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Transcriptional Analysis of the Flagellar Regulon of *Salmonella typhimurium*

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In *Salmonella typhimurium*, nearly 50 genes are involved in flagellar formation and function and constitute at least 13 different operons. In this study, we examined the transcriptional interaction among the flagellar operons by combined use of Mu dl(Ap^r Lac) *c*ts62 and Tn*10* insertion mutants in the flagellar genes. The results showed that the flagellar operons can be divided into three classes: class I contains only the *flhD* operon, which is controlled by the cAMP-CAP complex and is required for expression of all of the other flagellar operons; class II contains seven operons, *flgA*, *flgB*, *flhB*, *fliA*, *fliE*, *fliF*, and *fliL*, which are under control of class I and are required for the expression of class III; class III contains five operons, *flgK*, *fliD fliC*, *motA*, and *tar*. This ordered cascade of transcription closely parallels the assembly of the flagellar structure. In addition, we found that the *fliD* defect enhanced expression of the class III operons. This suggests that the *fliD* gene product may be responsible for repression of the class III operons in the mutants in the class II genes. These results are compared with the cascade model of the flagellar regulon of *Escherichia coli* proposed previously (Y. Komeda, J. Bacteriol. 170:1575–1581, 1982).

The bacterial flagellum is composed of three structural components, a basal body, a hook, and a filament. The filament extends into the extracellular space and is connected by the hook to the basal body embedded in the cell membrane. Genetic analysis of flagellar mutants have revealed that there are nearly 50 genes involved in flagellar formation and function in both *Salmonella typhimurium* and *Escherichia coli* (24, 25). Intergeneric complementation analysis revealed functional homology in the flagellar genes between these two organisms (8, 21, 39). Genes responsible for flagellar formation are called *flg*, *flh*, *fli*, or *flj*. Except for a few genes, mutants defective in these genes are nonflagellate and some of them produce presumptive precursor structures of the flagellum (35, 36). There are three kinds of genes responsible for flagellar function, including flagellar rotation (*mot*), chemotaxis (*che*), and transmembrane signal transduction of chemotactic stimuli (*tar*, *trg*, *tsr*, etc.) Mutants defective in these genes produce flagellar structures indistinguishable from those of the wild-type strain. Most of the flagellar genes are clustered in three regions of the bacterial chromosome, termed regions I, II, and III. These clustered genes constitute 14 and 13 different operons in *E. coli* (16, 32) and *S. typhimurium* (22), respectively. Flagellar genes of *S. typhimurium* are summarized in Fig. 1.

In *E. coli*, Komeda (14) constructed fusions of most of the flagellar operons to the *lac* genes by using Mu dl(Ap^r Lac) bacteriophage developed by Casadaban and Cohen (5). This phage contains the *lac* genes with no promoter, and its integration in a gene can result in rescue of expression of the *lac* genes due to the promoter of that gene. By using these operon fusions, he examined the transcriptional interaction among the flagellar operons and proposed the cascade model of the flagellar regulon. In his model, the flagellar operons are divided into six classes, depending on their transcriptional hierarchy. Recently, DNA sequence (2, 10) and biochemical (1) analyses suggested that the expression of the flagellar regulon may involve the participation of an alternative sigma factor which binds to core RNA polymerase.

In *S. typhimurium* there have been several reports which suggested that expression of the flagellar genes is controlled in a similar cascade mode. For example, Suzuki and Iino (34) reported that mRNA specific for *fliC*, which encodes flagellin, the component protein of filament, could not be detected in any nonflagellate mutants examined. However, an overall structure of the flagellar regulon has not yet been established in this organism. In our previous work (22), we isolated Mu dl(Ap^r Lac) *c*ts62 and Tn*10* insertion mutants in almost all of the flagellar genes in this organism. In this study, by combined use of these two kinds of insertion mutants, we have examined the transcriptional interaction of flagellar genes. On the basis of the observed interactions, we proposed a cascade model of the flagellar regulon in which the flagellar operons are divided into three classes with respect to transcriptional hierarchy.

Flagellar gene symbols used in this report follow the new unified nomenclature for *S. typhimurium* and *E. coli* (11), and each flagellar operon is named after the gene which is transcribed first in that operon (22).

