

# Properties of Adenyl Cyclase and Cyclic Adenosine 3',5'-Monophosphate Receptor Protein-Deficient Mutants of *Escherichia coli*

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Several spontaneous *cya* and *crp* mutants of *Escherichia coli* have been selected as clones simultaneously resistant to phage lambda and nalidixic acid and characterized. Both *cya* and *crp* mutants have been found to grow as cocci with increased doubling times. They have increased resistance to some mutagens (methylmethanesulfonate, ultraviolet light, gamma rays), antibiotics (nalidixic acid, ampicillin), phages (lambda, T6), sublethal heat and hypotonic shock, and decreased resistance to neutral detergents (sodium dodecyl sulfate, sodium deoxycholate), a protein synthesis inhibitor (streptomycin), and a respiratory inhibitor (sodium azide). The nature of changes in cell parameters indicate fundamental alterations in the envelope structure of the *cya* and *crp* mutant cells. The new *cya* and *crp* mutants have been found to be multiply carbohydrate negative and non-motile in conformity with similar previously isolated mutants. Studies of revertants and  $\phi 80$  *cya*<sup>+</sup> and  $\phi 80$  *cya* transductants indicated that the pleiotropic phenotype is related to a single mutational event at the *cya* or the *crp* locus in the mutants.

The cyclic adenosine 3',5'-monophosphate (cAMP) and its receptor protein-deficient mutants of *Escherichia coli* have been isolated and characterized previously (3, 25, 27, 31, 39). It has been demonstrated that all mutations deficient in cAMP lie between *ilv* and *metE* genes at 75 min of the genetic map of *E. coli* (3, 39) and inactivate the gene *cya* which codes for the enzyme adenyl cyclase (3, 25). All the cAMP receptor protein mutants (*crp*) have lesions at 64 min between *strA* and *aroB* genes (27). Both *cya* and *crp* mutants have been found to be pleiotropic (26). The mutant cells are deficient in utilizing a wide range of compounds as carbon sources (24-26), impaired in adsorbing lambda bacteriophage (40), and devoid of flagella (39). In addition, they contain lower levels of some cytochromes (5). The inability of *cya* and *crp* mutants to utilize lactose and arabinose as carbon sources has been shown to be due to blocks in the initiation of transcription of operons containing genes determining permeation and degradation of these compounds (9, 18). This is most likely the mechanism by which other phenotypes are also regulated by *cya* and *crp* products.

In this paper we describe characterization of some new *cya* and *crp* mutants isolated by

application of a powerful and highly selective procedure. The new mutants are similar to the previously described mutants for all the properties that we tested. As a result of this study several more properties of *cya* and *crp* mutants have been identified which indicate their general resistance to killing by different kinds of mutagens and other stressful conditions principally due to alterations in the cell envelope.

## MATERIALS AND METHODS

**Media and growth conditions.** Bacterial cultures were grown in tryptone broth (TB) containing, per liter, 10 g of tryptone (Difco) and 5 g of NaCl. Phage lysates were also made in this medium. TB medium supplemented with 20% sucrose + 0.01 M MgSO<sub>4</sub> · 7H<sub>2</sub>O or 1 mg of DL-alanine per ml was used to study the effects of the supplements on bacterial morphology. Colony counts were made on tryptone agar (TA) plates prepared with TB medium containing 2% agar (Patel Chest Institute, Delhi). Tryptone plates and tryptone top agar (0.8% Difco agar) were used for phage assays. TA medium supplemented with nalidixic acid (Nal; Sigma; 5 µg/ml), streptomycin sulfate (Pfizer; 1 µg/ml), ampicillin (Cadila Laboratories; 2 µg/ml), sodium dodecyl sulfate (SDS; 5 mg/ml), sodium deoxycholate (DOC; 2.5 mg/ml), or sodium azide (20 µg/ml) was used for diagnosing sensitivity of bacterial strains to inhibi-

tory agents. Semisolid TA medium (0.3% Difco agar) in tubes was used for detecting motility of bacteria. The minimal medium of Davis and Mingioli (8) was supplemented with agar (Patel Chest, 2%), required a carbon source (2 mg/ml) and thiamine hydrochloride (1  $\mu$ g/ml), and was used to make minimal medium plates. Eosin methylene blue (EMB) agar plates (7) containing different sugars (0.5%) were also used to test sugar fermentation capabilities of bacterial strains. Liquid and solid media were supplemented with  $10^{-3}$  M cAMP (Sigma) when required. All bacterial and lambda phage dilutions were made in 0.01 M  $\text{MgSO}_4$ . Growth conditions were aerobic and at 37 C unless otherwise specified.

**Bacterial and bacteriophage strains.** The *E. coli* strains used in this study are listed in Table 1. A description of *E. coli* phage strains used is given in Table 2. Lysates of lambda strains were prepared on the bacterial strain TC600, whereas those of other phages were prepared on the bacterial strain CA8000.

All lysates were titered on strain CA8000.

**Preparation of lysogens.** Bacteriophages  $\phi 80$  *cya*<sup>+</sup> and  $\phi 80$  *cya-854* were introduced as prophages in bacterial strains. Suspensions containing phages were applied to lawns of different bacterial strains to obtain plaques. The centers of the plaques were picked with a loop and streaked on TA plates. Single colonies obtained were tested for  $\phi 80$  immunity and spontaneous release of  $\phi 80$  by cross-streaking against a lysate containing  $\lambda$ imm<sup>80</sup>cl and strain CA8000. Suspected lysogenic colonies were purified and tested for the nature of phages released after ultraviolet (UV) induction.

