7. R. H. Ku and R. E. Billings, *Arch. Biochem. Biophys.* **247**, 183 (1986).
8. O. W. Griffith and A. Meister, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4668 (1985).
9. M. J. Meredith and D. J. Reed, *J. Biol. Chem.* **257**, 3747 (1982).
10. D. J. Kane *et al.*, unpublished data.
11. A. R. Rosenkranz *et al.*, *J. Immunol. Methods* **156**, 39 (1992).
12. I. Morel *et al.*, *Free Radical Biol. Med.* **13**, 499 (1992).
13. P. E. Starke and J. L. Farber, *J. Biol. Chem.* **260**, 86 (1985).
14. R. G. Kilbourn *et al.*, *Biochem. Biophys. Res. Commun.* **172**, 1132 (1990).
15. U. C. Garg and A. Hassid, *J. Clin. Invest.* **83**, 1774 (1989).
16. D. J. Kane *et al.*, unpublished data.
17. E. B. Gralla and D. J. Kosman, *Adv. Gen.* **30**, 251 (1992).
18. An 850–base pair (bp) fragment containing the open reading frame of the human *bcl-2* complementary DNA (cDNA) was ligated into the expression vector pAD4 containing the strong promoter of the alcohol dehydrogenase gene and the LEU2 selectable marker, and this construct was used for transformation of yeast. A vector without the 850-bp *bcl-2* fragment was used as a control. Four strains of yeast were transformed with the two constructs: the parental strain (DBY746; **α**, *leu2-3, 11 his3Δ1 trp1-298a ura3-52*); *sod1* (EG118; **α**, *leu2-3, 11 his3Δ1 trp1-298a ura3-52*; *sod1ΔA::URA3*); *sod2* (EG110; **α**, *leu2-3, 11 his3Δ1 trp1-298a ura3-52*; *sod2Δ::TRP1*); and *sod1sod2* (EG133; **α**, *leu2-3, 11 his3Δ1 trp1-298a ura3-52*; *sod1ΔA::URA3 sod2Δ::TRP1*). The lithium acetate method was used for transformation (*23*). After transformation and selection in medium lacking leucine, yeast were quantified by an assay of the optical density at 600 nm, then equal numbers were streaked in each of six plate sections and grown for 5 days in room air or in an atmosphere of 100% O_2. As a control, Bcl-2 was expressed in a histidine auxotroph; no growth was detected on His^- plates. Enhanced growth of *sod2* mutants expressing Bcl-2 was demonstrated by (i) growth on glycerol plates in 21% oxygen (no growth detected for pAD4-transformed *sod2* mutants) and (ii) enhanced plating efficiency on glycerol in 21% oxygen (0 for pAD4 control compared with 4×10^3 for Bcl-2–expressing *sod2* mutants and 5×10^5 for wild-type *S. cerevisiae*). Enhanced growth of *sod1* mutants expressing Bcl-2 was demonstrated by (i) growth on dextrose in reduced (~10%) oxygen (no growth detected for pAD4-transformed *sod1* mutants) and (ii) increased growth in liquid culture; optical densities were compared for *sod1* mutants transformed with pAD4 with those transformed with pAD4–*bcl-2*. After 6 hours in culture, the optical density of the pAD4-transformed *sod1* mutants was 4.4, in comparison with 7.2 for those transformed with pAD4–*bcl-2*.
19. M. D. Jacobson *et al.*, *Nature* **361**, 365 (1993).
20. M. B. Hansen, S. E. Nielsen, K. Berg, *J. Immunol. Methods* **119**, 203 (1989).
21. W. L. Richards *et al.*, *Exp. Cell Res.* **159**, 235 (1985).
22. K. Yagi, *Biochem. Med.* **15**, 212 (1976).
23. H. Ito, Y. Fukuda, K. Murata, A. Kimura, *J. Bacteriol.* **153**, 163 (1983).
24. We thank M. Cleary for the *bcl-2* cDNA; P. Mellon for the GT1-7 cells; J. Colicelli for the pAD4 yeast expression vector; H. Land for the pBabe expression vector; L. Ignarro for the nitro-L-arginine methyl ester and *S*-nitroso-*N*-acetylpenicillamine; S. Clarke, C. Clarke, R. Weiss, and C. Kayalar for thoughtful discussions; and K. Moghaddami for administrative assistance. Supported by NIH grant NS27812 (D.E.B.), NIH grant GM 28222 (J.S.V.), and a Margaret E. Early Medical Research Trust grant (E.B.G.).