MATERIALS AND METHODS

Bacteria and phage. All the bacterial strains used were derivatives of *S. typhimurium* wild-type strain LT2. Tn*10* insertion mutants in the flagellar genes were derived from LT2 or KK1004 (LT2 Δ*fljAB*) and were described previously (20, 22). Tn*10* insertion mutants in *cya* and *crp* were PP1002 and PP1037 (29), respectively, which were provided by K. E. Sanderson. Mu dl insertion mutants in the flagellar genes were derived from KK1005 (KK1004 *galE*) and were described in the previous report (22). Only the Lac$^+$ insertion strains were used in this study. In order to construct strains carrying both Mu dl and Tn*10* insertions in two different genes, Tn*10* insertion mutations were introduced into the Mu dl insertion strains by P22-mediated transduction, which was performed by using P22HT*int* phage as described previously (22, 23).

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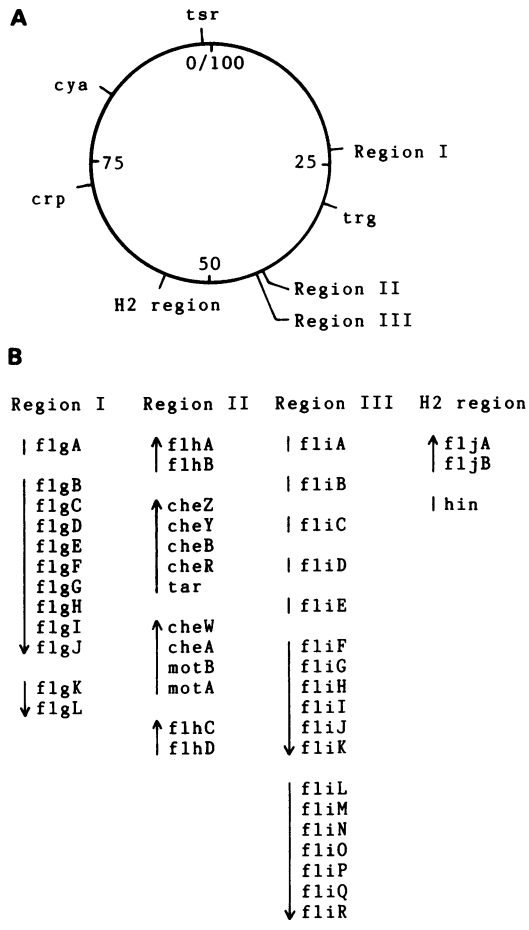

FIG. 1. Flagellar genes in *S. typhimurium* (11, 21, 24, 25, 29). (A) Flagellar genes are shown on the chromosomal map. Genes responsible for catabolite gene activation, *cya* and *crp*, are also indicated. (B) The operon structure of clustered flagellar genes are indicated by arrows.

Media and culture conditions. Minimal medium, minimal agar plate, L broth, and L broth agar plate were prepared as described previously (22, 23). Ampicillin and tetracycline were used at final concentrations of 25 and 20 μg/ml, respectively. 5-Bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal) was used at a final concentration of 25 μg/ml. Throughout this study, bacterial cells were grown at 30°C.

β-Galactosidase assay. β-Galactosidase activities in toluene-treated cells were assayed by the method of Miller (26). The enzyme units reported here were the average of at least five independent experiments. The results of the individual assays were all within 20% of the reported averages. The endogenous β-galactosidase activity of the parental strain KK1005 was 1.0 U under the conditions used in this study.

RESULTS

Effect of *cya* and *crp* mutations on the expression of flagellar genes. The production of the flagellum in *E. coli* and *S. typhimurium* has been shown to be under positive control from the cAMP-CAP complex (17, 30). In order to confirm this, we introduced a *cya*::Tn*10* or *crp*::Tn*10* mutation into Mu d1 insertion mutants in flagellar genes and expression of the *lacZ* gene in the resulting double-insertion mutants was examined on minimal agar plates containing X-Gal (Table 1). As expected, they were all Lac$^-$, indicating that transcription of all the flagellar genes are positively controlled by cAMP-CAP complex.

