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Characterization of *Escherichia coli* Flagellar Mutants That are Insensitive to Catabolite Repression

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In *Escherichia coli*, the synthesis of the flagellar organelle is sensitive to catabolite repression. Synthesis requires the presence of the cyclic adenosine monophosphate receptor protein (Crp) and 3',5'-cyclic adenosine monophosphate (cAMP); i.e., mutants that lack Crp or adenylcyclase (Cya) synthesize no flagella. We isolated and characterized a series of mutants (*cfs*) that restored flagella-forming ability in a Crp⁻ strain of *E. coli*. The mutations in these strains were transferred onto episomes and they were then introduced into a variety of other strains. The presence of the mutation resulted in flagella synthesis in Cya⁻ and Crp⁻ strains as well as in the wild type grown under conditions of catabolite repression. Deletion analysis and other genetic studies indicated that: (i) the *cfs* mutations had a dominant effect when they were in the transconfiguration in merodiploids; (ii) they occurred in or very close to the *flaI* gene; and (iii) their expression required the presence of an intact *flaI* gene adjacent to the *cfs* mutation. Biochemical studies showed that the synthesis of at least two flagellar polypeptides, the hook subunit and an amber fragment of flagellin, were absent in strains that carried a *cya* mutation. Their synthesis was depressed in strains grown under conditions of catabolite repression. The presence of the *cfs* mutation restored the specific synthesis of these two polypeptides. We suggest that the formation of the *flaI* gene product is the step in flagellar synthesis that is catabolite sensitive and requires cAMP. We propose a regulatory function for the product of the *flaI* gene.

It has been recognized for some time that the synthesis of the flagellar organelle of *Escherichia coli* is sensitive to catabolite repression. Cultivation of Fla⁻ strains in the presence of catabolite-repressing substrates such as glucose prevents the synthesis of flagella (1), and this effect can be reversed by the addition of exogenous cyclic 3',5'-adenosine monophosphate (cAMP) (2). Mutants lacking adenylcyclase activity (Cya⁻) produce little or no cAMP and are unable to synthesize flagella unless an exogenous supply of cAMP is provided (11). In addition to cAMP, another regulatory protein, the cAMP receptor protein (Crp) (3), is required, and mutants defective in this function are also unable to synthesize flagella. To investigate the mechanism of this effect, we sought mutants of *E. coli* in which the synthesis of flagella was insensitive to catabolite repression, i.e., constitutive mutants. They were obtained by selecting motile clones from an *E. coli* strain that was phenotypically Fla⁻ because of the presence of a *crp* mutation. Several such constitutive mutants were isolated, and the genetic alteration was shown to lie in a region of the *E. coli* chromosome known to contain previously defined flagellar genes. We report our characterization of these mutations and offer a proposal for the mechanism of the regulation of flagellar synthesis by cAMP.

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MATERIALS AND METHODS

Media. Tryptone broth contained, per liter of distilled water: tryptone (Difco), 10 g; NaCl, 5 g; and thymine, 0.1 g. L broth contained, per liter of distilled water: tryptone, 10 g; NaCl, 10 g; yeast extract (Difco), 5 g; and glucose, 2 g. Glucose was added aseptically after autoclaving. L agar plates were prepared by adding 1.5% agar (Difco) to L broth. Motility agar plates were prepared by adding 0.35% agar to tryptone broth. Thymine (0.1 g) was added to each liter of the above media where cultivation of a Thy⁻ strain was required.

Minimal medium contained, per liter of distilled water: K_2HPO_4, 11.2 g; KH_2PO_4, 4.8 g; $(NH_4)_2SO_4$, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $Fe_2(SO_4)_3$, 0.5 mg; glucose, 5 g; and thiamine, 1 ml. The $MgSO_4 \cdot 7H_2O$, glucose, and thiamine were added aseptically after autoclaving. Amino acid and thymine, if required, were added to a final concentration of 100 mg/liter

before autoclaving. Minimal motility plates were prepared by substituting glycerol for glucose and adding 0.35% agar to minimal medium. Minimal agar plates were prepared by adding 1.5% agar to minimal medium. MacConkey maltose and MacConkey lactose plates were prepared by adding 10 g of lactose or maltose and 40 g of MacConkey agar base (Difco) to 1 liter of distilled water. For cultivating bacteria under catabolite-repressing conditions, D-glucose, D-gluconic acid (sodium salt), and D-mannitol were added at a concentration of 5 g/liter each to either tryptone medium or minimal salts medium. Since Crp$^-$ and Cya$^-$ strains do not grow on minimal medium with glycerol as a carbon source (5) and grow poorly on tryptone medium, 5 g of D-glucose per liter was added to minimal medium and 0.5 g of D-glucose per liter was added to tryptone medium to insure adequate growth. When it was necessary to add cAMP to growth medium or motility plates, cAMP (Sigma Chemical Co.) was added to a final concentration of 10^{-2} M and the pH was adjusted to 7.0 with 0.1 N NaOH before sterilizing. Cultivation of bacteria on these media was at 37 C unless otherwise noted.

