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Adenosine 3',5'-Cyclic Monophosphate-Deficient Mutants of *Vibrio cholerae*

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Two adenosine 3',5'-cyclic monophosphate (AMP)-deficient mutants of *Vibrio cholerae* (biotype El Tor) were successfully isolated by nitrosoguanidine treatment followed by penicillin screening for pleiotropic sugar-negative clones. Exogenous cyclic AMP is required for the fermentation of sucrose, trehalose, fructose, maltose, and mannose but not of glucose, as well as for the formation of normal flagella and specific somatic antigens. A striking characteristic of the mutants is their growth behavior at higher temperatures. They cannot grow on TCBS selective plates at 37 C or higher unless they are provided with a supply of exogenous cyclic AMP, although they are capable of producing colonies on the same medium, even without cyclic AMP, at temperatures lower than 30 C. Since the mutants are converted to spheroplasts, spindle forms, and spiral filaments in cyclic AMP-free media at 37 C, and this phenomenon is stopped by the addition of cyclic AMP or a combination of 20% sucrose and 0.2% magnesium chloride, it is assumed that cyclic AMP is essential for the synthesis of the cell wall of *V. cholerae* at higher temperatures.

It is well understood that adenosine 3',5'-cyclic monophosphate (cyclic AMP) is an important intracellular component for metabolic regulation in both eukaryotic and prokaryotic cells. In higher animals, the role of cyclic AMP is revealed by its ability to activate protein kinases (8, 25), resulting in successful changes in various enzyme activities in the mediation of hormonal effects (20) and in other cellular functions (11, 19).

In bacteria, cyclic AMP controls gene activation. Recent papers indicate that cyclic AMP is necessary not only for the initiation of the transcription of adaptive enzymes (5, 16, 27), but also for the formation of flagella (26) and lysogenization of temperate phages in *Escherichia coli* and *Salmonella typhimurium* (9, 10).

On the other hand, it was reported that an application of cholera enterotoxin to the lumen of the small intestine of animals activated adenylate cyclase in mucosal epithelial cells, resulting in a stimulated fluid production to the lumen (12, 22), although nothing is known about the role of cyclic AMP in the cholera vibrio itself. This paper deals with isolation and characterization of cyclic AMP-deficient mutants of *Vibrio cholerae* (biotype El Tor).

MATERIALS AND METHODS

Bacteria. *V. cholerae* E1 and E2, both of which are biotype El Tor, were isolated in the Philippines in 1964 and kept in our laboratories as stab cultures in diluted, semisolid nutrient agar tubes at room temperature with occasional subculture.

Media. L broth (15), peptone-water (10 g of polypeptone, 5 g of sodium chloride, and 1,000 ml of distilled water, adjusted to pH 6.0, 7.0, or 8.0 with potassium hydroxide solution), and M9 medium (4) were used as liquid media. Nutrient agar (Eiken Chemical Co., Ltd., Tokyo, Japan), TCBS agar (Eiken; Oxoid Ltd., London, England, 6), and BTB-sugar agar (nutrient agar, 0.002% bromothymol blue, and 1% appropriate sugar) were employed as plating media. TTC-double sugar agar, which was used for counterselection of pleiotropic sugar-negative mutants, was prepared by supplementation of sterilized nutrient agar with sterile solutions of triphenyltetrazolium chloride, maltose, and mannose to give final concentrations of 50 μg/ml, 1%, and 1%, respectively, followed by heating at 100 C for 30 min. Bromocresol purple semisolid agar (Eiken) containing 1% sugar and blood agar containing 5% sheep erythrocytes were used for the sugar fermentation test and hemolysin production assay, respectively.

Chemicals. Cyclic AMP, dibutyryl cyclic AMP, 2'-*O*-monobutyryl cyclic AMP, and [^{6}N]monobutyryl cyclic AMP were provided by the Asahi Chemical Industry Co., Ltd., Nobeoka, Japan. Crystalline penicillin G was a gift from Shionogi Pharmaceutical Co., Ltd., Osaka, Japan. Pararosanilline hydrochloride and pararosanilline acetate, employed for the flagella stain, were products of Eastman Kodak Co., Ltd., Rochester, N.Y. [α-^{32}P]adenosine triphosphate, used for the adenylate cyclase assay, was purchased from the Radiochemical Centre, Amersham, England.