**Selection and classification of *cya* and *crp* mutants.** Twenty-three independent cultures of the strain CA8000 were grown to about  $2 \times 10^8$  cells per ml, infected with  $\lambda$ v2v1v3 phage at a multiplicity of infection (MOI) of 8 to 10, and plated directly and after a 1:10 dilution on TA plates containing 5  $\mu$ g of NaI/ml. After 64 to 72 h of incubation at 37 C, two colonies derived from each culture were streaked on NaI plates and incubated at 37 C. One colony from one streak per culture was then tested for sensitivity to lambda by streaking against  $\lambda$ v2v1v3, for ability to utilize lactose and maltose on minimal media and by streaking on suitably supplemented EMB media, and for motility by stabbing semisolid agar. The colonies that proved lambda resistant, incapable of fermenting lactose and maltose to the same extent as wild type, and unable to swarm in semisolid agar were isolated and purified further by streaking on TA medium. This phenotype was exhibited by more than 40% of the  $\lambda^R$  + NaI<sup>R</sup> colonies tested. From among the isolated clones those that did not grow on minimal medium with glucose as the carbon source were not included in this study. The rest were again subjected to tests for sensitivity to lambda, lactose and maltose utilization, and motility in media supplemented and unsupplemented with cAMP. The clones that behaved like the parental strain when supplied with cAMP were identified tentatively as *cya* mutants and the rest as *crp* mutants. Of the five *cya* mutants, two were found to be distinguishable from others in their leakiness deduced from the fact that they formed pink colonies on EMB-lactose and -maltose plates, in contrast to

TABLE 1. Bacterial strains

Strain	Sex	Chromosomal genotype	Origin
CA8000	Hfr	<i>thi</i> $\lambda^-$ <i>relA1</i>	J. Beckwith (3)
CA8306	Hfr	<i>thi</i> , <i>cya-854</i> <sup>a</sup>	J. Beckwith (3)
1-23	Hfr	<i>thi</i> , <i>crp</i>	J. Beckwith (personal communication)
TC600	F <sup>-</sup>	<i>thr</i> , <i>leu</i> , <i>thi</i> , <i>supE</i> <sup>+</sup> , <i>tsx</i>	T6 <sup>R</sup> derivative of C600, C. R. Fuerst (personal communication)
TC600-1-1	F <sup>-</sup>	<i>thr</i> , <i>leu</i> , <i>thi</i> , <i>supE</i> <sup>+</sup> , <i>tsx</i> <i>lamB</i> (424 cIts)	$\lambda^R$ mutant of TC600 lysogenized with 424, this paper
ED54	Hfr	<i>thi</i> , <i>cya-4</i>	Spontaneous mutants of CA800, this paper
ED55	Hfr	<i>thi</i> , <i>cya-5</i>	
ED73	Hfr	<i>thi</i> , <i>cya-6</i>	
ED56	Hfr	<i>thi</i> , <i>crp-4</i>	
ED66	Hfr	<i>thi</i> , <i>crp-5</i>	
ED67	Hfr	<i>thi</i> , <i>crp-6</i>	
ED50-1	Hfr	<i>thi</i> ( $\phi 80$ <i>cya</i> <sup>+</sup> )	Lysogenized with phage $\phi 80$ derivatives, this paper
ED50-2	Hfr	<i>thi</i> ( $\phi 80$ <i>cya-854</i> )	
ED54-1	Hfr	<i>thi</i> , <i>cya-4</i> ( $\phi 80$ <i>cya</i> <sup>+</sup> )	
ED54-2	Hfr	<i>thi</i> , <i>cya-4</i> ( $\phi 80$ <i>cya-854</i> )	
ED56-1	Hfr	<i>thi</i> , <i>crp-4</i> ( $\phi 80$ <i>cya</i> <sup>+</sup> )	
ED56-2	Hfr	<i>thi</i> , <i>crp-4</i> ( $\phi 80$ <i>cya-854</i> )	
ED54-9	Hfr	<i>thi</i> , <i>cya-4</i> , revertant of <i>cya-4</i>	Spontaneous revertant of <i>cya-4</i> , this paper
ED56-3	Hfr	<i>thi</i> , <i>crp-4</i> , Sup of <i>crp-4</i>	Spontaneous revertant of <i>crp-4</i> , this paper

<sup>a</sup> *cya-854* is a deletion mutation.

the near white colonies of other mutants and the very dark pink colonies with metallic haze of the wild type. These mutants have been saved for further studies. All the remaining *cya* and *crp* mutants were lysogenized singly with  $\phi 80$  *cya*<sup>+</sup> and  $\phi 80$  *cya*-854 phages, and properties of the lysogens were studied. Since  $\phi 80$  *cya*<sup>+</sup> lysogens of all *cya* mutants acquired parental phenotype and since  $\phi 80$  *cya* lysogens of all *cya* and *crp* mutants and  $\phi 80$  *cya*<sup>+</sup> lysogens of all *crp* mutants retained the mutant phenotype, the appropriateness of the mutant classification was confirmed.

**Isolation of revertants.** Ten-milliliter cultures of strains ED54 and ED56 were grown to a density of about  $10^8$  cells/ml. The cells were washed once with 0.01 M MgSO<sub>4</sub> and plated directly on minimal agar containing lactose as the sole carbon source, and then the plates were incubated at 37 C for 3 days. One-hundred Lac<sup>+</sup> revertant colonies of strain ED54 and 48 of strain ED56 were picked up randomly and streaked on lactose-minimal plates. Single colonies from streaks were tested for the different characteristics of the original mutations. Strains ED54-9 and ED56-3, the revertants of strains ED54 and ED56 selected for inclusion in this study, were found to be similar to strain CA8000 in all the properties considered here. Frequencies of such pleiotropic revertants were estimated to be about  $1.1 \times 10^{-6}$  for strain ED54 and  $2 \times 10^{-6}$  for strain ED56. The remaining kinds of obviously not true revertants have been saved for further studies.

