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Fusions of Flagellar Operons to Lactose Genes on a Mu *lac* Bacteriophage

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Previous studies have defined 29 genes necessary for synthesis of the *Escherichia coli* flagellar apparatus. This study analyzed the transcriptional control of flagellar genes, using Mu d (Ap^r *lac*) phage to generate flagellar mutants by insertion. These mutants contained operon fusions of flagellar genes to the *lac* genes of the Mu d phage and allowed the measurement of flagellar operon expression by detection of beta-galactosidase activity. These fusion mutants expressed the enzyme activity constitutively, and an autogenous regulation mechanism was not revealed. Lambda transducing phages carrying these chromosomal *fla-lac* fusions were also isolated and used to examine the effect of different *fla* mutations on expression of each flagellar operon. The results showed that flagellar operons are divided into six classes: (class 1) the *flbB* operon, which controls all of the other flagellar operons; (class 2) the *flaU* and *flbC* operons, which are controlled by the *flbB* operon gene products and are not required for the expression of other *fla* operons; (class 3) the *flbA*, *flaG*, *flaD*, *flaN*, *flaB*, and *flaA* operons, which are under *flbB* operon control and are required for the expression of other *fla* operons; (class 4) the *flaZ* operon, which is controlled by the gene products of the group 1 and 3 operons and is required for *hag* transcription; (class 5) the mocha and *flaS* operons, which are controlled by the gene products of the group 1 and 3 operons; and (class 6) the *hag* operon. These results are discussed with respect to the possible assembly sequence of the *fla* gene products.

Bacterial flagella have attracted the attention of investigators in various fields of biology. The formation of bacterial flagella is becoming a unique subject for studies of both cellular regulatory mechanisms and molecular bases of morphogenesis (7, 19). Genetic analysis has proved to be a valuable tool in defining the processes or the products involved in flagellar formation. Studies of *Escherichia coli* K-12 flagellar mutants have revealed that there are 29 genes (*fla*, *flb*, *hag*) required for synthesis of the flagellar apparatus (9, 19) and that there are an additional 11 genes involved in various aspects of flagellar function, including energy transduction (*mot* products) and integration of sensory information (*che* products) (13, 20). The *E. coli* K-12 flagellum is composed of three parts: filament, hook, and basal structure. The filament, which extends into the extracellular space, is connected by the hook to the basal structure embedded in the cell membrane. The intact flagellum has been shown to have twelve polypeptides when purified and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5).

This flagellar regulon contains significant features as follows. (i) The flagellar genes for completion of the structure comprise about 3% of the genes of the *E. coli* genome. Why are there so many dispensable genes? The high number may suggest the complex regulation mechanism. (ii) All of the flagellar genes have been defined by isolation of flagellumless phenotype mutants. The Fla$^-$ defects are recessive to wild-type alleles (although there are some mutation-specific exceptions). Thus, these *fla* genes have been suggested to contribute to the appearance of flagella. (iii) The phenotypes of Fla$^-$ mutants may constitute a hierarchy of flagellar constituent protein assembly or synthesis. None of the Fla$^-$ (flagellumless) mutants has a flagellar filament. The *hag* gene (coding for the flagellar filament constituent protein, flagellin) mutants have hook-basal body structure without a distal flagellar filament. The *flaK* (coding for hook subunit protein) mutants can form only a basal body structure, not a flagellar filament structure (22). The *flaM* (coding for one of the basal body constituent proteins) mutants do not have a filament or hook, but form the presumptive precursor structure of the basal body.

Most flagellar gene defects fail to transcribe the *hag* gene coding for flagellin which is a constituent protein of the flagellar filament (8). All of the *Salmonella fla* mutants have been

shown not to contain mRNA specific for flagellin (21). In analogy to the above-mentioned Fla$^-$ phenotypes, and since synthesis of the flagellar filament is probably the last step in flagellar assembly, it seemed possible that flagellar assembly was carried out by sequential expression of the genes that code for the flagellum, beginning with the basal body, then the hook, and finally the filament. If so, the synthesis of interacting flagellar proteins should be tightly coordinated for effective assembly. The absence of even one *fla* gene product would result in failure of the transcription of the *hag* gene because of blockage of this presumptive interaction network. In accordance with these ideas, I tried to analyze the expression of flagellar genes by using the in vivo gene fusion technique developed by Casadaban and Cohen (3). These fusion strains enabled me to analyze the interaction of flagellar genes at the transcriptional level.

MATERIALS AND METHODS

Chemicals, media, and assays. The chemicals, media, and assays used have been described (8). β-Galactosidase was assayed according to Miller (11), and 1 U was defined as 1 nmol of *o*-nitrophenol released per min.

Bacteria and phage strains. Table 1 describes the *E. coli* K-12 strains used, and Table 2 shows fusion strains isolated. Other strains were as previously described (9). The most stable mutants were chosen as representatives of each cistron if multiple mutants were available. Amber flagellar mutants are underlined in Table 3. The *flbC1101* and *-1102* mutants were isolated as heat survivors of *flbC*::Mu d (Ap^r *lac*) mutants and are postulated to be deletion mutants. The other flagellar mutants in Table 3 were missense revertible mutants. Figure 1 depicts the flagellar genes in *E. coli* K-12 identified so far (9, 10). These 29 genes responsible for flagellar formation have been shown to be divided into 12 transcriptional units. For the sake of convenience, each transcriptional unit is here called an operon; for example, the *flaU* gene is designated the *flaU* operon when transcription is concerned, although the operon has only one gene. The same applies to the *flaZ*, *flaN*, *flbC*, and *hag* genes. The mocha operon (18) was also included in this study. The 13 transcriptional units were numbered sequentially, beginning with the *flaU* operon (as 1) clockwise around the *E. coli* chromosome to the *flaA* operon (as 13). The operon number is defined as shown at the top of the promoter-proximal gene in Fig. 1. All genetic techniques were according to Miller (11). λ *fla*$^+$ phages used for mapping were as described previously (9).