Mutagenesis and isolation of cyclic AMP-deficient mutants. Nitrosoguanidine treatment was carried out by a method described previously (26). *V. cholerae* E1 and E2 were cultured overnight at 37 C with shaking in M9 minimal medium (pH 7.2) containing 1% maltose and 1% mannose as carbon sources. The cells harvested by centrifugation were washed twice with, and resuspended in, a 0.5 volume of 0.1 M citrate buffer (pH 5.5), and treated with 25 μg of nitrosoguanidine per ml at 37 C for 60 min without shaking. The phenotypic expression of mutations was allowed by culturing the treated cells in L broth containing 1% maltose and 1% mannose at 37 C for 90 min with shaking. The cells were again spun down, washed twice with, and resuspended in, 5 volumes of M9 medium containing maltose and mannose, and incubated at 37 C for 240 min to eliminate the endogenous nutrients of the auxotrophs. Afterward, 50 μg of penicillin G was added per ml, and pleiotropic sugar-negative clones were enriched by overnight incubation at 37 C with shaking. On the next day, the cells were washed twice with, and resuspended in, drug-free M9 medium and spread on the TTC-double sugar plates. Red (fermentation negative) colonies that appeared 24 h after the incubation period were purified on the same medium and streaked on BTB-agar containing 1% maltose and 1% mannose, on which a paper disk soaked in a 0.1 M aqueous solution of cyclic AMP was placed. Further sugar fermentation patterns of the mutants were tested by the stab culture method, using bromocresol purple semisolid agar tubes with and without cyclic AMP. Indol production was tested by the cholera red method (17), and hemolysin production was assayed with blood agar plates.

Flagella stain. The parental *V. cholerae* E2 strain and its cyclic AMP-deficient mutants E2511 and E2751 were cultured in L broth with or without 2 mM cyclic AMP at 27 C for 6 h without shaking. The cells were spun down after the addition of 0.1 volumes of neutralized formaline and washed three times with distilled water. The cells were then fixed on a glass slide, stained by the method of Leifson (14), and examined with a microscope.

Test for somatic antigens. The test vibrios were cultured overnight in L broth (pH 7.2) with or without 1 mM cyclic AMP at 27 and 37 C without shaking. Volumes (1 ml) of homogeneous cultures were transferred into small test tubes after being heated in boiling water for 120 min and cooled rapidly. A 0.1-ml volume of diluted anti-cholera O factor serum (Ogawa and Inaba types, Toshiba Chemical Co., Ltd., Tokyo, Japan) was added to each tube and incubated at 30 C for 120 min, and the O-agglutination was scored macroscopically.

Test for the temperature-sensitive growth of cyclic AMP-deficient mutants of V. cholerae. To avoid contamination by *cya*$^+$ revertants, the cyclic AMP-deficient mutants E2511 and E2751 were subcultured on TCBS selective plates (Oxoid) free from cyclic AMP at 27 C for 24 h and confirmed to be pure clones. The cells were scraped with a wire loop and suspended in sterile peptone-water (pH 8.0) to give a Klett reading of 10 (ca. 10^8 cells per ml) using a no. 66 filter (Klett Manufacturing Co., Ltd., New York). The suspension was diluted 100-fold with fresh peptone-water. Volumes (0.1 ml) of diluted suspension were inoculated into test tubes containing 5 ml of peptone-water (pH 8.0) with or without 1 to 10 mM cyclic AMP, 20% sucrose, and/or 0.2% magnesium chloride. The growth of each tube was measured turbidometrically 18 h after the incubation at 27, 30, 33, 37, and 42 C by employing a multiple water bath-shaking incubator (model Monomal Shaker NT, Taiyo Co., Ltd., Tokyo, Japan).

RESULTS

Isolation and characterization of cyclic AMP-deficient mutants of V. cholerae. The viable cell number of *V. cholerae* E2 was decreased from 1.9×10^9/ml to 2.8×10^8/ml by the nitrosoguanidine treatment. A subsequent decrease to 1.6×10^6 cells per ml was observed after penicillin screening. About 500 subclones which appeared on the TTC-double sugar plates as red colonies were purified and streaked on BTB-agar plates containing maltose and mannose, on which paper disks immersed in a 0.1 M solution of cyclic AMP were placed.