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Sensing Structural Intermediates in Bacterial Flagellar Assembly by Export of a Negative Regulator

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The ability of a regulatory protein to sense the integrity of the bacterial flagellar structure was investigated. In response to a defective hook–basal body complex, the anti-σ^{28} FlgM protein inhibits flagellin transcription. In cells with a functional hook–basal body complex, the flagellin genes are transcribed normally and the FlgM protein is expelled into the growth medium. In strains with a defective hook–basal body structure, FlgM is absent from the media. The presence of flagellin protein in the media is substantially reduced in strains carrying a FlgM-LacZ protein fusion, suggesting that the fusion is blocking the flagellar export apparatus. These results suggest that the FlgM protein assesses the integrity of the flagellar hook–basal body complex by itself being a substrate for export by the flagellar-specific export apparatus.

In both prokaryotes and eukaryotes, developmental pathways involving the synthesis and assembly of structures often proceed through structural intermediates. The synthesis of the flagellar organelle in bacteria provides a model system to study the coordination of transcriptional regulation with the assembly of structural intermediates. The flagellum is composed of a basal body structure, located in the cell membrane, and an extracellular hook, which acts as a flexible joint to which the flagellar filament is attached (Fig. 1). The genetics and biogenesis of the bacterial flagella have been recently reviewed (*1*). Components of the basal body complex are assembled into a structure that includes a motor driven by a proton pump, a switch complex that determines the direction of flagellar rotation, a rod structure that transverses the two membranes and the cell wall, ring proteins embedded within the membranes that stabilize the structure, and a flagellar-specific export apparatus at the cytoplasmic base. Assembly of the extracellular component of the flagellum involves the transport of individual components through a pore in the basal body structure (*2*). A flagellar-specific export pathway (*3*) determines which proteins are exported by what may be an ordered assembly mechanism (*1*). The hook subunits are exported and assembled to complete the hook–basal body structure, and then the export apparatus is possibly modified to stop the export of hook subunits and begin the export of flagellin subunits to be assembled into the flagellar filament (*4*).

The genes required for flagella synthesis and chemotaxis in the bacterium *Salmonella typhimurium* are organized into a regulatory hierarchy of three classes (*5*). Each class is required for expression of the next. The early class includes two master regulatory genes that respond to global signals such as cAMP (adenosine 3′,5′-monophosphate) levels (*6*, *7*), and when induced they turn

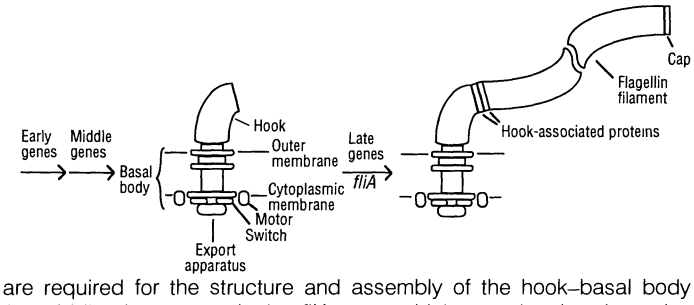

Fig. 1. Biosynthesis of the *S. typhimurium* flagellar structure. The genes required for synthesis of flagella are grouped into three classes (*5*). The early class encodes positive activators required for the expression of the middle class genes. The middle class genes are required for the structure and assembly of the hook–basal body complex (*1*). Included in the middle class genes is the *fliA* gene, which encodes the alternative sigma factor, σ^{28} (*8*). This sigma factor is specific for the transcription of the late class genes, which encode the flagellin filament genes, hook-associated proteins, and the flagellar cap protein. The *flgM* gene is transcribed from both middle and late class promoters (*16*).