Strains. Most of the *E. coli* K-12 strains used in this study are shown in Table 1 and are derived from strain MS1350, with which most of our earlier flagellar studies were done. The preparation of strain MS1350 has been previously described (9).

Construction of Cya$^-$ and Crp$^-$ strains. Hfr strains 5333 (Crp$^-$) and 5336 (Cya$^-$) were used to transfer the *crp* and *cya* genes to strain MS1350. Strains 5333 and 5336 are derived from strain HfrH and have an origin of transfer represented at 0 min on the *E. coli* genetic map (3, 5). Matings were performed in L broth with approximately equal proportions of donor and recipient bacteria (2×10^8 donor and recipient cells), and incubation was for 2 h without shaking in a 10-ml volume in a 250-ml Erlenmeyer flask. The mating mixture was washed and concentrated in 0.1 ml of minimal salts medium and spread on four minimal agar plates supplemented with histidine, thymine, and thiamine. Selection was for Arg$^+$ recombinants of strain MS1350, and the donor was counterselected by the addition of 200 μg of streptomycin sulfate per ml (Calbiochem). Since the *cya* marker (75 min) is closely linked to the *argE* gene (79 min), many of the Arg$^+$ recombinants should be Cya$^-$. Transfer of the *crp* mutation by conjugation and selection for Arg$^+$ recombinants is less likely since *crp* is located at 64 min on the *E. coli* genetic map adjacent to the *strA* marker and the selection process excludes the introduction of the donor's allele of *strA* gene. In fact, about 50% of the small colonies selected as Arg$^+$ recombinants by conjugation with strain 5336 carried the *cya* marker, whereas less than 1% of the Arg$^+$ recombinants obtained by conjugation with strain 5333 carried the *crp* marker. The recombinants were tested for their ability to grow on minimal medium supplemented with glycerol as an energy source and their inability to ferment maltose or lactose on maltose or lactose MacConkey agar plates (5). One of the Cya$^-$ and Arg$^+$ derivatives of strain MS1350 was saved and named strain MS1823. One of the Arg$^+$ Crp$^-$ recombinants was saved and named strain MS1822. Neither strain MS1822 or strain MS1823 synthesized flagella as determined by electron microscopic examination and by the ability to swim on tryptone motility agar supplemented with 0.5 g of D-glucose per liter. Addition of cAMP (10^{-2} M) restored both motility and the ability to utilize maltose and lactose to strain MS1823 but not to strain MS1822.

TABLE 1. *Description of strains used*

Strain	Relevant characteristics	Source
5333	Hfr *crp*	I. Pastan (3)
5336	Hfr *cya*	I. Pastan (5)
MS1350	F$^-$ *uvrC, galU, sup*$^+$, λ^-, *str*^{r}, *thy, argE*	This laboratory (9)
JC1553	F$^-$ *leu, his, argG, met, str*^{r}, *lac, mal, xyl, mtl, recA*	A. J. Clark
MSF1338	F *his*$^-$, *hag207, uvr*$^+$, *zuf*$^+$, *fla*$^-$ for all flagellar genes between *his* and *aroD* in strain JC1553	This laboratory (9)
KL96	Hfr, *his*$^+$ transfer as proximal marker, *thi*, λ^-, *str*^{s}	E. Low
MS1822	*crp arg*$^+$ derivative of strain MS1350	This laboratory
MS1823	*cya arg*$^+$ derivative of strain MS1350	This laboratory
MS1825	Fla$^-$ derivative of MS1822	This laboratory
MS1827	Fla$^-$ derivative of MS1822	This laboratory
MS1828	Fla$^-$ derivative of MS1822	This laboratory
MS1829	Fla$^-$ derivative of MS1822	This laboratory
MS1381	*hag912, flaE694* derivative of strain MS1350	This laboratory (8, 9)
MS912	*hag912* derivative of strain MS1350	This laboratory (9)
MS987	*hag987* derivative of strain MS1350	This laboratory (9)
MS1800	F1829 in strain JC1553	This laboratory (7)
MS1883	F1900 in strain JC1553	This laboratory (7)
MS1884	F1943 in strain JC1553	This laboratory (7)
MS1885	F1955 in strain JC1553	This laboratory (7)
MS1869	*cya* derivative of strain MS1381	This laboratory
MS1871	*cya* derivative of strain MS912	This laboratory
MS1872	*cya* derivative of strain MS987	This laboratory