Thus, two subclones, designated E2511 and E2751, were obtained from *V. cholerae* E2 as cyclic AMP-responding mutants (Fig. 1). The mutants produced large yellow colonies (sugar fermentation positive) around the disk and small colorless colonies (fermentation negative) at other locations. Additional sugar fermentation patterns were examined with bromocresol purple semisolid agar tubes with or without 1 mM cyclic AMP. Although both mutants were incapable of utilizing any sugar other than glucose in the absence of cyclic AMP, they fermented sucrose, maltose, mannose, trehalose, and fructose in addition to glucose in the presence of cyclic AMP. These results coincide with the sugar fermentation patterns of adenylate cyclase-deficient mutants of *E. coli* and *S.*

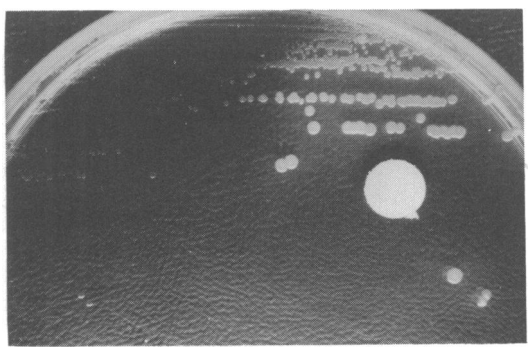

FIG. 1. *Cyclic AMP-deficient mutant of V. cholerae (biotype El Tor) E2751 on a BTB-maltose-mannose plate containing a paper disk soaked in 0.1 M aqueous solution of cyclic AMP.*

typhimurium with few exceptions. Furthermore, a preliminary experiment on adenylate cyclase mutants by the method of Tao (24) revealed that the mutants retained much lower activity than the parent strain. Accordingly, one can assume that the above-described cyclic AMP-responding clones of *V. cholerae* are adenylate cyclase-deficient mutants. Since spontaneous reverse mutations occurred with a frequency of approximately 10^{-7} per cell after 18-h of culture in cyclic AMP-free media, it was postulated that the mutants possessed point mutations in the *cya* gene.

Flagella formation and cyclic AMP in cyclic AMP-deficient mutants of V. cholerae. During scoring of sugar fermentation paterns, it was immediately evident that the growth of the mutants was completely limited to the line of the stab culture in the cyclic AMP-free medium, whereas those of parent strain and of the mutants grown with 1 mM cyclic AMP swarmed through the semisolid medium. This indicates that cyclic AMP is indispensable for the expression of *V. cholerae* motility just as observed in *E. coli* and *S. typhimurium* (26). The flagella stain was performed, by the Leifson method (14), on the parent (*cya*$^+$) and mutant (*cya*) strains grown in L broth with and without cyclic AMP for a rather short period at 27 C. The parent cholera vibrios possessed polar flagella regardless of the presence of exogenous cyclic AMP, whereas the mutants produced no flagella, or sometimes straight ones, in the absence of cyclic AMP and normally waved flagella in the presence of 2 mM cyclic AMP (Fig. 2).

Requirement of cyclic AMP for production of specific somatic antigens. The cyclic AMP-deficient mutants of *V. cholerae* produced neither Ogawa- nor Inaba-type somatic antigen in cyclic AMP-free media at 27 or 37 C. The mutants grown with 1 mM cyclic AMP, however, were confirmed to possess the Ogawa-type antigen, which was the specific somatic antigen of their parent, regardless of culture temperatures (Table 1).

It was almost impossible to perform a reliable slide agglutination test with the cells grown on agar plates not containing cyclic AMP, because those cells were spontaneously agglutinable.

As indicated in Table 2, the mutants also required exogenous cyclic AMP for production of indole (induction of tryptophanase) and hemolysin.

