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Use of Streptomycin and Cyclic Adenosine 5'-Monophosphate in the Isolation of Mutants Deficient in CAP Protein

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A method is described for selective isolation of mutants deficient in CAP protein necessary for the expression of catabolite-sensitive operons in *Escherichia coli*.

Cyclic adenosine 5'-monophosphate (AMP) is required to obtain maximal transcription of the catabolite-sensitive operons (3). Single-step mutants unable to express all of these operons have been isolated (4, 5). One class of these mutants lacks the enzyme adenylate cyclase and therefore cannot synthesize cyclic AMP. The inability of these mutants to grow on a variety of carbohydrates such as glycerol, lactose, maltose, rhamnose, and arabinose can be overcome by the addition of cyclic AMP to the medium. The second class of mutants lacks CAP protein, necessary for the specific stimulation by cyclic AMP of the catabolite-sensitive operons. These mutants cannot grow on glycerol, maltose, arabinose, lactose, and rhamnose even in the presence of cyclic AMP.

We wish to describe a procedure for the selective isolation of CAP-deficient mutants (*crp*$^-$) and some of their properties. The method of selection is based on the observation made in this laboratory (2) that cyclic AMP enhances streptomycin lethality in *Escherichia coli*. It has been shown that at low concentrations of streptomycin, glucose repressed the bactericidal effect of the antibiotic, and this repression was overcome by cyclic AMP. Thus, streptomycin at a concentration of 2.5 μg/ml had no effect on cells growing on glucose as the sole carbon source, but it became bactericidal when cyclic AMP was added to the medium. The requirement for the enhancing effect of cyclic AMP on streptomycin lethality was the presence of functional CAP protein. The effect of cyclic AMP on streptomycin lethality for *E. coli* CA8000 and its adenylate cyclase (*cya*$^-$) and *crp*$^-$ mutants is illustrated in Fig. 1.

We assumed that addition of streptomycin and cyclic AMP to mutagenized cultures of *E. coli* ought preferentially to kill all cells with functional CAP protein and enrich for mutants with defective CAP. Reconstruction experiments with mixtures of *E. coli* strains with functional CAP protein or adenylate cyclase mutants and strains with deficient CAP protein at a ratio of 10^5 to 1 showed this approach to be workable.

E. coli, B, W, and CA8000 were used for the selection of *crp*$^-$ mutants. The procedure was as follows: the cells were grown overnight in 5 ml of nutrient broth, sedimented, and diluted 100-fold in fresh medium. When the cultures were in exponential phase at a density of 10^8 cells/ml they were washed twice in 5 ml of citrate buffer (0.1 M citrate buffer, pH 5.5). Nitrosoguanidine was added to a final concentration of 50 μg/ml to cells resuspended in 5 ml of citrate buffer. The cells were incubated at 37 C in a water bath for 30 min. After 30 min, the cells were spun down and washed once in 5 ml of phosphate buffer (0.1 M phosphate buffer, pH 7.0). One-tenth milliliter of the washed-cell suspension was incubated overnight in 5 ml of nutrient broth. The overnight culture was diluted and spread on minimal medium containing glucose and streptomycin (2.5 μg/ml). The plates were then spotted in the center with a drop of 0.05 M cyclic AMP. Parental strains grew as a lawn on these plates with a zone of complete growth inhibition around the spot of cyclic AMP. Mutagenized cultures gave rise to numerous colonies which grew within the inhibition zone around the spot of cyclic AMP. These colonies were picked and used for gridding tetrazolium master plates which contained tryptose blood agar base and two sugars, arabinose and maltose. Mutants incapable of metabolizing both of the two sugars gave rise to red colonies on these plates. Except for occasional streptomycin-resistant mutants, all mutagenized cells gave rise to red colonies which were shown to be as sensitive to streptomycin as the parental strains.