Isolation of flagellar gene fusions with the lac genes. Mu d (Ap^r *lac*) phage insertion mutants were isolated by the method described by Casadaban and Cohen (3). This Mu d *lac* phage contains an ampicillin resistance gene and, near the *S* end of the Mu genome, *lac* genes with no promoter. Integration of the Mu d (Ap^r *lac*) phage in a gene can result in rescue of expression of the *lac* genes due to the promoter of that gene. Accordingly, *fla-lac* (or *flb-lac*, *hag-lac*, or *mot-lac*) fusions were selected by isolation of nonmotile Lac$^+$ mutants produced by insertion of the Mu d (Ap^r *lac*) phage within various *fla* genes (or *flb*, *hag*, or *mot* genes) in the appropriate orientation. A fresh saturated L-broth culture of strain YK405 or YK410 was supplemented with 10 mM $MgSO_4$ and $CaCl_2$ and then infected with Mu d phage for 20 min at 30°C without shaking. The multiplicity of infection (MOI) of the phage was 1 by calculation from plaque-forming units of a lysate, which was heat-induced (42°C, 15 min) from a culture of strain MAL103. To allow the expression of ampicillin resistance, the mixture was diluted with a 10-fold volume of L broth and incubated with shaking for 4 h at 30°C. Then flagellar mutants were selected by resistance to the flagellotropic phage chi, strain XJ1 (6). The mixture was plated in a tryptone broth overlay with the chi phage at an MOI of 5 on a MacConkey agar plate containing 1% lactose and 25 μg of ampicillin per ml. The ampicillin-resistant (Ap^r), chi phage-resistant, and lactose-fermenting colonies were picked up. Three cycles of single-colony isolation were performed. Mu d (Ap^r *lac*) phage insertion flagellar mutants were saved if transduction to recover the Fla$^-$ (or Flb$^-$, Mot$^-$, or Hag$^-$) defects resulted in a simultaneous loss of Mu d (Ap^r *lac*) phage immunity, Lac$^+$ phenotype, and Ap^r phenotype. In this isolation procedure of the *fla*::Mu d (Ap^r *lac*) mutants, I assumed that the flagellar genes were expressed with high enough activity to permit fermentation of lactose on MacConkey agar plates with lactose. Although this assumption was introduced a priori, I could isolate a whole set of Mu d-induced Fla$^-$ Lac$^+$ mutants with defects of representative flagellar genes.

TABLE 1. *E. coli* K-12 strains

Strain		Relevant genotypes	Reference
MC4100	F$^-$	*araD139 ΔlacU169 rpsL thi flbB*	2
MAL103	F$^-$	*araD139 ΔlacU169 rpsL thi flbB ara*::Mu d (Ap^r *lac*) (Mu cts)	3
YK405	F$^-$	*araD139 ΔlacU169 rpsL thi nalA thyA pyrC46*	8
YK410	F$^-$	*araD139 ΔlacU169 rpsL thi nalA thyA pyrC46 his*	8

Isolation of λ strains carrying flagellar gene-*lac* fusions. Figure 2 depicts the presumptive arrangement of genes adjacent to a *fla-lac* fusion joint. λ pl(209) phage (2) was used to lysogenize these fusion strains. Insertion was preferentially at the *fla*::Mu d (Ap^r *lac*) site due to the *lac* gene homology (Fig. 2d) or Mu phage gene homology (Fig. 2c), because λ pl(209) phage has no other integration system (2). The Mu d (Ap^r *lac*)-defective phage has temperature-sensitive immunity and still retains the ability to kill its host at high temperature (41°C). Thus, the resulting lysogen of a *fla-lac* fusion strain was grown at 41°C to collect the survivors that were free from the intervening Mu d prophage. As expected from the gene sequence of Fig. 2e, main classes of the survivors had a phenotype that was sensitive to ampicillin, was able to ferment lactose, and contained a lambda prophage. Heat survivors of lysogens having either of the gene arrangements shown in Fig. 2c and 2d were expected to have the same chromosomal arrangement. Lambda phages

TABLE 2. *fla*::Mu d (Ap^r *lac*) mutants with Lac$^+$ Fla$^-$ (or Mot$^-$) phenotype[a]

flaU	*3440* (0.71)						
flbA	*3443* (0.79)						
flaW							
flaV	*3407* (1.7)	*3451* (0.48)	*3491* (1.2)	*3494* (1.6)			
flaK	*3404* (1.6)	*3412* (0.30)	*3417* (0.86)	*3419* (0.22)	*3420* (2.1)	*3429* (0.42)	*3480* (1.2)
flaX	*3410* (1.3)	*3439* (1.7)					
flaL	*3408* (0.28)	*3425* (2.1)	*3465* (0.56)	*3495* (0.24)			
flaY	*3413* (1.7)	*3422* (0.84)	*3444* (1.7)	*3478* (1.3)			
flaM	*3434* (1.2)	*3470* (1.0)					
flaZ	*3449* (0.68)						
flaS	*3452* (0.24)						
flaT	*3475* (1.2)	*3484* (1.4)					
flaH	*3433* (0.80)	*3441* (0.61)	*3462* (0.34)	*3483* (0.48)			
flaG	*4318* (0.39)						
motA or *B*	*3401* (0.36)	*3477* (0.41)	*3487* (0.65)				
flaI	*4323* (0.26)	*4329* (1.5)	*4333* (1.8)				
flbB	*4330* (0.35)	*4331* (0.89)	*4332* (1.5)	*4334* (0.57)			
flaD	*4337* (1.1)	*4338* (0.72)					
hag	*3421* (0.70)	*4327* (3.1)					
flbC	*3415* (0.43)	*3426* (0.70)					
flaN	*4326* (0.74)						
flaB	*3416* (1.2)	*3418* (0.61)	*3428* (0.55)	*3435* (0.56)	*3446* (1.3)	*3456* (0.77)	*3467* (1.0)
flaC	*3411* (0.22)	*3427* (0.51)	*3466* (0.38)				
flaO							
flaE	*3423* (0.85)	*3464* (0.75)					
flaA	*3406* (0.47)	*3414* (1.1)	*3431* (0.40)	*3461* (0.88)	*3496* (0.65)		
flaR	*3469* (0.53)						
flaQ							
flaP							

[a] Flagellar mutants induced with Mu d (Ap^r *lac*) phage are shown by allele numbers and the *fla* gene in which the insertion occurred. The *fla* genes are situated clockwise on the *E. coli* chromome from top to bottom. The β-galactosidase activity of each mutant was measured at late log phase (1.3×10^9 cells/ml) in minimal glycerol medium. Values in parentheses indicate units per microgram of protein (average of at least three measurements).

