Molec. gen. Genet. 176, 343–350 (1979)

MGG

Involvement of Cyclic AMP and Its Receptor Protein in the Sensitivity of *Escherichia coli* K 12 toward Serine

Excretion of 2-Ketobutyrate, a Precursor of Isoleucine

Jacques Daniel[1] and Antoine Danchin[2]

[1] Unité de Biochimie cellulaire, Département de Biochimie et Génétique Microbienne, Institut Pasteur, 28, rue du Docteur Roux, F-75724 Paris Cedex 15, France
[2] Institut de Biologie physico-chimique, 13, rue Pierre et Marie Curie, F-75005 Paris, France

Summary. A relationship between serine-induced growth sensitivity and the cAMP-CAP complex is established. Mutants of *Escherichia coli* K 12 deficient either in the *cya* or *crp* gene function exhibit a resistant phenotype on serine media although they harbor a *rel*A allele normally leading to sensitivity toward serine. The presence of a *crp** allele in a cya_{Δ}*rel*A background restores the sensitivity phenotype, while the analysis of serine resistant mutants selected from a *crp** cya_{Δ}*rel*A strain shows that the mutation leading to resistance is located at, or very near, the *crp* gene, giving a more or less Crp^- phenotype. In addition *crp** cya_{Δ}*rel*A strains excrete large quantities of 2-ketobutyrate when grown on glucose M63 medium. This excretion is unambiguously linked to the presence of the *crp** allele and is correlated with an enhanced threonine deaminase activity. Besides, the complex regulation exerted on the acetolactate synthase activities is discussed.

Introduction

It has long been suspected (Alföldi and Kerekes, 1964) that an interrelationship exists between the otherwise unrelated metabolic pathways which lead to the production of the one-carbon amino acids (serine, methionine and glycine) and the branched-chain amino acids (isoleucine, leucine, valine). More precisely it has been observed that *Escherichia coli* K 12 growth is inhibited by an excess of serine and that this inhibition is relieved by addition of exogenous isoleucine. Mutants in the stringent coupling between translation and transcription were shown to be extremely sensitive to serine because of the lack of derepressibility of the *ilv* operons after addition of excess serine (Uzan and Danchin, 1978). Several mutants hypersensitive to serine were also isolated and found to behave as relaxed strains (i.e., lack of accumulation of ppGpp and synthesis of stable RNAs after amino acid starvation) (Danchin, 1977) although their genetic defect does not map in the well-known *rel*A gene. Conversely mutants which show extreme resistance to serine were also selected and characterized (Danchin and Dondon, submitted to Molec. Gen. Genet.).

In the present study, we describe a new class of hypersensitive or hyperresistant mutants which result from mutations in the adenyl cyclase and cyclic AMP receptor protein system. We thus establish that a clear relationship exists between growth inhibition induced by serine and the system involved in catabolite repression. In addition we show that the mutants which are particularly sensitive to serine excrete large quantities of a product which can revert growth inhibition of a *rel*A strain by serine. Identification of this product as 2-ketobutyrate, a precursor of isoleucine, is described and the possible mechanism of this excretion is discussed.

Materials and Methods

Strains and Media. All strains used in this study are listed in Table 1. Minimal medium (M63), rich medium (LB) and EMB medium are described in Miller (1974). SD medium is M63 supplemented with serine, methionine, glycine, leucine (1 mM each), thiamine (5 µg/ml) and 0.5% sodium deoxycholate. The carbon source is as indicated (Danchin and Dondon, submitted to Molec. Gen. Genet.).

Transductions. Generalized transductions were performed with P_1 *vir* according to the method of Castellazzi et al. (1972). In particular the phage was systematically ultraviolet treated with about 900 $erg \cdot cm^{-1}$ before transduction. For the transduction of the *crp** allele (from strain CA8404) into cya_{Δ} strain (CA8306), selective medium was the minimal medium (M63) containing maltose as the carbon source. In order to check that transductants received