Thermosensitive growth of cyclic AMP-deficient mutants of V. cholerae. The cyclic AMP-deficient mutants of *V. cholerae* could form colonies on TCBS selective plates free from cyclic AMP at 27 C but not at 37 C,

TABLE 1. *Antigenicity of cyclic AMP-deficient mutants of V. cholerae grown with and without 1 mM cyclic AMP*

Culture temp (C)	O-agglutination			
	With cyclic AMP		Without cyclic AMP	
	To anti-Ogawa	To anti-Inaba	To anti-Ogawa	To anti-Inaba
27	+++	–	–	–
37	+++	–	–	–

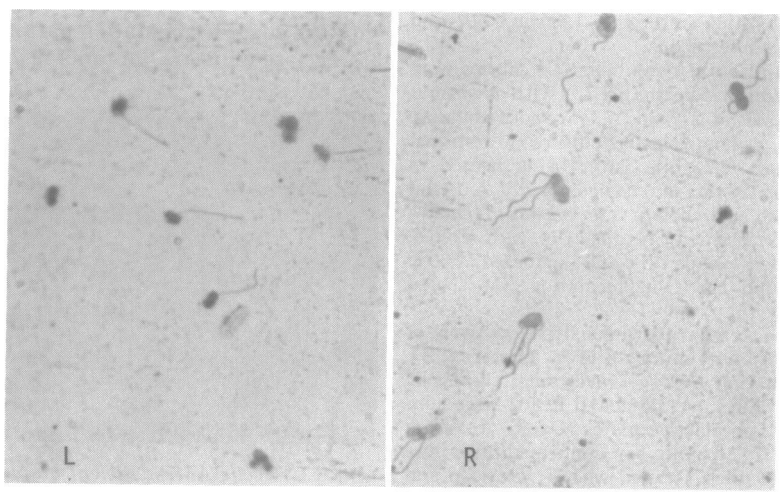

FIG. 2. *Flagella stain of the cyclic AMP-deficient mutant of V. cholerae E2751. L, Grown in L broth not containing cyclic AMP; R, grown in L broth containing 2 mM cyclic AMP.*

TABLE 2. *Pleiotropy of cyclic AMP-deficient mutants of V. cholerae (biotype El Tor)*

Strain	Sugar fermentation		Other phenotypes
	Require cyclic AMP	Not require	Require cyclic AMP
E2511	Sucrose, maltose, mannose, fructose, trehalose	Glucose	Flagella formation, specific O-antigen, indole production, hemolysin production (?)
E2751	Sucrose, maltose, mannose, fructose, trehalose	Glucose	Flagella formation, specific O-antigen, indole production, hemolysin production (?)

although they were capable of growing either at 27 or 37 C on the same medium supplemented with cyclic AMP (Fig. 3). The growth of the mutants was also sparse at 37 C on nutrient agar plates or in peptone-water not containing cyclic AMP. The mutant cells were converted to spheroplasts, spindle forms, and spiral filaments under such conditions (Fig. 4); however, they retained normally curved vibrio forms at 27 C with and without cyclic AMP and at 37 C with cyclic AMP. Addition of exogenous cyclic AMP and different culture temperatures did not influence the morphology of the parent (*cya*$^+$) vibrio. The temperature-dependent requirement of cyclic AMP for the normal vibrio form was influenced markedly by hydrogen ion concentrations in the media. The most drastic morphological changes were observed at pH 8.0, moderate changes were observed at pH 7.0, and the least drastic changes were observed at pH 6.0 (Fig. 5).

Table 3 presents generation times and cell morphologies of the mutants grown in peptone-water (pH 8.0) with and without 1 mM cyclic AMP at various temperatures. The increase in the generation time and abnormality of cell form became more prominent in media without cyclic AMP as culture temperatures increased.

Figure 6 indicates the influence of various amounts of cyclic AMP on the growth of mutants in peptone-water (pH 8.0). The mutants could grow only at low temperatures (around 30 C) in the absence of cyclic AMP when a small inoculum (ca. 2.0×10^4 cells per ml) was employed, although growth at higher temperatures was possible if increased amounts of exogenous cyclic AMP were provided. The mutants could grow even at 42 C when the medium was supplemented with 10 mM cyclic AMP.