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on expression of the middle class of genes. The middle class genes are required for the structure and assembly of the hook–basal body complex (Fig. 1). Included in the middle class of flagellar genes is the *fliA* gene, which encodes an alternative sigma factor, σ^{28} (8), required specifically for transcription of the late class of genes (5). The late class includes flagellin, chemotaxis, and motility genes, as well as two hook-associated protein (HAP) genes and a gene for the cap protein that is found at the distal end of the flagellum (5). The flagellin subunits are assembled between the HAPs attached to the end of the hook structure and the cap protein (9). Flagellar structures missing the HAP proteins or the cap protein do not assemble flagella, and excrete flagellin subunits into the medium (10). In addition to this structural role in flagellar assembly, cap-defective mutants are derepressed for expression of the late class of flagellar genes (5).

The regulation of genes in the flagellar biosynthetic pathway may involve the ability of the negative regulatory protein FlgM to respond to defects in the hook–basal body structure (11). When a middle class gene involved in the structure or assembly of the hook–basal body is defective, the FlgM protein prevents expression of the late class of genes. It has been reported that FlgM prevents late gene expression of the flagellin genes by interacting with the σ^{28} factor specific for late gene transcription (12). It was not clear how the FlgM protein could assess the integrity of the hook–basal body structure in order to control late gene expression in strains with a functional hook–basal body complex and prevent late gene expression in strains with a defective hook–basal body complex. One model predicts that when the hook–basal body complex is completed and competent for export and assembly of flagellin subunits, it also becomes competent for export of the FlgM negative regulator (13). This would provide a means for FlgM to sense structure and effect regulation accordingly. Here, evidence is presented in support of this model.

Regulation of late gene expression is determined by the relative levels of σ^{28} and

Table 1. Effect of FlgM and FliA levels on flagellin transcription in wild-type flagella backgrounds and in strains defective in genes required structure or assembly of the hook–basal body complex (*fla*$^-$). Transcription from the flagellin (FljB) promoter occurs in an otherwise wild-type strain (strain 1). In strains defective in the formation of the hook–basal body complex (*fla*$^-$ strains, strain 2), transcription of flagellin genes is inhibited by the action of the FlgM protein (11), which inhibits the σ^{28} protein required for transcription of the flagellin gene (8, 12). Transcription is restored either by the loss of the FlgM negative regulator or by overexpression of the *fliA* gene that encodes the σ^{28} protein (strains 3 and 4). Overexpression of the *flgM* gene results in loss of flagellin expression in a wild-type strain (strain 5).

Strain	Genotype*	Flagellin transcription†
1	*fljB-lac*	+
2	*fla*$^-$ *fljB-lac*	–
3	*flgM*$^-$ *fla*$^-$*fljB-lac*	++
4	p*fliA*$^+$/*fla*$^-$*fljB-lac*	++
5	p*flgM*$^+$/*fljB-lac*	–

*The *fla*$^-$ alleles include mutants in the following genes: *flgA-J*, *flhA*, *flhB*, and *fliE-R*. †Flagellin transcription was measured by production of β-galactosidase from a *fljB-lac* operon fusion present in all strains tested on lactose-tetrazolium indicator plates (11). The actual β-galactosidase activities are as follows (in Miller units): Strain 1, 260 units; strain 2, 19 units; strain 3, 1700 units; strain 4, 3600 units; strain 5, 17 units. The plasmid carrying the *fliA* gene used in this study is pMS531 (14). The plasmid carrying the *flgM* gene used in this study is pMH71 (11).