**Growth measurements.** Each strain was grown overnight in 5 ml of TB medium. Each of these cultures was diluted (between 1:40 to 1:100 depending on the strain) into fresh TB medium grown in a shaking water bath to about  $10^8$  cells per ml. These pregrown cultures were diluted 1:10<sup>5</sup> in fresh TB medium maintained at 37 C and growth rate was determined by making periodic measurements of colony-forming units (CFU) per milliliter. For this purpose 1-ml aliquots were withdrawn, chilled in an ice bath, and plated directly or after a 1:10 dilution; 0.1 ml of the sample was used per plate. The plates were incubated at 37 C for 2 days and the colonies were counted.

**Test for Nal resistance.** Appropriate dilutions of cultures grown to about  $2 \times 10^8$  cells per ml were spread on TA plates containing different concentrations of Nal. Plates were examined for the number of colonies after 3 days of incubation at 37 C.

**UV and gamma irradiation.** Cultures grown to  $2 \times 10^8$  to  $3 \times 10^8$  cells per ml were diluted 1:100 into 0.01 M MgSO<sub>4</sub> and distributed into 2-ml aliquots in petri dishes (diameter 95 mm) for UV irradiation and in 10-ml screw-capped glass tubes for gamma irradiation. The ice-cold sample tubes were gamma irradiated in <sup>60</sup>Co Gamma Cell 200 (Atomic Energy of Canada, Ltd.). A 15-W Phillips germicidal lamp fitted 20 inches (ca. 51 cm) above the sample platform was used as the UV source. After various times of irradiation, cells were diluted and assayed for colony-forming ability. All operations for the UV experiments were carried out in dim red light.

**Methyl methane sulfonate treatment.** Ten-milli-

TABLE 2. *Bacteriophage strains*

Name	Origin
$\lambda$ <sup>+</sup>	W. F. Dove (16)
424 cIts	S. Streicher
$\lambda$ cl <sup>+</sup> h <sup>424</sup>	Selected from a cross between $\lambda$ <sup>+</sup> and 424 cIts as a turbid recombinant on TC600-1-1 at 32 C, this paper
$\lambda$ clh <sup>424</sup>	Clear plaque-forming derivative of $\lambda$ clh <sup>424</sup> this paper
$\lambda$ imm80cI	W. F. Dove (16)
P1vir	E. Meynell
T1	A. Singh
T4	A. Guha
T6	C. R. Fuerst
T7	W. Szybalski
$\lambda$ v2v1v3	M. Ptaschne (16)
$\phi 80$ <i>cya</i> <sup>+</sup>	J. Beckwith (3)
$\phi 80$ <i>cya</i> -854	J. Beckwith (3)

liter logarithmic cultures were pelleted and suspended in 1 ml of 0.01 M MgSO<sub>4</sub>. The suspensions were diluted 100-fold into a prewarmed solution of 0.2% methyl methane sulfonate (MMS) (Eastman Organic Chemicals) in 0.01 M MgSO<sub>4</sub>. Incubation was carried out at 37 C without shaking. At different times from the transfer of cells to MMS, samples were taken into cold diluent and plated for CFU per milliliter.

**Thermal injury.** The cells in cultures grown to about  $2 \times 10^8$  cells per ml were diluted 1:100-fold in TB medium maintained at 50 C. At short intervals, samples were removed to an ice bath, diluted with chilled diluent, and plated to estimate viable cells per milliliter.

**Phage sensitivity.** Cultures grown to about  $2 \times 10^8$  cells per ml were divided into 2-ml aliquots and infected with different phages at an input multiplicity of infection (MOI) of about 20 to 35. After 30 min of incubation at 37 C without shaking, the phage bacterial mixtures were diluted and assayed for surviving CFU per milliliter.

**Assays for phage adsorption.** To study adsorption of lambda phage, 10-ml cultures at about  $5 \times 10^8$  cells per ml were sedimented, resuspended in 0.01 M MgSO<sub>4</sub> at  $10^8$  cells per ml, and infected with phage at an input MOI of  $2.3 \times 10^{-4}$ . These mixtures were incubated at 37 C for 15 min and then centrifuged to sediment bacteria. The percentage of phage adsorption was estimated by determining infective centers in the pellet and free plaque-forming units (PFU) in the supernatant fluid. The indicator was strain TC600.

Phage T6 adsorption was studied by infecting 1 ml of TB cultures containing about  $5 \times 10^8$  cells with  $10^8$  particles. The mixtures were retained for 15 min at 37 C in a water bath shaker. The percentage of phage adsorption was determined by plaque assays of infected cultures before and after addition of chloroform. All phage dilutions were performed in TB medium. Strain CA8000 was used as indicator.

**Growth in DOC medium.** Ten-milliliter portions of TB medium supplemented with various concen-

trations of DOC were inoculated with  $10^7$  cells from log-phase pregrown cultures. After 4 h of incubation without shaking at 37 C, the CFU per milliliter were measured. The effect of DOC on growth was expressed in terms of percentage of viable cells in DOC-containing cultures using plain cultures as the controls.