Transcriptional interaction of the flagellar operons. Tn*10* insertion mutations in flagellar genes were introduced into strains carrying fusions of flagellar genes to the *lac* genes, and expression of the *lacZ* gene in the resulting double-insertion mutants was examined on minimal agar plate containing X-Gal. The results are summarized in Table 1. Insertions in the different genes in the same operons gave the same results, which suggests that the internal signals of transcription within the polycistronic operons, if any, may not be so significant in our experimental system used here. Based on the positive interaction of transcription, the flagellar operons can be divided into three classes. Class I comprises only the *flhD* operon. This operon did not require functions of any other flagellar genes for its transcription, and genes in it were required for expression of all of the other flagellar operons. Class II includes seven operons, *flgA*, *flgB*, *flhB*, *fliA*, *fliE*, *fliF*, and *fliL*. Their expression required the functions of genes in the class I operon, and their functions are required for expression of the class III operons. Class III comprises five operons, *flgK*, *fliD*, *fliC*, *motA*, and *tar*. Their expression needed functions of the class I and class II operons, and their functions were not responsible for expression of the other operons.

Tn*10* insertion mutations in some of the class II genes, such as *flgA*, *flgH*, *flgI*, *flgJ*, and *fliK*, were found to allow the class III operon to be expressed partially. Even the null mutants in these genes, including Tn*10* insertion ones, produce flagella at a low frequency, probably because these gene products are not absolutely required for flagellar formation and function (our unpublished results). Therefore, it is likely that this partial expression may be due to leakiness of the mutations.

One surprising finding from this experiment was that the *fliD* mutation enhanced expression of the other class III operons. This suggests that the gene product of the *fliD* operon may have an activity to repress the class III operons, although all the genes responsible for flagellar formation, including the *fliD* gene, have been identified as the positive factors for the appearance of the flagellar structures. We propose that this repressible activity be designated as *rflA* (repression of flagellar operons).

Quantitative assay of β-galactosidase. In order to obtain quantitative data on expression of the *lacZ* gene, the activity of β-galactosidase was measured in representative fusion strains. For this purpose, we used strains which carried Mu d1 insertions in most promoter-proximal genes available in the individual operons. For representatives of *lac* fusion strains in the *fliL* and *tar* operons, we used Mu d1 insertion mutants in the *fliM* and *cheR* genes, respectively, because we had not established Mu d1 insertion mutants in either *fliL* or *tar*. For those of the other operons, we used strains carrying Mu d1 insertions in the genes which are transcribed first in the corresponding operons. First, the activities in the strains carrying no Tn*10* insertion mutation were compared. In general, the operons, gene products of which have been known to constitute flagellar structures (the *flgB*, *fliD*, *flgK*, and *fliC* operons) or to be involved in flagellar function (the *motA* and *tar* operons), had higher activity, although the *flhD* operon showed an exceptionally high activity.

We also measured the activity in the double-insertion mutants. The results shown in Table 2 are essentially equivalent with those obtained by the qualitative experiment in

TABLE 1. Expression of the *lacZ* gene fused to promoters of the flagellar operons[a]

