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Genetic Characterization of Mutations Which Affect Catabolite-Sensitive Operons in *Escherichia coli*, Including Deletions of the Gene for Adenyl Cyclase

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Sixty-two spontaneous mutations have been characterized which reduce the level of expression of catabolite-sensitive operons. These mutations appear to affect either the *crp* (catabolite gene activator protein) or *cya* (adenyl cyclase) loci. No new loci have been discovered. Deletions of the *cya* gene do not remove an essential function. ϕ80 transducing phage for the *cya* gene have been used to do recombination and complementation studies on *cya* mutants.

The bacterium *Escherichia coli* is able to utilize a wide range of compounds as carbon or nitrogen sources. Many of these compounds are metabolized by inducible pathways. In all cases which have been examined, these pathways are dependent on the substrate for induction and, in addition, appear to be dependent on the levels of adenosine-3',5'-cyclic monophosphate (cAMP) in the cell (13, 17). The best-studied example of control of an inducible pathway is that of the genes determining lactose (*lac*) metabolism. In this case, it has been shown that cAMP and a protein factor, catabolite gene activator protein (CAP), are required, in addition to ribonucleic acid (RNA) polymerase, for the initiation of transcription of the *lac* operon (4, 5, 7, 20). Preliminary genetic evidence suggests that the promoter of the *lac* operon is divided into two at least partially distinct sites, one the site of CAP action and the other the site of RNA polymerase interaction (1).

Although the basic aspects of this model have been demonstrated in an in vitro transcription system, certain pieces of information have raised the possibility that the mechanism of control may be more complex. For instance, in an in vitro protein-synthesizing system, expression of the *lac* and arabinose (*ara*) operons, both of which are dependent on cAMP, are significantly stimulated by guanosine-tetraphosphate (4, 19). Furthermore, repression in the in vitro transcription system is not complete (4, 7).

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So far, two genetic loci have been identified which determine the cAMP control of inducible operons in *E. coli*. The phenotype of mutants in these loci are generally pleiotropically negative for the inducible pathways, including, for example, those for lactose, arabinose, and maltose (*mal*) metabolism. One of these loci, *crp*, codes for the CAP factor (5, 17) and at least one mutation in the other, *cya*, has been shown to lack adenyl cyclase (13). The pleiotropic phenotype of the *cya* mutants is reversed by addition of cAMP to growth media, whereas that of the *crp* mutants is not.

In this paper we describe the isolation and characterization of a considerably larger number of pleiotropic mutants than have been described before. Genetic mapping and complementation studies with these mutants give no evidence for any additional loci involved in CAP-cAMP control. As a result of these studies, we have been able to show that at least one *cya* mutation is a deletion, indicating that adenyl cyclase is not essential for cell growth. In addition we describe the isolation of ϕ80 high-frequency transducing phages which carry the *cya* locus.

MATERIALS AND METHODS

Bacterial strains. The genotypes and derivation of the strains used are listed in Table 1.

Chemicals. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (XG) was obtained from Cyclo Chemical Corp.; isopropyl-β-D-thiogalactoside (IPTG) was from Schwarz-Mann; and *N*-methyl-*N*-nitrosoquanidine (NG) was from Aldrich Chemical Co.

TABLE 1. *Characteristics of the strains used*[a]

Strain	Sex	Characters	Origin
CA8000	Hfr Hayes	*thi*	J.B.
I-7019	F⁻	*aroB*	L. Gorini
IO-7004	F⁻	*ilv metE*	L.S.
LS-680	F⁻	*trp his cya* 283	L.S.
IO-7011	F⁻	*trp his metE ilv*	E.B.

[a] Abbreviations are as follows: *thi*, *aro*, *ilv*, *met*, *trp*, *his*, requirements for thiamine, aromatic metabolites, isoleucine and valine, methionine, tryptophan and histidine, respectively.

Media. Tetrazolium agar was as described by Ohlsson et al. (11). Liquid cultures were grown in LB broth (9). Minimal medium both liquid and solid was M63 (9). Minimal glucose agar containing XG (2) was used here to distinguish high and low levels of *lac* operon expression.

Phage lysates and transduction. The making of P1 *vir* lysates, P1 transduction, and ϕ80 lysates are as described by Miller (9).