**Morphological studies.**  $\text{MgSO}_4$  (0.01 M) suspensions of colonies grown on solid media and turbid liquid cultures were used to make dried films of cells on microscope slides. Staining was done by Burke's modification of the Gram method (7); the cells were observed with a Leitz microscope. The pictures were projected to obtain 141,000-fold enlargements and the boundaries of 50 cells of each strain were drawn to estimate dimensions. By assuming the cells to be short cylinders with flat ends and smooth surface, the data obtained were used to calculate estimates of average surface areas and volumes of the cells of the different strains.

## RESULTS

**Growth properties of the mutants.** The new set of spontaneous *cya* and *crp* mutants described here have sugar utilization properties very similar to those already noted for mutants of these types. The mutants exhibited very little capacity to utilize arabinose, xylose, mannose, maltose, and lactose. Furthermore, on TA these mutants formed small, smooth-appearing colonies very different from the big-sized, rough, often sectorized colonies formed by the wild type. They were not temperature sensitive and formed colonies with equal efficiency at 30, 37, and 42 C. The growth rate of the mutants in TB as well as in minimal liquid medium was slower. The doubling time of mutants in TB at 37 C was found to be lengthened by 27 to 35% of that for the wild type. It was 36 min for strain ED56, 35 min for strain ED54, and 27 min for the wild-type strain. Addition of  $10^{-3}$  M cAMP to ED54 cultures reduced its doubling time to 26 min.

**Resistance of the mutants to Nal, MMS, UV, and gamma rays.** In addition to Nal (against which the mutants were selected), the *cya* and *crp* strains were also found resistant to lethal actions of a number of mutagenic agents (32) including MMS, ethyl methane sulfonate, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, UV, and gamma rays.

The Nal-resistant nature of the *cya* and *crp* mutants was revealed to us by the results of a separate study (unpublished data) in which a large number of Nal-resistant mutants of *E. coli* K-12 were isolated and characterized. About 2% of the Nal-resistant mutants of an HfrH derivative (*met thi*) were found to exhibit a pleiotropic carbohydrate-negative phenotype typical of *cya*

and *crp* mutants. This finding and the already known property of resistance of *cya* and *crp* mutants to lambda bacteriophage (40) formed the basis for using simultaneous selection for resistance to Nal and lambda phage as a procedure for enriching *cya* and *crp* mutants (see Materials and Methods).

The Nal and MMS resistance of the mutants ED54 and ED56 and wild type is compared in Fig. 1 and 2, respectively. For both the agents, the mutants were found to be much more resistant. The level of resistance of strain ED54 (*cya*) was lowered when its culture was grown with  $10^{-3}$  M cAMP supplementation. In contrast, strain ED56 (*crp*) was not affected by an exogenous supply of cAMP (data not shown).

The UV survival curves for strains ED54 and ED56 and the wild type are shown in Fig. 3. In contrast to the exponential curve obtained for the wild type, the mutant curves were prominently convex. The mutants were about two times more resistant to UV than the wild-type strain as judged from doses that reduced the survivors by 90% ( $D_{10}$ ) over the exponential part of the curves.

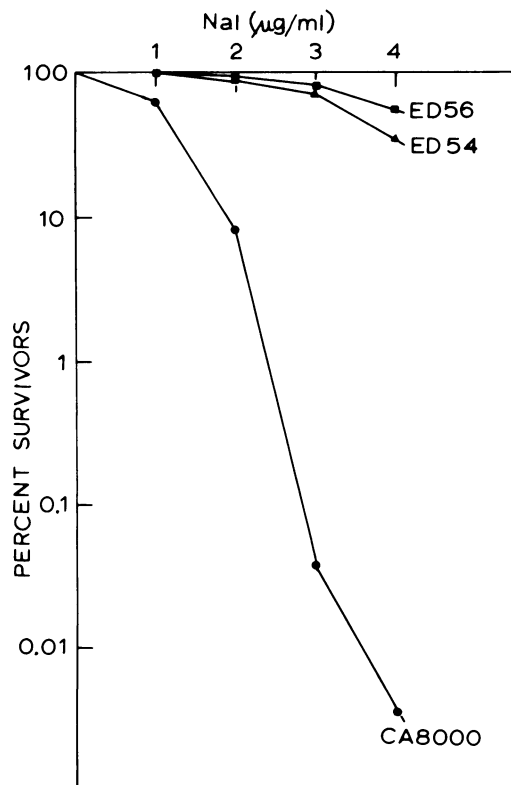


FIG. 1. Colony-forming abilities of the wild-type, *cya*, and *crp* strains on TA containing Nal.

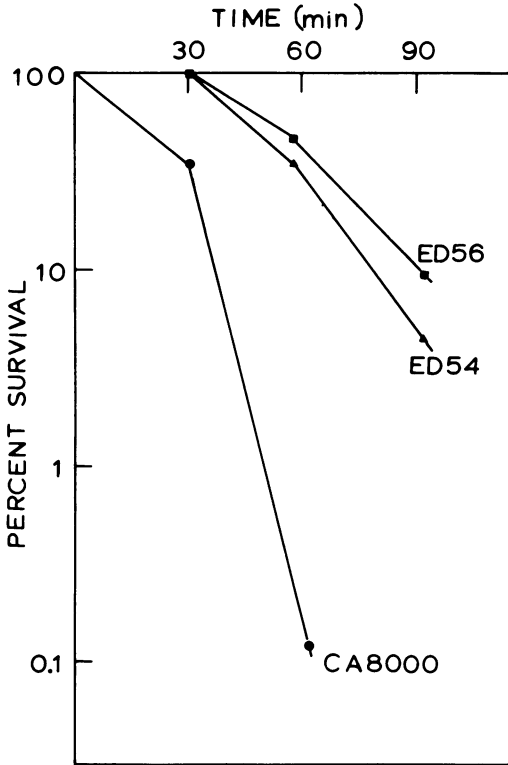


FIG. 2. Effect of methyl methane sulfonate on colony-forming abilities of the wild-type, *cya*, and *crp* cells.