Cyclic AMP was replaced with a combination of sucrose and magnesium chloride (Fig. 7). The mutants were capable of multiplying at temperatures up to 42 C in the medium supplemented with 20% sucrose and 0.2% magnesium ion but not with cyclic AMP. In this case, the effect of exogenous cyclic AMP was negligible. Not only the combination of sucrose and magnesium ion but also each compound alone could replace cyclic AMP to some extent (Fig. 8). Contrary to the cyclic AMP-deficient mutants, the wild-type *cya*$^+$ *V. cholerae* E2 could grow in a wide range of culture temperatures without cyclic AMP or osmotic stabilizers (Fig. 9). It was thus postulated that cyclic AMP is an essential compound for the cell wall synthesis of *V. cholerae*, especially at high temperatures.

Response to cyclic AMP derivatives of cyclic AMP-deficient mutants of V. cholerae. Cyclic AMP-deficient mutants of *V. cholerae* responded similarly to cyclic AMP, 2′-*O*-monobutyryl cyclic AMP, [^{6}N]monobutyryl cyclic AMP, and 2′-*O*-[^{6}N]dibutyryl cyclic AMP in their sugar fermentation, flagella formation, production of specific somatic antigens, and temperature-sensitive growth. Biological activity of dibutyryl cyclic AMP, however, was somewhat lower than other derivatives. It was suggested that *V. cholerae* could convert dibutyryl cyclic AMP to monobutyryl cyclic AMPs or cyclic AMP, because dibutyryl cyclic AMP has been known to be biologically inactive for adenylate cyclase-deficient mutants of *E. coli* and *S. typhimurium* (26), for stimulation of in vitro transcription of the *gal* operon of *E. coli* (1), and for activation of mammalian protein kinases (3).

DISCUSSION

The role of cyclic AMP has been well estab-

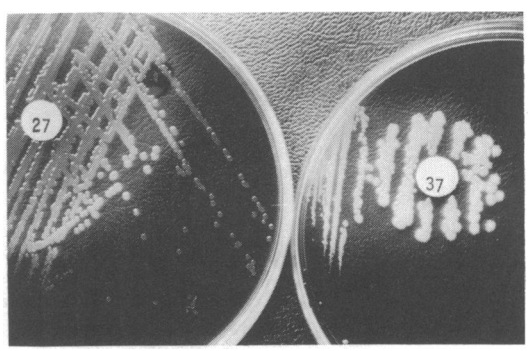

FIG. 3. *Colonies of the cyclic AMP-deficient mutant of V. cholerae E2751 on TCBS selective plates containing paper disks immersed in 0.1 M cyclic AMP. 27: Incubated at 27 C for 24 hours. 37: Incubated at 37 C for 24 hours.*

lished in the regulatory mechanism of adaptive enzymes of *E. coli* and *S. typhimurium* (5, 16, 27), although only a little knowledge has been accumulated on other functions of this novel compound (9, 10, 26) and almost nothing is known for species other than the *Enterobacteriaceae*. We compare here the pleiotropic phenotype of cyclic AMP-deficient mutants of *V. cholerae* to those of *E. coli* and *S. typhimurium*. A summary of biological characteristics of cyclic AMP-deficient mutants of various bacteria is listed in Table 4.

For sugar fermentation of enteric bacilli, cyclic AMP is usually not required for utilization of glucose, fructose, and trehalose, which are considered to be fundamental energy sources of organotropic bacteria. Since cyclic AMP is a key substance for production of adaptive enzymes, it may be assumed that enzymes involved in such basic metabolism are constitutively produced or that each step is doubly controlled by both constitutive and adaptive fractions of an enzyme. Kundig and Roseman (13) reported that enzyme IIs of the phosphotransferase system of *E. coli* were sugar-specific membrane components, most of which were inducible (except those for glucose, fructose, and mannose, which seemed to be constitutive). We, however, recently isolated a new adenylate cyclase mutant from *E. coli* R1 (*metB*, Hfr) which lacked the constitutive but not the adaptive fraction of enzyme II for glucose, and found that this mutant fermented glucose only in the presence of exogenous cyclic AMP (Table 4; T. Yokota and S. Kuwahara, manuscript in preparation). From these results,