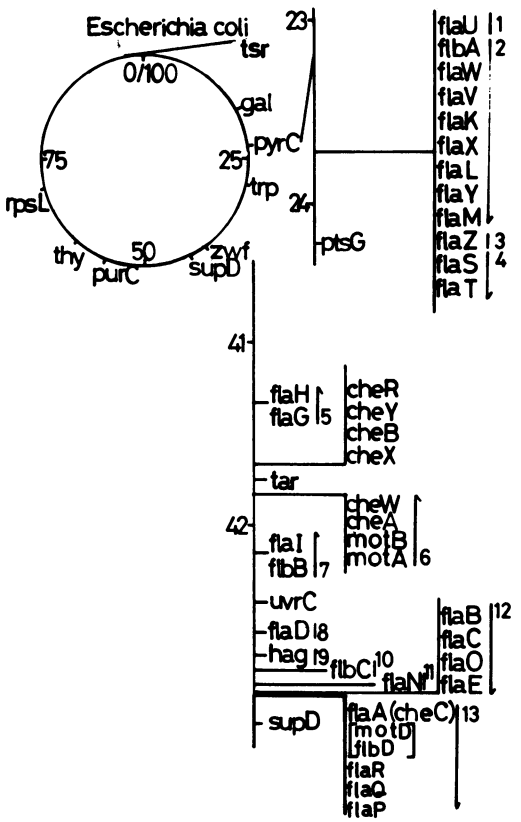

FIG. 1. Flagellar genes in *E. coli* K-12. *E. coli* flagellar genes are shown on the chromosomal map (9). The number to the right of each operon is the operon number as defined in the text.

induced from these clones by UV irradiation were plated in a tryptone broth overlay with strain YK410 on MacConkey plates containing 1% lactose. Red plaque formation on this medium indicated *lac* transducing phage capable of expression of the *lac* genes. Lambda phages that can make red plaques were found at a frequency of about 1% of the plaques that appeared on this plate. Existence of a flagellar gene promoter on these λ *lac* phages was confirmed according to the following observations. (i) All of the flagellar operons were positively controlled by the *flbB* operon products. When an *flbB5019*::Tn*10* mutation (polar to *flaI* gene) was introduced to *fla-lac* fusions representing all flagellar operons except the *cheB*, *flbB*, *tar*, and *tsr* operons, which were not examined, the double mutants lost the ability to ferment lactose. Therefore, the *flbB* or *flaI* product (or both) appeared to be responsible for expression of the other flagellar genes. The *hag* gene has also been shown to be transcribed under *flbB* and *flaI* control (8). (ii) As expected from Fig. 2e, the λ phage should have part of a flagellar gene. The existence of this material was demonstrated by infecting the λ *fla-lac* phage onto Fla$^-$ mutants and looking for the formation of motile recombinants on swarm agar.

The *flbB* operon was exceptional because it was unknown whether the operon was regulated by its own products. A genetic approach initially described by Berman and Beckwith (1) was used to show that the presumptive λ *flbB-lac* phage really had the promoter of the *flbB* operon (Fig. 3). λyk71 (or -72) originated from strain YK4329, which is a *flaI*::Mu d (Ap^r *lac*) mutant. Accordingly, the presumptive λ *flbB-lac* phage (λyk71 or -72) would have an intact *flbB* gene and its promoter. The phage could complement all of the FlbB$^-$ mutants available, as expected. As described by Berman and Beckwith (1), the λ *flbB-lac* phage could be lysogenized onto an *flbB*::Mu *cts* mutant (strain YK3386), and some resulting Fla$^+$ lysogens were Lac$^-$ by interruption of the transcription from the *flbB* promoter. This observation strongly suggested the existence of the *flbB* promoter on the λ *flbB-lac* phage (λyk71 or -72).

The λ phages carrying the *fla-lac* fusions were named as follows. The serial number of the flagellar operon carried by the fusion phage was followed by an isolation number which did not exceed 9 (see Fig. 1 for definition of operon number). For example, λyk11 is a transducing phage that has a *flaU-lac* fusion and was the first such isolate; λyk132 has a *flaA-lac* operon fusion and was the second isolate.

Assay of β-galactosidase after infection of λ *fla-lac* (or *flb-lac* or *mot-lac*) phage onto flagellar mutants. The flagellar mutant was grown with shaking at 30°C in minimal medium containing 5×10^{-3} M glycerol as the carbon source. The culture was grown until it reached a concentration of 1.3×10^9 cells/ml. $MgCl_2$ was added to the culture to 10^{-3} M. Each λ *fla-lac* (or λ *flb-lac* or *mot-lac*) phage was adsorbed to a 0.1-ml portion of the culture for 10 min at 30°C without shaking (MOI = 5). Nine volumes of minimal medium was added to each sample, which was incubated for 90 min at 30°C with shaking. The infected cells were mixed with toluene and shaken at 37°C for 40 min. The β-galactosidase activity was assayed as described above. In this assay, Z buffer was replaced with minimal medium. Specific activity was expressed by dividing the units by the initial cell mass of the sample.

RESULTS

Isolation of *fla-lac* fusion. Many flagellar mutants produced by insertion of the Mu d (Ap^r *lac*) phage were isolated. The arrangement of the *lac* genes (*lacZ*, *lacY*) in the Mu d (Ap^r *lac*) phage enables fusion to the gene or operon into which the phage is inserted. The *lacP* (promoter) region has been removed and the *lac* genes are aligned at the *S* end of the Mu phage genome (3) such that in one insertion orientation *lac* transcription can be affected by flagellar gene promoters. Therefore, the flagellar mutants that have a phenotype of Ap^r, Mu immunity, and Lac$^+$ are fusion mutants of flagellar genes to the *lac* genes. The fusions are operon fusions according to Casadaban's definition (2). These mutants behaved as usual Mu phage-induced flagellar mutants; that is, they were stable and exhibited polarity effects on downstream genes in the same operon. To characterize these fusion