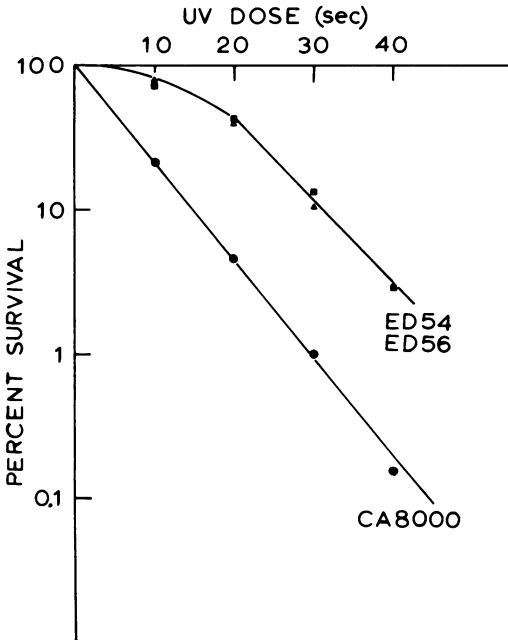


FIG. 3. UV survival curves of the wild-type, *cya*, and *crp* strains.

In Fig. 4 the gamma-ray survival curves of strains ED54 and ED56 and the wild type are shown. The comparison of  $D_{10}$  values of the curves suggested that the mutants were about 1.7 times more resistant to gamma rays than the wild type.

**Resistance of the mutants to thermal stress and osmotic shock.** Sublethal heating produces several kinds of lesions in bacteria (37) which if not repaired may lead to cell killing. One of the reasons for cell death may be the unrepaired breaks in deoxyribonucleic acid (DNA) somehow induced as a consequence of thermal stress (4). A correlation between sensitivities to ionizing radiation and sublethal heating has been demonstrated (4). Since the *cya* and *crp* mutants were found to be more resistant to gamma rays than the wild type, it was of interest to see if the mutants differed from wild type in their thermal tolerance as well. A comparison of the kinetics of thermal death at 50 C of the wild type and strains ED54 and ED56 (Fig. 5) revealed that, indeed, the mutants were more resistant to heat than the wild type.

*E. coli* cells under thermal stress are known to carry large surface blebs (29), to lyse spontaneously at low frequencies (28), and to be hypersensitive to lysis by sodium ethylenediaminetetraacetate and lysozyme (28), indicating building up of higher than physiological internal osmotic pressure. These are also generally the characteristics of cells undergoing osmotic shock (2). This correlation may be used to suggest that the survival from both thermal stress and

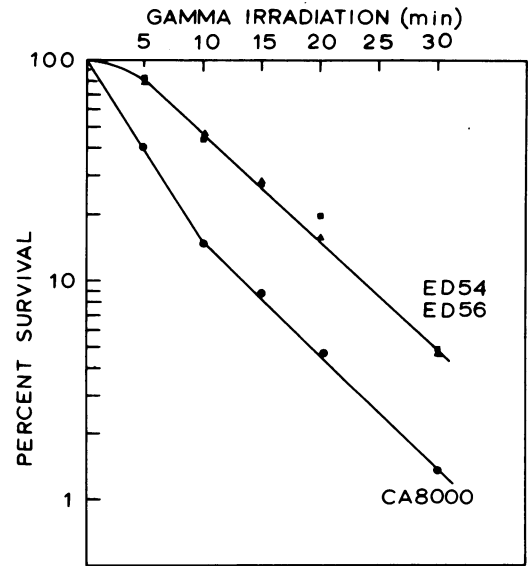


FIG. 4. Gamma-ray survival curves of the wild-type, *cya*, and *crp* strains.

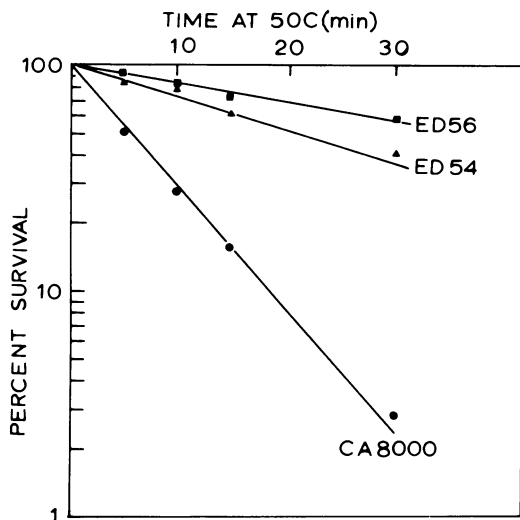


FIG. 5. Effect of heating at 50 C on colony-forming abilities of the wild-type, *cya*, and *crp* strains.

osmotic shock requires reduction in differential osmotic pressure without injury to the cell. According to this idea, the *cya* and *crp* cells which were found to have increased thermal resistance should also have increased osmotic shock resistance. This was tested by studying the effects of hypotonic shock in water at 37 C on the colony-forming abilities of the wild type, ED54, and ED56 strains (Table 3). As expected, the mutants were found to be more resistant to hypotonic shock than the wild type. This result indicated that in mutant cells either the internal osmotic pressure was maintained at low levels or the envelopes were of high flexibility and rigidity. Thus it was of interest to examine cell envelope characteristics of the mutants.