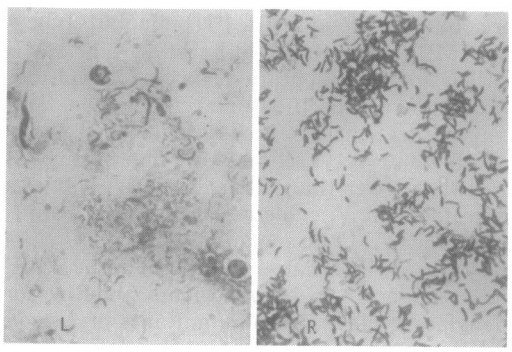

FIG. 4. *Cell morphology of the cyclic AMP-deficient mutant of V. cholerae E2751 grown at 37 C on nutrient agar plates (pH 8.0). L, Grown without cyclic AMP; R, grown with 5 mM cyclic AMP.*

TABLE 3. *Growth behavior of the cyclic AMP-deficient mutant E2751 of V. cholerae in peptone-water (pH 8.0) at various temperatures in the presence and absence of cyclic AMP*[a]

Culture temp (C)	Generation time (min)		Cell morphology	
	With 1 mM cAMP	Without cAMP	With 1 mM cAMP	Without cAMP
27	180	210	Normal	Normal
30	96	120	Normal	Mix[b]
33	80	190	Normal	Filament
37	140	>500	Normal	Spheroplast
42	>500		Filament	Ghost

[a] Inoculum size, 2.0×10^6 cells per ml.

[b] Normal vibrio forms were mixed with several filamentary cells.

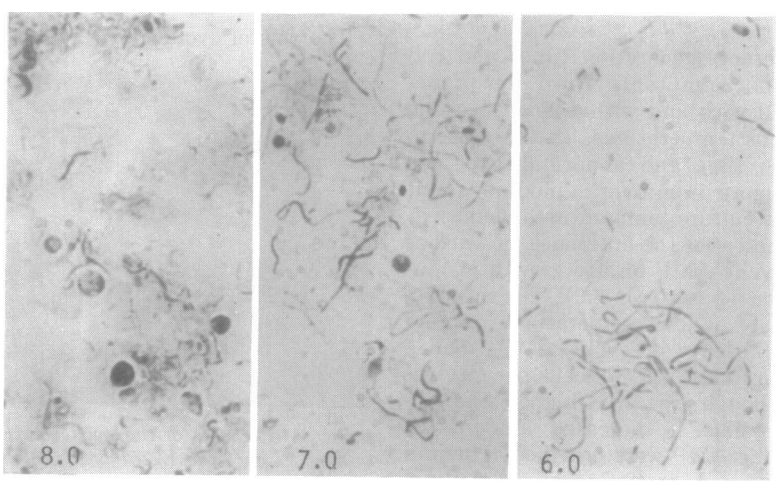

FIG. 5. *Cell morphology of the cyclic AMP-deficient mutant of V. cholerae E2751 grown in peptone-water at various pHs and free from cyclic AMP. Culture temperature, 37 C. Numbers indicate pH of peptone-water.*

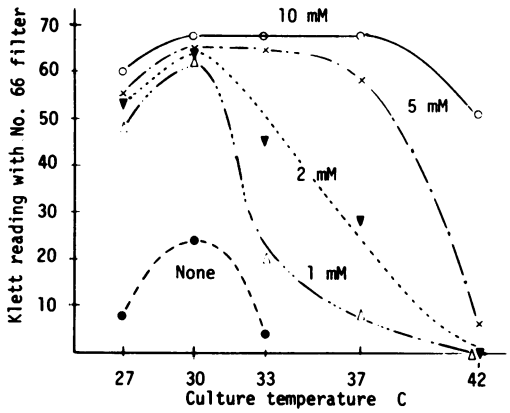

FIG. 6. *Growth turbidity of V. cholerae E2751 (cya) at various temperatures in peptone-water (pH 8.0) supplemented with various amounts of cyclic AMP. Inoculum size, 2.0 × 10⁴ cells per ml. Figures indicate concentrations of cyclic AMP added.*

