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Isolation and Characterization of an *Escherichia coli* Mutant Affected in the Regulation of Adenylate Cyclase

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A mutant, *cyaR1*, affecting regulation of adenylate cyclase expression or activity is described. It was obtained as a thermoresistant revertant of a strain harboring a thermosensitive transcription termination factor, rho (*rho-15*). This mutant failed to synthesize adenosine 3',5'-phosphate and exhibited a carbohydrate-negative phenotype. A secondary mutation at the *crp* locus (*crpC*) restored the ability of the mutant to synthesize adenosine 3',5'-phosphate, enabled the expression of catabolite-sensitive operons, and conferred on the strain an extreme sensitivity to catabolite repression. In addition, we showed that the *crpC* mutation restored the pleiotropic carbohydrate-positive phenotype even in a Δ*cya* background. We interpret this to mean that the adenosine 3',5'-phosphate receptor protein regulates negatively either the activity or synthesis of adenylate cyclase and that the *cyaR1* mutation is either in a regulatory protein or a regulatory site of adenylate cyclase.

We have recently shown that a specific class of double mutants of *Escherichia coli*, which carries the defective transcription termination protein rho (6) and lacks the cAMP receptor protein CAP (*rho-crp* mutants), displays a carbohydrate-negative character and can give rise to pleiotropic carbohydrate-positive pseudorevertants. Most of these mutants were shown to exhibit catabolite repression (10). Since such revertants could not be isolated in a *crp-rho*$^+$ background, we inferred that rho might be involved in the regulation of the expression of catabolite-sensitive operons. In addition, we found that almost all of the pleiotropic carbohydrate-negative thermoresistant revertants from a strain that carried the *rho-15*(Ts) allele map at the *crp* locus, whereas less than 3% were shown to be adenylate cyclase deficient (*cya*). This contrasts with the normal spontaneous distribution of *crp* and *cya* mutants in a *rho*$^+$ background (one third *crp*, two thirds *cya*), and it was challenging to speculate that the presence of the *rho-15* allele had allowed the selection of regulatory mutants in the adenylate cyclase expression or activity. One such *rho-15-cya* double mutant is studied in the present paper. From this mutant we isolated a series of carbohydrate-positive pseudorevertants to characterize their behavior with respect to catabolite repression. The most prominent feature of the work we present here is that in these pseudorevertants an additional mutation, which maps at the *crp* locus, restored cAMP synthesis in our *rho-cya* background. We interpret this to mean that the corresponding *cya* alteration is a regulatory mutation.

MATERIALS AND METHODS

Strains and growth media. The bacterial strains used in this work were *E. coli* K-12 derivatives and are listed in Table 1.

The strains were grown in LB or minimal medium 63 (14) supplemented with the required amino acids (100 μg/ml), thiamine (5 μg/ml), shikimic acid (100 μg/ml), and either glucose (0.4%) or succinate (0.2%) as carbon source.

Induction and assay of enzymes and phage λ receptor. The bacteria were grown at 41°C, separately induced for β-galactosidase (10^{-3} M isopropyl-β-D-thiogalactoside), amylomaltase, phage λ receptor (10^{-2} M maltose), and tryptophanase (10^{-2} M tryptophan). After five to six generations, β-galactosidase and tryptophanase were assayed in toluene-treated bacterial suspensions according to Pardee et al. (15) and Suelter et al. (25), respectively; amylomaltase was assayed according to Schwartz (22) in sonically disrupted bacterial extracts. One unit was defined as the amount of enzyme that converted 1 nmol of substrate per min at 28°C. Phage λ receptor was assayed as described by Randall-Hazelbauer and Schwartz (18).

cAMP assay. Exponential cultures grown at 41°C were diluted to obtain 500 μg (dry weight) of bacteria per ml. The bacterial suspensions were heated for 5 min at 100°C, and the total amount of cAMP was determined by a standard radioimmunoassay (24) according to the instructions of the supplier.

Transductions. Generalized transductions were performed with P1 *vir* phage as described by Miller (14). Plates were incubated at 41°C.