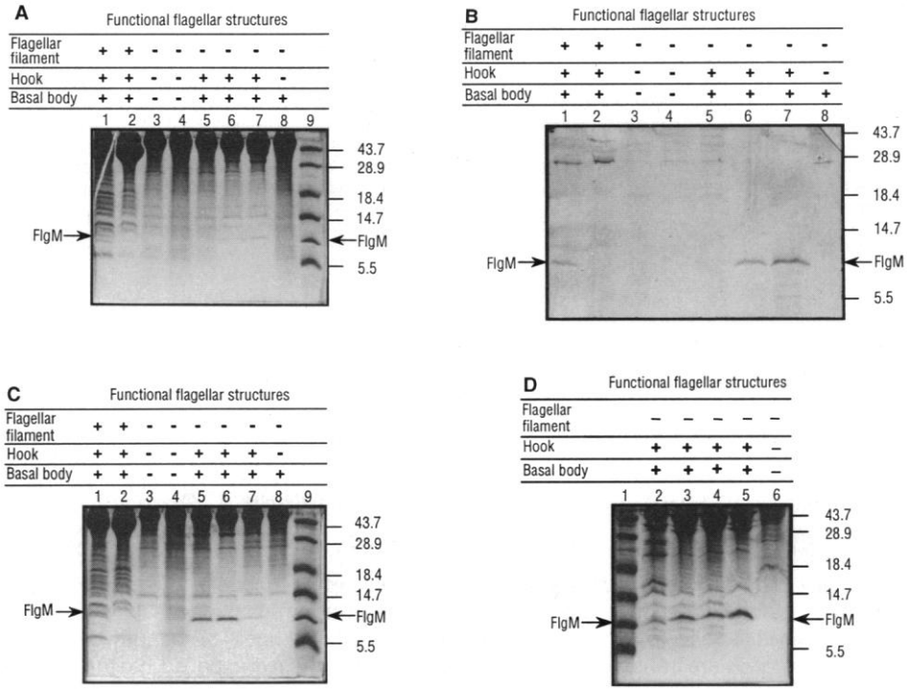

Fig. 2. Export of FlgM protein into the media (30). All strains carry a Mud-*lac* fusion in the *fljB* flagellin gene (11). Sample preparation and gel running conditions were as described (30). FlgM protein was purified as described (12) and added to the molecular size markers. (**A**) FlgM protein is present in the spent growth media of the wild-type strain (lane 1), a strain defective in both flagellin genes, *fljB* and *fliC* (lane 6), and a strain defective in the gene that codes for the flagellar cap *fliD* (lane 7). FlgM is transcribed from both σ^{28}-dependent and σ^{28}-independent promoters (16); however, there is no FlgM protein visible in the Δ*fliABCD* strain (lane 5), which is missing the σ^{28} gene (*fliA*), both flagellin genes, and the cap gene. Approximately 80% of *flgM* gene transcription is from the σ^{28}-dependent promoter (16). FlgM protein is absent in the spent growth media of both a strain defective in basal body formation (Δ*flgGHIJKL*, lane 3), which was found to express high amounts of FlgM protein (16), and a strain defective in hook formation (*fliK*, lane 8). Mutant strains that do not produce FlgM protein, *flgM* (lane 2) and Δ*flhCD* (lane 4), as expected do not exhibit a FlgM band in the spent growth media. (**B**) The spent growth media was also examined on the same set of strains as in (A) by protein immunoblot analysis with antibody to purified FlgM protein (31). (**C**) The spent growth media was also examined on the same set of strains as in (A), except that the strains carried a FliA-producing plasmid (pMS531) (14) and that 25 μg of protein was loaded in each lane as compared to 50 μg loaded in each lane for (A). This gave identical results as in (A) except that, by visual determination, more FlgM protein was found in the media for strains lacking flagellin genes (lanes 5 and 6) even though one-half of the amount of protein was loaded in (C). The presence of the *fliA*$^+$-containing plasmid complements the *fliA* mutation [compare (A) and (C), lanes 5]. Overexpression of σ^{28} caused by the presence of this plasmid leads to increased transcription of the *flgM* gene (16). NH_2-terminal amino acid sequencing (24) was done on the band in the p*fliA*$^+$/Δ*fliA-D* strain [(C), lane 5] that comigrates with FlgM and was found to be SIDXTSPLKP (33); this is identical to the NH_2-terminal sequence predicted for the *flgM* gene product (17) except that the NH_2-terminal methionine is missing. (**D**) Some FlgM protein was present in the spent growth media of a hook mutant strain (*flgE*, lane 2). FlgM protein was also found in the spent media of HAP mutant strains (*flgK* and *flgL*, lanes 3 and 4), in apparently smaller amounts than were found in a strain defective in both flagellin genes, *fljB* and *fliC* (lane 5). FlgM was not found in the media of a strain defective in the flagellar export pathway (*fliI*, lane 6). Purified FlgM protein (1 μg) was loaded with molecular size markers. Molecular size markers are indicated on the right (in kilodaltons).

