhD* and *flhC*, determine whether flagella are made. Expression of the FlhD and -C proteins is dependent on the general state of the cell. In addition, these proteins are required for normal cell growth. The *flhDC* operon is regulated in response to environmental stimuli such as cyclic AMP levels and temperature (23, 53), heat shock (50), osmolarity and membrane biosynthesis (22, 38, 50, 51), and the H-NS protein (4). In addition, mutants of the *flhDC* operon affect cell division (45). The FlhD and FlhC proteins make up a heteromultimeric complex that acts as a transcriptional activator to stimulate transcription from promoters of class 2 flagellar genes by σ^{70}-containing RNA polymerase (32). The FlhDC complex binds a ~40-bp region upstream of the −35 promoter sequence and is thought to interact with the C-terminal region of the α subunit of RNA polymerase to initiate transcription (32, 33).

Class 2 genes (Fig. 1) encode the proteins needed for the structure and assembly of the basal body-hook (BBH) structure as well as the *fliA* regulatory gene (25). The *fliA* gene encodes an alternative sigma transcription factor, σ^{28}, necessary for transcription of most class 3 genes (41). In addition,

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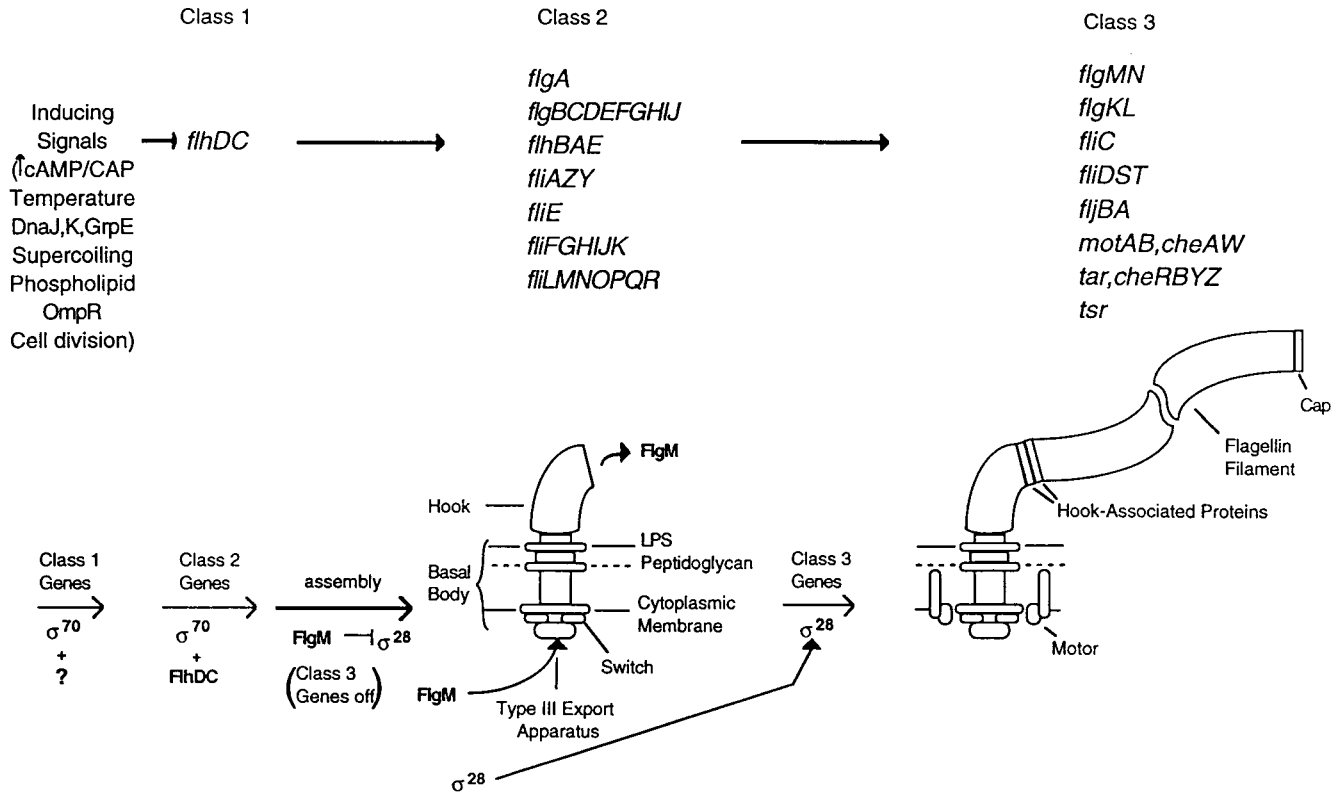

FIG. 1. Morphogenesis of the flagellar organelle is coupled to regulation of transcription in the flagellar regulon. Class 1 gene expression is regulated in response to a variety of environmental signals. Class 2 genes require the class 1 gene products for expression. Class 2 genes code for proteins involved in the structure and assembly of the BBH complex, a structural intermediate in organelle assembly, and two regulatory factors, FlgM and σ^{28} (product of the *fliA* gene). The class 3 promoters are dependent on the alternative sigma factor, σ^{28}, for promoter recognition by RNA polymerase and transcription. However, the FlgM protein inhibits σ^{28}-dependent transcription in the absence of a completed BBH structure. The BBH acts as an anti-FlgM factor by exporting FlgM out of the cell, thereby relieving inhibition of class 3 promoter transcription. The class 3 genes are expressed, and flagellar biosynthesis is completed. Class 3 genes encode the flagellin filament proteins, HAPs Cap (see text), and factors required for the chemosensory response. cAMP/CAP, cyclic AMP/catabolite gene activator protein; LPS, lipopolysaccharide.

σ^{28}-containing RNA polymerase will transcribe from promoters of several class 2 genes, including the structural gene for σ^{28} itself (*fliA*) (34).

There are class 3 genes expressed from both FlhDC-dependent and σ^{28}-dependent promoters. These include hook-associated protein (HAP) genes located at the hook-filament junction, the flagellar cap gene (encoding Cap), and the *flgM* regulatory gene (13, 29). Following hook assembly, the HAP-1, HAP-2, and Cap subunits are added to the end of the hook (35). These proteins are necessary for polymerization of the long external filament structure. The filament subunits initiate polymerization between the HAP-2 and Cap structures (35). Even though the HAP- and the Cap-encoding genes are expressed from FlhDC-dependent promoters, their protein products are not exported for assembly at the tip of the growing structure until after completion of the BBH.

Genes expressed solely from σ^{28}-dependent promoters encode the flagellin subunits (FliC and FljB) whose products make up the long external filament, the proteins required for chemotactic signal transduction, and the flagellar motors. The entire flagellum structure has a hollow center through which subunits transverse to polymerize at the tip of the growing organelle (10). Flagellin subunits polymerize at the cap-filament junction at the distal end of the growing filament (21).

It has been shown in both *E. coli* and *S. typhimurium* that if any of the class 2 genes are defective or absent, the class 3 flagellin genes are not expressed (11, 25). This is due to the action of the *flgM* gene product, which prevents σ^{28}-dependent transcription of the class 3 genes in the absence of a functional BBH complex (42). In the presence of a functional BBH complex, FlgM is found in the external medium, and its presence outside the cell is dependent on the cell's ability to assembly the BBH intermediate structure (20, 26). This finding led to the hypothesis that FlgM is exported through a completed BBH structure into the medium. Coupling export of FlgM through a completed BBH structure provides a simple mechanism to inhibit σ^{28}-dependent transcription prior to BBH completion. Upon BBH completion, export of FlgM relieves inhibition of σ^{28}-dependent transcription (the class 3 genes are transcribed), and flagellar assembly is completed. In this manner, FlgM is able to sense the completion of the BBH structure. This is probably necessary for at least two reasons. FlgM-mediated repression of σ^{28}-dependent transcription prevents the intracellular accumulation of flagellin, which might be toxic. Also, flagella can be sheared off, in which case FlgM is presumably exported, thereby allowing the immediate transcription and synthesis of more flagellin. This mechanism provides a system of gene regulation that can sense the development of the flagella and satisfies requirements for new filament protein subsequent to flagellar completion and following filament loss.

There are two separate secretory pathways utilized in BBH formation (Fig. 2). With the exception of the P- and L-rings, components assembled beyond the cytoplasmic membrane are exported through a type III flagellar export pathway through a hollow core within the organelle. These include the rod and

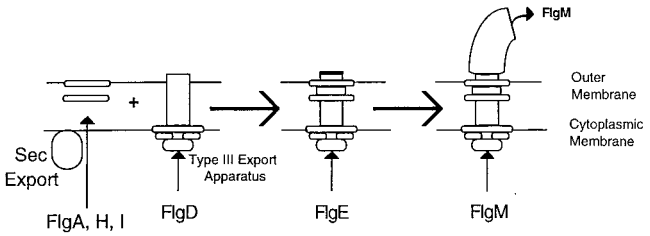

FIG. 2. Final stages of BBH assembly (24). Two extracytoplasmic protein export pathways are utilized for completion of the BBH structure. The flagellum-specific type III export system is required for assembly of the rod and extracellular components of the flagellum. The P- and L-rings are the only components of the flagellum known to possess a cleavable signal peptide and have been shown to be transported into the periplasm by the general secretory pathway of the cell (16, 17). The FlgD protein acts as a scaffold for the hook subunits (40).

hook structures; however, the hook will not polymerize until the P- and L-rings are assembled around the rod (24). The second pathway is via the general secretory pathway (39) and is required for export of P- and L-ring subunits (16, 17). FlgH and FlgI are the only proteins within the flagellar regulon with known *sec*-dependent signal sequences and are presumed to be secreted across the cytoplasmic membrane by the general secretory pathway into the periplasm, where they assemble into the P- and L-rings of the flagellum (16, 17). The FlgA protein is required for P-ring assembly (24, 57). FlgA has a deduced N-terminal hydrophobic stretch of amino acids proposed to be a signal sequence, although that was not verified experimentally (28).