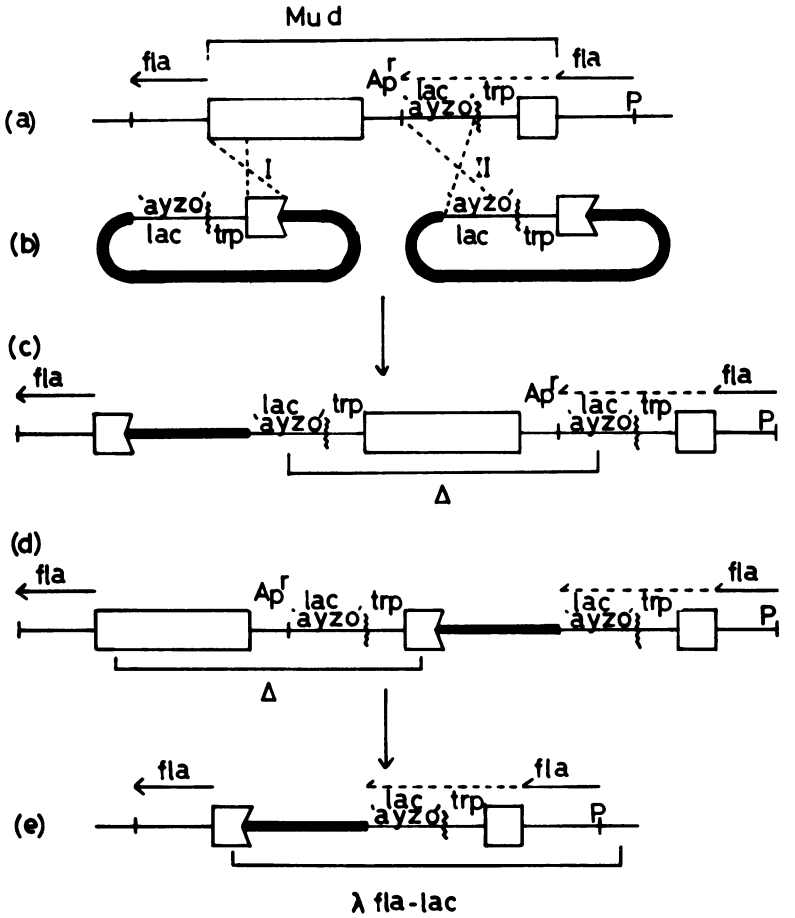

FIG. 2. Isolation of λ phage carrying *fla-lac* fusion. (a) A *fla*::Mu d (Ap^r *lac*) mutant was infected with (b) λpl(209) phage, which was integrated by using Mu gene homology (in I) or *lac* gene homology (in II). (c) A λpl(209) lysogen integrated with the phage, using Mu gene homology. (d) A λpl(209) lysogen integrated by using *lac* gene homology. (e) Resulting chromosomal DNA after heat induction of Mu d phage of the lysogen in (c) or (d). The deletion mutant was Lac$^+$ Amp^s and imm λ$^+$. Symbols: —, bacterial chromosome; ←, direction of transcription with promoter (shown as P); ⇠, transcriptional reading-through from the *fla* promoter; }, fusion joint; □, Mu phage gene; ■, lambda phage gene; Δ, possible deletion after heat induction.

mutants, complementation analysis was performed by using *fla*-transducing phages (11) and also P1 phage-mediated transduction with tester strains carrying defined mutations (9, 16).

Expression of the *lac* genes fused to flagellar promoters. Since the *lac* genes of these fusion mutants are transcribed from *fla* gene promoters, the expression of the *lacZ* gene should reflect the activity of the *fla* promoter. The specific activity of the β-galactosidase in the fusion mutants was measured (Table 2). These fusion mutants expressed β-galactosidase activity constitutively. The activity varied among the different mutants carrying Mu d (Ap^r *lac*) insertions in the same gene, perhaps because the DNA sequences between the *fla* promoter and the Mu d insertion sites can differ from one strain to another and affect the efficiency of transcriptional read-through.

I also isolated some Mu d insertions into *fla* genes in the antitranscription orientation. Strain YK4335 (*flbC*::Mu d [Ap^r *lac*] in "wrong" orientation) had 5.3×10^{-2} U per μg of protein. The activity corresponded to the basal level of parental YK410 (4.8×10^{-2} U per μg of protein). Other antitranscriptional insertions expressed almost the same activity. Accordingly, the fusions (in Table 2) expressed β-galactoside activity by transcriptional read-through from *fla* gene promoters.

Effects of Fla$^-$ mutations on expression of *fla-lac* fusions. The fusion strains described above made it possible to analyze interactions of flagellar genes at the transcriptional level. The tran-

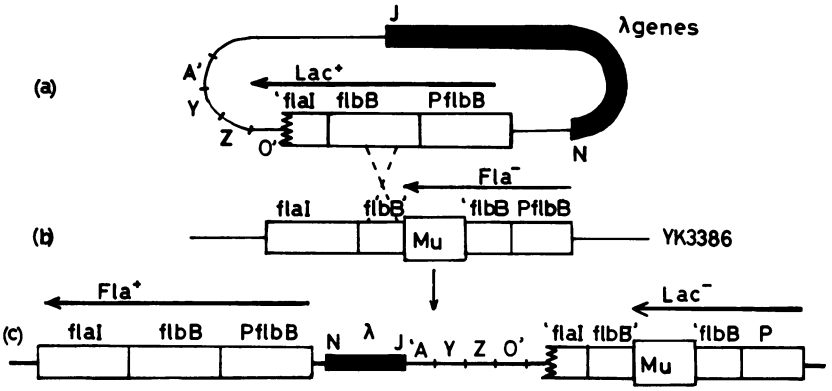

FIG. 3. The *flbB-flaI* genetic material carried by a fusion-transducing phage. A transducing phage carrying a presumptive *flbB-lac* fusion was used to infect strain YK3386 carrying a Mu *cts* prophage at the *flbB* locus (b). A single reciprocal crossover event in the indicated *flbB* gene sequences generated a recombinant that becomes Fla^+ (restoration of both *flbB* gene activity and polarity to *flaI*) as well as Lac^- (c). No *flbB* material being promoter proximal from the *flbB3386*::Mu *cts* mutation in *flbB-lac* or the *flbB* genetic material distal to the fusion makes it impossible to obtain Fla^+ Lac^- transductants.

scriptional control of the *hag* gene has already been examined (8). To elucidate the transcriptional interactions of flagellar genes, λ phages carrying the *fla-lac* fusions were isolated as described in Materials and Methods and used to examine β-galactosidase expression in various *fla* mutant hosts.