Reversion. Reversion of mutants with NG was tested by spreading 0.1 ml of a saturated broth culture of the strain on maltose minimal agar and putting a crystal of NG in the middle of the plate.

β-Galactosidase assays. Assays were done after growth of cultures to a density of 2×10^8 bacteria per ml in glucose minimal media containing 2×10^{-3} M IPTG (12). Specific activities were calculated by the method of Beckwith et al. (2).

Isolation of mutants. Cultures of independent clones of strain CA-8000 were grown to a density of 2×10^8 bacteria/ml in LB broth. A 0.1-ml amount of a 10-fold dilution of these cultures was mixed with 0.1 ml of a λvir (2×10^{11} phage/ml) lysate, and the mixture was incubated at 37 C for 20 min. This mixture was then spread on arabinose-maltose tetrazolium agar giving approximately 100 λ^R colonies.

Isolation of ϕ80 cya transducing phages. A lysogen containing the defective prophage ϕ80d ϕ3*ilv* (Soll, manuscript in preparation), integrated in the *ilv* region, was used to generate ϕ80d*cya*$^+$ phage. A low-frequency transducing lysate prepared for this strain was used to transduce LS680 (multiplicity of infection = 20 plaque-forming units/cell) for the ability to grow on maltose as sole carbon source. The non-revertibility of the *cya* 283 allele with respect to growth on maltose enabled us to examine 10^{11} phage per petri dish. Mal$^+$ colonies which appeared at a frequency of 2×10^{-11} were tested for their ability to produce high-frequency transducing (HFT) lysates for the *cya*$^+$ marker. Approximately half could do so. Twelve of these HFT lysates were kept; among these, four retained the *ilv* marker on the prophage.

Recombination of cya mutants onto ϕ80 cya. *ilv*$^-$ derivatives of *cya* mutants were constructed by transducing strain IO7011 to *met*$^+$ with P1 lysates grown on a number of the *cya* mutants. Transductants which were *ilv*$^-$*cya*$^-$ were then transduced to *ilv*$^+$ by spotting a drop of the ϕ80 *ilv*$^+$*cya*$^+$ lysate onto a lawn of the recipient on glucose-minimal agar. *Ilv*$^+$ transductants were purified. Samples of broth cultures of these *ilv*$^+$*cya*$^+$/*ilv*$^-$*cya*$^-$ heterogenotes were spread along with 10^{10} λvir phage on glucose-minimal agar containing XG. On this agar we select to hold the ϕ80*ilv*$^+$ and can screen for low levels of *lac* operon expression, reflecting a *cya* defect. λ^R colonies which were pale blue on this medium were presumed to be due to homogenotization to ϕ80 *ilv*$^+$*cya*$^-$/*ilv*$^-$*cya*$^-$ which would make the strains Mal$^-$ (λ^R) and Lac$^-$ (pale blue on XG). The nature of the strains was verified by ultraviolet light induction of the ϕ80 and demonstration of HFT lysates for *ilv*$^+$.

Complementation studies. *cya* and *crp* mutants grow more slowly on all media we have tested. There is, therefore, a strong selection for revertants during growth. In general, for most of our experiments we allow as little growth of a *cya*$^-$ strain as possible. For complementation studies, we constructed and tested heterogenotes of the genotype ϕ80*ilv*$^+$*cya*^-_a/*ilv*$^-$*cya*$_b$ by the following steps. (i) Single colonies of the *ilv*$^-$*cya*^-_b (*trp*$^-$, *his*) strains were suspended in two drops of LB broth, and one drop of HFT ϕ80 *ilv*$^+$*cya*$_a$ was added. The adsorption mixture was incubated at 37 C for 20 min. (ii) A drop of this mixture was streaked out on glucose-minimal media containing histidine and tryptophan. (iii) Four *ilv*$^+$ colonies from each transduction were purified on the same selective media. (iv) Four colonies of each of the *ilv*$^+$*cya*^-_a/*ilv*$^-$*cya*^-_b heterogenotes were then streaked out on arabinose-tetrazolium agar to determine whether the presence of the two *cya* alleles restored a higher level of *ara* operon expression.

