JOURNAL OF BACTERIOLOGY, Feb. 1996, p. 899–901 Vol. 178, No. 3
0021-9193/96/$04.00+0

Negative Regulation by FliD, FliS, and FliT of the Export of the Flagellum-Specific Anti-Sigma Factor, FlgM, in *Salmonella typhimurium*

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The *fliD* operon of *Salmonella typhimurium* consists of three flagellar genes, *fliD*, *fliS*, and *fliT*, and is transcribed in this order. It has been shown that an *fliD*::Tn*10* mutation causes an excess export of the flagellum-specific anti-sigma factor, FlgM, resulting in an overexpression of flagellar class 3 operons. In this study, using gene-disruption mutants in the individual genes in the *fliD* operon, we showed that mutations in any one of the genes in the operon enhanced both FlgM export and the expression of flagellar regulon. This indicates that all three genes in the operon are involved in the negative regulation of FlgM export.

The flagellum of *Salmonella typhimurium* is composed of a basal body, a hook, and a filament. More than 50 genes are required for flagellar formation and function (17, 19). According to the cascade model of the flagellar regulon, the flagellar operons are grouped into three classes, 1, 2, and 3, with respect to their relative positions in the transcriptional hierarchy (16). The class 2 operons are positively controlled by activator proteins, FlhD and FlhC, which are encoded by the class 1 genes (18). The class 3 operons are controlled positively by FliA and negatively by FlgM (1, 14). FliA is a flagellum-specific sigma factor encoded by one of the class 2 genes, *fliA* (20). FlgM is believed to act as an anti-sigma factor (21) and is encoded by the *flgM* gene, which is transcribed from both class 2 and class 3 promoters (2, 11). The FliA-FlgM regulatory system also controls class 2 expression (14), but the molecular mechanism underlying this control is still unknown. In the flagellar regulon, the sequential expression of the flagellar operons is coupled with the assembly process of the flagellar structure (16). All of the genes involved in formation of the hook-basal body complex belong to class 2, and those involved in filament assembly and flagellar function belong to class 3. This coupling is achieved by a mechanism whereby FlgM is exported outside the cell through the hook-basal-body structure formed by the products of the class 2 genes (7, 11).

The *fliD* operon consists of three flagellar genes, *fliD*, *fliS*, and *fliT* (10, 22), and is transcribed in this order from both class 2 and class 3 promoters (13). The *fliD* gene encodes a capping protein of the filament (4, 8) which inhibits the free release of newly exported flagellin molecules and facilitates their polymerization onto the growing filament tips (5, 9). Therefore, *fliD* mutants produce hook-basal-body structures lacking the filament portion and excrete flagellin monomers into the culture media (3, 6). In contrast, the *fliS* and *fliT* genes are shown to be dispensable for flagellar formation (22). However, the *fliS* mutant shows an impaired flagellin export and produces flagellar structures with short filaments. On the other hand, the *fliT* mutant produces flagellar structures indistinguishable from those produced by a wild-type strain.

In a previous report, we showed that an *fliD*::Tn*10* mutation upregulated the transcription of the class 3 operons and postulated that the *fliD* operon might contain a negative regulator gene termed *rflA* (16). Recently, we found that FlgM export is enhanced by the *fliD*::Tn*10* mutation and proposed that the *rflA* function may be attributed to the flagellar cap protein encoded by the *fliD* gene (11). However, we could not exclude the possibilities that either *fliS* or *fliT* corresponds to the *rflA* gene and that the RflA$^-$ phenotype of the *fliD*::Tn*10* mutant may be caused by the polar effect of the Tn*10* insertion. This study was performed to test these possibilities.