Tn10 insertion	Mud1(Ap^r Lac)cts62 insertion																																					
	flhD(3425)	flhC(3424)	flgA(3401)	flgB(3402)	flgC(3403)	flgD(3404)	flgE(3405)	flgF(3406)	flgG(3407)	flgH(3408)	flgI(3409)	flgJ(3410)	flhB(3414)	flhA(3413)	fliA(3426)	fliE(3429)	fliF(3430)	fliG(3431)	fliI(3433)	fliJ(3434)	fliK(3435)	fliM(3437)	fliN(3438)	fliO(3439)	fliP(3440)	fliQ(3441)	fliR(3442)	fliD(3428)	flgK(3411)	flgL(3412)	fliC(3427)	motA(3423)	motB(3422)	cheA(3421)	cheW(3420)	cheR(3418)	cheB(3417)	cheY(3416)
None	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
cya	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
crp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
flhD(2040)		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
flhC(2009)	+		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
flgA(2640)	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	±	±	±	±	±	±	±	±	±	±
flgB(2085)	+	+	+		-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
flgC(2705)	+	+	+	+		-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
flgD(2043)	+	+	+	+	+		-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
flgF(2708)	+	+	+	+	+	+	+		-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
flgG(2008)	+	+	+	+	+	+	+	+		-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
flgH(2059)	+	+	+	+	+	+	+	+	+		-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	±	±	±	±	±	±	±	-	-	-
flgI(2141)	+	+	+	+	+	+	+	+	+	+		-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	±	±	±	±	±	±	±	-	-	-
flgJ(2017)	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	±	±	±	±	±	±	±	-	-	-
flhB(2019)	+	+	+	+	+	+	+	+	+	+	+	+		-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
flhA(2136)	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
fliA(2091)	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
fliE(2024)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
fliF(2092)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
fliG(2119)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
fliH(2135)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
fliI(2046)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
fliJ(2700)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
fliK(2143)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	±	±	±	±	±	±	±	±	-	-	-
fliM(2071)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
fliN(2736)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
fliO(2873)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		-	-	-	-	-	-	-	-	-	-	-	-	-	-
fliP(2053)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
fliQ(2130)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		-	-	-	-	-	-	-	-	-	-	-	-
fliR(2110)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		-	-	-	-	-	-	-	-	-	-	-
fliD(2601)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		*	*	*	*	*	*	*	*	*	*
flgK(2944)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		-	+	+	+	+	+	+	+	+
flgL(2087)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+
fliC(2604)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+
motA(2930)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		-	-	-	+	+	+
motB(2049)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		-	-	+	+	+
cheA(2051)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		-	+	+	+
cheR(2811)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		-	-
cheB(2078)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		-
cheY(2014)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
cheZ(2668)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] Single colonies of each strain grown on L broth agar plates were transferred onto minimal agar plates containing X-Gal, and the Lac phenotype was examined after incubation at 30°C. Although the incubation periods were varied (from 5 to 24 h) depending on the transcriptional activity of the *lac* fusion strains, they were the same among the strains derived from the same *lac* fusion strains. Blue color developed in and around the colony was categorized by using the following symbols: +, blue color development; ±, less blue color development; −, no color development; *, dark-blue color development. The transcriptional orientation of polycistronic operon is indicated by the arrow. Numbers in parentheses indicate KK numbers of the strains used in this experiment.

which minimal agar plate containing X-Gal was used. The *fliD* mutation was found to enhance two to six times the expression of the class III operons.

DISCUSSION

By using fusions of flagellar operons to the *lac* genes, we have analyzed the effects of mutations in flagellar genes on the expression of each flagellar operon. The operon fusions were produced by insertion of Mu d1 at various flagellar genes, and Tn*10* insertion mutations were introduced into these fusion strains. In our experimental system, we could not examine the autogenous regulation of flagellar operons because the *lac* fusion strains used were defective in the genes at which Mu d1 inserted and in the downstream genes in the same operons. However, the results shown in Tables 1 and 2 suggest the positive fashion of regulation and categorize flagellar operons into three classes. On the basis of these results, we constructed a hypothetical scheme for the interaction of flagellar operons in the flagellar regulon of *S. typhimurium* (Fig. 2A). Only the *flhD* operon belongs to class I. This operon contains two genes, *flhD* and *flhC*, both of which have been postulated to regulate in a positive fashion expression of all of the other flagellar genes (31). Furthermore, expression of this operon has been thought to be controlled positively by cAMP-CAP complex (17, 30). Our results clearly confirmed these presumptions. Except for *fliA*, which is responsible for filament formation, all of the genes in the class II operons have been shown to be involved in hook-basal body complexes in the morphogenic pathway of flagellar structure (35, 36). On the other hand, genes in the

TABLE 2. Activity of β-galactosidase in the fusions of flagellar operons to the *lac* genes[a]