Recombination studies. The colonies obtained from the purification of step iii above were suspended in 1 ml of LB broth at 37 C and allowed to grow to approximately 2×10^8 bacteria/ml. Titration of a number of cultures showed that the cell concentration varied between 1.25×10^8 and 2.0×10^8 *ilv*$^+$ cells/ml. A couple of drops of each of these cultures were put on a grid on the inside of the cover of a plastic petri dish. A replicator with 16 prongs was dipped into these drops, and they were transferred to glucose and maltose-minimal agar. The plates were incubated at 37 C for 48 h. The amount of material carried over by the prongs was calibrated and found to correspond to approximately 0.05 ml. The number of colonies on the maltose plates was observed. Since there is preferential growth of *cya*$^+$ recombinants and revertants in these cultures, measurements of recombination *frequencies* are unreliable, except for the negative results.

RESULTS

Isolation of the mutants. We have previously described a method for the detection of *crp* or *cya* mutants after mutagenesis of a wild-type strain (17). In this paper, we described a selective technique for enriching for such mutants, which allows us to detect spontaneous mutants. We were particularly interested in screening spontaneous mutants since the proportion of deletions among them should be

relatively high (16). Many direct selections exist for the elimination of catabolic pathways. One of these derives from the finding that adsorption of the bacteriophage λ to *E. coli* is dependent on some aspects of the functioning of the pathway for maltose catabolism (15). Among λ resistant (λ^R) mutants selected with *E. coli* is a high proportion which is simultaneously Mal^-. The procedure we have devised is to select λ^R derivatives of a wild-type strain of *E. coli* on solid tetrazolium indicator agar containing arabinose and maltose. On this medium, we hoped that only λ^R Mal^- which are also Ara^- will show up as red or pink colonies as opposed to the white carbohydrate-positive colonies.

One hundred and forty independent cultures of strain CA-8000 (crp^+cya^+) were treated in this way. Potential pleiotropic carbohydrate utilization mutants were detected in most of these cultures. In general, we selected those colonies from the tetrazolium agar which appeared to have even slightly less efficient sugar utilization than the rest of the colonies. These strains were then further characterized by testing for growth on arabinose, maltose, lactose, and glucose medium. Only those which were unable to grow on one of these sugars were selected for further analysis since genetic studies would otherwise be difficult. As a result, we have a collection of 67 mutant strains which represent at least 56 independent mutations. These mutants fall into the classes indicated in Table 2.

We purposely processed mutants which did not exhibit a completely negative phenotype for the sugars used in order to have leaky mutants among those characterized. Four which exhibited the most leaky phenotype ($Lac^+Ara^+Mal^-$ or $Lac^+Ara^-Mal^+$) were assayed for their β-galactosidase levels. Interestingly, all four showed fully induced β-galactosidase levels of about 22% of a wild-type control (Table 3).

Since our selections are carried out on rich agar media, strains could have been detected which were auxotrophic due to either side effects of the mutations or due to deletion of neighboring genes along with deletion of the gene in question. Among the 67 mutants, one was found not to grow on glucose minimal medium. When this mutant was assayed for β-galactosidase levels after growth in broth, it was found to have levels identical to that of the parent strains. Thus, the indicator agar reaction of this colony does not appear to reflect an impairment of the CAP-cAMP system.

Chromosomal location of pleiotropic mutants. Our approach to mapping the mutations isolated was to determine whether the mutations could be localized in the known region of either the *crp* or *cya* locus. The *crp* locus is located between the *strA* gene and the *aroB* gene (6), while *cya* has been mapped between *ilv* and *metE* (18). What we have done is to make P1 lysates of all mutant strains and to transduce an $aroB^-$ and an ilv^- strain to prototrophy. The transductants are then screened to see whether any have also picked up the carbohydrate-negative character as an unselected marker. By these tests, of the 67 mutants 20 were linked to *aroB* and 42 to *ilv*. Four of the strains were shown to carry mutations in the *malA* locus, since a $\phi 80malA^+$ (8) transducing phage restored the mal^+ character, while the Ara character of the mutants was indistinguishable from wild type. Thus, no new loci have been revealed in the characterization of these mutants.