Effects of the *fliD*, *fliS*, and *fliT* mutations on expression of the class 3 flagellar operons. The *fliD*::Tn*10* mutant and the gene-disruption mutants in which the individual genes in the *fliD* operon are specifically inactivated by the insertion of the kanamycin resistance gene cassette (*kan*) have been described previously (16, 17, 22). They were KK2601 (*fliD*::Tn*10*), KK1391 (*fliD*::*kan*), KK1392 (*fliS*::*kan*), and KK1393 (*fliT*::*kan*). Into these strains, the chromosomal *lac* fusion with one of the class 3 operons, *tar*, was introduced by transduction from KK1105 (14), and β-galactosidase enzyme activity was measured with the resulting transductants as described previously (16) (Table 1). As reported previously (16), the *fliD*::Tn*10* mutation enhanced the expression of the fusion gene. To our surprise, it was found that all of the mutants in the individual genes resulted in overexpression of the fusion gene by more than fourfold the level of the wild type. In order to exclude the possibility that the *rflA* gene corresponds to *fliT* and that the *kan* insertion into the *fliD* or *fliS* gene exerts a polar effect on the expression of the downstream *fliT* gene, a recombinant plasmid carrying only the *fliD* or *fliS* gene was introduced by transformation into KK1391 or KK1392 carrying the *tar-lac* fusion gene and β-galactosidase enzyme activity was assayed with the resulting transformants. In both mutants, introduction of the plasmids reduced *tar-lac* expression to the wild-type level (data not shown). Similarly, introduction of the plasmid carrying the *fliD* gene could restore the motility of the cells of KK1391 to the wild-type level (data not shown). These results indicate that the *kan* insertions did not exert a polar effect in these mutants. Therefore, we conclude that all of the three genes in

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TABLE 1. Effects of mutations in the *fliD* operon on *tar-lac* expression

Mutation in the *fliD* or *flgM* operon[a]	*tar-lac* expression with *flhB* allele[b]:	
	flhB$^+$	*flhB*::Tn*10*
None (wt)	41	8
fliD::Tn*10*	309	ND[c]
fliD::*kan*	304	7
fliS::*kan*	236	7
fliT::*kan*	164	7
flgM311	368	327

[a] Strains used for each mutation: wild type (wt), KK1004 (17); *fliD*::Tn*10*, KK2601 (16, 17); *fliD*::*kan*, KK1391 (22); *fliS*::*kan*, KK1392 (22); *fliT*::*kan*, KK1393 (22); and *flgM311*, KK1311 (11).

[b] The chromosomal *tar-lac* fusion gene was introduced by transduction from KK1105 (14). The *flhB*::Tn*10* mutation was introduced by transduction from KK2019 (16, 17). *tar-lac* expression levels are expressed as the β-galactosidase enzyme activities (in Miller units) as described previously (16).

[c] ND, not done.

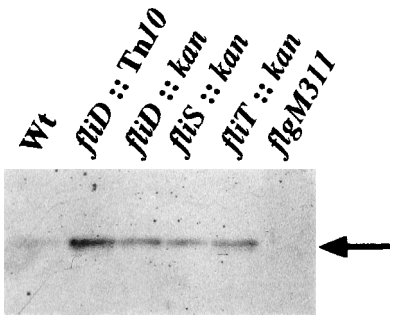

FIG. 1. FlgM export from *S. typhimurium* cells. The cells were grown overnight in minimal medium containing 0.3% Casamino Acids (Difco) and 0.2% glycerol. Aliquots of the culture containing 10^9 cells were clarified by centrifugation, and the supernatant fluid was treated with 10% trichloroacetic acid. The precipitated proteins were separated by SDS-PAGE, and the FlgM protein on the gel was visualized by Western blotting with a specific polyclonal antibody. The arrow indicates the position of the FlgM protein. Strains used for each mutation: wild type (Wt), KK1004; *flgD*::Tn*10*, KK2601; *fliD*::*kan*, KK1391; *fliS*::*kan*, KK1392; *fliT*::*kan*, KK1393; and *flgM311*, KK1311.

the *fliD* operon may be directly or indirectly involved in the *rflA* function.

FlgM export in the *fliD*, *fliS*, and *fliT* mutants. Because the *fliD*::Tn*10* mutation did not affect expression of the *fliA* operon (16), it is unlikely that the gene products of the *fliD* operon might be involved in negative regulation of the *fliA* gene. The RflA$^-$ phenotype of the *fliD*, *fliS*, or *fliT* mutant was not observed in the hook-basal-body (*flhB*::Tn*10*) mutant background (Table 1). This indicates that, unlike the *flgM* gene, none of these three genes encodes a negative regulator protein for the class 3 transcription.