TABLE 1. *Bacterial strains*

Strain	Genotype	Origin
AB1528	F′ *thi-1 ilvC7 argE3 his-4* DE62 *galK2 lacZ4 supE44*? F′16 P013 AB313 *ilv*$^+$ *cya*$^+$	CGSC[a]
X8605 M	F$^-$ *trpR trp-lacZ tonB rpsL ΔlacU169 metB rho-201 ilv*::Tn*5*	B. Colonna
AB1528 16K	F′ *thi-1 ilvC7 argE3 his-4* DE62 *galK2 lacZ4 supE44*? F′ 16 P013 AB313 *ilv*::Tn*5 cya*$^+$	AB1528 × P1 X8605 Kan^r
CAA8306	Hfr H *thi-1 relA1 Δcya glp*$^+$	(13)
G61	Hfr G6 *aroB his*	M. Schwartz
SA1030	F$^-$ *his rpsL gal3*	(6) via E. Brody
MI183e	F$^-$ *thi leu metE proC purE trp lysA xyl lac rpsL tonA*	Institut Pasteur
PP2010	F$^-$ *xyl Δcya argH1 ΔlacX74 srl*::Tn*10 recA*	19a
PP2010 16K	F′ *xyl Δcya argH1 ΔlacX74 srl*::Tn*10 recA* F′ 16 *ilv*::Tn*5*	PP2010 × AB1528 16K Kan^r
PP7811	F$^-$ *xyl argH1 his* λ	5a
PP7810	F$^-$ *xyl ilv A215 argH1 his* λ	(10)
PP7814	F$^-$ *xyl ilv A215 argH1 his malA* λ	Spontaneous Mal$^-$ λ *vir*^{r}
PP7815	F$^-$ *xyl ilv A215 argH1 his aroB* λ	PP7814 × P1 G61 Mal$^+$
PP7860	F$^-$ *xyl Δcya argH1 his* λ	PP7810 × P1 CAA8306 Ile$^+$
PP7812	F$^-$ *xyl rho-15(Ts) argH1 his* λ	(10)
RCC1	F$^-$ *xyl rho-15(Ts) argH1 his* λ *cyaR1*	Spontaneous thermoresistant from PP7812
RCC2	F$^-$ *xyl rho-15(Ts) argH1 his* λ *crp RCC2*	Spontaneous thermoresistant from PP7812
PP7100	F$^-$ *xyl aroB ilv A215 argH1 his* λ$^-$ λ *i21cts*	λ heteroimmune curing
PP7000	F$^-$ *xyl aroB ilvA215 argH1 his* λ$^-$	λ *i21* cured
PP7200	F$^-$ *xyl aroB ilv A215 argH1 his* λ *cI857 xis*	λ *cI857 xis* lysogenization
PP7200 T1	F$^-$ *xyl aroB ilv A215 argH1 his Δatt* λ *bio* λ$^-$	Spontaneous excision of λ *cI857 xis*
PP7210	F$^-$ *xyl aroB ilv A215 argH1 his gal3*	PP7200 T1 × P1 1030 Bio$^+$
PP7220	F$^-$ *xyl aroB metE argH1 his gal3*	PP7260 × P1 MI183e Mal$^+$
PP7260	F$^-$ *xyl aroB Δcya argH1 his gal3*	PP7210 × P1 CAA8306 Ile$^+$
PP7244	OF$^-$ *xyl aroB rho-15(Ts) cyaR1 argH1 his gal3*	PP7210 × P1 RCC1 Ile$^+$
PP7246	F$^-$ *xyl crpRCC2 cyaR1 rho-15(Ts) gal3 argH1 his*	PP7244 × P1 RCC2 Aro$^+$
PP7247	F$^-$ *xyl aroB cyaR1 argH1 his gal3*	PP7220 × P1 RCC1 Met$^+$
CGR20	F$^-$ *xyl rho-15(Ts) cyaR1 argH1 his* λ *crpC20*	Spontaneous carbohydrate-positive from RCC1
CGR21	F$^-$ *xyl rho-15(Ts) cyaR1 argH1 his* λ *crpC21*	
CGR22	F$^-$ *xyl rho-15(Ts) cyaR1 argH1 his* λ *crpC22*	
CGR24	F$^-$ *xyl rho-15(Ts) cyaR1 argH1 his* λ *crpC24*	
PP7000/CGR20	F$^-$ *xyl aroB rho-15(Ts) cyaR1 argH1 his* λ$^-$	PP7000 × P1 CGR20 Ile$^+$
PP7000/CGR21	F$^-$ *xyl aroB rho-15(Ts) cyaR1 argH1 his* λ$^-$	PP7000 × P1 CGR21 Ile$^+$
PP7000/CGR22	F$^-$ *xyl aroB rho-15(Ts) cyaR1 argH1 his* λ$^-$	PP7000 × P1 CGR22 Ile$^+$
PP7000/CGR24	F$^-$ *xyl aroB rho-15(Ts) cyaR1 argH1 his* λ$^-$	PP7000 × P1 CGR24 Ile$^+$
CGR40	F$^-$ *xyl crpC20 rho-15(Ts) cyaR1 argH1 his* λ$^-$	PP7000/CGR20 × P1 CGR20 Aro$^+$
CGR41	F$^-$ *xyl crpC21 rho-15(Ts) cyaR1 argH1 his* λ$^-$	PP7000/CGR21 × P1 CGR21 Aro$^+$
CGR42	F$^-$ *xyl crpC22 rho-15(Ts) cyaR1 argH1 his* λ$^-$	PP7000/CGR22 × P1 CGR22 Aro$^+$
CGR51	F$^-$ *xyl aroB Δcya argH1 his* λ$^-$	PP7000 × P1 CAA8306 Ile$^+$
CGR52	F$^-$ *xyl aroB Δcya argH1 his* λ$^-$	PP7000 × P1 CAA8306 Ile$^+$
CGR49	F$^-$ *xyl crpC20 Δcya argH1 his* λ$^-$	CGR51 × P1 CGR20 Aro$^+$
CGR50	F$^-$ *xyl crpC24 Δcya argH1 his* λ$^-$	CGR52 × P1 CGR24 Aro$^+$
CA8404	Hfr H *thi rpsL Δcya crp**	J. Beckwith (20)
CGR53	F$^-$ *xyl crp** *rho-15(Ts) cyaR1 argH1 his* λ$^-$	PP7000 × P1 CA8404 Aro$^+$
CGR57	F$^-$ *xyl crpC20 Δcya argH1 his* λ$^-$ *rpsL*	Spontaneous streptomycin resistant from CGR49