Isolation of catabolite repression-insensitive mutants. Isolation of mutants insensitive to catabolite repression was attempted by selecting Fla$^+$ clones of strains MS1822 and MS1823. Tryptone motility plates supplemented with 0.5 g of D-glucose per liter were incubated with separate clones of strains MS1822 and MS1823 on a narrow zone across the center of the plate. Motile clones of bacteria were picked, streaked, and tested for fermentation of maltose and lactose and for the ability to use glycerol as an energy source. To insure that each Fla$^-$ (Crp or Cya$^-$) mutant arose independently, only one mutant from each selection plate was saved. These mutations will be referred to as *cfs*, for constitutive flagellar synthesis. Thus a Cfs$^+$ strain does not make flagella in the presence of catabolites or in the absence of cAMP (Cya$^-$) or Crp, whereas a Cfs$^-$ does make flagella even in the apparent absence of cAMP and Crp.

Hfr mapping. To determine whether the *cfs* muta-

tions are near the known flagellar genes, we tested for His$^+$ recombinants resulting from the conjugation of Hfr KL96 (point of origin at 40 min with *his*$^+$, the proximal marker transferred) with the Fla$^-$ derivatives of MS1822. The matings were performed as before except that the donor-to-recipient ratio was 1:10 and matings were carried out for 30 min before termination by shearing.

Transfer of genetic alterations to F elements. Since it was necessary to test the influence of the *cfs* mutations on strains with different genetic backgrounds, a method was devised for transferring these mutations to an F element. F1338, which carries the flagellar genes in the *his-aroD* region of the *E. coli* chromosome (9), was used for this purpose. Strain MS1338 was mated as before except in approximately equal proportions with the Fla$^-$Crp$^-$ (Rec$^+$) mutant strain for 30 min, after which the conjugation mixture was washed and concentrated in minimal salts medium. These bacteria were spread on minimal agar, which selected for His$^+$ exconjugants and counterselected the donor by amino acid deprivation. More than 100 of the merodiploid His$^+$ clones were pooled and used as donors to transfer the F elements to strain JC1553 (RecA) in a mating like that above, except that donor bacteria were selected against by the elimination of thymine from the minimal agar plates. If a recombination event had occurred, the mutation on the episome would now be in a stable configuration in strain JC1553, and this could act as a repository strain. These His$^+$ exconjugant clones of strain JC1553 were then mated on L agar plates, as previously described (7), with strains MS1823 and MS1822 to test for the acquisition by the F element of the *cfs* mutation. Approximately 2% of the clones of repository strain JC1553, harboring F element 1338, had acquired the ability to donate a factor that would enable strains MS1822 and MS1823 to synthesize flagella (as judged by the ability of His$^-$ strains MS1822 and MS1823 to swim on minimal motility agar plus glucose). The first *cfs* mutation was mobilized in this manner. To mobilize mutations from other Cfs$^-$ strains (and from MS1829 again), a similar approach was used except with a derivative of F1338 that carried a specific *flaI* gene mutation (*flaI9216*).

Deletion analysis. Two techniques were developed to select F elements with deletions in the flagellar genes. They have previously been described as the isolation of "type 1" and "type 2" F elements (7). Strain JC1553, harboring an F factor that carried one of the *cfs* mutations, F1829 (7), was spread across the center of a tryptone motility plate containing 5 g each of D-glucose, D-gluconate, and D-mannitol per liter. Motile swarms were selected, and many of them now carried deletions on the F element extending through some of the flagellar genes in the *his-aroD* region. The deletions arise because a locus between *his* and the flagellar genes must be deleted to eliminate the excessive accumulation of mucopolysaccharide product by this merodiploid strain. This accumulation of mucopolysaccharide interferes with the synthesis of flagella (9). The F element carrying the *cfs* mutation must retain it since motility is tested for under catabolite-repressing conditions. Thus, there exists a selective pressure for the retention of the F element itself. F elements so obtained were designated "type 1." Another method was used to obtain "type 2" F elements, and this procedure utilizing selective pressure for the deletion of the exogenote *hag* locus and has also been described previously (7). Type 2 F elements delete more extensively in the flagellar region due to the lack of requirement for the retention of any of the flagellar genes. The extent of the deletion on the episome was determined as previously described (7).

Introduction of specific gene defects. The technique for isolation of the specific episomal flagellar point mutations has been described previously (6) and includes mutagenesis of the F-element donor, immediate transfer of the F element to RecA Fla$^-$ mutant strains, and subsequent selection of Fla$^+$ merodiploid exconjugants. The Fla$^+$ exconjugants frequently carry an F element with a point mutation in the same gene as the endogenote point mutation. They can be checked by the appropriate complementation tests.