Hook completion is detected by the *fliK* gene product. In the absence of FliK, hook growth continues and extended hooks, called polyhooks, are formed (43, 52, 56). Polyhook mutants are nonmotile, but suppressors of *fliK* null mutations (motile revertants) which map to the *flhB* locus have been isolated (15, 27). Since FlhB is part of the type III export machinery, it is thought that FliK senses completion of the hook and then signals the export machinery to change specificity from class 2 proteins that make up the BBH to class 3 proteins, including FlgM, FlgK, FlgL, FliD, FliC, and FljB (63).

Given that ring assembly utilized a different export system than the rest of the basal body assembly, it seemed possible that ring completion might be sensed by the flagellar regulatory system by a different mechanism than the rest of the basal body assembly. In this study, a novel regulatory locus, the *flk* locus, that regulates flagellin gene expression in response to P- and L-ring assembly was identified. A genetic and molecular characterization of the *flk* gene and its product is described. The *flk* gene had not been described before, and given its chromosomal location distinct from those of all known flagellar genes (52 min), *flk* is likely to be involved in cellular processes other than flagellar assembly. We present a model suggesting that Flk plays a role in the transition from the export of hook subunits to the export of class 3 proteins including flagellin by sensing P- and L-ring assembly. Thus, the role of Flk is to ensure the efficient transition in the export apparatus from hook subunits to flagellin subunits, thereby reducing the time required for flagellar biosynthesis to occur. This would imply that the speed of flagellar assembly is an important survival advantage.

MATERIALS AND METHODS

Strains. 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 as described previously (8, 11).

Plasmid constructions. p322KS and p322SK were constructed in order to place a multiple cloning site (MCS) flanked by T3 and T7 promoters into pBR322 for subsequent isolation of *flk* clones into medium-copy-number vectors. The MCS from pBSIISK− (Stratagene) on a 173-bp *Bss*HII fragment was blunt ended with Klenow enzyme and was subsequently ligated into an *Eco*RI-*Nru*I blunt-ended site with mung bean nuclease of pBR322 (6). The notations KS and SK denote the orientation of the MCS.

Isolation of chromosomal *flk*::Tn*10*dCm and *flk*::Tn*10*dTc insertion mutants. Insertions of Tn*10*dCm and Tn*10*dTc in the *flk* gene of *S. typhimurium* were first described as an uncharacterized class of flagellar regulatory mutations affecting class 3 gene expression in a *flgI* mutant strain (11). An insertion in the *pdxB* gene was isolated by transducing strain TR5661 to AroC$^+$ with a P22 lysate grown on pooled cells containing randomly inserted Tn*10*dTc transposons. AroC$^+$ transductants were screened for cotransduction of Tn*10*dTc-encoded tetracycline resistance and for pyridoxine auxotrophy.

Isolation of the *flk*$^+$ gene of *S. typhimurium*. Genomic DNAs containing Tn*10*dCm insertions in *flk* were isolated and cloned into a medium-copy-number vector. Restriction fragments containing the partial *flk* gene from these clones served as DNA probes to hybridize a *flk*-enriched plasmid library made from *purF*::Mud-P22 phage DNA. Potential full-length clones were then checked for complementation in a *flk* mutant background.

Isolation of *flk*::Tn*10*dCm clones was done as follows. Genomic DNA from strain TH2859 containing the *flk* gene with a Tn*10*dCm insertion was isolated as described previously (46). The genomic DNA was partially digested with *Sau*3A1, and 5-kb DNA fragments were agarose gel purified and ligated into *Bam*HI-cut p322SK vector. The ligation mix was transformed into MC1061, and chloramphenicol-resistant (Cm^r) colonies were selected. Potential *flk*::Tn*10*dCm clones were identified by restriction mapping and partially sequenced. One subclone, pJK205, was subsequently used as a DNA probe for the isolation of full-length *flk* clones.

Full-length *flk* clones were screened from a *flk*-enriched plasmid library as follows. Isolation of phage DNA from a *purF*::Mud-P22 lysogen (TH878) was performed as described previously (64). The genomic DNA was partially digested with *Sau*3A1, and 3- to 5-kb DNA fragments were agarose gel purified, ligated into *Bam*HI-cut p322KS vector, and transformed into *E. coli* DH5α. A *Hin*dIII-*Bam*HI 0.5-kb DNA fragment from pJK205 was radiolabeled with [α-^{32}P]dCTP by using a Random Prime labeling kit (Boehringer Mannheim Biochemicals), and the transformants were screened by colony hybridization as described previously (46). Potential clones were transformed into TH2858, a *flk* mutant, and screened for an ability to complement the *flk* mutation on triphenyl tetrazolium chloride (TTC)-lactose (Lac) indicator plates (31).

Isolation of *lac* transcriptional fusions to the *S. typhimurium flk* gene. Transcriptional fusions of the *lac* operon to the *flk* promoter were made using the MudJ-*lac* fusion vector by the transitory *cis*-complementation method (19). MudJ refers to the transposon-defective *lac* operon fusion vector Mu d1734 (Km *lac*) (14). P22 lysate grown on the MudJ donor strain TT10288 was used to transduce an *S. typhimurium* strain harboring the *flk*$^+$ plasmid, pJK232, to MudJ-encoded kanamycin resistance (Km^r). P22 lysates prepared on pools of Km^r transductants were used to transduce wild-type strain LT2 to both plasmid-encoded ampicillin resistance (Ap^r) and MudJ-encoded Km^r. The Ap^r Km^r transductants were screened for loss of *flk*$^+$-complementing activity. In a *flk*::Tn*10*dTc ring mutant, the P_{fliC}-*cat* strain is Cm^r. Introduction of pJK232 complements the *flk* null mutation, resulting in a Cm^s phenotype. If a MudJ transposon inserts into the *flk*$^+$ gene in pJK232, the MudJ-containing plasmid will no longer complement the chromosomal *flk* mutation, resulting in Ap^r Km^r Cm^r transductants. Putative plasmid *flk*::MudJ fusions were screened for linkage to the plasmid and expression of *lac*.

Isolation of the *flk*$^+$ gene of *E. coli* and construction of a *flk*::Ω (Cm^r) mutant. The *E. coli pdxB*$^+$ and *flk*$^+$ genes were contained in a 4.4-kb *Eco*RI fragment isolated from Kohara phage λ407. The *pdxB*$^+$-*flk*$^+$ fragment was ligated into the *Eco*RI site of vector pBR322 to give plasmid pTX277, which complemented the pyridoxine requirement of *pdxB* mutants and was the source of DNA for sequencing *flk* and the region downstream. The *Eco*RI fragment from pTX277 was recloned into the *Eco*RI site of vector pGEM4Z (Promega Corp.) to give plasmid pTX446. Plasmid pHP45Ω-Cm (44) was digested with *Bam*HI, and the fragment containing the Ω (Cm^r) cassette was ligated into the *Bgl*II site in the middle of the *flk* coding region in pTX446 to give plasmid pTX447. pTX447 was linearized by *Bam*HI digestion, and the *flk*::Ω (Cm^r) gene was crossed into the chromosome of a *recBC sbc* mutant to give strain TX3430 (59). The *flk*::Ω (Cm^r) mutation was transduced into *E. coli* K-12 W3110 prototroph NU426 by P1 *vir* bacteriophage to give strain TX3447.

DNA sequencing of *flk* clones. The *flk* gene was sequenced by a transposon-based DNA sequencing strategy as described previously (55). The part of the *E. coli flk* gene not reported previously (48) was determined by automated DNA sequencing of both DNA strands as described before (59). DNA sequence analysis was performed by using the DNA Strider program (37) and the Genetics Computer Group sequence analysis software package (9).