[a] *E. coli* Genetic Stock Center, Yale University.

Reagents. Reagents were obtained from the following companies: the cAMP reagent kit was from Institut Pasteur Production; isopropyl-β-D-thiogalactoside was from Sigma Chemical Co., St. Louis, Mo.; and cAMP was from Boehringer Mannheim Corp., France S. A., Meylan, France. All other chemicals were from E. Merck AG, Darmstadt, Germany.

RESULTS

Isolation and characterization of *rho-cya* mutants. *rho-cya* mutants were isolated from strain PP7812, which carries the *rho-15* (Ts) allele (6), by selecting for growth on MacConkey maltose plates at a nonpermissive temperature (44°C). Clones appeared with a frequency of 2.10^{-7}. Of these, 20% were phenotypically Mal$^-$ and subsequently shown to exhibit a pleiotropic carbohydrate-negative character. Of 192 such mutants, 5 were thought to be *cya* mutants since addition of cAMP restored the carbohydrate-positive phenotype at 30°C. One such mutant, RCC1, was kept for further study. The corresponding *cya* mutation (*cyaR1*) was localized by P1 transduction with respect to the *ilvA, rho,* and *metE* loci and mapped with respect to the known Δ*cya* gene (5). To characterize the presence of *rho-15* in the transductants, we made use of a *gal3* background (polar IS*2* insertion at the *galETK* promoter [1]). Table 2 summarizes the results obtained. Although all *cyaR1* Ilv$^+$ transductants from P1 grown on strain RCC1 carried the parental *rho-15* allele, it was possible to separate *rho-15* from *cyaR1* when transductions were performed selecting for Met$^+$: of 48 Met$^+$ clones analyzed, 3 were found to be *cyaR1 rho*$^+$ recombinants. Transductions between *cyaR1* and Δ*cya* for Cya$^+$ recombinants in both directions yielded a very low number of Cya$^+$ bacteria, suggesting that the two loci are tightly linked. When P1 grown on RCC1 was transduced into a Δ*cya* strain, the recombinant Cya$^+$ clones were found to harbor the *rho-15* mutation. The tentative gene order is therefore *ilvA rho-15* Δ*cya cyaR1 metE,* which is in agreement with Das et al. (6) and others (27a), but at variance with results obtained by Inoko et al. (12), who found the order *ilv cya rho* (see also Bachmann and Low [3]). The *cyaR1* mutation was found to be recessive: we crossed strain PP7244 (*cyaR1*) with PP2010 16K harboring a *cya*$^+$ kanamycin-resistant episome, and we selected for kanamycin-resistant exconjugants, which were subsequently shown to be Mal$^+$.

Further characterization of *rho-cya* double mutant RCC1 showed that: (i) it did not synthesize detectable amounts of cAMP (see Table 4), thus indicating the absence of adenylate cyclase activity, and (ii) addition of cAMP restored the growth thermosensitivity of the *rho-cya* double mutant, indicating that the *rho*(Ts) allele was still present. This latter point is illustrated in Fig. 1: plates containing cAMP in the center were grown at 30 and 41°C. One can see a halo of growth inhibition at 41°C, whereas at 30°C cAMP did not affect growth.