From the master plates the colonies were

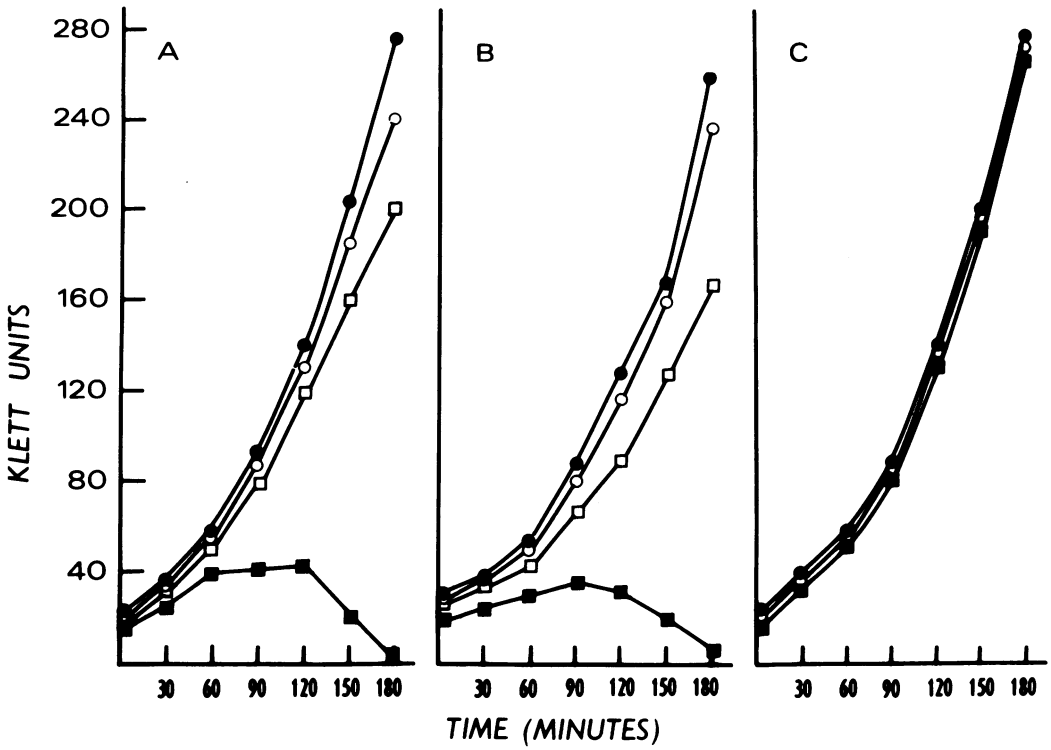

FIG. 1. *The effect of cyclic AMP on streptomycin lethality in E. coli CA8000 strain and its cya⁻ (CA7903) and crp⁻ (7901) mutants. The strains were obtained through the courtesy of D. Arditti. The cells were grown exponentially in a minimal medium supplemented with vitamin B_1 and glucose, 0.4%, to a density of 10^8 cells/ml. The cells were then divided into four portions. One portion served as a control, (●); other portions received: streptomycin, 2.5 μg/ml, (○); cyclic AMP, 5×10^{-3} M, (□); streptomycin, 2.5 μg/ml + cyclic AMP, 5×10^{-3} M, (■). (A) parental strain, (B) cya⁻ mutant, (C) crp⁻ mutant. Growth was followed in a Klett photometer. Cell lysis coincided with the loss of cell viability.*

replicated onto plates with minimal medium containing glucose, glycerol, rhamnose, lactose, or maltose as the sole carbon source. One-half of these plates were spread with 0.2 ml of 0.05 M cyclic AMP before replica plating. More than 95% of the colonies failed to grow on any of the above plates, except for plates with glucose, regardless of whether or not cyclic AMP was present in the medium. Plates with cyclic AMP allowed the growth of adenylate cyclase mutants.

We have also assayed the various mutants for their ability to synthesize β-galactosidase and tryptophanase (Table 1). The enzymes were assayed as previously described (1). All the mutants tested exhibited very low levels of both the enzymes, ranging from 1 to 5% of the parental strains. The synthesis of β-galactosidase and tryptophanase was not stimulated by the addition of cyclic AMP. The inability of the mutants to synthesize β-galactosidase was shown to be due to their failure to transcribe the *lac* operon. Direct hybridization assay has been used to study the synthesis of *lac* messenger ribonucleic acid (RNA) in the various mutants. In the hybridization assay, RNA was extracted from *E. coli* cultures which had been pulse-labeled with [^{3}H]uridine during induction with isopropyl-β-D-thiogalactopyranoside (IPTG) (5 mM) in the presence of cyclic AMP (5 mM), and a uniform amount of RNA was annealed to a large excess of deoxyribonucleic acid (DNA) prepared from $\lambda CI_{857}S_7plac$. Non-lac interactions between pulse-labeled RNA and λDNA were minimized by the addition of a large excess of unlabeled RNA extracted from lac deletion *E. coli* strain W4032. The percentage of pulse-labeled RNA hybridized to λDNA was taken as a measure of *lac*-specific counts in each preparation. The hybridization assay was performed as previously described (1). In uninduced strains of *E. coli* B, W, and CA8000, the *lac* messenger RNA content of the cell constituted about 0.03 to 0.04% of the total pulse-labeled RNA and rose to 0.7 to 0.8% after induction under derepressed conditions. When the differ-