Primer extension. Total RNA was isolated as described previously (46). Primer Flk214 (5′ TGG AGG CTG TGC AGG GGC GCC GGA GAT GGG 3′) was radiolabeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (New England Biolabs) and hybridized to 100 μg of total RNA. The reaction was extended by using avian myeloblastosis virus reverse transcriptase (Promega), and

TABLE 1. Bacterial strains used

Strain	Genotype	Reference or source[a]
S. typhimurium		
fabB1		18
PY13518	*leuA414 hsdSB* Fels$^-$[F′114(Ts) *lac*$^+$ *zzf-20*::Tn*10 tetA*::MudP]	64
PY13759	*leuA414 hsdSB* Fels$^-$[F′114(Ts) *lac*$^+$ *zzf-20*::Tn*10 zzf-3555*::MudQ]	64
PY13818	*purF*::MudQ *leuA414 hsdSB* Fels$^-$	P. Youderian
SJW107	Δ*fliK1107 fljB*^{e,n,x} *hin*(*vh2*)	S. Yamaguchi
SJW108	Δ*fliK1108 fljB*^{e,n,x} *hin*(*vh2*)	S. Yamaguchi
SJW113	Δ*fliK1113 fljB*^{e,n,x} *hin*(*vh2*)	S. Yamaguchi
SJW198	Δ*flgH2136 fljB*^{e,n,x} *hin* (*vh2*)	S. Yamaguchi
SJW200	Δ*flgI2453 fljB*^{e,n,x} *hin*(*vh2*) (contains another uncharacterized *fla* allele)	S. Yamaguchi
SJW203	Δ*flgHI958 fljB*^{e,n,x} *hin*(*vh2*)	S. Yamaguchi
SJW204	Δ*flgE1204 fljB*^{e,n,x} *hin*(*vh2*)	S. Yamaguchi
SJW1376	Δ*flgI1376 fljB*^{e,n,x} *hin*(*vh2*)	S. Yamaguchi
SJW1473	Δ*flgE2122 fljB*^{e,n,x} *hin*(*vh2*)	S. Yamaguchi
SJW1529	Δ*flgA1529 fljB*^{e,n,x} *hin*(*vh2*)	S. Yamaguchi
SJW1590	Δ*flgE2229 fljB*^{e,n,x} *hin*(*vh2*)	S. Yamaguchi
TH2150	*fljB5001*::MudJ Δ*flgG-L2157*	11
TH2164	*flgI2002 fljB5001*::MudJ	11
TH2413	*flk-5206*::Tn*10*dCm	11
TH2512	*flgA5210*::Tn*10*dTc	11
TH2541	*flk-5212*::Tn*10*dTc	11
TH2592	*fljB5001*::MudJ	
TH2858	*flgI2002 fljB5001*::MudJ *flk-5212*::Tn*10*dTc	
TH2859	*flgI2002 fljB5001*::MudJ *flk-5206*::Tn*10*dCm	
TH3227	*ataA*::[P22 *sieA Kn-9* $P_{fliC(-435-+6)}$−*cat*]	M. Chadsey
TH3281	*fljB5001*::MudJ *flgM5222*::MudCm	
TH3282	*fljB5001*::MudJ *flk-5212*::Tn*10*dTc	
TH3283	*fljB5001*::MudJ *flgM5222*::MudCm *flk-5212*::Tn*10*dTc	
TH3296	*fljB5001*::MudJ Δ*flgG-L2157 flk-5212*::Tn*10*dTc	
TH3342	*pdxB*::Tn*10*dTc	
TH3441	*fljB5001*::MudJ Δ*flgA1529*	
TH3442	*fljB5001*::MudJ Δ*flgH2136*	
TH3444	*fljB5001*::MudJ Δ*flgI1376*	
TH3445	*fljB5001*::MudJ Δ*flgHI958*	
TH3451	*fljB5001*::MudJ Δ*flgA1529 flk-5212*::Tn*10*dTc	
TH3452	*fljB5001*::MudJ Δ*flgH2136 flk-5212*::Tn*10*dTc	
TH3454	*fljB5001*::MudJ Δ*flgI1376 flk-5212*::Tn*10*dTc	
TH3455	*fljB5001*::MudJ Δ*flgHI958 flk-5212*::Tn*10*dTc	
TR705	*aroC5 hisT1529 hisC3714*	
TR5661	*aroC5 rpsL1*	
TR5663	*purF145 rpsL1*	
TT627	*pyrC7 rpsL1* [F′114(Ts) *zzf-20*::Tn*10*]	7
TT628	*pyrC7 rpsL1* [F′114(Ts) *zzf-21*::Tn*10*]	7
TT5866	*hisT290*::Tn*5*	J. Roth
TT10288	*hisD9953*::MudJ *hisA9944*::Mud1	19
E. coli		
BL21(DE3)	F$^-$ *ompT* r_B^- m_B^-	J. Dunn, Brookhaven
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ*lacU169* [ϕ80d *lac* Δ(*lacZ*)*M15*]	55
DPWC	*supE42 recA*-Δ(*Sac*II-*Eco*RI) Δ*srl*::Tn*10* (Tcs)	55
JGM	F$^-$ *araD139* Δ(*ara-leu*)*7696* Δ*lacX74 galU galK hsdR2 mcrB1 rpsL* Tn*5 seq-1*	55
MC1061	F$^-$ *araD139* Δ(*ara-leu*)*7696* Δ*lacX74 galU galK rpsL thi*	55
NU426	W3110 *sup* (Am) prototroph	C. Yanofsky
TX3447	NU426 *flk*::Ω (Cm^r) (in *Bgl*II site of *flk*)	

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

the transcript products were processed and electrophoresed on a 6% denaturing polyacrylamide gel as described previously (1).

Construction of truncated and full-length *flk* under the P_{trc} promoter. Truncated and full-length *flk* genes were placed downstream of the P_{trc} promoter as follows. Primers Flk1 (5′ ACA GAA TTC GCA TGC ATC CCA TCT CCG3′) and Flk2 (5′ ACA GAA TTC TTA GGT TTC AAT CAT CGG C3′) for the truncated *flk* gene and primers Flk1 and Flk3 (5′ACA GAA TTC TTA AGA AAC CAG CCA GAA A3′) for the full-length *flk* gene were used to PCR amplify 946- and 1,024-bp fragments, respectively, from pJK232. The PCR product ends were filled in with Klenow enzyme, digested with *Eco*RI, agarose gel isolated, and ligated into *Eco*RI-digested pBSIISK− (Stratagene) to make pJK293 (truncated *flk*) and pJK284 (full-length *flk*). pJK293 and pJK294 were digested with *Sph*I, blunt ended with mung bean nuclease, and digested with *Eco*RI, and fragments of 946 bp (truncated *flk*) and 1,024 bp (full-length *flk*) were agarose gel isolated and ligated into ptrc99A (Pharmacia) digested with *Nco*I, blunt ended with Klenow, and then digested with *Eco*RI. This placed the *flk* genes under the P_{trc} promoter in constructs pJK296 (truncated Flk [amino acids 1 to 307]) and pJK297 (full-length Flk [amino acids 1 to 333]).

Overproduction of Flk protein. Plasmids for T7 polymerase-dependent expression of *flk* were made as follows. pJK232 was digested with *Xba*I and *Eco*RI, blunt ended with Klenow enzyme, and religated with T4 ligase to make pJK262. This placed the *flk* gene downstream of the T7 promoter. pJK262 was digested with *Hpa*I and *Hin*dIII and blunt ended with Klenow enzyme, and the 5.5-kb fragment was religated with T4 ligase to make pJK263. This placed the T7 promoter within 125 bp of the translational start codon of the *flk* gene. pJK262 and p322KS (vector control) were transformed into *E. coli* BL21(DE3) (Nova-

TABLE 2. Expression of *fljB-lac* in various flagellar mutant backgrounds

Genotype	Lac phenotype[a]
fljB-lac	+
flgI fljB-lac	−
flgI flgM::Tn*10*d *fljB-lac*	+
flgI flk::Tn*10*d *fljB-lac*	+
Δ*flg*(*G-L*) *fljB-lac*	−
Δ*flg*(*G-L*) *flgM*::Tn*10*d *fljB-lac*	+
Δ*flg*(*G-L*) *flk*::Tn*10*d *fljB-lac*	−

[a] Phenotype was scored on TTC-Lac indicator medium.

gen), which has the T7 polymerase under control of the *tac* promoter and can be induced by the addition of isopropylthiogalactopyranoside (IPTG). Cells were grown at 37°C in 1× E minimal medium containing 0.2% glycerol and 100 μg of ampicillin per ml and supplemented with all amino acids except methionine. The cells were grown to a Klett value of 35, 1 mM IPTG was added, and incubation continued for 15 min. Rifampin was added to 200 μg/ml to prevent transcription from cellular RNA polymerase (58). [^{35}S]methionine-cysteine protein labeling mix (NEN) was added at 50 μCi/ml, and the mixture was incubated for 5 to 15 min. Cells were isolated by centrifugation, washed in buffered saline, and resuspended in 50 μl of 1× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer (30). Equal counts per minute of each sample were electrophoresed onto a 10% Tricine SDS-polyacrylamide gel (47); the gels were dried and autoradiographed with Hyperfilm-MP (Amersham).

β-Galactosidase assays. β-Galactosidase assays were performed in triplicate on mid-log-phase cells as described previously (36). β-Galactosidase activities are expressed as nanomoles/minute/unit of optical density at 650 nm/milliliter.

Nucleotide sequence accession numbers. The nucleotide sequence data presented in this report have been assigned GenBank accession numbers U77618 (*S. typhimurium* sequence) and U76961 (*E. coli* sequence).