When transduced into a *rho*$^+$ background, the *cyaR1* mutation reverted at a frequency of 10^{-6}, suggesting that it is a point mutation; it did not revert, however, in the *rho-15*(Ts) background. This was probably due to the deleterious action of the *rho-15* protein, which exerts a strong counterselecting action in a *cya*$^+$ background.

Isolation and characterization of carbohydrate-positive revertants from the *rho-cyaR1* mutant. An overnight culture of strain RCC1 grown on LB medium at 41°C was centrifuged, suspended in minimal medium 63, and plated on maltose minimal agar plates supplemented with eosin-methylene blue (10^9 bacteria per plate). After 48 h of incubation at 41°C, clones appeared at a frequency of about 10^{-7}. All Mal$^+$ revertants tested for growth on lactose or arabinose as carbon source also exhibited a Lac$^+$ Ara$^+$ phenotype. Four of such pleiotropic carbohydrate-positive pseudorevertants were kept for functional analysis.

In a first set of experiments we measured the level of expression of three enzymes, β-galactosidase, amylomaltase, and tryptophanase, and that of phage λ receptor, all known to be sensi-

TABLE 2. *Mapping of cyaR1*

Donor	Relevant marker	Recipient	Relevant marker	Marker selected	Marker scored	Cotransduction (%)
RCC1	*cyaR1 rho-15*	PP7210	*ilvA*	*ilvA*$^+$	*cyaR1*	30
CAA8306	Δ*cya*	PP7210	*ilvA*	*ilvA*$^+$	Δ*cya*	35
RCC1	*cyaR1 rho-15*	PP7210	*ilvA*	*ilvA*$^+$	*rho-15*	65
PP7220	*metE*	PP7260	Δ*cya*	*cya*$^+$	*metE*	20
RCC1	*cyaR1 rho-15*	PP7220	*metE*	*metE*$^+$	*cyaR1*	40
RCC1	*cyaR1 rho-15*	PP7220	*metE*	*metE*$^+$	*rho-15*	35
RCC1	*cyaR1 rho-15*	PP7260	Δ*cya*	*cya*$^+$	*rho-15*	100[a]

[a] Measured on three clones only. The recombination frequency between Δ*cya* and *cyaR1* was very low, about 0.5% of the frequency of Δ*cya ilvA* recombination.

tive to catabolite repression, under two extreme conditions: in the presence of glucose (condition of severe catabolite repression) and in the presence of succinate (condition of derepression). Table 3 compares the original strains PP7811 (*rho*$^+$), PP7812 [*rho-15*(Ts)], and RCC1 [*rho-15*(Ts) *cyaR1*] with the spontaneous carbohydrate-positive derivatives of RCC1. As expected for a *cya* strain, none of the catabolite-sensitive systems was expressed in strain RCC1. In the carbohydrate-positive mutants grown in succinate medium, the three enzymes and the phage λ receptor were expressed at high levels. When grown in the presence of glucose, in three out of the four revertants the rates of synthesis were severely reduced compared to those obtained in succinate, indicating that these strains were sensitive to catabolite repression. One of the strains, CGR22, seemed to be resistant to catabolite repression: the syntheses of the three enzymes