Potential deletions of the cya gene. To determine whether any of the mutants charac-

TABLE 2. *Phenotype and genotype of mutants*[a]

Genotype	Phenotype (no.)[b]			
	$Lac^-Ara^-Mal^-$	$Lac^+Mal^-Ara^-$	$Lac^+Mal^+Ara^-$	$Lac^+Mal^-Ara^+$
cya	19	7	11	5
crp	7	12	0	1

[a] The existence of the two classes of mutants, $Lac^+Mal^+Ara^-$ and $Lac^+Mal^-Ara^+$, in the *cya* gene is somewhat puzzling. For both cases, the growth on maltose or arabinose was very slight, although it was repeatedly observed. Transduction of the mutants into a different genetic background resulted in a more stringent carbohydrate-negative phenotype. All transductants tested failed to grow on arabinose, while the previously Mal^+ mutants grew, albeit even less well, on maltose.

[b] There were also four mal^- mutants and one auxotroph.

TABLE 3. *Rate of β-galactosidase synthesis in leaky cya mutants*[a]

Mutant no.	Phenotype	Enzyme levels[b]
Wild type		100
272	$Lac^+Mal^+Ara^-$	21
462	$Lac^+Mal^+Ara^-$	22
852	$Lac^+Mal^+Ara^-$	21
881	$Lac^+Mal^-Ara^+$	23

[a] Assays were done on two independent cultures with less than 10% average error. Cultures of *cya* mutants were checked to insure that there were no revertants accumulated, by spreading samples on arabinose-maltose tetrazolium agar.

[b] Measured as percentage of fully induced wild type.

terized were due to deletions, we have studied their reversion properties. Both *crp*$^-$ and *cya*$^-$ mutants can revert to Ara$^+$ or Lac$^+$ by mutations in their respective operons. Mal$^+$ revertants are less frequent and consistently exhibit Lac$^+$, Ara$^+$ phenotype. The failure to find Mal-specific revertants may be due to the fact that there are two separate *mal* loci (14). At any rate, it appeared to us that reversion on maltose-minimal agar could be a good indication of whether or not a deletion existed in a particular strain.

We have tested all strains for reversion to Mal$^+$ and have found only three (201, 283, 854) which fail to revert. The reversion was not seen, even in the presence of the mutagen, NG. The mutations in all three strains were linked to *ilv* and were presumed to be *cya* mutations. In a subsequent section, we demonstrate genetically that at least one of these strains does carry a deletion of the *cya* gene. No potential *crp* deletions were found.

Isolation of ϕ80 cya transducing phage. The genetic analysis of *cya* mutants described in the following sections was greatly facilitated by the isolation of ϕ80*cya* transducing phages. We have isolated these phages, starting with a strain in which a ϕ80*ilv* transducing phage is integrated in the *ilv* region. This approach to the isolation of transducing phages was described by Konrad et al. (B. Konrad, J. Kirschbaum, and S. Austin, in press). Lysates of this strain were used to transduce one of the presumed *cya* deletion strains, and Mal$^+$ transductants selected. The use of the *cya* deletion strain is crucial since there is tremendous selection for the growth of revertants over the mutant with point *cya* and *crp* mutants. The background of revertants makes it very difficult to detect any rare ϕ80 *cya*$^+$ transductants.

From such transductions we have characterized 12 ϕ80 *cya*$^+$ HFT lysates of which four also carry the *ilv* locus.

Complementation studies of the cya locus. We wished to determine whether all the mutations linked to *ilv*, and which we have tentatively called *cya* mutants, defined a single gene or a more complex locus. To do this, we have constructed diploid strains of the genotype ϕ80 *ilv*$^+$*cya*^-_a/*ilv*$^-$*cya*^-_b. These diploid strains were then streaked on arabinose-maltose tetrazolium agar to see whether any mutants complemented to restore a wild-type (Ara$^+$Mal$^+$) phenotype. Although, occasionally in these streaks we could easily see rare Ara$^+$Mal$^+$ colonies which were probably recombinants, all diploid strains maintained the Ara$^-$Mal$^-$ phenotype. The complementation studies involved 20 *cya* mutants including two mutants which had the phenotype Lac$^+$Ara$^-$Mal$^-$, one Lac$^+$Ara$^+$Mal$^-$, and Lac$^+$Ara$^-$Mal$^+$. They also included the three presumed *cya* deletion mutants. The mutants are the same ones used for recombination studies in Table 4 and, in addition, mutants 252, 591, and 131.