Tn10 insertion	Mud1(Ap^{r}Lac)cts62 insertion												
	flhD	flgA	flgB	flhB	fliA	fliE	fliF	fliM	fliD	flgK	fliC	motA	cheR
None	1.0 (190)	1.0 (27)	1.0 (16)	1.0 (3.4)	1.0 (6.7)	1.0 (2.2)	1.0 (3.2)	1.0 (6.6)	1.0 (38)	1.0 (30)	1.0 (18)	1.0 (53)	1.0 (21)
cya	0.34												
crp	0.31												
flhD		0.17	0.11	0.29	0.35	0.23	0.18	0.21	0.09	0.19	0.13	0.11	0.19
flgA	1.0		1.2	0.95	1.0	1.2	1.1	1.0	0.43	0.46	0.47	0.45	0.45
flgB	1.0	0.63		0.60	0.94	0.83	0.92	0.81	0.25	0.18	0.16	0.14	0.20
flhB	0.98	0.75	0.81		0.83	0.67	0.83	0.82	0.22	0.25	0.23	0.18	0.21
fliA	0.87	0.61	0.77	0.55		0.78	0.81	0.69	0.15	0.23	0.14	0.10	0.12
fliE	0.85	0.83	0.98	0.73	0.84		0.96	0.94	0.24	0.27	0.18	0.16	0.23
fliF	0.97	0.91	1.1	0.67	0.63	0.81		0.80	0.27	0.22	0.13	0.14	0.26
fliM	1.1	0.95	0.90	0.69	0.68	0.92	0.73		0.23	0.23	0.13	0.15	0.25
fliD	0.94	1.1	1.1	1.3	0.75	0.89	0.93	1.1		3.2	2.7	2.2	6.2
flgK	1.1	0.87	0.77	0.73	0.95	0.78	1.0	0.92	1.3		0.71	0.92	1.1
fliC	0.95	0.61	0.74	0.80	1.0	1.1	0.92	1.0	0.52	0.51		0.62	0.73
motA	0.98	0.83	0.96	1.0	0.95	1.0	0.88	0.96	0.86	0.95	1.1		1.1
cheR	1.0	1.1	0.98	0.99	1.0	1.1	0.99	1.1	0.97	1.0	1.1	0.97	

[a] Activity of β-galactosidase is expressed as a value relative to that of the fusion strain carrying no Tn*10* insertion mutation. The actual value of β-galactosidase enzyme units is shown in parentheses. Strains used were the same as in Table 1.

class III operons are responsible for formation of the filament, the final step of flagellar assembly (the *flgK*, *fliD*, and *fliC* operons) or function of the complete flagellum (the *motA* and *tar* operons). These facts indicate that the cascade of the flagellar regulon closely parallels the assembly hierarchy of the flagellar structure. Therefore, we propose that classes I, II, and III be called the early, middle, and late flagellar operons, respectively (Fig. 2A).

Our scheme presented here is slightly different from that of *E. coli* described by Komeda (14). In our model, the flagellar regulon consists of only one sequential pathway with three steps of transcription (Fig. 2A). On the other hand, according to the Komeda scheme, the flagellar regulon shows a branching cascade in which the flagellar operons are divided into six classes, classes 1 through 6 (Fig. 2B). It is possible that this difference may reflect species specificity between *S. typhimurium* and *E. coli*, because it is conceivable that the organisms differ in the details of the regulatory hierarchy. However, according to the Komeda model, the *flgJ* gene (class 4) should be expressed only after the operons responsible for formation of hook-basal body structures (class 3) were expressed, which is inconsistent with the results showing that function of the *flgJ* gene is required for the very early stage in the morphogenic pathway of hook-basal body (35, 36). In our earlier work (22), we showed that the *flgJ* gene of *S. typhimurium* does not form an independent operon but belongs to the *flgB* operon. This result was supported further by a recent DNA sequence analysis of the upstream region of the *flgJ* gene (12). If we assume that the *flgJ* gene might belong to the *flgB* operon in *E. coli* as well as in *S. typhimurium*, the schemes of the flagellar regulon would be very similar between these two organisms. Correspondences of the classes between the two schemes are as follows (the former is of the Komeda scheme, and the latter is of ours): class 1 to class I, classes 3 and 4 to class II, classes 5 and 6 to class III. Of the class 2 operons, *flgA* belongs to class II and *fliD* belongs to class III. However, we believe that our classification is correct even in *E. coli*, because at least one *flgA* mutation of *E. coli* has been shown to reduce expression of the class 5 and class 6 operons (15) and the upstream region of *fliD* of *E. coli* displays the consensus sequence for the class III promoter (see Fig. 3).