Send offprint requests to. Dr. A. Danchin

0026-8925/79/0176/0343/$01.60

Table 1. Bacterial strains used in this study

Strain	Relevant genetic markers and derivation	Origin
FB8	F^- prototrophic	F. Blasi
FB8r	*relA* from FB8	Uzan and Danchin (1976)
KL 14	Hfr *thi, relA*	Through M. Springer from CSHL
GT549 ($\lambda dthr_c$)	F^- *araD 139*, $lac_\Delta U$ *169*, *rpsL*, *thi*, *rel*A *(thr C-lacZ$^+$)$_{1-1}$* fusion, $\lambda dthr_c$	I. Saint-Girons
GT550 ($\lambda dthr_c$)	F^- *araD 139*, $lac_\Delta U$ *169*, *rpsL*, *thi*, *rel*A *(thrC – lacZ$^+$)$_{1-2}$* fusion, $\lambda dthr_c$	,,
CA8306	*relA*, cya_Δ, *thi*	J. Beckwith
GY2615	*relA*, *cya*, *thi*	,,
AB3059	Hfr, *thi1*, *leu6*, *ilvD132*, *thuA41*, *thyR23*, *lacZ4*, *rpsL*, λ^-, cya_{ns}, *sup?* (unmapped amber suppressor)	P. Howard-Flanders through CGSC (cya_{ns}, *sup* = this work)
CA8307	*relA*, *crp*, *thi*	J Beckwith
CA8404	*relA*, cya_Δ, crp^*_1, *rpsL*, *thi*	D. Sabourin and J. Beckwith (1975)
TIT304	*relA*, cya_Δ, crp^*_2, *rpsL*, *thi*	A. Dessein and al. (1978)
BM615; BM616; BM617	*mal*$^+$ transductants from CA8306 × P_1 CA8404: *relA*, cya_Δ, crp^*_1, *thi*	This work
BM618; BM619	*mal*$^+$ transductants from CA8306 × P_1 CA8404· *relA*, cya_Δ, crp^*_1, *rpsL*, *thi*	,,
BM617-a	smg glucose resistant derivative from BM617 *relA*, cya_Δ, *crp*, *thi*	,,
BM618-a	smg glucose resistant derivative from BM618 *relA*, cya_Δ, *crp*, *rpsL*, *thi*	
BM6160	Trimethoprim resistant from BM616 *relA*, cya_Δ, crp^*_1, *thy*, *thi*	,,
BM6161	*relA*, cya_Δ, crp^*_1, *argA*, *thi* from BM6160 by cotransduction *thy*$^+$-*argA*	,,
BM6162	*relA*, cya_Δ, crp^*_1, *argA*, *lysA*, *thi* from BM6160 by cotransduction *thy*$^+$-*argA-lysA*	,,
BM6163	cya_Δ, crp^*_1 from BM6161 by cotransduction *argA*$^+$, *relA*$^+$	,,
BM6164	cya_Δ, crp^*_1, *lysA*, *thi* from BM6162 by cotransduction *argA*$^+$-*relA*$^+$	,,
ALSO	*ilvA*, *argH*, $trpE_{ns}$, *relA*, *xyl*, *lac*, $thyA^{ts}$	,,
ALS25	*ilvC*, *argH*, $trpE_{ns}$, *relA*, *xyl*, *lac*, $thyA^{ts}$	,,
ALS26	*ilvD*, *argH*, $trpE_{ns}$, *relA*, *xyl*, *lac*, $thyA^{ts}$	,,

the *crp** allele, the particular appearance of an intense green sheen when streaked on a EMB lac medium was used.

Growth Inhibition by Serine in Liquid Medium. After overnight growth of *relA* strain FB8r in M63 medium with glucose as the carbon source, bacteria were transferred to the same fresh medium at a cell concentration of about 7×10^7 per ml and allowed to divide about 3 times. Bacteria were then transferred to the same medium containing in addition 1 mM L-serine, at a cell concentration of $1{,}2 \times 10^7$ per ml (if not otherwise stated). Turbidity at 650 nm was followed in a Zeiss spectrophotometer for 4 to 5 h. Care was taken to maintain a thorough aeration at 37° C.

Preparation of Culture Filtrates. After overnight growth in M63 medium with glucose as the carbon source (and the required metabolites) cultures were diluted into the same medium at a cell concentration of about 7×10^7 per ml (5 to 25 ml) and allowed to grow to 2.5 to 3×10^8 cells per ml. Cultures were then immediately filtered through a nitrocellulose membrane (Nalgene filter unit) and filtrates kept frozen at −30° C.