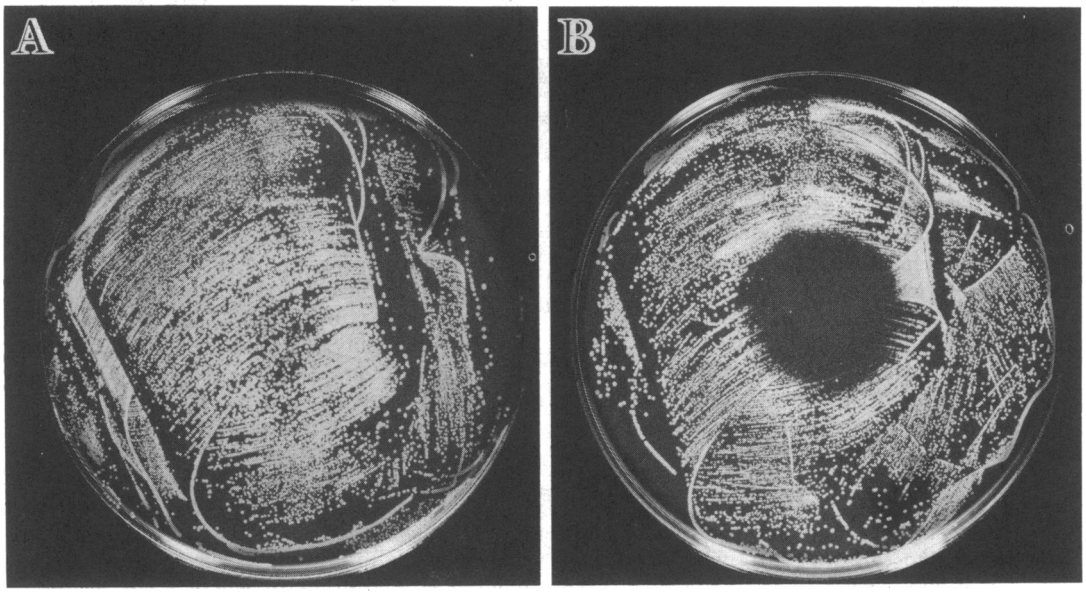

FIG. 1. *Effect of exogenously added cAMP on the growth of strain RCC1. A stationary-phase culture growing in LB medium was spread on LB plates. A 25-μl amount of a 2.10^{-1} M solution of cAMP was placed in the center of each plate. Plates were incubated at 30°C (A) and at 41°C (B).*

TABLE 3. *Expression of β-galactosidase, tryptophanase, amylomaltase, and phage λ receptor in the pleiotropic carbohydrate-positive pseudorevertants of rho-15(Ts) cyaR1 mutants as compared to the parental strains*

Strain	Carbon source	Rates of synthesis (U/mg [dry weight] of bacteria) of:			Molecules of phage λ receptor (receptor/bacteria)
		β-Galactosidase	Tryptophanase	Amylomaltase	
PP7811	Glucose	2,900	<4	<0.5	100
	Succinate	11,356	421	200	1,200
PP7812	Glucose	1,500	<4	<0.5	70
	Succinate	10,000	320	138	80
RCC1	Glucose	155	9	<0.5	8
	Succinate	—[a]	—	—	—
CGR20	Glucose	1,789	50	<0.5	113
	Succinate	5,231	127	81	1,400
CGR21	Glucose	1,428	15	<0.5	120
	Succinate	5,657	103	103	1,050
CGR22	Glucose	2,956	27	69	2,510
	Succinate	2,450	35	98	2,250
CGR24	Glucose	1,542	30	<0.5	70
	Succinate	5,817	168	71	1,170

[a] —, No growth in succinate.

and of the phage λ receptor were not significantly different in glucose as compared to succinate.

Functional and genetic analysis of the revertants. The isolation of carbohydrate-positive pseudorevertants exhibiting catabolite repression from a *rho-cya* double mutant prompted us to characterize the mutation responsible for this phenotype. All revertants retained their growth thermosensitivity at 41°C in the presence of cAMP (checked, as illustrated in Fig. 1, data not shown). This indicates that the *rho-15*(Ts) allele was still present in the mutants and suggests that they are not trivial *cya*$^+$ revertants. We determined cAMP levels in the pseudorevertants and, surprisingly, we found that in contrast to the original strain, RCC1, they did synthesize cAMP, although at reduced levels, as compared to the parental strain PP7000 (Table 4). The simplest interpretation—that the *cyaR1* mutation reversed—was eliminated by the result of transduction in an Ilv$^-$ strain for Ilv$^+$ character, using phage P1 grown on the mutants. Of the Ilv$^+$ transductants, 18 to 30% were pleiotropic carbohydrate negative. We measured cAMP levels in these transductants, and as can be seen in Table 4 (last four rows), none of the transductants which received the *cya* region of the carbohydrate-positive revertants synthesized detectable amounts of cAMP. Moreover, all transductants exhibited growth thermosensitivity in the presence of cAMP at 41°C. These data suggest that the *cyaR1* mutation isolated in the *rho-15*(Ts) strain affects the regulation of adenylate cyclase gene expression or enzyme activity.

TABLE 4. *cAMP levels in the pleiotropic carbohydrate-positive pseudorevertants of the rho-15(Ts) cyaR1 mutant and in the parental strains*

Strain	Relevant phenotype	Total cAMP concn (nmol/mg [dry weight] of bacteria)
PP7000	Rho$^+$ Cya$^+$ CAP$^+$	4.5
RCC1	Rho(Ts) Cya$^-$	<0.01
CGR20	Rho(Ts) Cya$^-$ Mal$^+$ Lac$^+$ Ara$^+$	1.3
CGR21	Rho(Ts) Cya$^-$ Mal$^+$ Lac$^+$ Ara$^+$	1.2
CGR22	Rho(Ts) Cya$^-$ Mal$^+$ Lac$^+$ Ara$^+$	2.4
CGR24	Rho(Ts) Cya$^-$ Mal$^+$ Lac$^+$ Ara$^+$	2
PP7000/CGR20	Rho(Ts) Cya$^-$ CAP$^+$	<0.01
PP7000/CGR21	Rho(Ts) Cya$^-$ CAP$^+$	<0.01
PP7000/CGR22	Rho(Ts) Cya$^-$ CAP$^+$	<0.01
PP7000/CGR24	Rho(Ts) Cya$^-$ CAP$^+$	<0.01