The extracellular FlgM levels were examined by Western blotting (immunoblotting) with the spent media of the *fliD*, *fliS*, and *fliT* mutants. The cells were grown overnight at 37°C in minimal medium as described previously (11), and the cultures were clarified by centrifugation. Proteins in the cell-free supernatants were precipitated with 10% trichloroacetic acid and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins on the gels were electrophoretically transferred onto a nitrocellulose membrane, and the FlgM protein was detected by Western blotting with a polyclonal antibody against FlgM as described previously (11). It was found that the amounts of FlgM in the spent media were much larger in all of the mutants in the *fliD* operon than in the wild-type strain (Fig. 1). This result does not necessarily mean that the intracellular FlgM level is lowered in these mutants. Because the autogenous control of the *flgM* gene maintains the synthesis of FlgM at a significantly low level (2, 11), we could not assess the intracellular FlgM concentration. However, the enhanced expression of the *tar-lac* fusion gene (Table 1) suggests that the intracellular FlgM concentration should be lower in these mutants than in the wild-type strain. Therefore, we conclude that the RflA$^-$ phenotype of all the mutants in the *fliD* operon is attributed to the enhanced FlgM export and that all the genes in the *fliD* operon are involved in negative regulation of FlgM export.

Number of flagella produced by the *fliS* and *fliT* mutants. Because the FliA-FlgM regulatory system controls expression of both class 2 and class 3 operons, FlgM depletion causes an enhanced expression of the whole flagellar regulon, resulting in the overproduction of flagellar structures (14). Therefore, we expected that the flagellar number should be increased in the *fliS* or *fliT* mutants. Cells grown exponentially in L broth were negatively stained with 1% phosphotungstic acid and observed by electron microscopy as described previously (22). As expected, the numbers of flagella produced in the *fliS* and *fliT* mutants were about twice as many as that in the wild-type strain and almost equivalent to that in the *flgM* mutant (Table 2).

Negative regulation of the FlgM export. In this study, we have shown that all three genes in the *fliD* operon are involved in the negative regulation of FlgM export. The *fliD* gene encodes the capping protein of the filament which prevents flagellin from being excreted into the medium (5). Because FlgM is believed to be exported through the same pathway as flagellin (7, 11), it is reasonable to assume that the FliD cap on the flagellar tip may also interfere with FlgM export. FliS and FliT are both believed to be cytoplasmic proteins (10). We showed previously that FliS is a positive factor for flagellin export and proposed that FliS may be a cytoplasmic chaperone specific for flagellin (22). Here, we have shown that FliS is also a negative factor for FlgM export. Therefore, FliS should exert the opposing roles of facilitating flagellin export and of inhibiting FlgM export. A total deletion of the *fliT* gene does not affect the flagellar structure (22), suggesting that, unlike FliD and FliS, FliT may be a factor specific for the negative regulation of FlgM export. Biochemical analysis will be required to understand the molecular mechanism underlying this negative regulation.

FlgM export through flagellar substructures is inhibited until the completion of hook assembly (11). Recently, we showed that one of the flagellar proteins, FlhB, is responsible for this inhibition process (15). The *fliD*, *fliS*, or *fliT* mutation does not affect this inhibition process (12), suggesting that FliD, FliS, and FliT may play roles in the inhibition of FlgM export only after the completion of hook assembly. Regulation by FliD may be an incidental feature of its function as a filament-

TABLE 2. Flagellar numbers produced by *S. typhimurium* cells

Mutation[a]	Avg no. of flagella/cell[b]
None (wt)	4.3
flgM311	7.9
fliS::*kan*	8.6
fliT::*kan*	8.8

[a] Strains used for each mutation: wild type (wt), KK1004; *flgM311*, KK1311; *fliS*::*kan*, KK1392; and *fliT*::*kan*, KK1393.

[b] The values are the averages of more than 200 cells.

capping protein, whereas regulation by FliS and FliT may be their primary function. It has been shown that FlgM depletion results in overproduction of flagellar structures and causes growth retardation of cells (14). This indicates that overproduction of flagella is deleterious to bacterial growth. Negative regulation of FlgM export by FliD, FliS, and FliT may avoid unnecessary overproduction of flagellar structures after the completion of hook assembly and during filament assembly.

Electron microscope facilities were provided by the Center for Gene Science, Hiroshima University.

This work was supported by grants from the Ministry of Education, Science, and Culture of Japan and from Electric Technology Research Foundation of Chugoku.

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