Cloning and Amplification of the Adenylate Cyclase Gene

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Received November 29, 1982

The gene (*cya*) responsible for adenylate cyclase activity of *E. coli* was cloned onto pBR322 and the hybrid plasmid carrying the *cya* gene was designated as pCAK-11. The hybrid plasmid was 6.0 Md in size and contained a 3.3 Md *Hind*III fragment derived from *E. coli* strain CA7902R chromosomal DNA. In various *E. coli* hosts, the hybrid plasmid coded for high adenylate cyclase activity. *E. coli* cells dosed with pCAK-11 accumulated a large amount of cAMP in both cells and broth. Especially marked accumulation of cAMP was found in a strain deficient in cAMP-receptor protein (CRP).

In both eukaryotes and prokaryotes, the intracellular response to extracellular stimuli is mediated by adenosine 3',5'-cyclic monophosphate (cAMP).^{1~3)} Although cAMP has various actions in prokaryotes, the mechanisms of these actions are little known except for its action at the transcriptional level in *E. coli*.⁴⁾ In *E. coli*, approximately 30 operons are supposedly controlled by cAMP.²⁾ For expression of these genes, the participation of cAMP and its receptor protein (CRP) are required as a positive⁴⁾ or negative⁵⁾ effector.

The intracellular level of cAMP, therefore, is one of the important factors for the elucidation of the mechanism of gene expression. In *E. coli* cells, the intracellular concentration of cAMP is considered to be controlled in various ways. They are (i) synthesis by the adenylate cyclase reaction, (ii) excretion of cAMP into the growth medium and (iii) degradation catalyzed by cAMP-phosphodiesterase (CPD). Recently, effects of CRP on both regulation of the intracellular level of cAMP and adenylate cyclase activity were also reported.^{6,7)}

For elucidation of the functions of cAMP

and for construction of a highly potent producer of cAMP, we first cloned the gene responsible for the adenylate cyclase activity onto pBR322 and introduced the hybrid plasmid, designated as pCAK-11, into various *E. coli* strains having deficiencies in genes responsible for the metabolism of cAMP. This article deals with the relationship between adenylate cyclase activity and the intra- and/or extra-cellular level of cAMP.

MATERIALS AND METHODS

Bacterial strains of *E. coli* K-12. Strains used and their genetic properties were listed in Table I. Strain CA7902 (Hfr *thi relA cya*), a mutant strain deficient in adenylate cyclase (*cya*⁻) activity, was a gift from M. Yamazaki. CA7902R was a revertant derived from CA7902 by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment according to the method of Adelberg *et al.*⁸⁾ Strain pp47, a mutant strain deficient in cAMP-receptor protein (CRP), was derived from strain pp6.

Preparation of DNAs. Chromosomal DNA was isolated from CA7902R (*cya*⁺) by the method of Miura.⁹⁾ Plasmid DNA was purified from the cleared lysates of CA7902 according to the procedure Clewell *et al.*¹⁰⁾

Construction of hybrid plasmids. Hybrid plasmids were

TABLE I. STRAINS USED

Strain	Relevant genotype	Source
CA7902	<i>cya</i> ⁻ , <i>crp</i> ⁺ , <i>cpd</i> ⁺ ^a	J. Beckwith
CA7902R	<i>cya</i> ⁺ , <i>crp</i> ⁺ , <i>cpd</i> ⁺	This work
pp6	<i>cya</i> ⁺ , <i>crp</i> ⁺ , <i>cpd</i> ⁺	I. Pastan
pp47	<i>cya</i> ⁺ , <i>crp</i> ⁻ , <i>cpd</i> ⁺	I. Pastan
Crooke's	<i>cya</i> ⁺ , <i>crp</i> ⁺ , <i>cpd</i> ⁻	(ATCC8739)

^a Gene for cAMP phosphodiesterase.

constructed using plasmid pBR322 as a cloning vector. pBR322 was cleaved with *Hind*III and treated with bacterial alkaline phosphatase as described by Ullrich *et al.*¹¹⁾ 3.0 µg of chromosomal DNA from CA7902R was digested with restriction endonuclease *Hind*III for 15, 30 and 60 min at 37°C and then ligated with 1.0 µg of pBR322 by *T*₄ DNA ligase according to the method of Tanaka and Weisblum.¹²⁾

Transformation. The whole ligation mixture thus prepared was used for the transformation of CA7902 (*cya*⁻) by the method of Kushner.¹³⁾ The method for the selection of transformants is described in RESULTS.