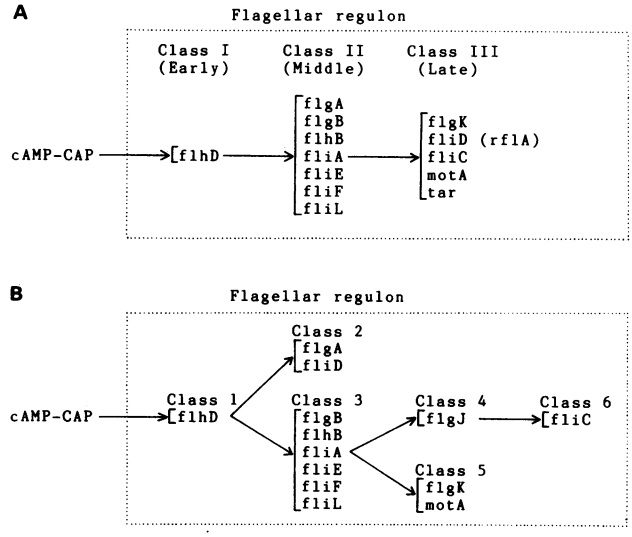

FIG. 2. Hypothetical schemes of the flagellar regulon showing the positive interaction of flagellar operons, which were drawn based on the results obtained in this study (A) or from descriptions by Komeda (14) (B). The positive interaction for transcription is shown by the arrow. Each flagellar operon is named after the gene which is transcribed first in that operon. Details are described in Discussion.

Recently, DNA sequence data on the upstream regions of *E. coli* and *S. typhimurium* flagellar operons have been accumulated. Comparison of these sequences revealed the promoter structures specific for flagellar operons which should be recognized by RNA polymerase containing an alternative sigma factor (2, 10). In the present work, we divided the flagellar operons into three classes. This suggests that there may be three different types of regulation of gene expression specific for each class. Because we can assume that this difference should be reflected by the difference in

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Class I
 E.flhD        CGGCGA CATC ACGGGGTGCGGTGAA ACCGCTAA AAATAAAGTTGGTTA

Class II
 E.fliL        TTGCGC TCAA GACGCAGGATAATTA GCCGATAA GCAGTAGCGACACAG
 S.fliL        TCGTGG ACAG GACACGGGATAATCA GCCAATAA GCAGTACCGAAACAG
 E.fliF        CCGGGA GTGA GTCTTGTTCCACTTT GCCAATAA CGCCGTCCATAATCA
 S.fliF           GGA GTGA GTCTTGTTCCACTTT GCAAAAAA CGCCGTCCACGATCA
 E.flhB        CACGTC ATAT CAGGCGGTCTGATAA GGCGATGA CGCCGCATCCGACAA
 S.flgA        TCTCCT CCGC AAATGGCAAAATTCA GCCGACAG CTTAAATGCCTTCAC
 S.flgB        TAGCGA CGCA TTTTGCGTTTATTCC GGCGATAA CGCGCGCGTGAAGGC
 S.fliA        ACCCTC TGTA GAAACGGATAATCAT GCCGATAA CTCATTTAACGCAGG

Class III
 S.flgK        AATTGC TCAA GTCCACGTAGTCGCT GCCGGAAT CAACGAGTATTGAAG
 E.fliD        TAAACG TAAA CTTTGCGCAATTCAG ACCGATAA CCCCGGTATTCGTTT
 S.fliD        TATCAT TAAA CTTTGCCTCCAGATT GCCGATAA CGCGCTTAACTACTG
 E.fliC        AAATTC TAAA GGTTGTTTTACGACA GACGATAA CAGGGTTGACGGCGA
 S.fliC        ATTTTC TAAA GTTCGAAATTCAGGT GCCGATAC AAGGGTTACGGTGAG
 S.fljB        AAATAG TAAA GTTTATGCCTCAACT GTCGATAA CCTGGATGACACAGG
 E.motA        AAGACG TAAA CTTTCCCAGAATCCT GCCGATAT TATCCCACAACTGCT
 E.tar         CAGCAA TAAA GTTTCCCCCCTCCTT GCCGATAA CGAGATCAACTTGTT
 S.tar         CAAAAG TAAA GTTATCGCCGCAGGT GCCGATAA CGTTGATAACTCGTT
 E.trg          GATCA TAAG TAATTACCGTCAAGT GCCGATGA CTTTCTATCAGGAGT
 E.tsr            TTA TAAA GTTTTTCCTTTCCAG GCCGAAAA TCTTGCATCGGTCCA