Concentration and Partial Purification of a Product Which Counteracts Growth Inhibition by Serine. 100 ml culture filtrates of strain CA8404 were evaporated to about 2 ml in a rotary evaporator. The concentrated fluid was then centrifuged at 4° C to remove salt cristals. The supernatant was mixed with 10 volumes methanol. After stirring on a vortex and centrifugation, the supernatant was evaporated to dryness in the rotary evaporator. The residue was redissolved in 2 ml distilled water ("concentrated filtrate") and kept frozen at −30° C. According to the test for measuring the counteracting effect on growth inhibition by serine, apparent yield was more than 50%. From resistivity measurements, we deduced that the purification of the desired product relative to salts was 18 to 25.

Measurements of Labeled Products Reacting with DNP Hydrazine. Cultures were grown and filtrates prepared according to the method described above Bacteria were grown in the presence of either ^{14}C-L-threonine [0.25 mCi/10 µM or 4 mCi/100 µM (CEA, France)] or ^{14}C-L-isoleucine [0.25 mCi/10 µM or 4 mCi/100 µM (CEA, France)] or ^{14}C-L-homoserine [0.5 mCi/100 µM (CEA, France)] 0.3 ml of a radioactive filtrate was mixed with 0.3 ml of a dinitrophenylhydrazine solution (0.1% in 2 N HCl). After 5 min at room temperature, 0.3 ml toluene was added. The mixture was then vigorously stirred on a vortex for 1 min. After separation of the two phases, 50 µl of the organic phase was counted for ^{14}C radioactivity in Bray's scintillator using an Intertechnique liquid scintillation counter.

Paper Chromatography. One dimensional descending chromatography was performed on Whatman n° 1 paper in a plastic tank. The migration distance was 13 cm Migration solvants were:

1) methanol. 2) ethanol; 3) propanol-1/H_2O (80:20), 4) propanol-1/pyridine-1 M/H_2O (75:5:20); 5) propanol-1/triethyl amine-1 M/H_2O (75:5:20); 6) propanol-1/acetic acid-0.25 M/H_2O (75:5:20). After drying, paper was cut into 0.5 or 1 cm long strips in the migration direction

a) To test the counteracting effect on growth inhibition by serine, paper strips were immersed in the culture medium as described below (see "Microcultures").

b) For counting ^{14}C radioactivity, paper strips were either directly immersed in toluene-POPOP-PPO scintillator or first eluted in water or M63 medium for 1 to 2 h and a sample then counted in Bray's scintillator.

c) In order to assay 2-keto acids, paper strips were immersed in 100 µl water for 1 to 2 h. Papers were then discarded and 0.2 ml of a solution of dinitrophenylhydrazine (0.1% in 2 N HCl) was added After 10 min at room temperature, 0 5 ml 96° ethanol and 0.5 ml 5 N sodium hydroxide were added. The mixture was then stirred on a vortex for 1 min. After 5 min optical density was read at 440 nm.

Chromatography on Thin-Layer Silica Gel Plates. One dimensional ascending chromatography was performed on thin-layer silica gel plastic plates (Schleicher and Schüll) in a Desaga tank. The migration distance was 13 cm. Migration solvants were: 1) methanol/H_2O (80·20); 2) propanol-1/H_2O (80:20); 3) butanol-1/H_2O (80 20); 4) dioxane/H_2O (80:20); 5) dioxane/H_2O (20:80); 6) dioxane/H_2O (95:5). After drying, the silica gel was scratched every 0.5 cm in the migration direction

a) To test the counteracting effect on growth inhibition by serine, silica gel was scratched off the plate and immersed in 400 µl M63 medium for 1 h at room temperature. After sedimentation of the silica gel, 200 µl were carefully withdrawn and used for microcultures (see below).

b) For counting ^{14}C radioactivity or testing for the presence of 2-keto acids, silica gel was scratched off the plate and immersed in 200 µl M63 medium. After 1 h, 50 to 100 µl were either pipetted into Bray's scintillator or used for dinitrophenyl hydrazone formation as described above (see "Paper chromatography").

When toluene extracted dinitrophenyl hydrazones were used as samples for chromatography, the migration solvent was dioxane. The control coloured spots were identified and the gel was then either scratched and eluted for radioactivity counting as described above, or more often used for autoradiography.