Measurement of polyhook protein. Polyhook protein (8) was measured by complement fixation using antipolyhook antibody. The polyhook mutant was grown in minimal medium with glycerol as carbon source. The cells were washed and resuspended in glycerol or in glucose-gluconate-mannitol (GGM) medium. They were grown for five generations and harvested. The cells were treated with 0.1% formaldehyde for 5 min. They were washed and used as antigen in an assay that included antipolyhook antibody (Ra923) at a 1:8,000 dilution.

Measurement of flagella. The influence of catabolite repression on the synthesis of flagella was determined in a manner identical to that used for polyhook protein measurement except that antiflagellar serum (no. 207) was used at a dilution of 1:15,000.

Measurement of internal hook subunit. Complement fixation assay with antihook subunit serum (no. 926) at a dilution of 1:800 was used to detect internal hook subunits. This antibody was prepared by immunizing rabbits with pure polyhook protein that had been dissociated by heating for 3 min at 100 C. It is specific for hook subunits and does not react with polymerized hook (polyhook or hook structures). To prepare cell extracts, 500 ml of late-log bacteria grown in tryptone broth were pelleted by centrifugation at 8,000 × *g* for 10 min. The pellets were resuspended in 20 ml of 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.0. The cell suspension was passed through a French pressure cell (American Instrument Co., Silver Spring, Md.) at 15,000 lb/in^2. The resulting suspension was clear but opalescent and was cleared of cell envelope debris by centrifugation at 8,000 × *g* for 10 min followed by high-speed centrifugation at 100,000 × *g* for 90 min. The resulting supernatant was heated at 100 C for 5 min, and the denatured protein was removed by centrifugation at 8,000 × *g* for 15 min and discarded. The resulting supernatant contained 1 to 2 mg of protein per ml and was saved for complement fixation analysis.

Measurement of hag amber fragment. Detection of the *hag* gene amber fragments was accomplished by a modification of the complement fixation assay for

whole flagella. The concentration of flagellar antigen (Hag 207) was adjusted to 0.5 μg/ml and antiflagellar antibody no. 207 was used at a dilution of 1:15,000. Flagellin or internal *hag* gene amber fragments were found to inhibit this reaction specifically by binding Hag 207 antibody. This inhibition was used to quantitate the amount of *hag* gene amber fragment in cell extracts (M. Silverman, Ph.D. thesis, 1972). The cell extracts of mutant strains with *hag* gene amber mutations used in this study were prepared in the same manner as the ones used for measurement of the hook subunit protein.

RESULTS

A series of motile derivatives of the Crp strain was selected. Each one was then tested for its ability to utilize maltose and lactose. About 60% of the clones regained the ability to use both sugars. The other clones that retained their inability to utilize the sugars, however, were motile. Four such clones were picked and designated MS1825, MS1827, MS1828, and MS1829 (Table 1). All motile clones derived from the Cya strain regained the ability to utilize maltose and lactose. This may be due to the high reversion rate of the *cya* mutation in strain 5336. The movement in tryptone motility agar of strain MS1829 is shown in Fig. 1. This mutant (and the other three as well) can also swim through motility agar to which catabolite-repressing substrates (glucose, gluconate, and mannitol) have been added, whereas movement of wild-type strain MS1350 is severely impaired (Fig. 1).

Linkage to his. His$^-$ recombinant clones resulting from the 30-min mating of Hfr strain KL96 with strains MS1825, MS1827, and MS1828, and MS1829 were examined for the retention of constitutive flagellar synthesis by testing the motility of cells derived from the His$^-$ recombinant clones on minimal motility agar (plus glucose). For strains MS1825, MS1827, MS1828, and MS1829, 60 to 69% of the His$^-$ recombinant clones no longer had the Fla$^-$ phenotype. Since the mutant character was lost concomitantly with the recombination of the *his* gene, we concluded that the genetic alteration in these mutant strains was probably linked to *his* and lay in the vicinity of the flagellar genes, i.e., those known to reside in the *his-aroD* region of the *E. coli* chromosome.

Transfer to F elements. The mutation in strain MS1829 was obtained on the F element as described in Materials and Methods. This allowed transfer of the mutation to other genetic backgrounds and tests of the properties conferred on the recipient strain by its presence. Transfer of the mutation into MS1350 enabled this strain to swim even when grown on plates containing GGM. When the episomes carrying the *cfs* mutations were transferred into strains MS1822 and MS1823, flagella were synthesized and the strains became motile. They were also motile when grown on GGM plates. Thus the mutations appear to be dominant and allow the synthesis of flagella under conditions of catabolite repression in the wild type and in Cya$^-$ and Crp$^-$ strains.