RESULTS

Identification of negative regulators of flagellin expression by insertion mutagenesis. We sought insertion mutations that relieved negative regulation in strains defective in only the ring assembly pathway of BBH assembly (a *flgI* mutant was arbitrarily chosen). These mutations were compared to the insertion mutations obtained in a strain defective in both ring assembly and in rod formation (Δ*flgG-L*). The *flgI* gene encodes the structural protein for the periplasmic ring component of the flagellum. A strain carrying both a *flgI* mutation and a *fljB-lac* transcriptional fusion is Lac$^-$ (11). This strain was mutagenized with transposons Tn*10*dTc and Tn*10*dCm (61). Insertion mutants were screened for loss of the negative regulatory effect of the *flgI* allele on *fljB-lac* expression. The results given in Table 2 demonstrate that screens for insertions resulting in a Lac$^+$ phenotype, allowing *fljB-lac* expression in a *flgI* mutant strain, yielded mutations in a novel regulatory locus in addition to the expected mutations in *flgM*, while previous screens in the Δ*flgG-L* mutant background yielded insertions only in the *flgM* locus. The novel negative regulatory locus was found by P22 cotransductional analysis to be unlinked to the *flg*, *flh*, *fli*, and *flj* flagellar gene clusters and was named *flk*. (Since as shown below *flk* represents a single-gene operon, it is referred to as *flk* and not *flkA* [i.e., there is no *flkB*].) Identical results were obtained in assays using a *fliC-lac* reporter for class 3 gene expression (data not shown).

The effect of flagellar mutations on expression of a *fljB-lac* transcriptional fusion are presented in Table 3. Only flagellar mutations in class 3 genes (the *flgK*, *flgL*, and *fliD* genes, which encode the HAPs and Cap, respectively, or a mutation in the alternate flagellin gene, *fliC*) have no effect on *fljB* gene expression. This negative regulation of *fljB* gene expression in any class 2 mutant background, except *fliA*, which encodes σ^{28}, is relieved by inactivation of the *flgM* negative regulatory gene (Table 3) (11). In addition, *fljB* was not expressed in class 1 mutant strains (*flhC* and *flhD*). This is expected since class 1 genes are required to transcribe the class 2 operons (25).

TABLE 3. Effects of *flgM* and *flk* on expression of the *fljB* flagellin gene in flagellar mutant strains on either TTC-Lac or Mac-Lac indicator plates

Strain[a]	Flagellar regulatory class	*fljB-lac* expression on:			
		Mac-Lac or TTC-Lac	TTC-Lac or Mac-Lac in *flgM* mutant strain	Mac-Lac in *flk* mutant strain	TTC-Lac in *flk* mutant strain
Wild type		+	+	+	+
flgA	2	−	+	+	+
flgB	2	−	+	−	−
flgC	2	−	+	−	−
flgD	2	−	+	+	−
flgE	2	−	+	+	−
flgF	2	−	+	+	−
flgG	2	−	+	+	−
flgH	2	−	+	+	+
flgI	2	−	+	+	+
flgJ	2	−	+	+	−
flgK	3	+	+	+	+
flgL	3	+	+	+	+
flhA	2	−	+	−	−
flhB	2	−	+	−	−
flhC	1	−	−	−	−
flhD	1	−	−	−	−
fljA	2	−	−	−	−
fliC	3	+	+	+	+
fliD	3	+	+	+	+
fliE	2	−	+	−	−
fliF	2	−	+	−	−
fliG	2	−	+	−	−
fliH	2	−	+	−	−
fliI	2	−	+	−	−
fliJ	2	−	+	−	−
fliK	2	−	+	−	−
fliL	2	−	+	−	−
fliM	2	−	+	−	−
fliN	2	−	+	−	−
fliO	2	−	+	−	−
fliP	2	−	+	−	−
fliQ	2	−	+	−	−
fliR	2	−	+	−	−

[a] Flagellar mutant alleles used, a gift from S. Yamaguchi, were previously described (11).

As for *flgM*, mutations in *flk* relieved repression of a *fljB-lac* fusion in a *flgI* mutant strain. The effect of *flk* on expression of the *fljB-lac* fusion in all individual flagellar mutant backgrounds was determined and is presented in Table 3. Unlike *flgM* mutants, mutants in *flk* had a regulatory effect on only a subset of the flagellar mutant strains. In addition, the subset of flagellar mutants in which the effect of *flk* was seen depended on the indicator medium used. Three lactose indicator media are routinely used: medium with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), MacConkey-lactose (Mac-Lac) indicator medium, and medium with TTC-Lac. Medium containing X-Gal is the most sensitive to low levels of β-galactosidase, whereas Mac-Lac requires higher levels of β-galactosidase to appear Lac$^+$ and TTC-Lac requires even higher levels of β-galactosidase to appear Lac$^+$. The introduction of an insertion mutation in *flk* resulted in loss of repression of the *fljB-lac* fusion in *flgA*, *flgH*,

and *flgI* mutant strains, evident as a Lac^+ phenotype on either TTC-Lac or Mac-Lac indicator plates. It did not relieve repression in the other class 2 flagellar mutant strains on TTC-Lac indicator medium. However, on Mac-Lac plates, loss of Flk relieved repression of BBH genes required after proximal rod formation, including the *flgD*, *flgE*, *flgF*, *flgG*, and *flgJ* genes in addition to the *flgA*, *flgH*, and *flgI* genes.

The relevant β-galactosidase activities are presented in Fig. 3. As shown previously, *fljB-lac* expression was inhibited in BBH mutants, and this inhibition is relieved by inactivation of FlgM (12). Loss of Flk significantly affected flagellin expression only in P- and L-ring mutant strains, but the effect was different from that seen with loss of FlgM. Loss of FlgM resulted in a full derepression of *fljB-lac* expression regardless of the presence of a BBH mutation. Loss of Flk brought *fljB-lac* expression back to the level measured for *fljB-lac* in a wild-type Fla^+ strain. The effect of a *flk* mutation on *fljB-lac* expression in the *flgD*, *flgE*, *flgF*, *flgG*, and *flgJ* mutant strains was only twofold, albeit this was enough for a change on Mac-Lac indicator plates to be seen. The significance of this twofold effect is unclear at this time. Identical results were obtained in assays using a *fliC-lac* reporter for class 3 gene expression (data not shown).

Genetic characterization of the *flk* locus. The new flagellar regulatory locus, *flk*, was roughly mapped on the *S. typhimurium* chromosome by Hfr mapping (7). Mapping experiments placed the *flk* locus at the 50-min region of the *S. typhimurium* genetic linkage map (data not shown). Linkage analysis by P22 transductional crosses to genetic markers in the region refined the mapping of the *flk* locus to 52 min on the standard linkage map between the *aroC* and *pdxB* genes. A detailed genetic map of the region is shown in Fig. 4. This map includes P22 cotransduction frequencies in the *flk* region.

Isolation of the *flk* gene from *S. typhimurium* using Mud-P22. Inhibition of *fliC* gene expression in a *flgI* mutant background is relieved when the *flk* gene is insertionally inactivated (Table 2). Libraries of chromosomal *S. typhimurium* DNA were cloned into a pBR322-derived plasmid vector. Clones were screened for the ability to complement a chromosomal *flk* null mutant and restore inhibition of *lac* expression (Lac^- phenotype) in a *flk flgI fljB-lac* background. Many plasmids which complemented the *flk* mutant phenotype were isolated. However, restriction analysis revealed that these plasmids contained different nonoverlapping regions of the chromosome. To determine which plasmid carried the *flk* region of the chromosome, DNA corresponding to the *flk* gene was isolated and used as a probe in hybridization experiments. The source of the probe was a plasmid that carried the *flk*::Tn*10*dCm insertion (see Materials and Methods). DNA flanking the insertion was used to probe plasmids for those that carried the *flk* locus. Surprisingly, none of the plasmids isolated as complementing the *flk* null phenotype hybridized to the *flk* probe. These plasmids probably include clones of the *fliA* gene, which when present in multicopy will overcome FlgM-mediated inhibition in BBH mutant strains (20).

To facilitate cloning of the *flk* locus, DNA localized to the *flk* region of the chromosome was specifically isolated following induction of a Mud-P22 insertion in the nearby *purF* locus. Mud-P22 is a derivative of P22 that lacks integrase functions so that upon prophage induction, it cannot excise (64). When induced, the defective P22 genome of the Mud-P22 insertion replicates in situ, amplifying both the Mud-P22 DNA and the adjacent chromosomal DNA of approximately 100 kb. A cotransduction frequency of 2 to 6% between *purF* and *flk* (Fig. 4) represents approximately 30 kb of chromosomal DNA between the two genes (62). This is within the 100 kb of flanking