The λ phage strains isolated were plated on Fla^- mutants representing all of the *fla* genes (see Fig. 1) on MacConkey agar plates containing 1% lactose with tryptone broth overlay. The plates were examined for plaque color after 20 h of incubation at 37°C. This analysis (Table 3) also included strain λyk95 carrying a *hag-lac* fusion isolated in a previous study (8). Table 3 shows the phenotype of the representative λ *fla-lac* phage from each operon. The other phage carrying each promoter had the same phenotype as the representatives. All phages but λyk31 (λ *flaZ-lac*) formed Lac^+ plaques when plated on Fla^- mutants with defects in the same gene as on the fusion phages. This result suggested that each operon did not need the gene products of its own operon for expression. When λyk31 was plated on an *flaZ* mutant (strain YK4402), the plaques appeared less red than on the $flaZ^+$ parent (strain YK410); however, the plaques did appear to be redder than those on an $FlbB^-$ ($FlaI^-$) mutant. This result suggested that the *flaZ* operon might need its own products for maximally effective transcription. None (except λ *flbB-lac*) of the phage strains could express the *lacZ* gene upon infecting mutants with defects in *flaI* or *flbB*, as expected since this property was used in identifying λ *fla-lac* during the original isolation. The results divided the flagellar operons into six classes (Fig. 4). The *flbB* operon needed no *fla* products for its expression, and the genes in it were responsible for expression of all the other operons. In the second group were the operons that needed *flaI* and *flbB* for their expression and that were not responsible for expression of other operons. These operons were *flaU* and *flbC*. In the third group were the operons that needed *flaI* and *flbB* functions for expression and that were also responsible for expression of other operons. These were *flbA*, *flaD*, *flaG*, *flaN*, *flaB*, and *flaA*. The fourth type, consisting of the *flaZ* operon, needed both *flaI* and *flbB* as well as the genes in the third group for expression and was responsible only for *hag* operon expression. The fifth group had the operons that needed both *flaI* and *flaB* as well as the genes in the third group for expression and that were not responsible for other expression. This group includes the mocha operon and the *flaS* operon. The sixth class (*hag* operon) needed the genes in the first, third, and fourth groups for its expression and was not responsible for other expression.

The results in Table 3 were obtained after examination of the *lacZ* expression of each operon fusion on individual *fla* mutations. Two special genes were included: the *flaE* and *motD* genes, members of the operons in the third class. Although they were expressed as members of these operons, when a *flaE* or *motD* mutant was used as host for the λ phages, these two genes did not behave as other members of these operons. The mutants with *flaE* defects have polyhooks and basal structures, but no flagellar filaments (15). The mutants with *flaE* defects supported the expression of the other operons except *flaZ* and *hag*. The *motD* gene is a mem-

TABLE 3. Expression of *lac* on *fla* mutants[a]

Host	λ *yk* strain												
	(p *flaU*) 11	(p *flbA*) 21	(p *flaZ*) 31	(p *flaS*) 41	(p *flaG*) 51	(p *motA*) 61	(p *flbB*) 71	(p *flaD*) 81	(p *hag*) 95	(p *flbC*) 101	(p *flaN*) 111	(p *flaB*) 121	(p *flaA*) 131
Fla$^+$	+	+	+	+	+	+	+	+	+	+	+	+	+
flaU4122,-4186	+	+	+	+	+	+	+	+	+	+	+	+	+
flbA2070,-2861	+	+	−	−	+	−	+	+	−	+	+	+	+
flaW971,-2841	+	+	−	−	+	−	+	+	−	+	+	+	+
flaV2022,-2023	+	+	−	−	+	−	+	+	−	+	+	+	+
flaK2008,-4151	+	+	−	−	+	−	+	+	−	+	+	+	+
flaX2033,-2058	+	+	−	−	+	−	+	+	−	+	+	+	+
flaL4118,-721	+	+	−	−	+	−	+	+	−	+	+	+	+
flaY4164	+	+	−	−	+	−	+	+	−	+	+	+	+
flaM4148,-2017	+	+	−	−	+	−	+	+	−	+	+	+	+
flaZ51,-1118	+	+	±	+	+	+	+	+	−	+	+	+	+
flaS4140,-2073	+	+	+	+	+	+	+	+	+	+	+	+	+
flaT4176,-2077	+	+	+	+	+	+	+	+	+	+	+	+	+
flaH4133,-4172	+	+	−	−	+	−	+	+	−	+	+	+	+
flaG4195	+	+	−	−	+	−	+	+	−	+	+	+	+
motA4174,-B4119	+	+	+	+	+	+	+	+	+	+	+	+	+
flaI4136,-4155	−	−	−	−	−	−	−	−	−	−	−	−	−
flbB4116,-4131	−	−	−	−	−	−	−	−	−	−	−	−	−
flaD4181,-867	+	+	−	−	+	−	+	+	−	+	+	+	+
hag4130,-4146	+	+	+	+	+	+	+	+	+	+	+	+	+
flbC1101,-1102	+	+	+	+	+	+	+	+	+	+	+	+	+
flaN4145,-4193	+	+	−	−	+	−	+	+	−	+	+	+	+
flaB4126,-4141	+	+	−	−	+	−	+	+	−	+	+	+	+
flaC4127,-4147	+	+	−	−	+	−	+	+	−	+	+	+	+
flaO1862	+	+	−	−	+	−	+	+	−	+	+	+	+
flaE694	+	+	−	+	+	+	+	+	−	+	+	+	+
flaA4160,-4166	+	+	−	−	+	−	+	+	−	+	+	+	+
flbD52	+	+	−	−	+	−	+	+	−	+	+	+	+
motD4117	+	+	+	+	+	+	+	+	+	+	+	+	+
flaR4150,-4170	+	+	−	−	+	−	+	+	−	+	+	+	+
flaQ4035,-4120	+	+	−	−	+	−	+	+	−	+	+	+	+
flaP4144,-4154	+	+	−	−	+	−	+	+	−	+	+	+	+

[a] The Lac phenotype was examined after infection of each λ *fla-lac* phage onto each flagellar mutant as shown in the text. The promoter carried on each λ *fla-lac* phage is described in parentheses at the top of the phage designation. Symbols: +, red color development in the plaque; ±, less red color development (see text); −, no color development. Underlined mutations are amber suppressible.