TABLE 1. *Some characteristics of E. coli mutants deficient in CAP protein*[a]

Strains	Induction with IPTG and tryptophan in the presence of cyclic AMP	% of pulse-labeled RNA hybridized to λ DNA	Differential rate of synthesis (% of wild type)		Growth on[b]				
			β-galactosidase	Tryptophanase	Glucose	Glycerol	Lactose	Arabinose	Rhamnose
E. coli B	Uninduced	0.03–0.04							
E. coli B	Induced	0.70–0.80			+	+	+	+	+
B-*crp*$_1^-$	Induced	0.03–0.04	2.5	2.0	+	–	–	–	–
B-*crp*$_2^-$	Induced	0.04–0.05	1.5	3.0	+	–	–	–	–
B-*crp*$_3^-$	Induced	0.03–0.04	3.0	2.0	+	–	–	–	–
B-*crp*$_4^-$	Induced	0.04–0.05	2.0	2.0	+	–	–	–	–
E. coli W	Uninduced	0.03–0.04							
E. coli W	Induced	0.80–0.85			+	+	+	+	+
W-*crp*$_1^-$	Induced	0.03–0.04	2.0	4.0	+	–	–	–	–
W-*crp*$_2^-$	Induced	0.035–0.04	5.0	3.0	+	–	–	–	–
W-*crp*$_3^-$	Induced	0.04–0.05	3.0	4.0	+	–	–	–	–
W-*crp*$_4^-$	Induced	0.04–0.045	5.0	3.0	+	–	–	–	–
E. coli CA8000	Uninduced	0.03–0.04							
E. coli CA8000	Induced	0.70–0.85			+	+	+	+	+
CA-*crp*$_1^-$	Induced	0.04–0.05	5.0	4.0	+	–	–	–	–
CA-*crp*$_2^-$	Induced	0.03–0.04	4.0	3.0	+	–	–	–	–
CA-*crp*$_3^-$	Induced	0.03–0.04	2.0	2.0	+	–	–	–	–
CA-*crp*$_4^-$	Induced	0.04–0.045	5.0	3.0	+	–	–	–	–

[a] The cells were growing exponentially in minimal media supplemented with glucose, 0.4%, at a density of 3 × 10^8 cells/ml when induced with L-tryptophan, 2 mM, and IPTG, 5 mM, in the presence of cyclic AMP, 5 mM, 2-^{14}C-leucine, 0.4 μCi/ml (specific activity 1 μCi/0.5 μmol), or [^{3}H]uridine, 2.5 μCi/ml (specific activity 23 Ci/mmol). For the determination of the differential rate of enzyme synthesis, samples were assayed for β-galactosidase, tryptophanase and for incorporation of radioactive leucine into trichloroacetic acid-precipitable material. Induced cells pulse-labeled with [^{3}H]uridine were used in hybridization assays.

[b] (+) Positive; (–) negative.

ent *crp*$^-$ mutants were induced with IPTG in the presence of cyclic AMP, the percentage of pulse-labeled RNA capable of hybridizing to λDNA was found to be within 0.03 to 0.04, the same percentage found in uninduced parental cultures.

The following experiments were carried out to demonstrate that the mutation was a single mutation and outside the respective operons affected. (i) **Reversion test.** Fresh cultures of mutants unable to metabolize glycerol, lactose, arabinose, maltose, and rhamnose were spread onto glycerol minimal plates. A small circle of filter paper immersed in a solution of nitrosoguanidine was placed in the center of each plate. After 48 h of incubation, few colonies grew around the filter paper disk. These colonies were picked and used for gridding minimal plates with glycerol. From these plates the colonies were replicated onto minimal plates with lactose, arabinose, maltose, and rhamnose as the sole carbon source. All glycerol revertants regained the ability to grow on each of the above carbohydrates. (ii) **Transduction with λh80dlac phage.** *crp*$^-$ mutants derived from the CA8000 strain were spread over the surface of lactose minimal plates to which lysates of λ*h80dlac* phage were applied. None of the *crp*$^-$ mutants tested could be complemented by the introduction of a second *lac* operon.

These results indicate that all the mutants isolated by means of the streptomycin-cyclic AMP method were deficient in a factor necessary for the stimulation by cyclic AMP of catabolite-sensitive operons.

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