Deletion analysis. Deletions in the F element, F1829, were used to define the relative position of the *cfs* mutations. Fig. 2 shows our present knowledge of the organization of the flagellar genes in the *his-aroD* region. Table 2 shows some of the F elements derived from F1829 and the effects of specific deletions on the expression of the Cfs$^-$ phenotype. The endogenote carries all of the structural genes for making functioning flagella, and the episomes carry the *cfs* genes that confer constitutivity, i.e., that allow flagellar synthesis in the presence of catabolites and in the absence of Cya and Crp. These abilities are lost when deletions are made in the *flaI-mot* region (region II) of the episome. Thus, for example, F1950, F1943, and F1967 lost constitutivity, whereas F1900, F1970, and F1971 all retained the ability. Figure 1 shows the relative motility of some of these strains. For example, strain MS1822 harboring F1971 was motile on tryptone motility agar and tryptone motility agar plus GGM (growth was faint on some plates in Fig. 1, making motility difficult to visualize). These results suggest that the *cfs* locus maps in region II close to *flaI* and *mot*. Further evidence is derived from the two methods of isolating deletions. Two hundred type I F elements were originally isolated and characterized (7). One important feature of their isolation is that there was selective pressure for the retention of motility under conditions of catabolite repression. Not one of these deletions extended into the *flaI* gene. Selection for type II deletions had no such specific selective pressure, and many deletions extended into and through the *flaI* gene. These deletions always resulted in the loss of motility under conditions of catabolite repression. Thus, it appears likely that the *cfs* locus is very close to or in the *flaI* gene itself.

Introduction of specific flagellar gene mutations. To clarify the relationship between the *cfs* locus and the *flaI* gene, a series of specific mutations was introduced on the episome. F element 1900 was used; it carries the *cfs* mutation derived from strain MS1829 and has the *flaI*, *mot*, *flaG*, and *flaH* genes but lacks all of the region III *fla* genes. First, the episome was introduced into strains carrying mutations in