Microcultures of Strain FB8r. 250 µl microcultures were performed in Kahn tubes (6 ml full content) with efficient stirring at 37° C. When paper strips were tested for any counteracting activity on growth inhibition by serine, care was taken not to let the paper strip prevent the aeration of the culture during growth After 3 to 4 h, 20 µl toluene and 20 µl sodium deoxycholate (1% in water) were added. Tubes were vigorously stirred on a vortex for 10 s and agitated at 37° C for 30 min. β-galactosidase assays were then performed on the whole toluenized suspensions. The rationale for using β-galactosidase activity measurements in order to detect any activity counteracting growth inhibition by serine in microcultures, comes from the following finding: when strain FB8r is grown in M63 medium in the presence of glucose as the carbon source, 1 mM IPTG as the inducer of the lactose operon, 1 mM L-serine and, in addition, different concentrations of a culture filtrate from strains CA8404 or TIT304 so as to generate different growth rates, one finds a linear relationship between the relative growth rate and the relative differential synthesis rate of β-galactosidase (Fig. 1). Thus in the presence of serine, β-galactosidase activity assayed after a certain induction time is not only dependent on cell mass but also on growth rate which are both modulated in the same direction by the anti-serine substances. Hence β-galactosidase measurements constitute a sensitive test for the detection of small amounts of those substances assayed on microcultures.

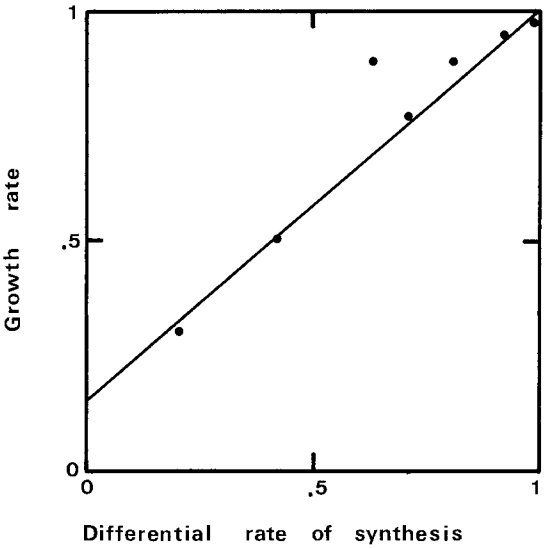

Fig. 1. Relationship between growth rate (in 1 mM serine) and differential synthesis rate of β-galactosidase in strain FB8r. Growth rate is plotted versus differential rate of synthesis of β-galactosidase. Results are given relative to culture without serine. Conditions are as described in Materials and Methods

β-Galactosidase Assay. β-galactosidase was assayed on toluenized bacterial suspensions as described by Pardee et al. (1959)

Threonine Deaminase Assay. Threonine deaminase was assayed using crude extracts prepared as described in Uzan and Danchin (1978) except that 0.1 mM isoleucine was usually added before sonication of bacteria.

Isoleucine Transaminase Assay. Isoleucine transaminase assay was performed according to Guardiola (1978) on the same crude extracts used for threonine deaminase assay.

Chemicals. Glucose and lactose were purchased from Merck; glycerol from Prolabo (Normapur); all L-amino acids (and in particular L-serine) from Merck; 2-ketobutyrate, 2-ketomethylvalerate, 2-ketoisovalerate (sodium salts) from Sigma. Most solvents used from chromatography were analytical reagent grade.

Results

Serine Sensitivity With Various Carbon Sources

As already established for most *Escherichia coli* strains, growth is transiently inhibited when serine is added to diluted cultures in glucose mineral medium. Moreover it was found that several metabolites, such as those related to the citric acid cycle, further enhance this inhibition (Uzan and Danchin, 1976). We thus tested the effect of different carbon sources on the sensitivity of strain FB8r toward serine (Table 2). The replacement of glucose by lactose, glycerol or glucose-6-phosphate increased the inhibitory effect of serine, whereas fructose did not appear to drasti-

Table 2. Growth inhibition by serine observed with various carbon sources and with glucose *plus* cyclic AMP.

Experimental conditions were as described in Materials and Methods. Strain FB8r was grown exponentially on the same carbon source than used for the sensitivity test. Cyclic AMP (3.3 mM final concentration) was added concomitantly with serine. No different doubling times were found in glucose and glucose *plus* cyclic AMP (in the absence of serine). Numbers represent the ratio of the doubling times in the presence and the absence of serine

Glucose	Fructose	Lactose	Glycerol	Glucose-6-phosphate	Glucose + cyclic AMP
2	1.9	> 5.5[a]	> 2.5[a]	> 5.5[a]	5

[a] This corresponds to a nearly complete arrest of growth. The precise doubling time under these conditions is difficult to measure since the starting optical density of the bacterial suspension is only 0.02 at 650 nm

Table 3. *cya* and *crp*$^-$ mutants obtained on smg media.