Restriction mapping of hybrid plasmid pCAK-11. Restriction mapping of hybrid plasmid pCAK-11 was carried out with various restriction endonucleases. The reaction conditions were the same as those described by Murata *et al.*¹⁴⁾ Agarose gel (slab type, 1.0%) electrophoresis was carried out using the same buffer as described by Helling *et al.*¹⁵⁾

Assay for adenylate cyclase activity. Adenylate cyclase activity was determined by the method of Tao *et al.*¹⁶⁾ *E. coli* cells were grown to the late log phase on Davis-Mingioli minimal medium¹⁷⁾ (glucose 1.0%) supplemented with 0.25% yeast extract and 0.4% polypeptone. The cells were harvested by centrifugation at 8,000 rpm for 10 min and washed once with 10 mM Tris-HCl buffer (pH 8.0) containing 10 mM MgSO₄. The washed cells were re-suspended in 10 ml/g wet wt. cells of 20 mM Tris-HCl buffer (pH 8.0) containing 10 mM MgSO₄, 0.25 mM ethylenediaminetetraacetate and 1.0 mM dithiothreitol. The cell suspensions were sonicated at 90 kHz for 30 sec at 0°C and then used as the enzyme sources. The adenylate cyclase reaction was carried out in a 1.0 ml mixture containing 50 mM Tris-HCl buffer (pH 9.0), 20 mM MgSO₄, 4.0 mM adenosine 5'-triphosphate (ATP) and cell extracts at 34°C for 30 min.

Measurement of cAMP. The cAMP formed in the reaction mixture was assayed by a radioimmunoassay using a cAMP assay kit obtained from Yamasa Shoyu Co., Ltd., Chiba, Japan. The cAMP content of cells was determined as follows. The cells (0.1 g wet wt.) in the late

log phase on Davis-Mingioli minimal medium supplemented with 0.02% yeast extract were extracted for cAMP with 20 ml of 6.0% perchloric acid for 10 min at 4°C. After neutralization with about 0.2 ml of 60% KOH, the mixture was made up 5.0 ml and centrifuged at 3,000 rpm for 10 min. The resultant supernatants were used for the determination of cAMP. The cAMP in the broth was determined using the culture filtrates. Protein was determined by the method of Lowry *et al.*¹⁸⁾

Chemicals. The chemicals used were all analytical grade reagents. Restriction endonucleases and *T*₄ DNA ligase were purchased from Takara Shuzo Co., Ltd., Kyoto, Japan.

RESULTS

Transformation and selection of hybrid plasmids containing the gene for adenylate cyclase

The criterion for the selection of hybrid plasmids carrying the *cya* gene was as follows. In *E. coli*, cAMP is indispensably required for the expression of the lactose operon.⁴⁾ Cells lacking in cAMP represent a Lac⁻ phenotype and give small white colonies on MacConkey-lactose agar plates.¹⁹⁾ On the other hand, cells transformed with a hybrid plasmid carrying the *cya* gene show a Lac⁺ phenotype and show red colour colonies on MacConkey-lactose agar plates. Therefore, the transformants harbouring the hybrid plasmid with the *cya* gene can be selected as the red colonies on MacConkey-lactose plates.

E. coli mutant CA7902 with a point mutation in the *cya* region was incubated with the whole ligation mixture consisting of pBR322 and *Hind*III restriction fragments of chromosomal DNA from CA7902R and spread on MacConkey-lactose agar plates supplemented with 20 µg/ml ampicillin. Transformants were selected as red colour-producing and ampicillin-resistant colonies. The frequency of the appearance of such colonies varied depending on the digestion time of chromosomal DNA. The numbers of transformants obtained for 15, 30 and 60 min digests of chromosomal DNA were 1, 18 and 5 per two thousand ampicillin-resistant colonies, respectively.