#### Altered phage sensitivity of the mutants.

The *cya* and *crp* mutants are known to be resistant to lambda phage (40). The mutants were tested for their sensitivity to several other phage strains. Under comparable conditions, whereas phages T1, T4, T7 and Plvir plated with high efficiency and produced normal-looking plaques on both wild-type and mutant strains, phage T6 plated normally on the wild type but produced ghost plaques with reduced efficiency on the mutants (data not shown). This indicated that the mutants were unable to propagate phage T6. Table 4 gives the results of an experiment in which cultures of strains ED54, ED56, and wild type were infected with phages T1, T4, T6, T7, and  $\lambda$ v2v1v3 individually at high MOI and frequencies of surviving CFU were determined. The mutants were found to tolerate phage T6 infection to an extent only

slightly lower than their tolerance for phage  $\lambda$ v2v1v3. Because lambda phage does not adsorb efficiently to *cya* and *crp* mutants, it was of interest to see whether T6 also did not adsorb to them. The results indicate that T6 adsorbed to the mutant cells poorly (Table 5).

**Patterns of sensitivity towards antibiotics, detergents, and other agents.** In certain *E. coli* mutants, phage resistance has been found to be associated with sensitivity to a number of antibiotics (34). On the other hand, several antibiotic-resistant mutants have been reported to be unusually sensitive to detergents (19, 33). Several kinds of biochemical lesions in the envelopes of such mutants have been identified (19, 34). When relative sensitivities of *cya* and *crp* mutants and wild type to antibiotics and

TABLE 3. Hypotonic shock stability of *cya*, *crp*, and wild-type strains<sup>a</sup>

Strain	Percentage of survival after hypotonic shock	
	Cells grown in TB + 20% sucrose	Cells grown in standard TB
CA8000	12	70
ED54	57	96
ED56	76	106

<sup>a</sup> Cells grown to logarithmic phase were diluted 1:100 in water at 37 C. After 5 min they were diluted in cold diluent and plated for CFU per milliliter. Initial titers were determined by use of chilled growth medium as the diluent.

TABLE 4. Sensitivity of the wild type and *cya* and *crp* mutants towards phage T1, T4, T6, T7, and  $\lambda$ v2v1v3

Phage <sup>a</sup>	Percentage of CFU surviving phage infection		
	CA8000	ED54	ED56
T1	$1.2 \times 10^{-5}$	$2.0 \times 10^{-5}$	$2.0 \times 10^{-5}$
T4	$3.4 \times 10^{-4}$	$7.3 \times 10^{-4}$	$7.0 \times 10^{-4}$
T6	$4.9 \times 10^{-4}$	3.1	7.6
T7	$7.1 \times 10^{-5}$	$4.9 \times 10^{-5}$	$6.5 \times 10^{-5}$
$\lambda$ v2v1v3	$3.5 \times 10^{-4}$	42	100

<sup>a</sup> Input MOI was between 19 to 31 PFU/cell.

TABLE 5. Adsorption of phage T6 and  $\lambda$ v2v1v3 to the wild type and *cya* and *crp* mutants

Phage	Percentage of input PFUs not adsorbed		
	CA8000	ED54	ED56
T6	10	91	96
$\lambda$ v2v1v3	4	94	98

detergents were studied, the mutants were found to be more resistant towards ampicillin and more sensitive towards streptomycin, SDS, and DOC (Table 6; Fig. 6). There was little protective effect of 500  $\mu$ g of DL-alanine/ml on DOC-caused inhibition of bacterial streaks on solid media.

The mutant bacteria showed increased sensitivity towards sodium azide, a respiratory inhibitor.

**Morphology of the mutants.** Since a number of penicillin-resistant mutants of *E. coli* are known to harbor morphological aberrations (15, 19, 35), and since *cya* and *crp* mutants were found to be ampicillin resistant, it was of interest to examine the morphology of *cya* and *crp* bacteria. The mutant cells grown on tryptone (Difco)-based liquid and solid media were observed to be coccobacilli approaching coccil shape (spherical), in contrast to rod-shaped cells of the wild type (Fig. 7; Table 6). The diameter of the mutant cells was noted to be

greater than that of the control cells. In comparison with 1.8- $\mu$ m length and 0.7- $\mu$ m diameter of the wild-type cells, the corresponding dimensions of the strain ED54 and ED56 cells were 1.1  $\mu$ m and 0.9  $\mu$ m. The segregation of cells was apparently normal and chains or filaments were not observed in unusual frequencies in mutant cultures. The filamentous cells were actually more common in the wild type than in the mutants. The *cya* cells were found to assume a rod shape when grown in the presence of cAMP for at least two cell generations. Both *cya* and *crp* cells grew as rods of varying sizes when cultured in broth supplemented with 20% sucrose and 0.01 M  $\text{MgSO}_4$ . Under these conditions, the wild-type cells appeared as rods of much reduced size. Supplementation with 1 mg of DL-alanine/ml did not change the round shape of the mutant cells.