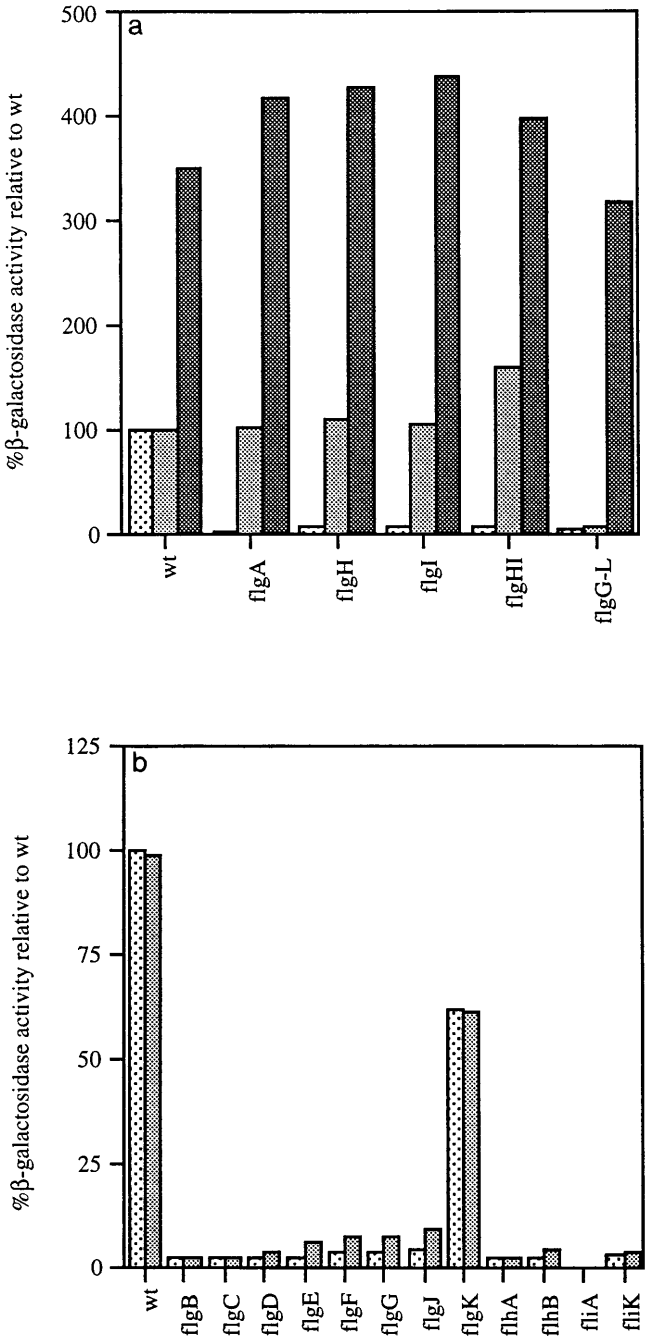

FIG. 3. Effects of Flk and FlgM on *fljB* expression in various flagellar mutant strains. Expression of *fljB* is measured from a *fljB5001*::MudJ insertion which is a transcriptional fusion of the *lac* operon. Expression of β-galactosidase is dependent on expression from the class 3 *fljB* promoter (11, 13). (a) Effect of either loss of Flk or loss of FlgM on expression in the different flagellar mutant strains shown. Symbols: ▨, flk^+; ▫, *flk*; ▩, *flgM*. (b) Effect of loss of only Flk on expression of *fljB* in the different flagellar mutant strains shown. Symbols: ▨, flk^+; ▫, *flk*. The following flagellar mutant alleles were used: Δ*flgA1529*, Δ*flgH2136*, Δ*flgI1376*, Δ*flgHI958*, Δ*flgG-L2157*, *flgB2164*, *flgC2036*, *flgD1156*, Δ*flgE2122*, *flgF2083*, *flgG2028*, *flgJ2076*, *flgK2408*, *flhA2007*, *flhB2033*, *fliA2087*, and Δ*fliK1107* (Table 1 and reference 11). The *flgM* allele used in panel a was *flgM5222*::MudCm; the *flk* allele used in both panel a and panel b was *flk-5212*::Tn*10*dTc. wt, wild type.

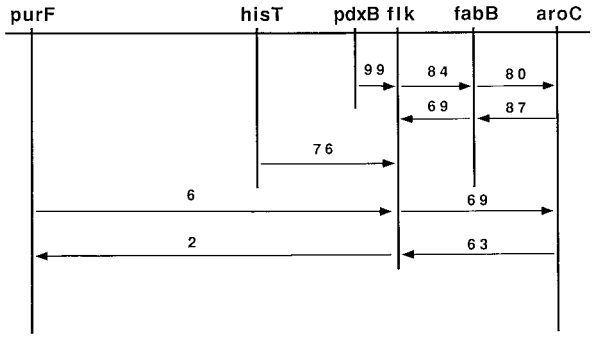

FIG. 4. P22-mediated cotransduction frequencies of the *flk* region of the *S. typhimurium* genetic map. A scaled portion of the 52-min region of the linkage map is illustrated.

chromosomal DNA packaged by induction of a Mud-P22 insertion in *purF*. A pBR-based plasmid bank was prepared from this DNA and screened for those that hybridized to a fragment adjacent to the Tn*10*dCm insertion in *flk*. Of five plasmids that hybridized to this fragment, one was able to complement a chromosomal *flk* null mutation. This plasmid, pJK232, was used for obtaining the DNA sequence of the *flk* gene.

Isolation of Tn*1000* insertions in the *flk* gene. To facilitate DNA sequence analysis of the *flk*-complementing region of plasmid pJK232, insertions of transposon Tn*1000* that lost the ability to complement a chromosomal *flk* null allele (55) were isolated in pJK232. Insertions of Tn*1000* into pJK232 were isolated as described in Materials and Methods. These plasmids were transferred into the *S. typhimurium* test strain TH2858 by electroporation and screened for loss of *flk*-complementing function. Thus, in strain TH2858, a plasmid carrying an intact and expressed *flk* gene converts the cells to a Lac^- phenotype whereas an insertion in the *flk* locus would allow the cells to remain Lac^+. Eleven different Tn*1000* insertions that resulted in a Flk^- phenotype were isolated in pJK232, and their sequences are shown in Fig. 5a. These were used for DNA sequence analysis. All of the insertions were within a 900-bp segment of the *flk*-complementing clone and therefore defined the *flk* locus.

DNA sequence of the *flk* gene of *S. typhimurium*. Once the *flk* locus in pJK232 was defined by Tn*1000* mutagenesis, primers that hybridize to either end of Tn*1000* were used to obtain DNA sequence flanking the different insertions. Using this method, a sequence of 1,313 bp was obtained (Fig. 5a). One large open reading frame (ORF) of 333 amino acid codons that includes all sites of the Tn*1000* insertion was observed within this sequence. This finding suggests that *flk* is a single gene. No apparent ORFs immediately downstream of *flk* were detected as potential genes in an operon with *flk*. Amino acids 314 through 332 represent a hydrophobic stretch of 18 amino acids which may represent a membrane-spanning domain.

DNA sequence of the *flk* gene from *E. coli*. A search of the sequence databases revealed that the 5′ end of the *E. coli flk* gene was originally identified as an ORF which shares its −10 promoter region with the overlapping, divergent promoter for the *pdxB* gene. The *pdxB* gene is required for de novo biosynthesis of the vitamin B_6-derived coenzyme pyridoxal 5′-phosphate. The sequence of the promoter-proximal part of *E. coli flk* was reported previously (48), and the remainder of the *E. coli flk* gene and the region downstream were sequenced as part of this study (Fig. 5b). The *E. coli flk* gene corresponds to an ORF of 331 amino acids. DNA sequence downstream of the *E. coli flk* gene revealed an ORF of 391 amino acids that is in the opposite orientation on the chromosome compared to *flk*; however, the stop codons for the two genes overlap. While the *S. typhimurium* sequence of this ORF was not fully determined, the C-terminal 45 amino acids of the ORF could be determined and were found to be 82% identical to the corresponding sequence from *E. coli* (not shown). These results suggest that *flk* is a single-gene operon flanking two genes, *pdxB* and an unidentified ORF, present in both species.

There is no evidence of coregulation between *flk* and either flanking genetic locus, although the overlap of the *flk* and *pdxB* promoters may somehow coordinate expression of these two divergent genes. An omega cassette imparting Cm^r was cloned into the *Bgl*II site near the middle of the *E. coli flk* coding sequence and crossed into the bacterial chromosome (60). The resulting *E. coli flk*::Ω (Cm^r) mutant TX3447 (Table 1) grew at the same rate as its isogenic flk^+ parent NU426 in LB at 37 or 44°C or minimal salts medium supplemented with glucose or glycerol at 37°C (data not shown). The amount of cellular PdxB protein was measured in the flk^+ parent and *flk*::Ω (Cm^r) mutant strain by quantitative Western immunoblotting using polyclonal antibodies prepared against affinity-tagged purified PdxB (66). The *flk*::Ω (Cm^r) mutation did not affect PdxB expression levels in bacteria grown in LB or minimal salts medium supplemented with glucose or glycerol at 37°C (data not shown). Thus, unlike many other divergent gene pairs (3), *flk* does not seem to regulate *pdxB* expression.

The deduced amino acid sequences for the *S. typhimurium* (333 residues, 37,032 Da) and *E. coli* (331 residues, 36,645 Da) Flk proteins are aligned in Fig. 6. The level of identity is relatively low at 61%, although there is 78% similarity. Both *E. coli* Flk and *S. typhimurium* Flk are rich in glutamine residues, containing 40 glutamine residues each, or 12 mol%. The significance of this is unclear. Hydropathy analysis of both proteins (Fig. 7) suggests that the proteins have similar three-dimensional structures. A striking feature of both proteins is the presence of a completely hydrophobic sequence of 18 amino acid residues, representing a potential membrane-spanning segment, at their very C termini. The presence of arginine residues immediately preceding the putative transmembrane segment would suggest that Flk is inserted into the cytoplasmic membrane via its C-terminal 18-amino-acid hydrophobic stretch facing into the cytoplasm (61). This C-terminal hydrophobic tail is essential for either the stability or function of Flk. An insertion on Tn*1000* which removes the C-terminal 13 amino acids of Flk (including 12 amino acids of the 18-amino-acid hydrophobic stretch) and results in a null phenotype was isolated.