Localization of the mutation responsible for the pleiotropic carbohydrate-positive character. For reasons which will be outlined in the Discussion section, we conjectured that the mutation responsible for the pleiotropic carbohydrate-positive character might affect the *crp* gene. To validate this hypothesis, we reconstructed the *rho-cyaR1* carbohydrate-positive revertants, using strain PP7000, which is an *ilvA aroB* derivative of strain PP7811. First we transduced for Ilv$^+$ character with P1 grown on RCC1. More than one third of the Ilv$^+$ strains were pleiotropic carbohydrate negative, exhibited growth thermosensitivity in the presence of cAMP at 41°C, and did not synthesize detectable amounts of cAMP (Table 4, last four rows). Then we transduced this strain for AroB$^+$ character, using P1 grown on three of the carbohydrate-positive pseudorevertants (CGR20, -21, -24). Of the AroB$^+$ clones approximately 25% were found to be pleiotropic carbohydrate positive. These cotransduction frequencies are compatible with a mutation in the *crp* gene or very tightly linked to it (3). This was confirmed by a more precise localization of one of the putative *crp* alleles (*crp C20*), which was mapped with respect to both the *aroB* and *rpsL* genes (Table

TABLE 5. *Mapping of crpC20*

Donor	Relevant marker	Recipient	Relevant maker	Marker selected	Marker scored	No. of colonies	Cotransduction (%)
CGR20	*crpC20 aroB*$^+$ *cyaR1*	PP7000	*aroB*	*aroB*$^+$	*crpC20*	38	37
CGR21	*crpC21 aroB*$^+$ *cyaR1*	PP7000	*aroB*	*aroB*$^+$	*crpC21*	96	23
CGR24	*crpC24 aroB*$^+$ *cyaR1*	PP7000	*aroB*	*aroB*$^+$	*crpC24*	96	21
CGR40	*crpC20 aroB*$^+$ *cyaR1*	PP7000/CGR20	*aroB cyaR1*	*aroB*$^+$	*crpC20*	100	25
CGR57	*rpsL crpC20 aroB*$^+$ Δ*cya*	PP7000/CGR20	*aroB cyaR1 rpsL*$^+$	*rpsL*	*aroB*$^+$	116	28
CGR57	*rpsL crpC20 aroB*$^+$ Δ*cya*	PP7000/CGR20	*aroB cyaR1 rpsL*$^+$	*rpsL*	*crpC20*	116	50

5). It must be noted that the revertants obtained by reconstruction differed in some aspects from the original *rho-cya* carbohydrate-positive pseudorevertants: they were cryosensitive for the carbohydrate-positive character, synthesized lower levels of cAMP in glucose medium, and were far more sensitive to catabolite repression (Table 6). This suggests that in the original strain (RCC1) some as yet unidentified mutation(s) abolished these secondary effects.

Functional analysis of the reconstructed strains. We measured the differential rates of β-galactosidase and tryptophanase synthesis in glucose and succinate media, and we determined in parallel the cAMP levels. The rates of β-galactosidase synthesis in the presence of glucose were extremely low as compared to those found in the parental strain (Table 6). This indicated a very strong catabolite repression effect, which was further substantiated by the finding that under these conditions tryptophanase synthesis could not be detected. However, in succinate medium the rates of synthesis of both enzymes were fully derepressed. The variations of cAMP concentrations seemed to be correlated with the variations of enzyme levels. This strong catabolite repression effect could be interpreted as being the result of low cAMP concentration in glucose medium. To verify this assumption we transduced the *crp* regions (*crpC*) from three of the original *rho-cyaR1* carbohydrate-positive pseudorevertants into a Δ*cya aroB rho*$^+$ strain. Among the AroB$^+$ transductants we found 18% pleiotropic carbohydrate-positive clones. We measured in these strains the rates of β-galactosidase synthesis in glucose and succinate media. The rates were also very low in glucose, and we found a 10- to 15-fold derepression in succinate medium (Table 7). It should be emphasized that in these strains no detectable amounts of cAMP could be found. From the data of Tables 6 and 7 one can see that the derepressed levels of β-galactosidase measured in succinate are higher in the *rho-cyaR1* than in the *rho*$^+$ Δ*cya* strains. We do not know whether this difference was due to the presence of the *rho*(Ts) allele or to the specific nature of the *cya* mutations. The catabolite repression effect was, nevertheless, similarly severe. One can therefore conclude that the catabolite repression observed in these strains was not due to variations in cAMP levels but rather to the specific nature of the *crp* mutation. This point was further strengthened by transducing the known cAMP-independent *crp* allele (*crp**; 20) in the *rho-cyaR1* strain and finding that the *rho cyaR1 crp** transductants neither exhibited catabolite repression nor synthesized cAMP. As a control we also constructed a *rho cyaR1 crp* strain where no cAMP synthesis could be detected (Table 8).