Consensus           (TAAA)      N15       GCCGATAA

Canonical           TTGACA      N17       TATAAT
                     -35                   -10
```

FIG. 3. DNA sequence comparison of the upstream regions of flagellar operons of *S. typhimurium* and *E. coli*. Sequences are aligned according to Helmann and Chamberlin (10) and Bartlett et al. (2). E. and S. preceding each gene symbol indicate that the gene originated from *E. coli* and *S. typhimurium*, respectively. Sequence data were adapted from the following references: E. *flhD*, E. *fliL*, E. *fliF*, and E. *flhB* (2); S. *fliL* (13); S. *fliF* and S. *flgK* (12); E. *fliD*, E. *fliC*, S. *fliD*, S. *fliC*, and S. *fljB* (37); E. *motA* (2 and 7); E. *tar* (18); S. *tar* (28); E. *trg* (3); E. *tsr* (4); S. *flgA*, S. *flgB*, and S. *fliA* from our unpublished results (our nucleotide sequence data will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession numbers D00497 for *fliA* and D00498 for *flgA* and *flgB*). The double-underlined sequence in E. *flhD* is a potential cAMP-CAP binding site (2). The boxed or underlined nucleotides indicate the start sites of transcription in vivo or in vitro, respectively, whose data were adapted from the following references: E. *fliL* (1 and 19); E. *fliC* and E. *fliD* (9); E. *tar* and E. *tsr* (1). Although we have not analyzed expression of the *trg*, *tsr*, or *fljB* operons in this study, these operons were included in class III in this figure. Details are described in Discussion.

the sequence of the upstream regulatory sites, we compared once again the preceding sequences of flagellar operons on the basis of our classification. The results are summarized in Fig. 3. The sequences TAAA and GCCGATAA with 15-base-pair spacers, which have been supposed to be the −35 and −10 promoter consensus sequences specific for flagellar operons (10), were found to be characteristic of the upstream regions of the class III operons. Recently, Arnosti and Chamberlin (1) isolated from *E. coli* cells an alternative sigma factor (σ^F) which restores specific transcription in vitro of the *tar* operon of *E. coli* when added to core RNA polymerase. More recently, we obtained several lines of evidence supporting the theory that σ^F is encoded by the *fliA* gene (manuscript in preparation). These results suggest that σ^F may be specific for transcription in vivo of the class III promoter. On the other hand, the preceding regions of the class II operons, except the *fliL* operon of *E. coli*, contain only the putative −10 sequence, which is consistent with the result reported by Bartlett et al. (2). The preceding region of the *fliL* operon of *E. coli* seems to contain both the putative −35 and −10 sequences. Although Arnosti and Chamberlin (1) showed that RNA polymerase containing σ^F can transcribe in vitro the *fliL* operon of *E. coli*, it is unlikely that this class III promoterlike sequence might be functional in the transcription in vivo, because the initiation site of transcription in vivo of this operon is different from that of transcription in vitro (Fig. 3) and the transcription in vivo of this operon does not require the function of the *fliA* gene (14). Therefore, we believe that the absence of the putative −35 sequence is characteristic of the class II promoter. The sequence data suggested that the gene products of *flhD* and *flhC* may act as sigma factors (2). If this is correct, it is possible that the presumptive sigma factors encoded by these genes may be specific for transcription of the class II promoter. The preceding region of the class I operon also contains the sequence similar to the putative −10 sequence. However, because occurrence of A and C at nucleotides 1 and 5 of the −10 sequence is quite unusual, as compared with the putative −10 sequences of the other flagellar operons, it seems unlikely that this sequence might participate in the promoter structure of this operon. For the final decision, it is necessary to determine the transcription start sites of all the flagellar operons. The preceding region of the class I operon also contains the potential cAMP-CAP binding site (Fig. 3). This feature is consistent with the result indicating that the transcription of this operon is positively regulated by *cya* and *crp* (Tables 1 and 2).