TABLE II. CHARACTERIZATION OF TRANSFORMANTS OBTAINED

Strain	Plasmid dependent Lac ⁺ phenotype ^a	Generation time on ^b		Adenylate cyclase activity ^c
		Lactose	Maltose	
CA7902	—	—	—	1
CA7902R	—	1.0	1.0	26
CA7902/pBR322	0	—	—	3
Transformant 1	0.87	4.5	—	0.01
2	0.01	4.0	—	3
3	0.88	4.5	3.0	7
4	1.00	6.0	4.0	2
5	1.00	1.0	—	3
11	1.00	1.0	1.0	28
12	1.00	1.0	1.0	24
13	1.00	1.0	1.0	22
14	1.00	1.0	1.0	18

^a The plasmid-dependent Lac⁺ phenotype was determined as follows. Crude extracts containing hybrid plasmids were prepared from the transformants and strain CA7902 was transformed with these extracts. The plasmid-dependent Lac⁺ phenotype was expressed as the ratio of [number of Lac⁺ phenotype]/[number of ampicillin-resistances].

^b Growth rates were determined on Davis-Mingioli minimal medium containing 10 µg/ml of ampicillin. In the case of strain CA7902R, this drug was omitted. Data represent the generation time in hours.

^c Adenylate cyclase activity was assayed as described in MATERIALS AND METHODS. Activity was expressed as pmol of cAMP formed per mg of protein per 30 min.

Characterization of transformants

To ascertain that the transformants obtained were carrying the *cya* gene, the following three (i~iii) experiments were carried out using nine red colour-producing colonies (Table II; transformants 1~5 and 11~14) obtained by transformation with the chromosomal DNA digested for 30 min with *Hind*III.

(i) Transforming activity of hybrid plasmids

Cleared lysates were prepared from these transformants and used for the re-transformation of CA7902. The cleared lysates from transformants 4, 5 and 11~14 could transform CA7902 to the Lac⁺ phenotype. On the other hand, the cleared lysates from transformants 1, 2 and 3 failed in the complete conversion of CA7902 to Lac⁺ phenotype (Table II).

(ii) Utilization of lactose and maltose

Utilization of maltose and lactose by these transformants was tested to check for contamination by lactose-constitutive strains, which can also produce a red colour on selection agar plates. Transformants 11, 12, 13 and 14 could grow on maltose as well as lactose

and they showed the same growth rates as that of the CA7902R (*cya*⁺) strain. Transformants 1, 2, 3 and 4 also could grow on lactose, but their growth rates were much lower than those of CA7902R and/or transformants 11, 12, 13 and 14. Transformant 5 also could grow on lactose and showed the same growth rate as that of CA7902R. But this strain could not utilize maltose.

(iii) Adenylate cyclase activity

Adenylate cyclase activities of red colour-producing colonies were determined. About 20- to 30-fold increases in adenylate cyclase activities were found in transformants 11, 12, 13 and 14 in comparison with that of CA7902. On the other hand, the activities of transformants 1, 2, 3, 4 and 5 remained at the same level as that of CA7902 or CA7902/pBR322.

Thus, four transformants (Transformants 11, 12, 13 and 14) showed the same phenotype in utilization of lactose and maltose and in adenylate cyclase activity. The hybrid plasmids harboured in these four transformants were also the same in size, though the data are not shown here. So, one (Transformant 11) of the

TABLE III. SIZE OF FRAGMENTS GENERATED BY DIGESTION WITH VARIOUS RESTRICTION ENDONUCLEASES

The conditions employed for the digestion were the same as those described by Murata *et al.*¹⁴⁾ *Bam*HI-*Hind*III and *Hind*III-*Pst*I double digestions were carried out under the conditions for *Hind*III. *Bam*HI-*Eco*RI double digestion was carried out under the conditions for *Eco*RI.

Restriction endonucleases	Size of fragments (Md)					Total size (Md)
<i>Hind</i> III	3.25	2.73				5.98
<i>Eco</i> RI	4.91	1.31				6.22
<i>Bam</i> HI	3.71	1.50	0.47			5.68
<i>Pst</i> I	5.07	0.61				5.68
<i>Bam</i> HI- <i>Hind</i> III	2.23	1.50	1.30	0.47	0.42	5.92
<i>Hind</i> III- <i>Pst</i> I	2.93	2.15	0.57			5.65
<i>Bam</i> HI- <i>Eco</i> RI	2.47	1.56	1.33	0.49		5.85

four transformants was selected and used for further characterization of the hybrid plasmid and the extra- and intra-cellular levels of cAMP.