TABLE 6. *Expression of catabolite-sensitive enzymes and cAMP levels in the reconstructed rho-15 cyaR1 carbohydrate-positive strains*

Strain	Relevant phenotype	Carbon source	β-Galactosidase		cAMP		Tryptophanase
			U/mg[a]	Succinate/glucose	nmol/mg[a]	Succinate/glucose	U/mg[a]
PP7000	Rho$^+$ Cya$^+$ CAP$^+$	Glucose	2,995	4.36	3.5	4.5	39
		Succinate	13,058		15.8		434
CGR40	Rho(Ts) Cya$^-$ Mal$^+$ Lac$^+$ Ara$^+$	Glucose	277	39	0.25	39	<6
		Succinate	10,792		9.8		229
CGR41	Rho(Ts) Cya$^-$ Mal$^+$ Lac$^+$ Ara$^+$	Glucose	688	17.6	0.36	18.5	<6
		Succinate	12,250		6.7		320
CGR42	Rho(Ts) Cya$^-$ Mal$^+$ Lac$^+$ Ara$^+$	Glucose	231	53	0.47	37	<6
		Succinate	12,367		17.4		130

[a] Units or nanomoles per milligram (dry weight) of bacteria.

TABLE 7. *Rates of β-galactosidase synthesis and cAMP levels in Δcya strains carrying the wild type and the crpC allele*

Strain	Relevant genotype	β-Galactosidase synthesis (U/mg [dry weight] of bacteria) with:		Total cAMP concn (nmol/mg [dry weight] of bacteria) with:	
		Glucose	Succinate	Glucose	Succinate
PP7860	Δ*cya crp*$^+$	120	—[a]	<0.01	—
CGR49	Δ*cya crpC20*	321	4,850	<0.01	<0.01
CGR50	Δ*cya crpC24*	270	3,702	<0.01	<0.01

[a] —, No growth in succinate.

TABLE 8. *Rates of β-galactosidase synthesis and cAMP levels in cyaR1 strains carrying different crp alleles*

Strain	*crp* allele	β-Galactosidase synthesis (U/mg [dry weight] of bacteria) with:		Total cAMP concn (nmol/mg [dry weight] of bacteria) with:	
		Glucose	Succinate	Glucose	Succinate
RCC1	*crp*$^+$	155	—[a]	<0.01	—
PP7246	*crpRCC2*	133	116	<0.01	<0.01
CGR53	*crp**	7,300	7,500	<0.01	<0.01
CGR40	*crpC20*	230	11,000	0.26	10.5

[a] —, No growth in succinate.

DISCUSSION

The essential point of the experiments presented in this paper was to show that a mutation located in the *cya* region affects the regulation of adenylate cyclase. This mutation, obtained in a *rho*(Ts) strain, renders the *rho-cya* double mutant deficient in adenylate cyclase activity, i.e., unable to synthesize cAMP. An additional mutation in the *crp* gene, or very tightly linked to it, restored the ability of the strain to synthesize cAMP and to express catabolite-sensitive operons. It was of interest to know whether the two effects were related, that is to say, whether restoration of cAMP synthesis in itself was responsible for the expression of catabolite-sensitive operons or whether the mutation had a dual effect: to restore adenylate cyclase activity and, independently, to promote expression of these operons. Our experimental results were straightforward: the two effects are indeed independent since in a Δ*cya* strain, in the absence of any detectable amounts of cAMP, this specific *crp* allele enabled the expression of catabolite-sensitive operons and, in addition, conferred on the strain an extreme sensitivity to catabolite repression. It was not very surprising that a specific *crp* mutant could display such a behavior. It has already been reported that *crp* mutants produce abnormally high levels of cAMP (9, 29), suggesting that CAP might be involved in the regulation of adenylate cyclase. Moreover, several *crp* mutants overcoming cAMP deficiency have been described: *crp** and *crp*^{s} mutants, exhibiting carbohydrate-positive character in a Δ*cya* background (7, 20, 26), and a *sup* mutant, suppressing the cAMP requirement of enzyme I mutants of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) for growth on a great number of carbohydrates (2). The *crpC* mutant(s) that we isolated, like the *crp** mutant, enabled the expression of catabolite-sensitive operons in a Δ*cya* background. However, they could be clearly distinguished from *crp** or *sup* by two specific effects: extreme sensitivity to catabolite repression and restoration of cAMP synthesis in the *rho-cyaR1* double mutant.