Demonstration of a deletion of the cya locus. We have done genetic recombination studies with *cya* mutants to see whether we could genetically demonstrate the deletion nature of mutants 201, 283, and 854. Again, diploids of the genotype ϕ80*ilv*$^+$*cya*^-_a/*ilv*$^-$*cya*$^-_{del}$ or the reciprocal were used. Cultures

TABLE 4. *Recombination between cya mutants*[a]

ϕ80 *cya* / Chromosomal *cya*	111	201	202	204	251	283	311	854	891	893	894
JB2								+			
111								+			
201		0		+		+	+	0	+	+	+
283	+	+	+	+	+	0	+	0		+	+
285						+		+			+
443						+		+			+
481						+		+			+
854		0	+	+		0	+	0	0	0	0
871						+		+			+
891						+		±			+
894						+		+			+
1311						+		+			+

[a] Crosses were carried out as described in Materials and Methods. A zero indicates a frequency of $<3 \times 10^{-8}$. Pluses do not necessarily indicate recombination, because of reversion problems described in Materials and Methods. The plus-minus of the 891-854 cross indicates that a few Mal$^+$ colonies were seen. These are probably due to revertants. Mutants 891, 893, and 894 come from the same original culture and may be identical.

of these strains were grown up, and fractions were spread on maltose-minimal agar. Controls were also done to correct for any reversion problems. The mutation in strain 854 failed to recombine with at least three independent *cya* mutants (Table 4). These latter mutants, however, did recombine with each other. As a result of these studies, we can say that strain 854 carries a *cya* deletion, and we believe, on the basis of the reversion studies, the same is true for strains 201 and 283.

We have previously concluded that the *lac* operon can function at a low rate (2–3% of wild type) in the absence of cAMP and CAP (1). At the time of those studies we did not have a *cya* mutation which we knew to be nonleaky. Having a *cya* deletion in hand, we considered it worth determining the level of expression of the *lac* operon. In a strain which is most likely to be completely defective in cAMP synthesis, the 2 to 3% of *lac* operon expression remains. β-Galactosidase levels in the wild type and mutants 201, 283, and 854 were 100, 2.7, 2.8, and 2.8, respectively. (The assays were done on three independent cultures of each strain, with an average error no greater than 7%.)

DISCUSSION

The studies described in the paper were initiated primarily to determine whether loci other than *cya* and *crp* were involved in the regulation of catabolite-sensitive operons. We have shown that all mutations which result in a pleiotropic carbohydrate-negative phenotype are linked to one of these two loci. In the case of *cya* mutations, we have demonstrated by complementation studies that all tested map in a single gene. Since similar studies were not carried out with the *crp* locus, we cannot say whether this locus is comprised of one or more genes.

These data do not rule out the possibility of another gene or genes being involved in this regulatory process. For instance, this hypothetical third gene may code for a product which is essential to cell growth and most mutations in it would be lethal. By analyzing leaky mutants, we hoped to rule out this possibility. We have studied such mutants which express the *lac* operon up to 23% of the normal levels. Another possibility is that the product of the gene in question has a stimulatory effect on the operons in question, but is not completely essential. Again, a thorough study of leaky mutants might have revealed such a gene. Finally, this hypothetical third gene could map close to the *crp* gene and thus be confused with *crp* mutants. Although we cannot rule out completely any of these possibilities, we feel it likely on the basis of these studies that only the *crp* and *cya* genes play major roles in the regulation of catabolite-sensitive operons.

We have conclusive evidence that one and probably three of our *cya* mutants carry deletions of the *cya* gene. We see no difference in general growth properties between these strains and any of the other non-leaky *cya* mutant strains. Measurements of cAMP concentrations in cultures of these mutants by W. Epstein indicate that they make very little of this metabolite (W. Epstein, personal communication). These results suggest, more conclusively than has been demonstrated heretofore, that cAMP is not essential for the survival of *E. coli*. Furthermore, the presumed complete absence of cAMP does not reduce *lac* operon expression below the 2 to 3% level described previously. This latter result is consistent with our suggestion that RNA polymerase can initiate transcription of the *lac* operon at a low rate in the absence of cAMP.

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