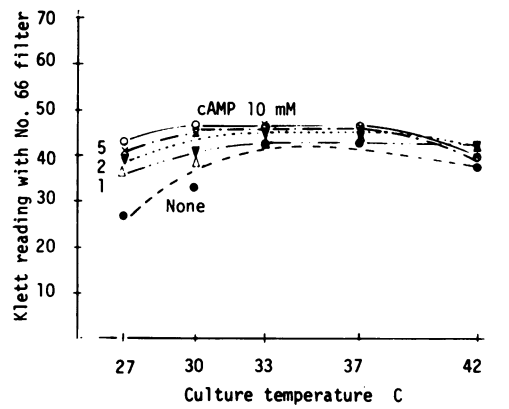

FIG. 7. *Growth turbidity of V. cholerae E2751 (cya) at various temperatures in peptone-water (pH 8.0) supplemented with 20% sucrose, 0.2% magnesium chloride, and various amounts of cyclic AMP. Inoculum size, 2.0 × 10⁴ cells per ml. Figures indicate concentrations of exogenous cyclic AMP.*

it is suggested that at least the glucose utilization pathway of *E. coli* is operated by the doubly controlled system. Unlike *E. coli* and *S. typhimurium*, cyclic AMP-deficient mutants of *V. cholerae* can ferment only glucose, not fructose and trehalose, in the absence of cyclic AMP. This evidence may mean that *V. cholerae* is a suitable microbe for studying the role of cyclic AMP in the utilization of basic energy sources in comparison to the *Enterobacteriaceae*.

The second area of interest in cyclic AMP-deficient mutants is the expression of motility. Yokota and Gots (26) reported that cyclic AMP is required for flagella formation of *E. coli* and *S. typhimurium*, both of which are peritrichous. This paper indicates that cyclic AMP is also essential for flagella formation in monotrichous microbes, i.e., *V. cholerae*. An interesting finding is that cyclic AMP-deficient mutants of *V. cholerae* sometimes produce straight flagella in the absence of cyclic AMP. Since flagella of vibrio species have sheaths (7), it may be that such straight flagella consist of only sheath materials which do not require cyclic AMP. The role that cyclic AMP plays in flagella formation of the *Enterobacteriaceae* and *Vibrio* species is

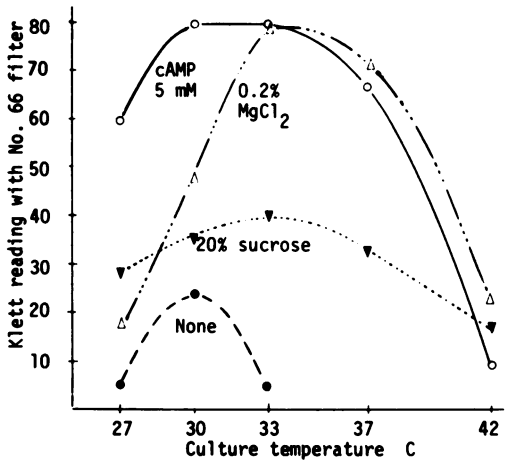

FIG. 8. *Growth turbidity of V. cholerae E2751 (cya) at various temperatures in peptone-water (pH 8.0) containing 20% sucrose, 0.2% magnesium chloride, or 5 mM cyclic AMP alone. Inoculum size: 2.0 × 10⁴ cells per ml.*

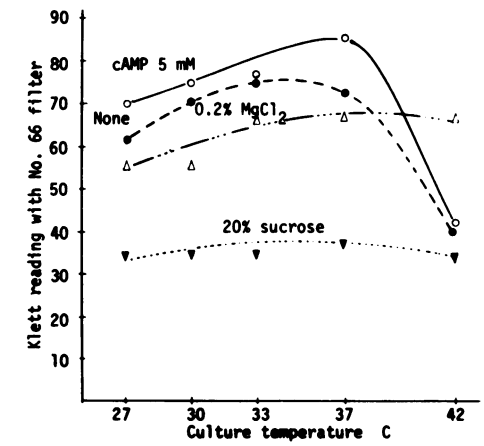

FIG. 9. *Growth turbidity of V. cholerae E2 (cya⁺) at various temperatures in peptone-water (pH 8.0) supplemented with 20% sucrose, 0.2% magnesium chloride, or 5 mM cyclic AMP. Inoculum size, 2.0 × 10⁴ cells per ml.*