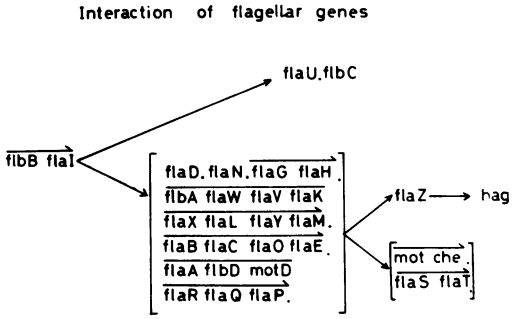

FIG. 4. Interaction of *fla* genes, showing a possible scheme of transcriptional control of flagellar genes based on the results shown in Table 3. The positive interaction for transcription is shown by the arrow. There is no interaction among the genes in the same parentheses. Details are described in the text. In this scheme, the *flaE* defect can express *flaS*, *flaT*, and mocha operon genes. The *motD* defect had no effect on transcription of other operons. These two genes are located at the position where the other members of their operon exist. Details are described in Discussion.

ber of the *flaA* operon and should be expressed as a member of it, like the other members. However, a mutant with a defect in *motD* had flagella and could support the expression of all of the other operons.

The examination of β-galactosidase of each Fla$^-$ mutant lysogenic for each λ *fla-lac* phage should clarify the effect of Fla$^-$ on the expression of other *fla* operons. The λ *fla-lac* phages do not have *attP.P'*, *int*, and *xis*. Strain YK4116 (*flbB*) lysogenic for λ *imm*^{434} phage, for example, was infected with each λ *fla-lac* phage. Thus, the lysogenization was expected to preferentially occur by using homology with the λ *imm*^{434} prophage. The resultant lysogen should have had a Lac$^-$ phenotype, as shown in Table 3. However, this was not the case. For example, the derivatives of strain YK4116 lysogenic for both λ *imm*^{434} and λ *hag-lac* (λyk95) phages had various Lac phenotypes: Lac$^-$, Lac$^\pm$, Lac$^+$, through Lac^{++}. The λ *hag-lac* phage had DNA segments derived from *E. coli trp*, Mu phage, and λ phage. The most likely lysogens would be inserted at *hag*, *trp*, or λ *imm*^{434} prophage genes. The insertions at *hag* and λ *imm*^{434} would have the same phenotype as that of strain YK4116 infected with the λ *hag-lac* phage (λyk95). The insertion in the *trp* operon would show *trp* promoter-directed *lac* expression. Therefore, addition of 10^{-4} M tryptophan to the culture of Lac$^+$ lysogens would result in a Lac$^-$ phenotype of some lysogens (which are inserted of λ *hag-lac* in the *trp* operon). I found some Lac$^+$ lysogens of this class, but I still found more Lac$^+$ (or Lac$^\pm$ or Lac^{++}) lysogens. Accordingly, I could not control the sites of their insertions and could not identify the clones which should be examined for β-galactosidase activity. The λ *fla-lac* phages seemed, for some unknown reason, to have a movable character around the *E. coli* genome. Investigation of this phenomenon is currently under way.

To obtain quantitative data on the β-galactosidase activity, I measured this activity after infection of each λ *fla-lac* phage onto the Fla$^+$ parent and each Fla$^-$ mutant. I tried many conditions to get a clear difference of the values, including changes in MOI, temperature, and medium. The Fla$^-$ mutants lysogenic for λ papa phage were also used for the assay as hosts for λ *fla-lac* infection. However, the conditions described in Materials and Methods gave the best resolution among the conditions tried (Table 4). The result supported the qualitative examination by the plating method.

DISCUSSION

A series of flagellar mutants carrying the Mu d (Ap^r *lac*) insertion in various flagellar genes has been constructed. These are operon fusion mutants (2) of flagellar genes to the lactose structural genes on the Mu *lac* phage. The degree to which the lactose genes are expressed depends on the amount of transcription that is not attenuated before reaching the *lac* genes. The *lac* expression is also limited by the efficiency of initiation of *lac* translation from the fused mRNA. Thus, the level of *lac* expression from a promoter is only a minimal measure of the transcription initiated at that promoter, and different levels of *lac* expression can result from different fusions to the same promoter. However, any transcriptional regulation of the gene whose promoter is fused to *lac* applies to expression of the fused lactose genes if the transcriptional control sites are intact. These fusion mutants would give us a powerful tool to analyze the flagellar regulon: (i) estimation of the activity of each flagellar gene promoter; (ii) finding of the effector for the regulon; (iii) examination of the expression of each operon; (iv) identification of regulatory mutants; and (v) the fact that the fusion is a good "gene" for an in vitro protein-synthesizing system, because the β-galactosidase is easy to assay.

As an initial step to understanding a control mechanism of the flagellar regulon, this study examined the β-galactosidase activity of each mutant and Fla$^-$ effects on each flagellar operon expression. The fusion mutants were produced by insertion of Mu d phage at various *fla* genes. If each operon is controlled by its own product(s), the enzyme activity of a given operon will disclose this mechanism through comparison of the activities after infecting λ *fla-lac* phages onto

TABLE 4. Activity of β-galactosidase of λ *fla-lac* infected onto each flagellar mutant[a]