Isolation and characterization of hybrid plasmid pCAK-11

To characterize hybrid plasmids harboured in transformants, the hybrid plasmid, designated as pCAK-11, was extracted from Transformant 11 (Table II) and purified by CsCl-ethidium bromide density gradient centrifugation. The results of restriction analysis of plasmid pCAK-11 are summarized in Table III.

From the results of restriction analysis, the size of this plasmid was determined to be 6.0 Md, indicating that the 3.25 Md (approximately 5 kilo base pairs) segment of chromosomal DNA was inserted into the *Hind*III site of pBR322. The size of this plasmid was calculated to be about 6.0 Md from an electron micrograph (Fig. 1). The DNA fragment cloned onto pBR322 had a single susceptible site for *Pst*I and *Eco*RI and two for *Bam*HI, but it contained no *Hind*III sites. Based on these results, the circular restriction map of hybrid plasmid pCAK-11 was determined as shown in Figure 2. Aiba independently cloned the *cya*

FIG. 1. Electron Micrograph of Hybrid Plasmid pCAK-11.

The procedure for electron microscopy was the same as that described by Kleinschmit *et al.*²⁰⁾ The photograph of plasmid DNA was taken at $\times 20,000$ magnitude. Contour length measurements were made on at least 9 molecules and the molecular weight was calculated taking pBR322 as an internal molecular weight standard.

FIG. 2. Circular Restriction Map of Hybrid Plasmid pCAK-11.

The restriction sites are drawn relative to their scale on a circular map. The numbers in the circle represent megadaltons (Md). , chromosomal DNA of CA7902R; , vector plasmid pBR322.

gene of *E. coli* onto pBR322 and designated this hybrid plasmid as pCA1. pCA1 seemed to be the same as pCAK-11 in both molecular size and restriction pattern. The details of hybrid plasmid pCA1 will be described elsewhere.

Adenylate cyclase activity and cAMP contents of transformants

E. coli CA7902 (*cya*⁻, *crp*⁺, *cpd*⁺), pp6

TABLE IV. EFFECT OF pCAK-11 ON ADENYLATE CYCLASE ACTIVITY AND cAMP CONTENTS

Strain	Relevant genotype ^a	Adenylate cyclase activity ^b (pmol/30 min/mg-protein)	cAMP content in ^b	
			Broth (nmol/ml)	Cells (nmol/g-wet wt. cells)
CA7902	<i>cya</i> ⁻ , <i>crp</i> ⁺ , <i>cpd</i> ⁺ ^c	2.8 (1.0)	trace (—)	0.17 (1.0)
CA7902/pCAK-11		27 (9.6)	0.1 (—)	0.24 (1.4)
CA7902R	<i>cya</i> ⁺ , <i>crp</i> ⁺ , <i>cpd</i> ⁺	28 (1.0)	0.2 (1.0)	0.40 (1.0)
CA7902R/pCAK-11		175 (6.3)	3.2 (16)	2.40 (6.0)
pp6	<i>cya</i> ⁺ , <i>crp</i> ⁺ , <i>cpd</i> ⁺	141 (1.0)	0.3 (1.0)	4.40 (1.0)
pp6/pCAK-11		269 (1.9)	0.8 (2.7)	6.40 (1.6)
pp47	<i>cya</i> ⁺ , <i>crp</i> ⁻ , <i>cpd</i> ⁺	239 (1.0)	50 (1.0)	14.0 (1.0)
pp47/pCAK-11		314 (1.3)	80 (1.6)	17.3 (1.2)
Crooke's	<i>cya</i> ⁺ , <i>crp</i> ⁺ , <i>cpd</i> ⁻	29 (1.0)	5.2 (1.0)	6.80 (1.0)
Crooke's/pCAK-11		140 (4.8)	8.2 (1.6)	6.80 (1.0)

^a Genotypes are listed in relation to cAMP.

^b Adenylate cyclase activity and cAMP contents of broth and of cells were assayed as described in MATERIALS AND METHODS. In parenthesis is shown the relative activity or content.

^c Gene for cAMP phosphodiesterase.

(*cya*⁺, *crp*⁺, *cpd*⁺), pp47 (*cya*⁺, *crp*⁻, *cpd*⁺) and Crooke's (*cya*⁺, *crp*⁺, *cpd*⁻) were transformed with hybrid plasmid pCAK-11 and adenylate cyclase activity and intra- and extra-cellular levels of cAMP in the transformants were determined. These results are summarized in Table IV.