FlgM in the cell (Table 1). A strain defective in the hook–basal body complex (*fla*$^-$) does not express flagellin (Table 1, strain 2) (*5*, *11*). This negative regulation is overcome by inactivation of the *flgM* gene (Table 1, strain 3) (*11*). In addition, flagellin expression is also restored in *fla*$^-$ strains by the introduction of a plasmid expressing the *fliA* (σ^{28}) gene from its own promoter (*14*) (Table 1, strain 4). These results support the idea that the relative levels of FlgM and σ^{28} are important in the regulation of late gene expression. Because FlgM negative regulation can be overcome by overexpression of the *fliA* gene, overexpression of the *flgM* gene may inhibit normal σ^{28} function. Indeed, introduction of a plasmid expressing the *flgM* gene from its own promoters (*15*) into the wild-type flagellar strains results in the inhibition of flagellin expression (Table 1, strain 5).

If the relative levels of FlgM and σ^{28} are important in late gene expression, regulation could be achieved by varying the amounts of FlgM. The FlgM anti-sigma factor inactivates σ^{28}-dependent transcription, in vitro, when FlgM is present in stoichiometric amounts with σ^{28} (*12*). One means to regulate FlgM amounts would be to export FlgM out through a functional hook–basal body structure, because it has been shown that *flgM* gene transcription does not increase in response to the hook–basal body structure (*16*). Thus, by exporting enough FlgM to reduce FlgM amounts with respect to σ^{28}, the late genes would be transcribed.

As a test of this model, various *S. typhimurium* strains were grown in a liquid medium, and the spent growth media was assayed for protein by polyacrylamide gel electrophoresis (PAGE). The results presented in Fig. 2 support the model that FlgM is exported through the flagellar structure into the medium. FlgM protein is present in the medium for strains that have a functional hook–basal body complex. These include a wild-type strain (Fig. 2A, lane 1) and strains that do not express either flagellin (*fliC* and *fljB*) (Fig. 2A, lane 6), the flagellar Cap (*fliD*) (Fig. 2A, lane 7), or the hook-associated proteins (Fig. 2D, lanes 3 and 4), although a functional *fliA* (σ^{28}) gene is required [Fig. 2, A (lane 5) and C (lane 5)]. Protein immunoblots performed on the gel in Fig. 2A with an antibody to FlgM confirmed that these bands represent FlgM protein (Fig. 2B). The FlgM protein was not found in either the spent medium of a *flgM* mutant strain (Fig. 2A, lane 2) or in the spent medium of strains defective in the hook–basal body complex, which are not export-competent for flagellin (Fig. 2A, lanes 3, 4, and 8). There was one exception, however: FlgM was found in the spent media in a hook mutant strain (Fig. 2D, lane 2), although at lower amounts than were seen for strains missing the hook-associated or flagellin proteins (Fig. 2D, lanes 3, 4, and 5). This finding was unexpected because no FlgM was found in a strain defective in hook-length control (Fig. 2, A and C, lanes 8). This may be because the allele of the hook structural gene used in this study is leaky. It will be necessary to obtain a deletion of the hook gene to determine whether FlgM is exported in such a mutant strain.

The presence of FlgM is enhanced in strains carrying a plasmid with a functional *fliA* gene and lacking the flagellin genes (Fig. 2C, lanes 5 and 6). Transcription of the *flgM* gene is substantially increased in the presence of this plasmid (*16*), presumably because the *flgM* gene is itself under the control of a σ^{28}-dependent promoter (*17*). Furthermore, in strains defective for both flagellin genes, FlgM would not compete with flagellin subunits for export, which would explain why more FlgM is found in the spent growth media.