The involvement of CAP in the expression of catabolite-sensitive operons is well documented (8, 23). But the modulation of the extent of catabolite repression is generally accounted for by the variations of the intracellular level of cAMP. Several lines of evidence suggest, however, that cAMP is not the unique regulator of catabolite repression since this regulation can take place in the absence of the nucleotide (7, 28). Our finding that a specific mutation in the *crp* region can bring about an extreme sensitivity to catabolite repression in the absence of cAMP argues in favor of the hypothesis that a specific effector, other than cAMP, might be responsible for the modulation of catabolite repression and that CAP would be involved in this regulation (7, 27). Such a model would readily account for the specific feature of the *crpC* mutants we described.

By far the most significant finding is the demonstration of a specific regulation of adenylate cyclase. The *rho-cyaR1* mutant we isolated did not synthesize cAMP; however, in the presence of the *crpC* mutation it regained the ability to synthesize reduced levels of cAMP in glucose medium. These levels remained low during the whole growth cycle and did not increase even after the arrest of growth (data not shown), in contrast to wild-type strains where a substantial rise in cAMP concentration occurred during the stationary phase of growth (16). It is noteworthy that in the mutants grown in succinate, cAMP levels could increase up to 40-fold, whereas in a *cya*$^+$-*crp*$^+$ strain the increase never exceeded 4- to 5-fold (Table 4 and 6).

To account for the regulation of adenylate cyclase activity several models have already been proposed; unfortunately, none of them seems completely satisfactory: it has been shown that glucose inhibits adenylate cyclase activity (11), yet this model cannot be easily reconciled with the different features of our mutants. There is indirect evidence that adenylate cyclase is activated by a phosphorylation carried out by enzyme I of PTS (17). However, a modulation of intracellular cAMP concentration has been observed in a strain with a partial deletion of the structural gene for enzyme I, indicating that the regulation of adenylate cyclase does not necessarily require the function of enzyme I (30). Another highly speculative model has been proposed, postulating a central regulatory protein which, in its phosphorylated form, could activate adenylate cyclase (21). This model would predict

that the activities of the components of the PTS would increase in parallel with cAMP production in a *crp* strain. This has been shown not to be the case (19).

Since very little is known about the genetic structures of *crp* and *cya* loci or the biochemistry of adenylate cyclase, one is reduced to speculations. We took three hypotheses into consideration and tried to fit them with already reported, as well as our present, results.

(i) Expression of the adenylate cyclase structural gene could be negatively regulated by CAP, exhibiting repressor function. This model, suggested previously (4), accounts well for the abnormally high levels of cAMP in *crp* mutants. It is, however, hardly compatible with the specific features of the *cyaR1* mutation, i.e., cAMP synthesis would not be restored in a *crp* background, unless we suppose that the modified CAP encoded by the *crpC* mutation is converted into an activator.

(ii) Adenylate cyclase expression could be positively regulated by an activator protein: CAP could interact with this protein preventing its activator function. According to this model, the *cyaR1* mutation would affect the regulatory protein, which remains inactive even in the absence of functional CAP. However, it could be switched from the inactive to the active state by interaction with a specifically altered CAP. This model predicts that the *cyaR1* mutation should be recessive in a $cya^+/cyaR1$ diploid. We found that this was the case.

(iii) Adenylate cyclase activity could be positively regulated via a regulatory subunit or another regulatory protein. CAP interacting, directly or indirectly, with the regulatory protein would prevent activation. Operationally, this model is not different from (ii), except that it would rather support an allosteric type of regulation. Supposing that the *cyaR1* mutation affected a regulatory subunit of adenylate cyclase, it could no longer associate with the catalytic subunit. A conformational transition of the mutated protein with a specifically altered CAP would entail restoration of activity.

Our experimental results neither definitely eliminate nor preferentially support any of these hypotheses. We are tempted, however, to favor the last one because of a certain analogy with the adenylate cyclase systems of animal origin.

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