Transcription of the *S. typhimurium flk* gene is independent of the flagellar regulatory hierarchy. To identify the promoter region for *flk* and determine if transcription was under the control of the flagellar regulatory hierarchy, a primer extension experiment was performed. If transcription was under flagellar regulation, then it should not occur in the absence of the FlhDC positive activators required for both class 2 and class 3 gene expression. Primer extension using a primer to the promoter-proximal coding sequence of the *flk* gene was used to map the transcriptional start site of the *flk* gene by reverse transcriptase-mediated extension of a complementary DNA product back to the start of the *flk* mRNA. RNA samples isolated from isogenic fla^+ and Δ*flhDC* mutant strains were used to determine the transcriptional start point in both strain backgrounds. The results of primer extension analysis presented in Fig. 8 demonstrate that the two strains use the same two start sites at adjacent positions 45 and 46 bp upstream

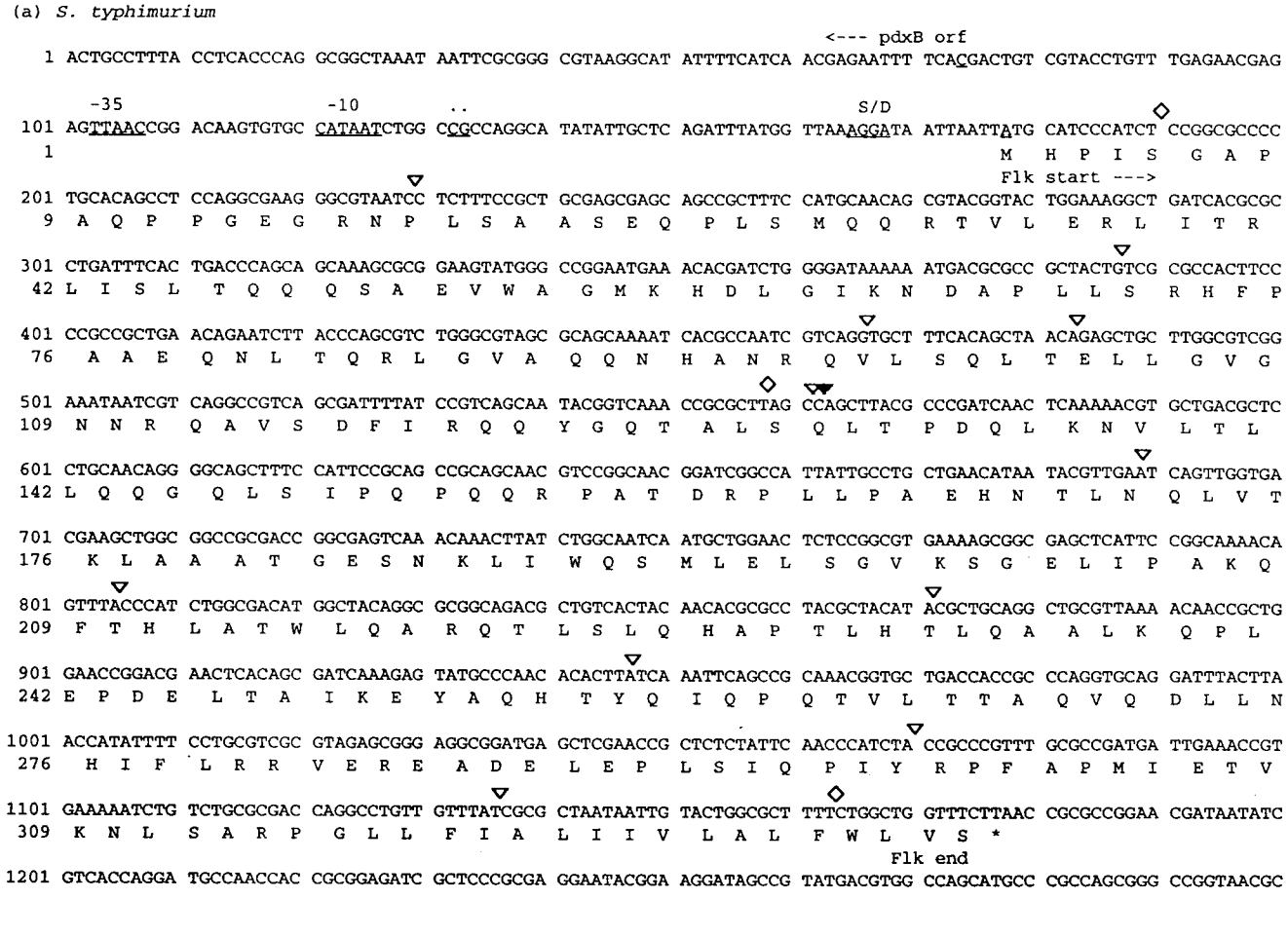

FIG. 5. Nucleotide sequences of the *flk* genes from *S. typhimurium* (a) and *E. coli* (b) and the flanking 5′ and 3′ sequences. The *flk* genes from both species are flanked by divergent ORFs. On the 5′ end of *flk* is the *pdxB* gene, which is required for pyridoxal 5′-phosphate biosynthesis, and the −10 regions of the *flk* and *pdxB* promoters overlap (48). On the 3′ end of *flk* is a hypothetical ORF corresponding to 391 amino acids from the *E. coli* sequence (not shown). The deduced amino acid sequences are shown below the corresponding DNA sequences. Potential ribosome binding sites (labeled S/D) are underlined, as are the presumed −10 and −35 promoter regions. The transcriptional start sites from the *S. typhimurium flk* gene are denoted by dots above the consecutive bases. Insertion mutants in the *S. typhimurium flk* gene (a) whose location has been determined by DNA sequence analysis are indicated by diamonds for MudJ insertions, open triangles for Tn*1000* insertions, and a closed triangle for a Tn*10*dCm insertion. The *Bgl*II site, into which an Ω (Cm^r) cassette was inserted in the *E. coli flk* gene to generate a null mutation of *flk* in *E. coli*, is labeled and underlined in panel b.

from the amino acid start codon. Given that the promoter is not affected by the *flhDC* mutation and that it matches the canonical σ^{70} promoter sequence, it is unlikely that transcription of the *flk* gene itself is regulated along with the class 2 and class 3 flagellar operons and may represent a novel class 1 flagellar gene. Moreover, the DNA sequences of the −35, spacer, and −10 regions of the *flk* promoter are exactly conserved in *S. typhimurium* and *E. coli* (Fig. 5a) (48). This conservation makes it likely that *flk* expression is controlled in the same way in these bacteria. In contrast, the −35 regions of the overlapping *pdxB* promoters are slightly different in the two bacteria and do not match the consensus.

In addition to primer extension analysis, Mud-*lac* operon fusions within the *flk* structural gene were isolated in plasmid pJK232. The three chosen were determined by DNA sequence analysis to be located in the N-terminal portion, middle portion, and C-terminal portion of the *flk* gene (Fig. 5). Expression of the *lac* operon in each fusion was unaffected by introduction of insertion mutations in either the *flhDC* operon or the *fliA* gene (data not shown). This result agrees with the primer extension data presented above.

T7 RNA polymerase-mediated expression of the Flk protein. To verify that the 333-amino-acid ORF encodes the *flk* gene product responsible for complementation of chromosomal null mutations in *flk*, in vivo expression of Flk was assayed. Plasmid pJK263 contains the *flk* gene downstream from a T7 RNA polymerase promoter. This plasmid was introduced into *E. coli* BL21(DE3). Strain BL21(DE3) contains the *lacI*$^+$ gene and a λ lysogen, DE3, with T7 RNA polymerase expressed from the *tac* promoter. Following induction of T7 RNA polymerase by IPTG and inhibition of the host's RNA polymerase by the addition of rifampin, a ^{35}S-labeled protein labeling mix was added to the cells, and the labeled protein products were analyzed by SDS-polyacrylamide gel electrophoresis.

^{35}S-labeled protein products obtained from pJK263 are shown in Fig. 9. The only difference between the vector-only control (p322KS) and pJK263 following Flk induction was the appearance of a protein with an apparent molecular mass of 35