**Properties of revertants and  $\phi 80$  *cya*<sup>+</sup> and  $\phi 80$  *cya* lysogens of the mutants.** The purpose of this study was to determine whether all the

TABLE 6. Characters of *cya* and *crp* mutants, their revertants, and transductants and wild type<sup>a</sup>

Strains	Utilization of lactose, maltose, and motility <sup>a</sup>	Resistance to UV, MMS, gamma rays, wet heat, and hypotonic shock <sup>c</sup>	Sensitivity to SDS, DOC, azide, and streptomycin <sup>d</sup>	Resistance to $\lambda$ and T6 <sup>e</sup>	Resistance to ampicillin <sup>d</sup> and NaI <sup>e</sup>	Colony morphology	Cell morphology <sup>b</sup>	Growth rate <sup>b</sup>
CA8000	+	S	R	S	S	Rough big	Rod	Normal
ED54, ED56	—	R	S	R	R	Smooth small	Round	Slow
ED50-1, ED50-2, ED54-1, ED54-9, ED56-3	+	S <sup>f</sup> , <sup>g</sup>	R <sup>h</sup>	S	S <sup>i</sup>	Rough big	Rod <sup>i</sup>	ND
ED54-2, ED56-1, ED56-2	—	R <sup>f</sup> , <sup>g</sup>	S	R	R <sup>i</sup>	Smooth small	Round	ND
CA8306, ED55, ED73	—	R <sup>f</sup>	S	R	R	Smooth small	Round	Slow
1-23, ED66, ED67	—	R <sup>f</sup>	S	R	R	Smooth small	Round	Slow

<sup>a</sup> Abbreviations: +, yes; —, no; R, resistant; S, sensitive; ND, not determined.

<sup>b</sup> Determined as described in Materials and Methods.

<sup>c</sup> Resistance was determined from survival curves.

<sup>d</sup> Determined by measuring growth of bacterial streaks on solid media containing inhibitors.

<sup>e</sup> Determined by streaking against a T6 lysate with titer of about 10<sup>7</sup> PFU/ml.

<sup>f</sup> Lysogens were generally more sensitive to these agents than nonlysogens.

<sup>g</sup> The response of these strains to hypotonic shock was not determined.

<sup>h</sup> ED54-1 and ED54-9 grew better than wild type on TA containing 50  $\mu$ g of azide per ml.

<sup>i</sup> Compared to wild type, these were more filamentous and had longer cells.

phenotypic changes described here were due to mutational events only in the *cya* and *crp* loci. The three types of evidence sought in affirmative were: (i) whether a class of revertants could be isolated in which the various phenotypes of

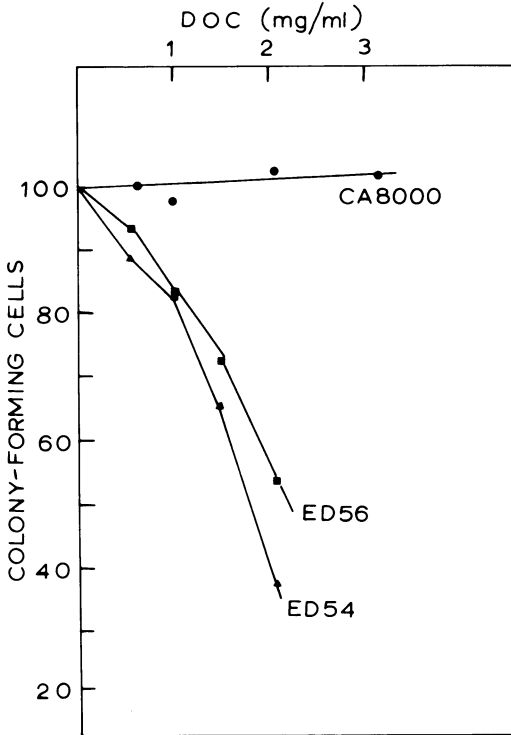


FIG. 6. DOC sensitivity of the wild type and *cya* and *crp* strains.

the mutants would revert together to wild type, (ii) whether *cya* mutants and not *crp* mutants will behave like wild type upon their lysogenization with  $\phi 80$  *cya*<sup>+</sup> and not with  $\phi 80$  *cya*, and (iii) whether *cya* mutants assume wild-type phenotype when supplied with cAMP exogenously. It was found that the frequency of the desired kind of revertants of strains ED54 and ED56 (Table 6) was in the range of  $10^{-6}$ . This value is very much higher than would be expected if the various phenotypic changes in the mutants were due to several independent mutations. Further,  $\phi 80$  *cya*<sup>+</sup> phage and not  $\phi 80$  *cya* could restore wild-type behavior of strain ED54 and other *cya* strains.  $\phi 80$  *cya*<sup>+</sup> lysogens of strains ED56 and other *crp* strains maintained the mutant phenotype. Also *cya* cells of cultures supplemented with  $10^{-3}$  M cAMP were found to have acquired the wild-type phenotype for all the following characters tested: colony and cell morphology, growth rate, sensitivity or resistance to Nal, ampicillin, DOC, and azide, motility, carbohydrate utilization, and phage sensitivity.