The model in which FlgM has the ability to be exported predicts that a fusion of FlgM to a bulky protein might maintain the signals for export, but fail to be exported. If the fusion protein was able to block the export apparatus, no other protein subunits would be exported and the cells would be nonmotile. As a test of this idea, fusions of the *Escherichia coli lacZ* gene, which encodes β-galactosidase, to the COOH-terminus of FlgM were constructed with the Mud-*lac* transposon (MudII1734) (*18*). Cells expressing these fusions were defective in motility as compared with cells of a wild-type strain (Fig. 3A). This motility defect was dominant to the presence of a *flgM*$^+$ gene (Fig. 3B). Revertants that regained motility arose and included cells that both lost and retained the FlgM-LacZ fusion as evidenced by behavior on lactose indicator plates (Fig. 3C). These results support the model that the FlgM–β-galactosidase fusion proteins block the flagellar export pathway and can interfere with flagellar assembly.

As a test of this hypothesis, flagellin (FliC) export was assayed in the presence of a FlgM-LacZ protein fusion (Fig. 4). In a cap mutant strain, FliC is released into the media (Fig. 4, lane 1). Either a *lacZ* operon fusion or protein fusion to the *flgM* gene was introduced into the cap mutant strain, and FliC release into the spent growth media was determined. The presence of a *flgM-lacZ* operon fusion did not affect FliC

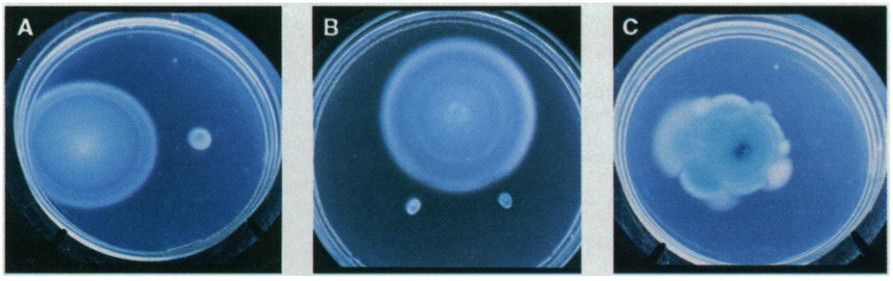

Fig. 3. Behavior of *S. typhimurium flgM* mutants on motility plates (*32*). (**A**) A Mud-*lac* translational fusion in the *flgM* gene (*16*) (right) has reduced motility as compared with that of the wild-type strain LT2 (left). (**B**) The reduced motility phenotype of the FlgM-LacZ protein fusion (bottom left) is dominant to the presence of a wild-type *flgM*$^+$ gene (bottom right). The wild-type strain (top) was included for direct comparison. (**C**) A strain carrying a FlgM-LacZ protein fusion reverts to motility (Mot$^+$). There are three classes of Mot$^+$ revertants. One class is Lac$^-$ on the chromogenic indicator for β-glactosidase activity and maps to the *flgM* locus. The other classes are Lac$^+$ in the presence of X-Gal and include revertants that map to the *flgM* locus and those that do not (*16*).

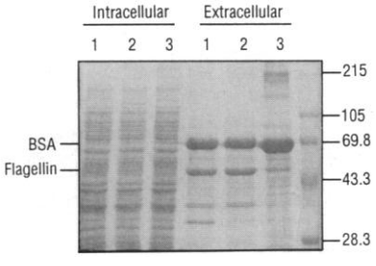

Fig. 4. Export of FliC flagellin protein into the media. All strains are defective in the cap protein (*fliD2380*). Sample preparation and gel running conditions were as described (*30*). FliC protein was identified by protein immunoblot analysis with antibody to FliC flagellin (Difco) that was purified by serum affinity chromatography (*32*). In the cap mutant strain, FliC is released into the media (lane 1). Release of FliC in the cap mutant strain was also assayed in the presence of either a *lacZ* operon fusion or a *lacZ* gene fusion to the *flgM* gene (lanes 2 and 3, respectively). The presence of a FlgM-LacZ protein fusion in the cell results in a reduction in the amount of FliC released into the media (lane 3). No substantial amount of flagellin was observed to accumulate intracellularly in these strains. These results support the model that the FlgM-LacZ fusion protein blocks the flagellar export pathway.

release (Fig. 4, lane 2), whereas the FlgM-LacZ protein fusion did reduce the amount of FliC protein found in the spent media (Fig. 4, lane 3). However, because there was no accumulation of flagellin intracellularly in the presence of the FlgM-LacZ protein fusion (Fig. 4, lane 3), it is still possible that expression of the flagellin gene was affected. This result is consistent with the model that a FlgM-LacZ protein fusion blocks the flagellar export pathway.