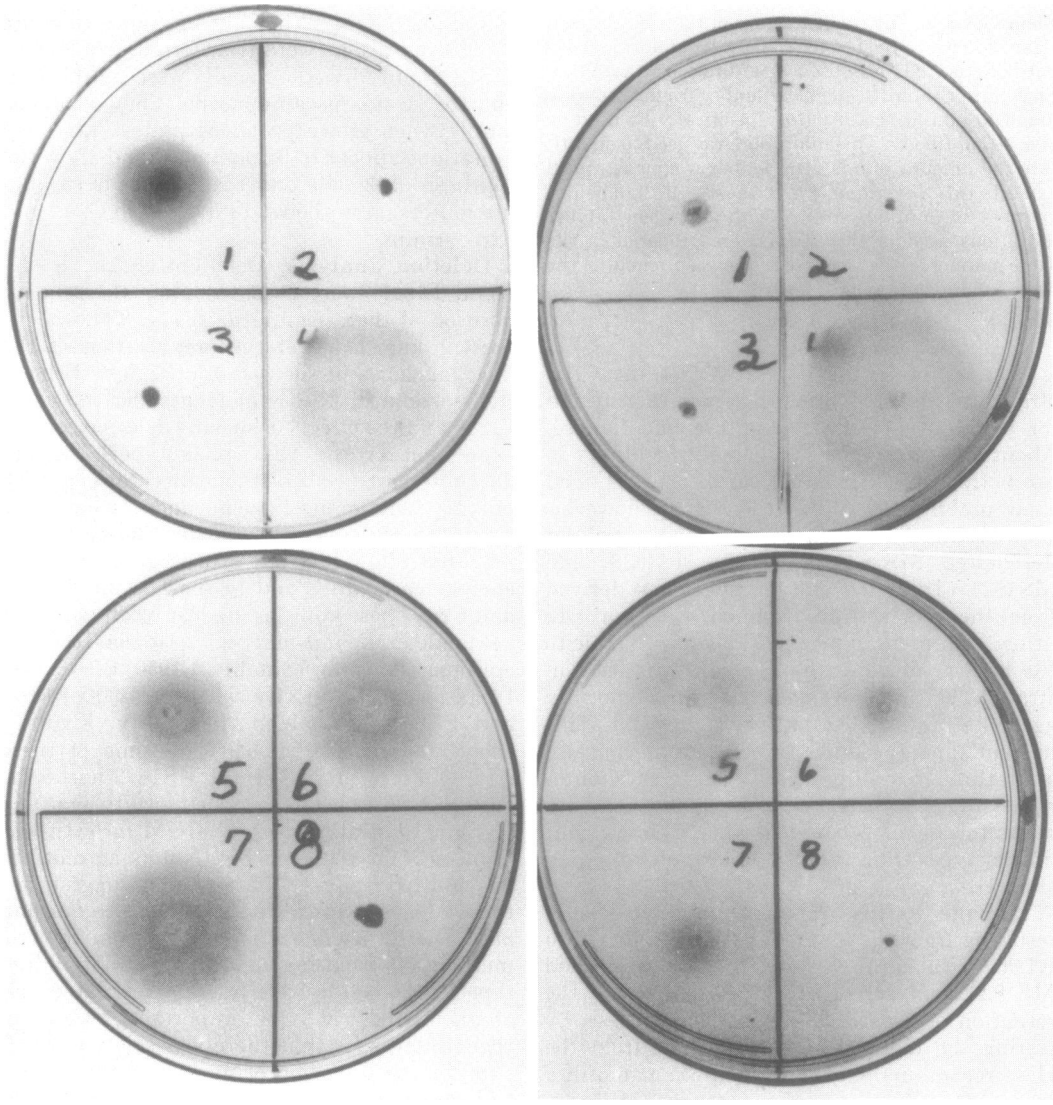

FIG. 1. *Movement of bacterial strains on tryptone motility agar (left plates) and Tryptone motility agar plus glucose, gluconate, and mannitol (right plates) after 6 h at 37 C. Bacterial strains are: (1) MS1350; (2) MS1822; (3) MS1823; (4) MS1829; (5) MS1822 harboring F1829; (6) MS1822 harboring F1900; (7) MS1822 harboring F1971; and (8) MS1822 harboring F1967.*

flaI, *mot*, or *flaH* on the endogenote (Table 3). All of these merodiploids were insensitive to catabolite repression. Thus mutations in these genes, when they are *trans* to *cfs*, have no effect on its expression. The strains were then treated with mutagen and Fla$^-$ mutants were selected. The mutations on the episomes in these strains were defined by complementation analysis. The episomes were then transferred into a RecA derivative of strain MS1350 and tested for their ability to allow the strain to swim on tryptone agar containing GGM (Table 3). Whenever the episome carried a *flaI* mutation, it lost its ability to confer resistance to catabolite repression. Mutations in the *mot* or *flaH* genes on the episome or in the endogenote had no effect. Thus, in order for the *cfs* mutation to function, it requires that the *flaI* gene in the *cis* position be intact. Further confirmation of these results was obtained by repeating this analysis with the other three mutant strains (MS1825, MS1827, and MS1828). First, the mutations were mobilized by transferring them to an F element carrying a *flaI* mutation (*flaI9216*). The F ele-

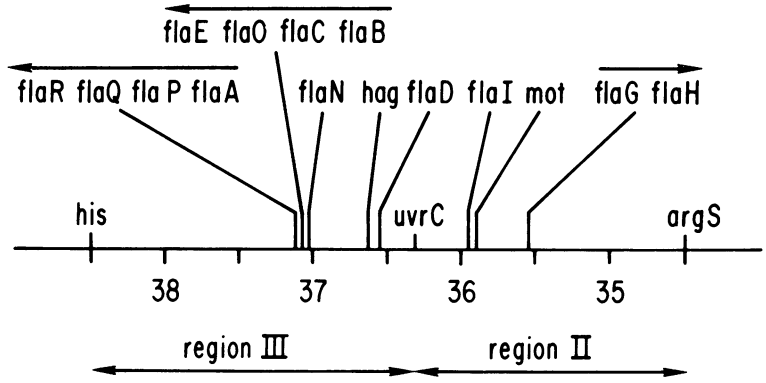

FIG. 2. *Summary of the position of flagellar genes on the E. coli genetic map. The arrows above groups of cistrons indicate organization of these cistrons into multicistronic transcriptional units with transcription in the direction shown by the arrows.*

TABLE 2. *Deletion analysis*

F element	Flagellar mutations and deletions	Recipient	Relevant mutation	Motility[a]	
				Tryptone	GGM
1338	None	1350	None	+	+
		1823	*cya*	−	−
		1822	*crp*	−	−
1829	*cfs*	1350		+	+
		1823	*cya*	+	+
		1822	*crp*	+	+
1900	*flaR,Q,P,A,E,P,C,B, N, hag, flaD*	1350		+	+
		1823	*cya*	+	+
		1822	*crp*	+	+
1970	*flaA,E,O,C,B,N, hag, flaD*	1350		+	+
		1823	*cya*	+	+
		1822	*crp*	+	+
1971	*flaB,N hag, flaD*	1350		+	+
		1823	*cya*	+	+
		1822	*crp*	+	+
1942	*flaR,Q,P,A,E,O,C,B, N, hag, flaD,I, mot, flaG,H*	1350		+	+
		1823	*cya*	−	−
		1822	*crp*	−	−
1943	*flaR,Q,P,A,E,O,C,B, N, hag, flaD,I, mot*	1350		+	+
		1823	*cya*	−	−
		1822	*crp*	−	−
1950	*hag, flaD,I, mot*	1350		+	+
		1823	*cya*	−	−
		1822	*crp*	−	−
1967	*flaR,Q,P,A,E,O,C,B, N, hag, flaD,I*	1350		+	+
		1823	*cya*	−	−
		1822	*crp*	−	−

[a] The episomes were transferred to the recipients, and the recipients were tested for motility (see Fig. 1).

