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NOTES

Requirement of Adenosine 3′, 5′-Cyclic Phosphate for Flagella Formation in *Escherichia coli* and *Salmonella typhimurium*

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Adenosine-3′,5′-cyclic phosphate (cyclic AMP) is absolutely required for flagella formation and, hence, motility in cyclic AMP-deficient mutants of *Escherichia coli* and *Salmonella typhimurium*.

Adenosine-3′5′-cyclic phosphate (cyclic AMP) is involved as an intracellular messenger in the mediation of a variety of hormonal effects in higher animals (6). Its role in bacterial physiology has been revealed by its ability to counteract catabolite repression (10), its requirement for the induction of a variety of enzymes (3), and its apparent necessity for the initiation of transcription at the promoter site (2, 9). Further confirmation of some of these effects was obtained with the isolation of an adenyl cyclase-deficient mutant of *Escherichia coli* by Perlman and Pastan (11) This mutant showed a pleiotropic deficiency. in the utilization of a variety of carbohydrates which could be phenotypically corrected by cyclic AMP. We have independently isolated similar mutants and find that cyclic AMP is not only necessary for the restoration of sugar fermentation, but is also absolutely required for the formation of flagella and hence motility.

Cyclic AMP-deficient mutants of *E. coli* were isolated from HfrH and HfrC strains as follows. Overnight cultures in Penassay Broth (Difco) were treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (150 μg/ml) by the method of Mise and Suzuki (8). Mutants pleiotropic for utilization of sugars as carbon sources and tryptophan as a nitrogen source were selected by penicillin treatment in a minimal salts medium (M-9) containing lactose, arabinose, and maltose as carbon sources and tryptophan as the only nitrogen source. After overnight incubation, the survivors were spread on nutrient agar plates containing triphenyltetrazolium chloride (50 μg/ml) and 0.5% each of lactose, arabinose, and maltose. Red (fermentation-negative) mutants which appeared after 24 hr of incubation were purified on the same media and tested for their sugar fermentation patterns by using EMB agar plates and bromocresol purple semisolid agar tubes (Difco broth base containing 0.3% agar) supplemented with 1% of the various sugars, with and without 10^{-3} M cyclic AMP (Sigma Chemical Co.). Mutants responding to cyclic AMP and having little adenyl cyclase activity have been assigned the genotype *cya*. The *cya* locus was found to map near *met E* (*manuscript in preparation*). A *cya* mutant of *Salmonella typhimurium* strain DB99 was found by similar fermentation screening of the unclassified pleiotropic sugar-negative mutants in the collection of David Berkowitz. This mutant had been isolated by Berkowitz from strain SL751, a multiauxotrophic, nonmotile mutant carrying a *flaA* mutation. For subsequent flagellar antigen analyses, it was necessary to convert this to *fla*$^+$. Strain GP8 (*fla*$^+$ *cya*) was obtained by transducing the *fla*$^+$ allele into strain DB99 using the selection method described by Stocker (12) and P22 transducing phage grown on strain *metB23*, a *fla*$^+$ donor. Strain CA7902 is a *cya* mutant of *E. coli*, isolated by J. Beckwith and obtained from him after our own isolations and characterizations were completed.

Among the pleiotropic sugar mutants isolated, strain GP1, derived from *E. coli* HfrH, shows a typical *cya* phenotype and the most marked deficiency in adenyl cyclase. In the absence of cyclic AMP, it is completely negative for the

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utilization of lactose, arabinose, maltose, mannitol, xylose, galactose, and glycerol, but positive for glucose. All sugars are strongly fermented in the presence of 10^{-3} M cyclic AMP. The *Salmonella* mutant, strain DB99, shows the same pattern except that arabinose utilization does not require cyclic AMP and it is naturally lactose-negative.

The effect on motility was discovered during scoring of the fermentation results in the semisolid stab cultures. It was immediately evident that the growth of the mutant was completely restricted to the line of the stab, whereas that of the wild type, or the mutant grown in cyclic AMP, swarmed through the semisolid medium. This effect was confirmed using Stocker's semisolid agar-gelatin motility medium (12). Figures 1 and 2 show the effect and Table 1 summarizes the results. The effect was obtained with all of the *cya* mutants at cyclic AMP concentrations higher than 10^{-5} M. Related compounds, such as adenosine triphosphate, adenosine diphosphate, AMP, adenosine, cyclic guanosine monophosphate, and dibutyryl cyclic AMP, at concentrations of 10^{-3} M had no effect on either motility or fermentation. Electron microscopy showed that the lack of motility was due to an absence of flagella rather than a paralysis, since no flagella could be found on hundreds of cells (strain GP1) cultured in the absence of cyclic AMP. The same strain cultured in the presence of cyclic AMP showed on the average of four flagella per cell (Fig. 3).