Host	λ *yk* strain												
	(p *flaU*) 11	(p *flbA*) 21	(p *flaZ*) 31	(p *flaS*) 41	(p *flaG*) 51	(p *motA*) 61	(p *flbB*) 71	(p *flaD*) 81	(p *hag*) 95	(p *flbC*) 101	(p *flaN*) 111	(p *flaB*) 121	(p *flaA*) 131
Fla$^+$ (yk410)	1.7	3.2	0.88	1.3	0.94	1.3	2.0	1.3	1.5	1.2	1.2	1.5	1.9
flaU4122	1.1	2.4	0.83	1.0	1.1	1.5	1.8	1.3	1.4	1.6	1.1	2.1	2.4
flbA4457	1.0	3.5	0.41	0.43	1.0	0.33	2.1	1.4	0.74	1.1	1.5	1.6	2.0
flaW4410	1.9	4.1	0.39	0.37	0.98	0.51	2.2	1.2	0.75	1.3	1.7	1.7	1.9
flaV4451	1.9	2.3	0.41	0.41	1.2	0.34	2.3	1.0	0.70	1.4	1.6	1.9	2.1
flaK4151	1.8	3.2	0.35	0.50	0.95	0.38	2.2	1.1	0.78	1.1	1.5	1.7	1.6
flaX4453	1.2	3.6	0.24	0.71	0.89	0.53	2.7	1.1	0.81	0.94	1.1	1.3	1.4
flaL4118	1.3	2.3	0.11	0.42	0.91	0.62	2.4	1.3	0.82	0.84	1.0	2.1	2.4
flaY4164	1.3	2.0	0.55	0.60	0.92	0.66	1.4	1.6	0.78	1.1	1.1	2.1	2.1
flaM4148	1.5	3.0	0.58	0.50	0.99	0.50	1.5	1.2	0.70	0.92	1.0	1.8	1.6
flaZ4402	1.4	2.4	0.60	0.87	0.88	1.2	1.6	1.0	0.75	1.2	2.1	1.9	2.4
flaS4140	1.8	2.1	1.2	1.3	1.1	1.2	2.3	0.98	1.6	1.9	1.7	1.3	2.3
flaT4176	1.5	2.0	1.5	1.3	1.2	1.1	2.3	1.1	1.4	1.6	1.4	1.0	2.9
flaH4172	1.4	3.6	0.31	0.44	0.94	0.33	2.2	1.1	0.75	0.93	1.7	1.2	2.0
flaG4195	1.0	3.5	0.25	0.41	1.1	0.38	2.1	1.0	0.60	0.95	1.0	1.0	1.8
mot4119	1.4	3.7	1.7	1.1	1.0	0.94	2.3	1.2	1.5	1.0	1.0	1.5	1.5
flaI4136	0.70	0.11	0.40	0.27	0.40	0.53	1.5	0.43	0.86	0.63	0.85	0.62	0.70
flbB4116	0.32	0.17	0.40	0.37	0.16	0.14	1.2	0.29	0.74	0.34	0.61	0.58	0.59
flaD4181	1.0	1.6	0.55	0.57	1.0	0.99	1.5	1.2	0.61	0.93	1.1	1.1	1.9
hag4146	1.5	1.6	0.98	1.2	1.1	0.98	2.0	1.2	1.7	1.2	1.8	1.3	1.2
flbC1101	2.0	1.9	0.95	1.4	1.1	1.1	1.1	1.1	1.6	1.1	2.2	1.0	1.2
flaN4193	1.4	1.3	0.39	0.41	1.1	0.36	1.5	1.3	0.75	1.4	1.5	1.7	1.9
flaB4126	1.2	2.0	0.35	0.42	1.0	0.42	1.6	1.0	0.70	1.4	0.98	1.2	2.0
flaC4127	1.8	2.6	0.32	0.39	1.2	0.41	1.3	0.98	0.60	1.5	2.2	1.5	1.2
flaO4112	1.4	3.1	0.31	0.64	0.98	0.49	1.6	1.0	0.46	1.4	2.2	1.0	1.4
flaE4105	1.1	1.6	0.33	0.63	0.92	0.58	2.2	1.0	0.90	1.2	1.7	1.2	2.1
flaA4160	1.3	2.2	0.31	0.46	1.1	0.24	2.4	1.1	0.80	1.3	2.1	1.8	1.4
flbD52	1.2	1.9	0.31	0.39	1.1	0.38	1.4	1.3	0.70	1.2	1.0	1.7	1.3
motD4117	1.6	0.99	0.99	0.70	1.0	1.1	1.4	1.1	1.1	1.3	1.0	1.3	1.8
flaR4150	1.2	1.5	0.43	0.40	1.0	0.62	2.3	1.2	0.65	0.95	1.1	1.8	1.3
flaQ4120	1.4	1.6	0.21	0.40	0.99	0.45	1.4	1.3	0.79	0.93	1.0	1.8	1.4
flaP4144	1.1	1.3	0.18	0.43	1.1	0.60	1.5	1.5	0.61	0.98	1.1	1.9	1.5

[a] The activity of β-galactosidase of λ *fla-lac* infected onto each flagellar mutants was measured as described in the text. Values are expressed as units per microgram of protein (average of at least three measurements).

Fla^+ cells with those onto corresponding Fla^- (belonging to each operon of the λ *fla-lac*) cells. None of the *fla* operons (except *flaZ*) required its own products for expression, because the enzyme activities observed were almost the same between the Fla^+ and the corresponding Fla^- cells. The *flaZ* promoter appeared to be an exception. The λ yk31 phage (λ *flaZ-lac*) showed more β-galactosidase activity after infection onto Fla^+ cells than onto $FlaZ^-$ cells. The *flaZ* promoter apparently required its own product for the most effective transcription. Thus, the enzyme activity in strain YK3449 (a *flaZ*::Mu d [Ap^r *lac*] mutant) would not really reflect the promoter activity of the *flaZ* operon. Accordingly, each *fla-lac* fusion would reveal the characteristics of a corresponding *fla* promoter except the *flaZ* promoter. One of the *hag-lac* fusions (strain YK4327) had the highest β-galactosidase activity. The flagellin constitutes the highest portion of the flagellar constituent proteins. The activity of strain YK4327 corresponded closely to that of fully induced β-galactosidase of strain YK421 (Lac^+ derivative of YK410). According to the analysis by O'Brien and Bennett (12), 1 μm of flagellar filament consists of about 2,200 flagellin molecules. Since strain YK410 has about five flagella which are at least about 5 μm long, it should synthesize about 55,000 flagellin molecules. Assuming that there are 20,000 molecules of β-galactosidase in a cell when *lacZ* expression is fully induced (4), strain YK4327 will also have as many molecules. This figure is compatible with that of flagellin molecules in strain YK410. It may suggest that the fused mRNA transcribed from the *hag* promoter is translated with the same efficiency as that from *lacP* in strain YK4327. Other mutants may represent the activity of each operon. However, the current scant knowledge about the *fla* gene products made it difficult to inspect each mutant.

This study analyzed the effects of *fla* mutations for transcription of flagellar gene operons. The analysis clearly showed that there are interactions among flagellar genes. Initially, the mocha operon and all of the *fla* operons (except the *flbB* operon) were shown to be positively controlled by the *flbB* operon gene products. Second, the *fla* operons had interactions at the transcriptional level. The results of Fla^- effects summarized in Table 3 categorize flagellar operons into six classes as described in the Results.