In the present study, we did not analyze the expression of five flagella-related operons, the *trg*, *tsr*, *fljB*, *fliB*, and *hin* operons, because we had not established fusions of these operons to the *lac* genes. The upstream sequences of the former three operons contain both TAAA and GCCGATAA consensus sequences with 15-base-pair spacers, which is characteristic of the class III promoters (Fig. 3). Because *trg* and *tsr* are responsible for flagellar function and *fljB* is responsible for filament formation (24, 25), the products of these three genes should function in the late stage of flagellar morphogenesis. These facts suggest that these three genes may belong to the class III operons. The *fliB* gene is involved in methylation of certain lysine residues of flagellin (33) and residues in region III (Fig. 1). Therefore, it is likely that *fliB* may also be one of the members of the flagellar regulon. The *hin* gene is involved in site-specific inversion of the promoter segment of the *fljB* operon, which is responsible for phase variation (40). The inversion has been shown to occur even in the *flhD* mutants (our unpublished results), which indicates that the expression of *hin* is controlled independently of the flagellar regulon.

Our scheme shown in Fig. 2 indicates that the expression of class III requires the functions of all of 27 genes of class II. However, it is unlikely that all of these gene products might act as activators for transcription of class III. In *E. coli*, Komeda (15) presented evidence that the positive controller of the later operons (classes 4, 5, and 6) is only the *fliA* gene among the class 3 genes and the *flgA* gene product functions as repressor whose action can be masked by its interaction with gene products of the class 3 operons. It is possible that only *fliA* may be the activator gene for class III in *S. typhimurium* as well as in *E. coli*, because among the mutants in the class II genes, only the *fliA* mutants produce a complete hook-basal body lacking a filament portion (35, 36). In this study, we used an *flgA*::Tn*10* mutant to analyze the effect of *flgA* defect on the expression of other operons. If the gene product of *flgA* has dual functions (one is a repressor for the expression of class III, and the other is an essential factor for formation of basal body), introduction of an *flgA*::Tn*10* mutation should enhance the expression of the class III operons because the Tn*10* insertion mutant is expected to show a null phenotype. However, the results shown in Tables 1 and 2 indicate that it also reduced expression of the class III operons. Therefore, we do not believe at present that the gene product of *flgA* might function as a repressor in *S. typhimurium*. On the other hand, we found that introduction of an *fliD*::Tn*10* mutation

enhanced expression of the class III operons. This indicates that the *fliD* gene or an unidentified gene downstream in the *fliD* operon may be involved in repression of the class III operons in the mutants in the class II genes. Expression of the flagellar genes in *Caulobacter crescentus* is also regulated in a cascade fashion (6). Recently, negative regulation was found to be superimposed on the positive control in the flagellar hierarchy of this organism (27, 38). Therefore, it is plausible that the negative regulation is a general mechanism to coordinate expression of flagellar genes and flagellar assembly in bacteria.

In the quantitative assay of β-galactosidase, we found that the operons whose gene products have been shown to constitute flagellar structures have activities about 10 times as high as those of the operons whose gene products have not yet been identified in the isolated flagellar structures (24). It is reasonable to assume that the flagellar genes whose products are required in larger quantities for flagellar assembly may be transcribed at higher rates. If so, transcription rates of flagellar genes could be coordinated with the amounts of protein products required for flagellar morphogenesis. On the other hand, the activity of the *flhD* operon was found to be exceptionally high, although neither of the gene products of this operon is the structural constituent of flagellar structure (24). It is possible that an unidentified gene encoding a hypothetical repressor for expression of the *flhD* operon might exist in the flagellar regulon. If so, the β-galactosidase activity in the *flhD-lac* fusion strain reported here might represent that of a derepressed state, because the hypothetical repressor gene should not be expressed in that fusion strain. Examination of autogenous regulation will give some clues to know the manner of transcriptional control of the *flhD* operon.

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