TABLE 4. *Pleiotropic phenotypes of cyclic AMP-deficient mutants*[a]

Species	Strain	Sugar fermentation		Other phenotypes	Reference
		Require cyclic AMP	Not require	Require cyclic AMP	
E. coli	GP1	Lac, Gal, Ara, Mal, Mtl, Gly, Xyl, Rha, Dul, Mel, Rbs	Glc, Fru, G-6-P, Tre	Flagella formation, lambda receptor, indole production	25
	CA7902	Lac, Gal, Ara, Mal, Mtl, Gly, Xyl, Rha, Dul, Mel, Rbs	Glc, Fru, G-6-P, Tre	Flagella formation, lambda receptor, indole production	21, 25
	5336	Lac, Ara, Mal, Mtl, Gly	Glc, Fru, *Gal*		17
	YE100	*Glc*, *Tre*, Lac, Gal, Ara, Mal, Mtl, Gly, Xyl, Rha, Rbs, Dul, Mel	Fru, G-6-P, F-6-P	Flagella formation, indole production	Isolated from *E. coli* R1
S. typhimurium	GP8	Gal, Mal, Mtl, Gly, Xyl, Dul, Mel, Rbs, Ara, Ins, Cit	Glc, Fru, G-6-P	Flagella formation, P22 lysogenization	2, 9, 25
V. cholerae	E2511	Suc, Mal, Man, *Fru*, *Tre*	Glc	Flagella formation, specific O-antigen, cell wall at 37 C	
	E2751	Suc, Mal, Man, Fru, Tre	Glc	Flagella formation, specific O-antigen, cell wall at 37 C	
Vibrio parahemolyticus	Ps126	Gal, Ara, Man, Mal, Mtl, *Tre*	Glc, Fru		22

[a] Abbreviations: Ara, arabinose; Cit, sodium citrate; Dul, dulcitol; Fru, fructose; Gal, galactose; Glc, Glucose; Gly, glycerol; Ins, inositol; Lac, lactose; Mal, maltose; Mel, melibiose; Mtl, mannitol; Man, mannose; Rbs, ribose; Rha, rhamnose; Suc, sucrose; Tre, trehalose; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate.

now under investigation.

The most striking characteristic of cyclic AMP-deficient mutants of *V. cholerae* is their growth behavior at higher temperatures. The mutants can not grow on TCBS selective plates and hardly multiply on or in nutrient agar plates and peptone-water at 37 C unless provided with a supply of exogenous cyclic AMP. One can assume, therefore, that cyclic AMP is an essential component for formation of the cell wall and/or cell membrane of *V. cholerae* at higher temperatures, so that the influence of cyclic AMP depletion is stronger with TCBS agar, which contains fairly large amounts of surface-active compounds (6) than ordinary media. In fact, the mutants are converted to spheroplasts, spindle forms, and spiral filaments showing incomplete formation of the cell wall at 37 C in cyclic AMP-free media. This assumption is further supported by evidence demonstrating that supplemented sucrose and magnesium in a medium can replace cyclic AMP for the growth of the mutants at high culture temperatures. Since more cyclic AMP is required for the growth of mutants at higher temperatures, it can be postulated that cyclic AMP is an intracellular mediator for bacterial adaptation not only to chemical changes but also to physical fluctuations in the environment. Unlike the requirement of cyclic AMP for cell wall formation, the production of specific somatic antigens of *V. cholerae* is unconditionally dependent on the presence of cyclic AMP at either low or high culture temperatures. Exactly how cyclic AMP functions in the formation of the normal surface architecture of *V. cholerae*

still remains to be answered. Biochemical studies on the role of cyclic AMP in the formation of the cell wall and somatic antigens of *V. cholerae* are in progress.

The last area of interest in cyclic AMP-deficient mutants of *V. cholerae* is the requirement of cyclic AMP for the production of cholera enterotoxin. Ohashi et al. and one of the authors recently found that the mutants produced cholera toxin only with cyclic AMP (personal communication). This may indicate that cyclic AMP works in a wide range of regulatory systems not only in the *Enterobacteriaceae* but also in *Vibrio*.

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