(b) *E. coli*

```
                                                                                                  <--- PdxB orf
   1 ACCGCGGTCA CCTCACCCAA ACGGCTAAAT AAGTCGCGGG CATAAGGCAT ATTTTCATCA ACAAGGATTT TCACGTTTGT GTTACCTGTA TGAGACGAGA

       -35                  -10                                                  S/D
 101 GTTAACCGGA CAAGTGTGCC ATAATCTCGC GGCCAGGCAT ACTTGCGAAG ATTTCAGGTA TAAGGATACG TAATGATACA ACCTATTTCC GGCCCTCCTC
   1                       -10                        -35                                 M  I  Q  P  I  S  G  P  P  P
                                                                                          Flk start ---->
 201 CTGGGCAACC ACCAGGTCAG GGAGATAATC TGCCGTCTGG CACGGGCAAT CAGCCTTTAT CCAGTCAGCA ACGTACTTCG CTGGAAAGCT TAATGACGAA
  11   G  Q  P  P  G  Q  G  D  N  L  P  S  G  T  G  N  Q  P  L  S  S  Q  Q  R  T  S  L  E  S  L  M  T  K

 301 AGTGACCTCA CTGACGCAAC AGCAAAGAGC AGAACTGTGG GCGGGTATCA GGCACGATAT TGGTCTGTCG GGAGATTCAC CGCTGCTTTC GCGTCACTTC
  44  V  T  S  L  T  Q  Q  Q  R  A  E  L  W  A  G  I  R  H  D  I  G  L  S  G  D  S  P  L  L  S  R  H  F

 401 CCTGCCGCTG AGCATAATCT GGCGCAACGT CTGCTGGCCG CGCAAAAAAG CCATTCTGCC CGCCAGCTTT TAGCGCAATT AGGGGAGTAT TTACGTCTGG
  77  P  A  A  E  H  N  L  A  Q  R  L  L  A  A  Q  K  S  H  S  A  R  Q  L  L  A  Q  L  G  E  Y  L  R  L  G

 501 GGAATAATCG TCAGGCGGTC ACGGATTATA TCCGTCATAA CTTTGGTCAG ACGCCGCTGA ATCAGCTCTC ACCGGAGCAA TTAAAAACCA TTCTCACCCT
 111   N  N  R  Q  A  V  T  D  Y  I  R  H  N  F  G  Q  T  P  L  N  Q  L  S  P  E  Q  L  K  T  I  L  T  L

 601 GTTGCAGGAA GGGAAGATGG TTATTCCGCA ACCACAGCAG CGCGAGGCGA CCGACCGTCC TTTATTACCG GCGGAGCACA ATGCGCTAAA ACAGCTGGTG
 144  L  Q  E  G  K  M  V  I  P  Q  P  Q  Q  R  E  A  T  D  R  P  L  L  P  A  E  H  N  A  L  K  Q  L  V
                                          BglII
 701 ACCAAACTTG CGGCGGCAAC GGGGGAACCC AGCAAACAGA TCTGGCAATC GATGCTGGAA CTTTCCGGGG TGAAAGATGG CGAGTTAATT CCAGCGAAAC
 177  T  K  L  A  A  A  T  G  E  P  S  K  Q  I  W  Q  S  M  L  E  L  S  G  V  K  D  G  E  L  I  P  A  K  L

 801 TGTTTAACCA TCTGGTGACC TGGCTACAGG CGCGTCAGAC GCTAAGCCAG CAAAATACGC CGACGCTGGA ATCACTACAG ATGACGCTAA AACAACCTTT
 211   F  N  H  L  V  T  W  L  Q  A  R  Q  T  L  S  Q  Q  N  T  P  T  L  E  S  L  Q  M  T  L  K  Q  P  L

 901 AGATGCCAGT GAACTGGCGG CGTTATCGGC ATATATCCAG CAAAAATATG GTCTTTCTGC GCAATCATCG CTTTCTTCTG CCCAGGCCGA GGATATTCTT
 244  D  A  S  E  L  A  A  L  S  A  Y  I  Q  Q  K  Y  G  L  S  A  Q  S  S  L  S  S  A  Q  A  E  D  I  L

1001 AATCAGCTTT ATCAACGGCG GGTTAAAGGG ATTGATCCGC GTGTTATGCA ACCGCTGCTT AATCCTTTTC CACCGATGAT GGACACGTTG CAAAATATGG
 277  N  Q  L  Y  Q  R  R  V  K  G  I  D  P  R  V  M  Q  P  L  L  N  P  F  P  P  M  M  D  T  L  Q  N  M  A

1101 CAACGCGTCC CGCGCTGTGG ATACTGTTAG TCGCGATTAT CCTGATGCTG GTCTGGCTGG TTCGTTAACC CCGACGAAAT GACAGTATCG TGACAATAAT
 311   T  R  P  A  L  W  I  L  L  V  A  I  I  L  M  L  V  W  L  V  R  *
                                                                  Flk end
1201 ACCCAGCACC GCAGAGATCG CCCCGGCAAG AAATACCGAA GAGTAACCAA ACGTGGTCGC CAGCATTCCC GCCAGCGGCC CGGAGACGCC GAGGGCGATA

1301 TCCTGAAACG CGGCGTAACC GCCCAGTGCG GTGCCGCGAA CTTGTGAGGG GACGCGTTTA ACCACCTCCA CGCCCAGCGC AGGAAAGATA AGCGAACATC

1401 CGGCTCCGGT TAACGCCGCG CCCGCTAATG CGACCCATGC ACCTGGGGCT TGCCAGAGCA GCAACAAGCC CACCGTTTCT ACAAGCAGAG AGACAATCGC

1501 CACTTTCACG CCGCCAAAAC GGTCCGGCAT CCAGCCAAAC ATGACGCGCA TCACGACAAA TGCGCCGCCA AACGCGGTAA GAGTAAAGCC CGCCATCGCC

1601 CATCCTTTGC TGGCAAAGTA GAGCGAAACG AAAGTCCCGA TAACAGCAAA ACCAACGCCT TGTAGTGCCA GACCTAACCC TGGTTTCCAG ATAAGCCCGA

1701 CAACGCTCCA CAGCGATGGA CGTTCTCCCG CCAGGGCCGG TACTTTGCGC ACTGTGCCGT TACAGGCCCA CGCCAGTACG GGTAATACCA TTGTGGTGAT

1801 CGCCAGTGCG GCAAAACCGT AATGGCTATG AATCAACAGG CCAAGCGGAG CACCAACAGC GAGGGCACCG TAAATCGCCA TTCCGTTCCA TGACATCACT

1901 TTGCCAGAGT GTTTTGGCCC TACGATGCCT AGTCCCCAGG TCAGAGCGCC TGTCAGTAAC TGGCTTTCAC CAAAGCCAAG AATCAAACGC CCGACGACCA

2001 ACAGAGCAAA TTTGAACGGT GCGGAGACAG GCAAAATCGC CGCCAGCAGC AACGCGCCGC CAGCCAGACC ACAAGCTAAC ATTCCCTGAA GCGCCGAACG

2101 TTTTGCACCA TATTGATCGG CCAGTCGCCC GGCGTAACCA CGCGTCAGCA CCGTAGCCAG AAACTGAATC CCGACGGCAA TGCCGACCAT GGTATTGCCA

2201 TAGCCCAGTT CATGATGAAC AAACAGCGGG ATAACCGGCA ACGGCAACCC TACGGTCATG TAGGTGAGAA AAACCGCAAA AGCGATGCGG AAGAGCGAAA
                                  <--- orf-1 start
2301 AATTGGCAGA AGATCGTGTT TCGGTTTGGC TTACAGCAGT CATGCATTAC TCCAGAATGC AGCGCAAGGC GAGGAGTATC CCCGTCTCAT CTCTCTGGTT

2401 TCAGGGTTAC AGTGCGTTGG CAGGATTTAA CGCGTACGTC TTTTCAGAAG GAAATCGACA AAGCGGGAAG TTTGCCTGGA ACCAGGCGAT TGTCAATGAT

2501 GTGAAAAAGG GAACCATCGG GTTCCCTTTT TGTGTTAGTG CCGGAGGCCG CATTGGCGCG TAACGTCGGA TGCGAGCCTG GCGCGCCTTA CCCGACCTAC

2601 GGCGAA
```

FIG. 5—*Continued.*

kDa. The predicted molecular mass of Flk is 37 kDa. A poly-His tag was fused to the N terminus of Flk in plasmids pJK264 and pJK266 such that His-tagged Flk is expressed from the T7 and *trc* promoters, respectively. (The *trc* promoter is a fusion of the *trp* and *lac* promoters and repressible by LacI [Pharmacia].) The apparent molecular mass of these fusions was 36 kDa, which was also less than the predicted mass of 39 kDa (data not shown).

```
Flk_S    1  MHPISG-APAQPPGEGRNPLSAASEQPLSMQQRTVLERLITRLISLTQQQSAEVWAGMKH
            ::|||| :|:||||:| |  |:.::|||| |||| ||.|:|:::.||||||.||:|||::|
Flk_E    1 MIQPISGPPPGQPPGQGDNLPSGTGNQPLSSQQRTSLESLMTKVTSLTQQQRAELWAGIRH

Flk_S   60  DLGIKNDAPLLSRHFPAAEQNLTQRLGVAQQNHANRQVLSQLTELLGVGNNRQAVSDFIR
            |:|:..|.||||||||||:||.||| .||..|..||:|.|.|.|.| :|||||||.|:||
Flk_E   62  DIGLSGDSPLLSRHFPAAEHNLAQRLLAAQKSHSARQLLAQLGEYLRLGNNRQAVTDYIR

Flk_S  120  QQYGQTALSQLTPDQLKNVLTLLQQGQLSIPQPQQRPATDRPLLPAEHNTLNQLVTKLAA
            :.:|||:|.||.|:|||.:|||||:|.: |||||||.||||||||||||.|.|||||||
Flk_E  122  HNFGQTPLNQLSPEQLKTILTLLQEGKMVIPQPQQREATDRPLLPAEHNALKQLVTKLAA

Flk_E  180  ATGESNKLIWQSMLELSGVKSGELIPAKQFTHLATWLQARQTLSLQHAPTLHTLQAALKQ
            ||||..| ||||||||||||.|||||| |.||.|||||||| |:.|||..|| .|||
Flk_E  182  ATGEPSKQIWQSMLELSGVKDGELIPAKLFNHLVTWLQARQTLSQQNTPTLESLQMTLKQ

Flk_S  240  PLEPDELTAIKEYAQHTYQIQPQTVLTTAQVQDLLNHIFLRRVEREADELEPLSIQPIYR
            ||::.||.|:..| |:.|.: :|. |..||.:|:||::: |||    .:::|  :||:..
Flk_E  242  PLDASELAALSAYIQQKYGLSAQSSLSSAQAEDILNQLYQRRV----KGIDPRVMQPLLN

Flk_S  300  PFAPMIETVKNLSARPGLLFIALIIVLALFWLVS  333
            ||:||::|:.|:..||:|::: : |:| |.|||.
Flk_E  298  PFPPMMDTLQNMATRPALWILLVAIILMLVWLVR  331
```

FIG. 6. Alignment of the deduced amino acid sequences from the *flk* genes of *S. typhimurium* (Flk_S) and *E. coli* (Flk_E). Identical amino acids are connected by vertical lines, conservative changes are indicated by dots (two dots for more conservative changes than indicated by single dots), and gaps are indicated by hyphens. A putative C-terminal membrane-spanning segment is highlighted in boldface for each sequence.