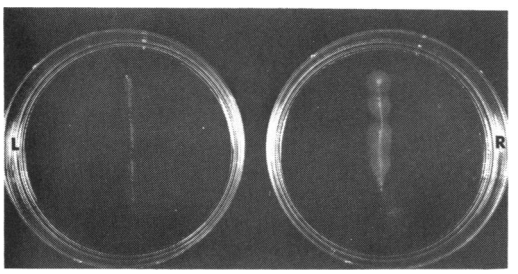

FIG. 1. *Motility of E. coli GP1 (fla+; cya) on gelatin-semisolid agar plates. Plate on left does not contain cyclic AMP; plate on right contains 1 mM cyclic AMP.*

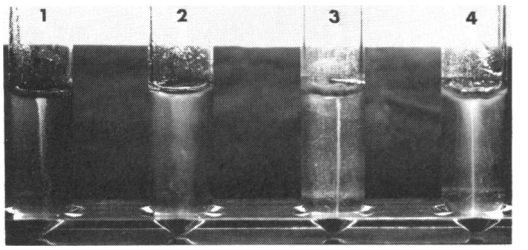

FIG. 2. *Motility of E. coli GP1 (fla+; cya) and its recombinant GP7 (fla+; cya) by stab culture method in a gelatin-semisolid agar medium. (Strain GP7 is a recombinant between E. coli GP1 and E. coli AB1157, a polyauxotrophic F strain (pro, his, thi, cya, strr.) Tube 1: strain GP1, no cyclic AMP in medium; Tube 2: strain GP1, plus cyclic AMP (1 mM); tube 3: strain GP7, no cyclic AMP in medium; tube 4: strain GP7, plus cyclic AMP (1 mM).*

Salmonella flagella were further analyzed by means of antigenic analysis and susceptibility to the flagellotropic "*chi*" phage (7). Agglutination was measured in small tubes after treating the cells with 0.3% Formalin and adding *Salmonella* H-factor antisera (Difco), phase 1 ("i") and phase 2 ("2"). Susceptibility to *chi* phage was determined by the overlay method using L-agar (5). The results obtained with strain GP8 are

TABLE 1. *Cyclic adenosine monophosphate (AMP) requirement for motility of cyclic AMP-deficient mutants (cya) of Escherichia coli and Salmonella typhimurium*

Organism	Strain	Genotype[a]		Motility[b] at cyclic AMP concentration (M) of				
		fla	*cya*	10^{-3}	10^{-4}	10^{-5}	10^{-6}	0
E. coli	Hfr-H	+	+	+	+	+	+	+
E. coli	GP1	+	−	+	+	±	−	−
E. coli	CA7902	+	−	+	±	−	−	−
S. typhimurium	met B23[c]	+	+	+	+	+	+	+
S. typhimurium	DB99	−	−	−	−	−	−	−
S. typhimurium	GP8	+	−	+	+	+	−	−

[a] Gene controlling flagella formation *fla*; gene for cyclic AMP requirement, *cya*.

[b] Motility determined by swarming stab culture semisolid agar-gelatin medium. The following chemicals were negative at a concentration of 10^{-3} M: adenosine, AMP, adenosine diphosphate, adenosine triphosphate, cyclic guanosine monophosphate, and dibutyryl cyclic AMP.

[c] *S. typhimurium* metB23 is the donor strain for the transduction of *fla*+ producing GP8 (*fla*+; *cya*) from DB99 (*flaA*; *cya*)