So far, cAMP is the only known extrinsic regulator that controls flagellar formation. The *cfs* mutation, which renders flagellar synthesis insensitive to catabolite repression, has been shown to control the *flaI* gene, which regulates expression of some of the flagellar genes examined (8, 14, 17–19). Our recent mapping results (9) suggest that since *flaI* and *flbB* comprise one operon, *cfs* may be a promoter mutation which affects expression of both genes. Thus, both the *flaI* and the *flbB* gene products were postulated to regulate in a positive fashion the expression of all of the other flagellar genes (17). This study extended the analysis of the role of the products of the *flbB* operon into all of the flagellar genes and clearly showed that the *flbB* operon gene products controlled transcription of all of the other flagellar operons. This study did not clarify the manner of interaction to the class 4, 5, and 6 operons. Isolation of the *flbB* operon products will reveal the molecular mechanism.

The results in Table 3 suggested the positive fashion of *fla* gene regulation. Since all of the *fla* genes are positive factors for the appearance of the flagella, an interaction scheme should contain features of positive interaction. A scheme for the interaction of flagellar genes was constructed based on the following assumptions (Fig. 4). (i) If a gene *A* defect results in failure of operon *B* expression (examined by the expression of *lacZ* on λ "*B*"-*lac* phage), the gene *A* product is required for transcription of the *B* operon, and the *B* operon is situated after *A* in the sequence. (ii) If the *C* operon is not expressed on hosts with a gene *D* and a gene *E* defect, and if *E* is situated after *D* as shown in (i), the interaction sequence is drawn as $D \rightarrow E \rightarrow C$. Each gene was situated self-consistently on the sequence with two exceptions. The *flaE* and *motD* genes were the members of those operons which were controlled by the *flbB* operon genes. Unlike the other members, the *flaE* defect had no effect on expression of group 5 operons (mocha and *flaS* operons), and the *motD* defect had no effect on transcription of any operons at all.

Recent electron microscopic observation (22) has revealed some precursor structures of a flagellum in the *fla* mutants. The mutants with defects in the *flaS*, *flaT*, *flbC*, and *hag* genes had hook-basal body structures. The mutants with *flaK* defects formed basal body structures. The mutants carrying *flaM*, *flaU*, *flaV*, and *flaY* defects had putative precursor structures of a basal body. Except for the *flaU* mutants, it was noted that defects of the genes in the late group (*hag*, *flaS*, *flaT*, and *flbC*) (Fig. 4) had the most complex precursor: the hook-basal body structure. I believe the scheme in Fig. 4 reflects at least some aspects of the interaction of flagellar gene products for their synthesis and assembly.

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LITERATURE CITED

1. **Berman, M. L., and J. Beckwith.** 1979. Fusions of the *lac* operon to the transfer RNA gene *tyrT* of *Escherichia coli*. J. Mol. Biol. **130**:285–301.
2. **Casadaban, M. J.** 1976. Transposition of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J. Mol. Biol. **104**:541–555.
3. **Casadaban, M. J., and S. N. Cohen.** 1979. Lactose genes fused to exogenous promoters in one step using a Mu-*lac* bacteriophage: *in vivo* proof for transcriptional control sequences. Proc. Natl. Acad. Sci. U.S.A. **76**:4530–4533.
4. **Cohn, M.** 1957. Contributions of studies on the β-galactosidase of *Escherichia coli* to our understanding of enzyme synthesis. Bacteriol. Rev. **21**:140–168.
5. **Hilmen, M., and M. Simon.** 1976. Motility and the structure of bacterial flagella, p. 35–45. *In* R. Goldman, T. Pollard, and J. Rosenbaum (ed.), Cell motility, vol. 3. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. **Icho, T., and T. Iino.** 1978. Isolation and characterization of motile *Escherichia coli* mutants resistant to bacteriophage χ. J. Bacteriol. **134**:854–860.
7. **Iino, T.** 1977. Genetics of structure and function of bacterial flagella. Annu. Rev. Genet. **11**:161–182.
8. **Komeda, Y., and T. Iino.** 1979. Regulation of expression of the flagellin gene (*hag*) in *Escherichia coli* K-12: analysis of *hag-lac* gene fusions. J. Bacteriol. **139**:721–729.
9. **Komeda, Y., K. Kutsukake, and T. Iino.** 1980. Definition of additional flagellar genes in *Escherichia coli* K-12. Genetics **94**:277–290.
10. **Kondoh, H., and H. Ozeki.** 1976. Deletion and amber mutants of *fla* loci in *Escherichia coli* K-12. Genetics **84**:403–421.
11. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **O'Brien, E. J., and P. M. Bennett.** 1972. Structure of straight flagella from a mutant *Salmonella*. J. Mol. Biol. **70**:133–152.
13. **Parkinson, J. S.** 1977. Behavioral genetics in bacteria. Annu. Rev. Genet. **11**:397–414.
14. **Silverman, M., P. Matsumura, R. Draper, S. Edwards, and M. I. Simon.** 1976. Expression of flagellar genes carried by bacteriophage lambda. Nature (London) **261**:248–250.
15. **Silverman, M. R., and M. I. Simon.** 1972. Flagellar assembly mutants in *Escherichia coli*. J. Bacteriol. **112**:986–993.
16. **Silverman, M., and M. Simon.** 1973. Genetic analysis of flagellar mutants in *Escherichia coli*. J. Bacteriol. **113**:105–118.
17. **Silverman, M., and M. Simon.** 1974. Characterization of *Escherichia coli* flagellar mutants that are insensitive to catabolite repression. J. Bacteriol. **120**:1196–1203.
18. **Silverman, M., and M. Simon.** 1976. Operon controlling motility and chemotaxis in *E. coli*. Nature (London) **264**:577–580.
19. **Silverman, M., and M. Simon.** 1977. Bacterial flagella. Annu. Rev. Microbiol. **31**:397–419.
20. **Silverman, M., and M. Simon.** 1977. Identification of polypeptides necessary for chemotaxis in *Escherichia coli*. J. Bacteriol. **130**:1317–1325.
21. **Suzuki, H., and T. Iino.** 1975. Absence of messenger ribonucleic acid specific for flagellin in nonflagellate mutants of *Salmonella*. J. Mol. Biol. **95**:549–556.
22. **Suzuki, T., and Y. Komeda.** 1981. Incomplete flagellar structures in *Escherichia coli* mutants. J. Bacteriol. **145**:1036–1041.