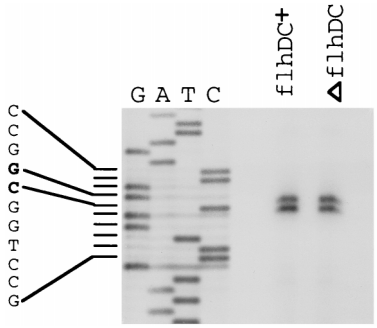

FIG. 8. Transcriptional start-site mapping for the *S. typhimurium flk* gene. Lanes G, A, T, and C, sequencing ladder. Primer extensions were measured in wild-type Fla^+ and an isogenic Δ*flhDC* mutant strain. The bases shown in boldface to the left are the transcriptional start sites.

DISCUSSION

The *flk* gene is a single-gene operon present in both *S. typhimurium* and *E. coli*. There was no other homology found in searches using either *flk* DNA or Flk amino acid sequences and available databases (BLAST, Prosite, GCG motifs, Fasta Swiss PROT, and Blocks).

The very C-terminal codon at the end of the hydrophobic tail is Ser-333 for *S. typhimurium* Flk and Arg-331 for *E. coli* Flk. Although formally possible, it seems unlikely that a cytoplasm-facing Flk protein could sense P- and L-ring completion through a single, periplasm-facing amino acid residue. We propose that the *flk* gene product is involved directly or indirectly in detecting completion of the P- and L-rings. The deduced amino acid sequence of Flk predicts a protein attached to the cytoplasmic membrane by a C-terminal hydrophobic tail. Whether the rest of Flk is within the cytoplasm or periplasm awaits availability of Flk antibodies or isolation and characterization of alkaline phosphatase protein fusions to Flk. Regardless of the orientation of Flk within the cytoplasm or the

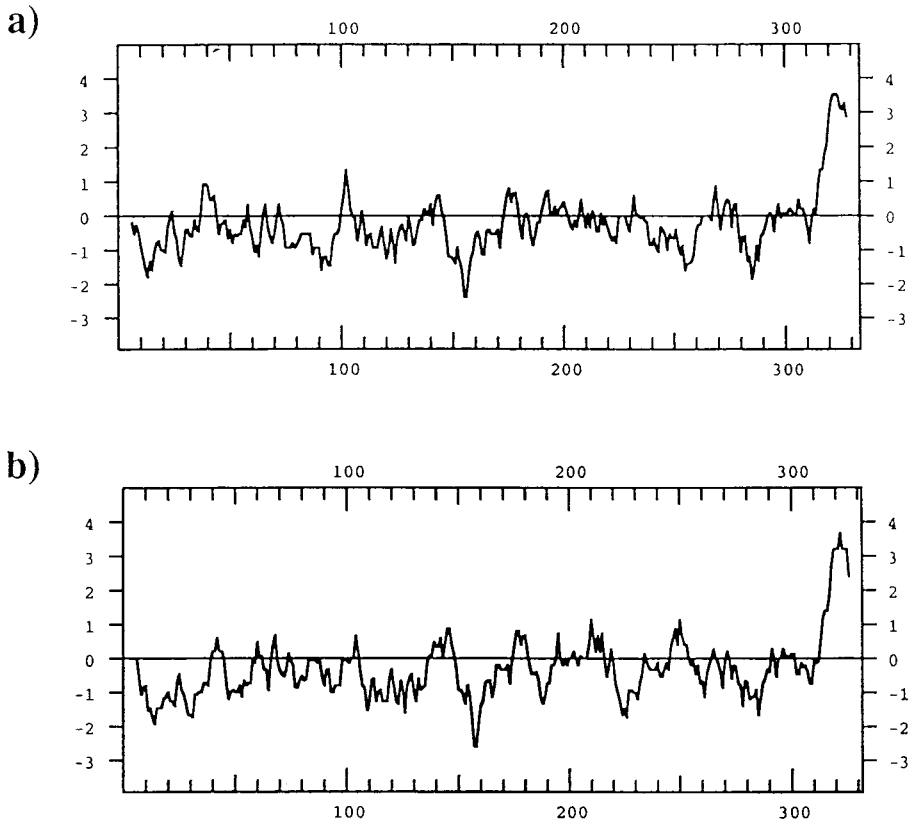

FIG. 7. Hydrophobicity plots for the deduced amino acid sequences from the *S. typhimurium* (a) and *E. coli* (b) *flk* genes.

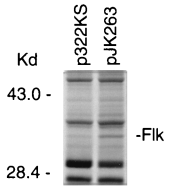

FIG. 9. Flk translated protein product from T7 RNA polymerase-mediated expression of the *flk* gene. Proteins were produced from wild-type strains carrying a vector-only control plasmid (p322KS) and plasmid pJK263, which has the *flk* gene placed downstream of the T7 promoter. The Flk protein is indicated. Positions of molecular mass markers are shown on the left.

periplasm, the fact that only one amino acid extends beyond the proposed membrane segment suggests that Flk might interact with at least one other protein in order to assess the state of P- and L-ring assembly and transmit this signal to affect class 3 transcription.

Mutations in the *flk* gene were isolated as null mutations that allowed class 3 gene expression in P- and L-ring mutant strains. If the hypothetical coregulatory protein exists, then a null mutation would have either no phenotype or a superrepressor phenotype. Suppose the role of Flk is to increase FlgM activity until ring completion. After ring completion, Flk activity is inhibited by the hypothetical sensor of ring completion. This results in a reduction in FlgM activity allowing some initiation of class 3 transcription prior to FlgM export by the completed BBH structure and possibly more efficient flagellar synthesis. If the hypothetical protein were mutated, then Flk would act as if the rings were never completed and FlgM activity would remain maximal. If this FlgM activity were significant compared to reduction in FlgM activity by export of FlgM through the BBH, then loss of the hypothetical coregulator would always keep FlgM activity high, resulting in the inhibition of class 3 gene expression. If the Flk-induced FlgM activity were not very significant, such that export of FlgM still resulted in derepression of class 3 gene expression, then loss of the coregulator would have no significant effect on class 3 gene expression. On the other hand, suppose the role of Flk is to decrease FlgM activity after ring completion and prior to hook completion, where FlgM is exported and its activity is further decreased. If the coregulator were defective, Flk would always sense that the rings were incomplete and FlgM would remain active until hook completion and export of FlgM. It is also possible that the coregulator is part of the basal body and that a null mutation would not allow FlgM export such that class 3 gene expression is always inhibited.

How does loss of Flk activity result in class 3 gene transcription in ring mutant strains but not in other BBH mutant strains? Recent results support a mechanism by which loss of Flk in P- and L-ring mutant strains results in a reduction in the levels of FlgM protein in the cell (21a). Reduction in FlgM protein levels could be due to decreased transcription, decreased translation, modification to an inactive form, increased turnover, or export through a basal body structure lacking rings. These possibilities are currently under investigation.

Is Flk uniquely part of the flagellar regulon? This is unlikely, given that the *flk* gene is not coregulated with other flagellar genes and a *flk* null mutant is still motile. It is more likely that Flk is of general importance to the periplasm. Further characterization of how Flk couples P- and L-ring assembly to regulation of class 3 gene expression is likely to elucidate what such a role might be.

The discovery that loss of the Flk protein results in derepression of σ^{28}-dependent transcription uniquely in strains defective in P- and L-ring assembly (*flgA*, *flgH*, and *flgI* mutants) supports a model where two signals are required for the export machinery to switch to class 3 export: one signal that the hook is complete via FliK and one signal that the P- and L-rings are complete via Flk. Once the flagellar export apparatus is converted from class 2 protein export to class 3 export, FlgM is removed from the cytoplasm, σ^{28}-dependent transcription of class 3 promoters occurs, and flagellar biosynthesis is completed.

A role for Flk in modulating FlgM levels in response to ring biosynthesis might be to fine-tune the efficiency of the assembly process in response to gene expression. By sensing that the rings were complete, the cell could act through Flk to reduce FlgM levels enough to initiate class 3 gene expression. Ring completion would signal that hook completion was imminent. By lowering FlgM levels enough to initiate some flagellin synthesis, a small pool of flagellin subunits would be immediately available for export upon hook completion. Otherwise, the cell might have to wait for hook completion followed by FlgM export before class 3 derepression occurs, and this would result in a small lag time between hook completion and initiation of filament assembly.

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