## DISCUSSION

This paper implicates cAMP and its receptor protein in expression of some additional cellular functions of *E. coli*. We have shown that the *cya* and *crp* mutants grow as cocci with longer doubling times and have increased resistance to some mutagens (MMS, UV light, gamma rays), antibiotics (Nal, ampicillin), phages (lambda and T6), and stressful conditions (sublethal heat, hypotonic shock), and decreased resist-

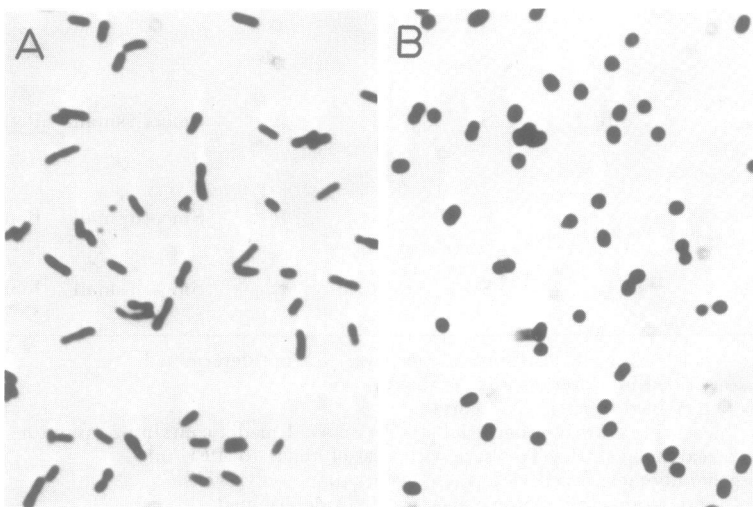


FIG. 7. Photomicrographs of bacterial cells at  $\times 2,000$ . (A) Parent strain; (B) ED54. Cells of ED56 looked like those of ED54.



ance to neutral detergents (SDS, DOC), a protein synthesis inhibitor (streptomycin), and a respiratory inhibitor (azide). We have noted a close correspondence between the characteristics of *cya* and *crp* mutants and of several other spherical mutants of *E. coli* isolated by chance, as conditional lethals or for their resistance to ampicillin (13, 15, 20, 23, 35). This has led us to suggest that most of the unusual parameters of *cya* and *crp* cells are manifestations of their defective envelope structure. This line of interpretation is discussed in more detail below.

The envelope of *E. coli* cells is known to be three layered (1, 30). The innermost layer is the cytoplasmic membrane of lipids and proteins. This layer contains, integrally or peripherally, among others, the proteins for electron transport, permease and chemoreception functions, enzymes for phospholipid and polysaccharide synthesis, and cytochromes. The middle layer is the peptidoglycan sacculus which adheres to cytoplasmic membrane on the inside. Linked to it on the outside is a layer of lipopolysaccharides present in complex with lipids and proteins. This layer is associated with phospholipases, phage and bacteriocin receptor proteins, and other polypeptides. The integrity, strength, and permeability of the three-layered cell envelope is likely to depend on the organization and interaction of its different components. The functional and biochemical analyses have previously shown the *cya* and *crp* mutants to be deficient in several carbohydrate-specific permeases and cytochromes. Their phage resistance pattern indicates that *cya* and *crp* cells must also be deficient in receptor proteins for lambda and T6. The spherical shape of *cya* and *crp* cells is expected to result from alterations in the pattern of mucopeptide linking in their peptidoglycan. Thus the structure of all three envelope layers of *cya* and *crp* mutants may actually be changed.

The resistance of *cya* and *crp* cells to thermal and osmotic stresses is indicative of better control of differential internal osmotic pressure by them. One way this might be achieved in mutant cells is if they maintained low internal osmotic pressure even under normal cultural conditions. The mutants may accomplish this by any of several possible mechanisms. Besides reducing the synthesis of polyamines and other molecules that raise the internal osmotic pressure, the mutants may restrict intake of certain kinds of carbon and nitrogen sources and other substances, or lose through leakage certain critical intracellular constituents (22). All such cell responses are likely to lower the growth rate

by retarding the division process (21). Alternatively, the slow growth rate of *cya* and *crp* cells may be a consequence of their unusual morphology (38). A critical configuration of cytoplasmic membrane appears necessary for the normal progression of cell division process triggered at the completion of chromosomal replication (6, 38).

Increased sensitivity of *E. coli* cells to neutral detergents is usually a result of increased permeability of the outer envelope layer (17). This might be the reason, at least partially, for the increased sensitivity of *cya* and *crp* cells to streptomycin and azide in addition to SDS and DOC. Because of increased permeability of the outer membrane, the *cya* and *crp* cells may be expected to be generally sensitive to antibiotics. In disagreement with this expectation, the *cya* and *crp* cells have increased resistance towards nalidixic acid and ampicillin. A possible explanation of ampicillin resistance of *cya* and *crp* mutants can be that because of their slow-growing nature they might escape the lethal action of ampicillin better than fast-growing cells, since it is known that nongrowing cells are not killed by penicillins (14). The mechanism of lethal action of Nal on *E. coli* is not known. The characteristics of the different classes of Nal-resistant mutants isolated in *E. coli* have also not provided any clue in this respect (11, 12). Slow growth and reduced intake of Nal appear to be two likely reasons for Nal resistance of *cya* and *crp* mutants, since Nal specifically inhibits DNA synthesis (10) and kills only those cells that are replicating DNA (36).

The acquisition of wide resistance against mutagenic agents by *cya* and *crp* mutants may be the result of at least three changes in the cell's parameters. First, the mutant cells may have increased target size if their slow growth is due to inordinate lengthening of cell division time in relation to DNA replication time. Second, the long doubling time of the mutants may allow increased repair of the mutagen-induced lesions. Finally, due to about 33% reduction in their surface to volume ratio the mutant cells will have less surface area exposed to the mutagens and thus are likely to receive lower levels of mutagen-induced primary damage.

The physiological explanations considered here for the various properties of *cya* and *crp* mutants are mostly experimentally testable. The properties of the *cya* and *crp* mutants are such that procedures can easily be designed for selection of mutants reverted for only one or a few phenotypic characteristics at a time. The study of revertants and their derivatives should

help not only in the analysis of the various metabolic pathways of *E. coli* controlled by cAMP, but also of the mechanism of morphogenesis.

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