Adenylate cyclase activity of CA7902/pCAK-11 was about 10-fold higher than that of CA7902 as a control. But, a marked increase in adenylate cyclase activity was not observed in the case of strains such as pp6 and pp47.

Intra- and extra-cellular contents of cAMP also increased on introduction of hybrid plasmid pCAK-11 into various recipient strains of *E. coli*. Especially marked extracellular accumulation of cAMP was observed for strain pp47 having a deficiency in cAMP-receptor protein (CRP). A strain deficient in cAMP-phosphodiesterase (CPD) activity (Crooke's) was also found to accumulate a large amount of cAMP in both the cells and broth. The introduction of hybrid plasmid pCAK-11 into Crooke's strain also resulted in an increase in adenylate cyclase activity.

DISCUSSION

The chromosomal segment carrying the *cya* gene of *E. coli* CA7902R was cloned onto pBR322. The following three facts indicated that this segment contained the *cya* gene. (i) pCAK-11 could transform CA7902 (*cya*⁻) to Lac⁺. (ii) Cells harbouring this hybrid plasmid could utilize both lactose and maltose. (iii) pCAK-11 gave rise to adenylate cyclase activity. However, the method we used for the selection of transformants was not specific for the hybrid plasmid carrying the *cya* gene. Spontaneous revertants of CA7902 harbouring a hybrid plasmid and several lactose-constitutive strains also show a red colour on selection plates. So, the properties of red-colour producing colonies that appeared on the selection plates were analyzed (Table II). As a result, four (Transformants 11, 12, 13 and 14 in Table II) were found to be intrinsic transformants carrying the hybrid plasmid with the *cya* gene. Besides these four strains, various transformants were obtained (Table II). But, they showed little adenylate cyclase activity, although they had interesting characters as to sugar utilization.

The cloning of the *cya* gene was also re-

ported by Clark *et al.*,²¹⁾ Roy *et al.*²²⁾ and Wang *et al.*,²³⁾ respectively. Clark *et al.* obtained the *cya* gene of *E. coli*, but they did not report on it in detail. Roy *et al.* presented a restriction map of the *cya* region on plasmid pLC 41.04. Wang *et al.* isolated the *cya* gene from *Salmonella* by the transformation of *E. coli* using λ gt4 phage as a cloning vector. They have concluded that 2.6 k.b.p. is essential for coding the *cya* gene of this organism. The size of the cloned segment obtained in our experiment was 5.0 k.b.p. This size seemed to be sufficient to cover the adenylate cyclase.

The increase in adenylate cyclase activity in transformants was host strain-dependent (Table IV). Five- to ten-fold increases in this enzyme activity were observed, when the strains (CA7902, CA7902R and Crooke's) having low adenylate cyclase activity were used as recipients. On the other hand, such an increase in activity was not observed, when the recipient strains (pp6 and pp47) had relatively high adenylate cyclase activity. The reason for this strain dependent activity of adenylate cyclase is now obscure. The expression of the *cya* gene on a hybrid plasmid may be controlled by various factors determining the contents of intra- and extra-cellular cAMP.

Increased levels of intra- and extra-cellular contents of cAMP were found in cells dosed with the *cya* gene. An especially marked increase in the extracellular content of cAMP was found in a strain deficient in cAMP-receptor protein (CRP). Although the relationship between the *crp*⁺ phenotype and control of the cAMP level is now obscure, the same results were also presented by many investigators.^{7,24,25)} A cAMP-phosphodiesterase (CPD) activity deficient strain (Crooke's) also showed high levels of extra- and intra-cellular accumulation of cAMP. Therefore, it was suggested that *crp*⁻ and *cpd*⁻ may be necessary for the accumulation of a large amount of cAMP.

From this standpoint, we are studying the construction of a mutant strain having a deficiency in both CRP and CPD for the production of a large amount of cAMP. We are

also carrying out detailed analysis of the effect of the *crp*⁻ phenotype on the accumulation of cAMP.

Acknowledgments. We thank Dr. M. Sakaguchi, the Research Institute for Food Science, Kyoto University, for his kind and critical suggestions for this work. We also want to express our sincere thanks to T. Higasa, the Research Institute for Food Science, Kyoto University, for taking the electron microscope pictures. Thanks are also due to Dr. M. Yamazaki, Faculty of Agriculture, Tokyo University, for generous sharing of strains.

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