One alternative model suggests that *flgM* gene transcription is regulated in response to structure (*16*). The results of studies on the regulation of *flgM* gene transcription and translation have shown that the *flgM* gene is transcribed from both middle and late class promoters and that *flgM* gene expression is not increased in strains defective in the hook–basal body complex (*16*). Thus, cells do not repress late gene transcription by increased expression of the *flgM* gene. Other models propose that FlgM protein is modified or degraded in response to structure or that FlgM protein is sequestered into the completed structure (*11*). To date, no modifying activity for FlgM has been described, nor has FlgM protein been found associated with the isolated flagellar structure. Still, these last three models remain formal possibilities and could account for FlgM-dependent regulation in addition to regulation by export.

It seems improbable that the role of FlgM is to sense a defective flagellar structure and prevent late gene transcription. It is more likely that FlgM plays a role in normal flagellar function and regulation. We suggest that FlgM protein is important in wild-type cells both for temporal control of flagellar assembly and for regulation of flagellar length. The *flgM* gene is expressed from both middle and late class promoters (*16*). After initial induction of the flagellar regulon, both FlgM and FliA are expressed, either preventing or reducing late gene expression in the absence of functional hook–basal body structures. This would prevent intracellular accumulation of flagellin subunits that may be deleterious to cell growth or to synthesis of the hook–basal body complex. When functional hook–basal body structures are assembled, FlgM is exported outside the cell, which would result in instantaneous transcription of the late genes at a time when their gene products are needed. At some point, a steady state of FliA and FlgM levels would be attained as a result of the fact that the *flgM* gene is transcribed by the same sigma factor that it inhibits (*17*) and that flagellar proteins are assembled normally in response to these levels. However, if at any time the cell is subjected to forces that shear off the flagella, accumulated intracellular FlgM would be immediately exported through the hook–basal body structures present, and transcription of the late genes would be initiated as a result of the presence of the FliA sigma factor and the absence of the FlgM anti-sigma factor. In the bacterial environment, it is likely that forces are often encountered that shear off the flagellar filament. The ability to respond quickly and resynthesize the full-length structure would restore motility rapidly and provide a survival advantage. In addition, FlgM protein may also serve to regulate flagellar length. As the flagella increase in size, it becomes increasingly more difficult for proteins, including flagellin subunits and FlgM, to travel down the length of the flagella. Thus, the flow of FlgM protein outside the cell would be reduced, resulting in inhibition of late gene expression and thus limiting the final size of the flagellar structures.