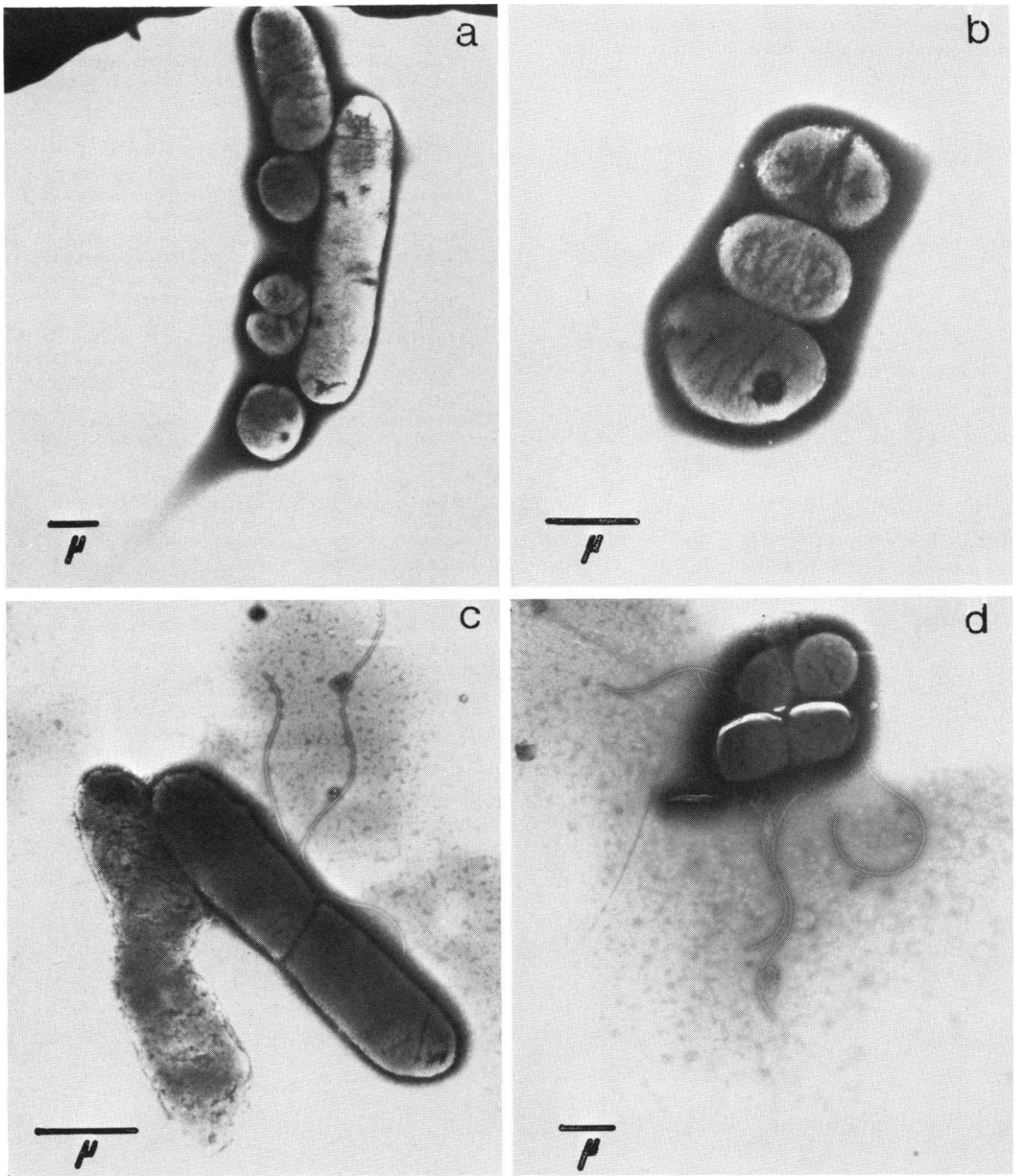

FIG. 3: *Electron micrographs of E. coli GP1 (negative stain). (a and b) Grown without cyclic AMP; (c and d) grown with 1 mM cyclic AMP.*

shown in Table 2. No flagellar antigens could be detected when the mutant was cultured in the absence of cyclic AMP, but after 150 min of incubation under growing conditions with 10^{-3} M cyclic AMP, phase 1 flagella antigen was detected. Phase 2 antigen was not detected. The wild type, *cya*$^+$, which is also the *fla*$^+$ donor used to construct strain GP8 showed both antigens, even without cyclic AMP. Susceptibility of the mutant to flagella phage *chi* could be demonstrated only when it had been both subcultured and plated with the phage in the presence of cyclic AMP. The *cya* locus maps near *metE* (*manuscript in preparation*) and hence cannot correspond to six of the known *fla* genes (A to F) since their chromosomal locations are known to map far

TABLE 2. *Effect of cyclic adenosine monophosphate (AMP) on production of flagella antigen and susceptibility to chi phage in parent and cya mutant strains of Salmonella typhimurium*

Strain	Cyclic AMP in subculture	H antigen agglutination		Efficiency of plating of *chi* phage[a]
		Phase 1	Phase 2	
met B23 (*fla*$^+$; *cya*$^+$)	None	+	+	1.0
	1 mM	+	+	1.0
GP8 (*fla*$^+$; *cya*$^-$)	None	−	−	$<1.0 \times 10^{-7}$
	1 mM	+	−	9.1×10^{-2}

[a] Efficiency of plating of *chi* phage was determined in L-agar medium containing 1 mM cyclic AMP in semisolid overlay.

from this site (4). The location of *flaG* is unknown (4) and it remains to be determined whether this gene is similar to *cya*.

Cyclic AMP is not required for the integrity of another bacterial appendage, the F pilus, as shown by the fact that the Hfr *cya* strain GP1 can still serve as a normal donor in conjugation and is still sensitive to male-specific phage R-17 in the absence of cyclic AMP.

Exactly how cyclic AMP functions in the formation of bacterial flagella remains to be answered. Its known role in the formation of inducible enzymes would suggest that it is probably involved in the formation of flagellar protein rather than polymerization. In view of the role of cyclic AMP in counteracting catabolite repression (10), it is of special interest to note that Adler and Templeton (1) found that the presence of glucose in a chemically defined growth medium prevented the synthesis of flagella in *E. coli*.

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