TABLE 3. *Effect of flagellar mutations on motility*

F element	Flagellar mutations and deletions	Recipient	Relevant mutation	Motility	
				Tryptone	GGM
1900	*flaR,Q,P,A,E,O,C,B, N, hag, flaD*	1350		+	+
		1823		+	+
1900	*flaR,Q,P,A,E,O,C,B, N, hag, flaD*	MS616	*flaH*	+	+
1900	*flaR,Q,P,A,E,O,C,B, N, hag, flaD*	MS92	*mot*	+	+
1900	*flaR,Q,P,A,E,O,C,B, N, hag, flaD*	MS778	*flaI*	+	+
1840	*flaR,Q,P,A,E,O,C,B, N, hag, flaD, H1840*	1350		+	+
		1823		+	+
1846	*flaR ·flaD, mot1846*	1350		+	+
		1823		+	+
1852	*flaR ·flaD, flaI1852*	1350		+	
		1823		−	
1842	*flaR ·flaD, flaH1842*	1350		+	+
		1823		+	+
1847	*flaR ·flaD, mot1847*	1350		+	+
		1823		+	+
1850	*flaR ·flaD, flaI1850*	1350		+	−
		1823		−	−

ment was introduced into the strains and then transferred to strain JC1553. Clones of the repository strain were tested, and all of the strains that had acquired the ability to transfer insensitivity to catabolite repression on the episome were shown to have also lost the *flaI9216* mutation. These new F elements were used to obtain deletions, and as before the type I deletions never extended into the *flaI* gene but deleted *flaR*, *flaQ*, *flaP*, *flaA*, *flaE*, *flaO*, *flaC*, *flaB*, *flaN*, *hag*, and *flaD*. Furthermore, over 60 specific mutations were introduced on the episome and characterized. In all cases only strains carrying the *flaI* mutations on the episome lost the insensitivity to catabolite repression. We therefore conclude that all four *cfs* mutations initially isolated are similar. They can act in a dominant fashion to allow the synthesis of flagella under otherwise restrictive conditions. In order to act, however, they require the presence of an intact *flaI* gene in the *cis* position.

Synthesis of flagellar components. To understand how the synthesis of flagella is regulated by cAMP, we measured several flagellar

components under catabolite-repressing conditions. Table 4 shows the effect of growth with and without added GGM. External flagellar filaments on strain MS1350 and external polyhook filaments on strain MS1381 were measured on formalin-treated cells. The cells were grown in glycerol medium and the culture was split; half of it was resuspended in glycerol medium and the other half in GGM medium. After growth for 3 h, the cultures were harvested and the flagellar components were measured. There was a marked decrease in flagellar filament, in polyhook as well as in internal pools of hook subunit protein, and in the amber flagellin gene product. It should be noted that internal pools of intact flagellin have not been detected. However, some strains carrying amber mutations in the *hag* gene accumulate internal pools of polypeptides that are smaller than intact flagellin but still have antigenic activity and can be measured by complement fixation. The isolation, characterization, and regulation of these fragments will be described elsewhere.

Cya$^-$ derivatives of strains MS1381, MS912, and MS987 were prepared. In the Cya$^-$ strain grown in the absence of cAMP, neither *hag* amber fragment nor hook subunit could be detected (Table 5). If exogenous cAMP was added, the levels of both of these components increased markedly. There was at least a 10- to 100-fold increase in each component. The Cya$^-$ derivative of strain MS1381 behaved in a similar fashion; i.e., in the absence of added cAMP, polyhooks could not be detected either by immunochemical or electron microscope techniques. However, after growth in the presence of cAMP, polyhooks were found.

Since the levels of cAMP within the cell appear to control the synthesis of both the hook protein and the *hag* gene amber fragment, and since the *cfs* mutations make the synthesis of flagella insensitive to the levels of cAMP, we introduced the *cfs* alteration into Cya$^-$ strains listed in Table 6 to see whether hook protein and *hag* gene amber fragment synthesis would be restored even in the absence of exogenously added cAMP. Whenever an episome carrying the *cfs* mutation was introduced, both the levels of hook subunit and *hag* amber fragment increased dramatically (Table 6). In the controls where the *cfs* locus or the *flaI* gene was deleted, there was no change in the level of flagella-related polypeptides.