REFERENCES AND NOTES

1. R. M. Macnab, *Annu. Rev. Genet.* **26**, 131 (1992).
2. T. Wagenknecht, D. J. DeRosier, S.-I. Aizawa, R. M. Macnab, *J. Mol. Biol.* **162**, 69 (1982).
3. C. J. Jones and R. M. Macnab, *J. Bacteriol.* **172**, 1327 (1990).
4. T. Suzuki and T. Iino, *ibid.* **148**, 973 (1981).
5. K. Kutsukake, Y. Ohya, T. Iino, *ibid.* **172**, 741 (1990).
6. T. Yokata and J. S. Gots, *ibid.* **103**, 513 (1970).
7. Y. Komeda, H. Suzuki, J.-I. Ishidsu, *Mol. Gen. Genet.* **142**, 289 (1975).
8. K. Ohnishi, K. Kutsukake, H. Suzuki, T. Iino, *ibid.* **221**, 139 (1990).
9. T. Ikeda, M. Homma, T. Iino, S. Asakura, R. Kamiya, *J. Bacteriol.* **169**, 1168 (1987).
10. M. Homma, H. Fujita, S. Yamaguchi, T. Iino, *ibid.* **159**, 1056 (1984).
11. K. L. Gillen and K. T. Hughes, *ibid.* **173**, 2301 (1991).
12. K. Ohnishi, K. Kutsukake, H. Suzuki, T. Iino, *Mol. Microbiol.* **6**, 3149 (1992).
13. D. Blair and S. K. Dutcher, *Curr. Opin. Genet. Dev.* **2**, 756 (1992).
14. An 884–base pair DNA fragment containing the *fliA* gene of *S. typhimurium* strain LT2 was obtained by polymerase chain reaction amplification with the FliAI and FliAII primers as described [M. N. Starnbach and S. Lory, *Mol. Microbiol.* **6**, 459 (1992)]. This fragment was ligated into the Sma I site of pBluescript KSII+ (Stratagene) to form pMS531 (M. Starnbach, personal communication).
15. M. Homma, K. Ohnishi, T. Iino, R. M. Macnab, *J. Bacteriol.* **169**, 3617 (1987).
16. K. L. Gillen and K. T. Hughes, unpublished results.
17. ———, *J. Bacteriol.* **173**, 6453 (1991).
18. B. A. Castilho, P. Olfson, M. J. Casadaban, *ibid.* **158**, 488 (1984).
19. H. J. Vogel and D. M. Bonner, *J. Biol. Chem.* **218**, 97 (1956).
20. R. W. Davis, D. Botstein, J. R. Roth, Eds., *Advanced Bacterial Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1980), pp. 207–210.
21. U. K. Laemmli, *Nature* **227**, 680 (1970).
22. M. Bradford, *Anal. Biochem.* **72**, 248 (1976).
23. H. Schagger and G. von Jagow, *ibid.* **166**, 368 (1987).
24. NH_2-terminal amino acid sequencing was done on an Applied Biosystems Model 447A Protein Sequencer. Samples were prepared as described (*25*).
25. P. Matsudaira, *J. Biol. Chem.* **262**, 10035 (1987).
26. E. Harlow and D. Lane, Eds., *Antibodies, A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988), pp. 519–521.
27. D. Masson and J. Tscopp, *Cell* **49**, 679 (1987).
28. B. Traxler and J. Beckwith, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10852 (1992).
29. W. N. Burnette, *Anal. Biochem.* **112**, 195 (1981).
30. Various mutant strains affecting flagellar biosynthesis were grown to an optical density of 0.8 in 1 liter of minimal E media (*19*) supplemented with 0.2% glycerol and a mixture of amino acids, vitamins, and nucleotides [pools 6 through 11 as described by R. W. Davis *et al.* (*20*)]. The cells were removed by centrifugation at 15,000*g*. Bovine serum albumin (50 mg; Boehringer Mannheim) was added to the supernatant as a carrier protein, followed by the addition of ammonium sulfate to 60% at 0°C. Protein was pelleted by centrifugation at 15,000*g* for 20 min, and the dried pellet was resuspended in loading buffer (*21*). Proteins were quantified (*22*) and electrophoresed on a 16.5% Tricine–SDS-polyacrylamide gel as described (*23*). Gels were stained with Coomassie brilliant blue-R, and FlgM protein was identified by NH_2-terminal sequencing (*24*).
31. M. Silverman and M. Simon, *J. Bacteriol.* **113**, 105 (1973).
32. FlgM protein was purified according to the method described by K. Ohnishi *et al.* (*12*). Rabbit antisera to FlgM protein were produced at Pocono Rabbit Farm and Laboratory Inc., Canadensis, PA. Antibodies were purified from the antisera by ammonium sulfate precipitation, followed by protein A chromatography (*26*) and affinity purification of FlgM-specific antibodies isolated according to the procedures described by D. Masson and J. Tschopp (*27*). The supernatant or whole-cell extracts were fractionated by Tricine–SDS-PAGE (*23*); the proteins electrotransferred onto Western polyvinylidene difluoride membranes (Schleicher & Schuell) in CAPS buffer (*25*). The membranes were incubated with antibodies to rabbit immunoglobulin G–alkaline phosphatase reaction (*28*) or incubated with ^{125}I-labeled protein A (New England Nuclear) (*29*) and the blot developed against x-ray film.
33. Single-letter abbreviations for the amino acid residues are as follows: D, Asp; I, Ile; K, Lys; L, Leu; P, Pro; S, Ser; and T, Thr. X, any amino acid.

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