TABLE 4. *Effect of GGM on flagellar component synthesis*

Strain	Flagellar component measured	Amt of component (μg)	
		−GGM	+GGM
MS1350	Flagellar filament	2.9[a]	1.3[a]
MS1381	Polyhook filament	1.7[a]	0.4[a]
MS912	*hag* gene fragment	33.3[b]	14.0[b]
MS987	*hag* gene fragment	23.7[b]	10.3[b]
MS987	Internal hook subunit	0.58[b]	0.23[b]

[a] Per milliliter of culture at 10^9 cells/ml.
[b] Per milligram of protein in French press extract.

DISCUSSION

The *cfs* mutations isolated in this study were found to lie in the *his-aroD* region of the *E. coli* chromosome and could be mobilized on F elements that harbor the flagellar genes. The F elements were used to transfer these mutations into various genetic backgrounds. Thus it was possible to show that the *cfs* mutations allow flagellar synthesis to occur in Cya$^-$ strains, Crp$^-$ strains, and strains grown in the presence of catabolite-repressing substrates. These mu-

TABLE 5. *cAMP control of flagellar component synthesis*

Strain	Flagellar component	Amt of component (μg/ml of protein in cell extract)	
		+cAMP	−cAMP
MS1871	*hag* gene amber fragment	10.6	<0.1
MS1872	*hag* gene amber fragment	24.0	<0.1
MS1871	Internal hook subunit	1.6	<0.05
MS1872	Internal hook subunit	0.5	<0.05

TABLE 6. *Influence of fla1829 on the production of flagellar components in merodiploid strains*

Strain	Genotype[a]		Flagellar component	Amt of component (μg/mg of protein in cell extract)
	Exogenote	Endogenote		
MS1873	F1900	1871	*hag* gene fragment	2.1
MS1875	F1950	1871	*hag* gene fragment	<0.1
MS1876	F1971	1871	*hag* gene fragment	2.4
MS1877	F1900	1872	*hag* gene fragment	19.6
MS1878	F1943	1872	*hag* gene fragment	<0.1
MS1879	F1955	1872	*hag* gene fragment	<0.1
MS1880	F1971	1872	*hag* gene fragment	33.0
MS1877	F1900	1872	Hook subunit	0.6
MS1878	F1943	1872	Hook subunit	<0.05

[a] See Table 1 for the explanation of the genotype of the endogenote and Table 2 for the genotype of the exogenote.

tations behave in a *trans*-dominant fashion; i.e., they confer insensitivity to catabolite repression when they are introduced into RecA wild-type strains. Deletion analysis showed that the mutations isolated were in or very near to the *flaI* gene. These mutations were furthermore related to the expression of the *flaI* gene.

Biochemical studies showed that two flagellar components, the hook protein subunit and the *hag* gene amber fragment, are sensitive to the levels of cAMP in the cell. Transfer of F elements carrying the *cfs* mutations to Cya$^-$ strains resulted in the synthesis of the hook subunit and the *hag* gene amber fragment.

These observations, taken together, suggest to us the following mechanism for the regulation of flagellar synthesis. (i) The *flaI* gene is part of a catabolite-sensitive operon for the flagellar system. The cAMP receptor protein in the presence of cAMP modulates the transcription of the flaI gene. (ii) The product of the *flaI* gene serves a "management" function by exerting a positive controlling effect on the expression of other flagellar genes such as the *hag* gene. Thus, when cAMP levels are reduced or the cAMP receptor protein is defective, the *flaI* gene is transcribed slowly and, therefore, little or none of the *flaI* product or *hag* amber product is made. Mutations like *cfs* release the transcription of the *flaI* gene from the requirement for the presence of cAMP and Crp. This mechanism is analogous to the one proposed for the lactose operon where promoter mutations release transcription of that operon from the requirement for cAMP and Crp (3, 10). However, the flagellar system is more complex since there are at least 20 genes necessary for flagellar formation and function. These are separated into at least six different operon-like units. We can imagine that the *flaI* gene product acts as promoter in all of these units or, more probably, that it activates one of these units which in turn produces a regulator for the next unit, and so on in a cascade fashion.

We do not know the product of the *flaI* gene, and since the specific mapping experiments necessary to define a promoter region have not yet been done, the explanation for the nature of the *cfs* mutation is only hypothetical. As might be expected from the above mechanism, *flaI* mutations that result in strains with a Fla$^-$ phenotype do produce the hook subunit protein. In fact, of the many classes of flagellar mutants that we have examined, only all of the strains with *flaI* gene mutations lack the hook subunit protein (Silverman and Simon, manuscript in preparation). Therefore, the *flaI* gene may specify a structural protein, the hook subunit, and the management function of the *flaI* gene product need not act only at the level of transcription of other flagellar genes. Determination of the identity of the *flaI* gene product should precede further speculation about the influence of the *flaI* gene product on the synthesis of flagellar components.

The finding of strains that show good motility on agar plates even in Cya$^-$ strains suggests that cAMP may not be necessary for chemotaxis. This suggestion is currently being tested.

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