The smg resistant isolates were tested for their capacity to grow on mineral media containing lactose, maltose, arabinose (for KL 14 and FB8r) or maltose [for GT549 ($\lambda d thr_c$) and GT550 ($\lambda d thr_c$)]. The response toward cyclic AMP on those sugars could discriminate between *cya* and *crp*$^-$ mutants

	Number of isolates	*cya*	*crp*$^-$
KL 14 on SD glucose medium	16	4	0
FB8r on SD glucose medium	48	10	1
GT549 ($\lambda d thr_c$) on glucose smg M63	7	1	0
GT550 ($\lambda d thr_c$)			
on glucose smg M63	13	2	1
on glucose smg deoxycholate M63	7	1	1

cally alter the response as compared with glucose. These results seem to exclude a direct correlation between the catabolite repression state and the level of growth sensitivity toward serine, since glycerol, fructose and lactose are known to elicit catabolite derepression whereas glucose and glucose-6-phosphate induce a strong repression state (Magasanik, 1961). Because of our genetical results (see below) we looked however for a possible modulating effect of cyclic AMP on the growth of strain FB8r in the serine minimal medium with glucose as the carbon source. As shown in Table 2, the cyclic nucleotide was found to markedly enhance growth sensitivity toward serine.

Involvement of Cyclic AMP

It has been shown that addition of methionine and glycine strongly increases the potential of serine as a growth inhibitor of *rel*A strains (Uzan and Danchin, 1976). Mutants resistant to a synthetic medium supplemented with 1 mM each of *s*erine + *m*ethionine + *g*lycine and designated smg (smg M63 medium) can be readily selected. Yet the frequency of the mutants thus obtained is about 10^{-4} at 37° C. In order to increase selectivity, we added leucine (1 mM) and 0.5% sodium deoxycholate to the smg M63 medium (the rationale for this procedure is given in Danchin and Dondon, submitted to MGG). Table 3 summarizes the phenotypes obtained using either strain KL14, FB8r, GT459 ($\lambda d thr_c$) or GT550 ($\lambda d thr_c$). The most prominent observation is that around 20% of the mutants appeared to be defective in the production of cyclic AMP. Mapping of two of these mutations indicated a 60% co-transduction with an *ilvA* marker. This is consistent with a defect in the adenyl cyclase.

The apparent relationship between the loss of adenyl cyclase activity and resistance to serine prompted us to test known alleles of the *cya* gene. Three of them, a point mutation, an amber mutation and a deletion, were transduced into a *rel*A background. Each mutation rendered the strain resistant to the smg M63 medium. Addition of exogenous cyclic AMP restored the sensitivity phenotype. Besides we observed that addition of cyclic AMP together with smg on glucose plates would not only inhibit the growth of *rel*A strains but also of stringent bacteria (Table 4).

A trivial explanation of our results could be that serine permeation is impaired in a *cya* background. However we found an even more efficient permeation of serine in a *cya* strain than in its wild-type counterpart (data not shown).

Involvement of the Cyclic AMP Receptor Protein

In order to test whether cyclic AMP is active, as such, we made use of some of the known alleles of the *crp* gene. The *crp* deficient mutants (*crp*$^-$) in a *rel*A background, were found to be resistant to smg (Table 4). Furthermore this phenotype was not altered by the addition of cyclic AMP to the medium. These results were in agreement with the finding that *crp*$^-$ mutants were also isolated when selection for smg resistance was applied to *rel*A strains (see Table 3).

The effect of another *crp* allele (*crp**) that leads to a modified cyclic AMP receptor protein which acts independently of cyclic AMP, was also tested. As shown in Table 4, *cya crp** strains were found to be (particularly) sensitive to smg when harboring the *rel*A mutation; yet the *rel*A$^+$ allele rendered these strains resistant to the same medium.

These results indicate that the cyclic AMP-CAP couple is responsible for the expression of serine sensi-

Table 4. Sensitivity of different mutant strains toward smg, with or without cyclic AMP

Sensitivity toward smg was tested on solid 63 glucose B_1 at 37° C for 24 to 48 h, as described by Uzan and Danchin (1976) Cyclic AMP concentration was 3 mM

		relA	*relA, cya*	*relA, crp*$^-$	*relA, cya, crp*, rpsL*	*relA, cya, crp**		*cya, crp**
						rpsL		
	FB8	FB8r KL14	CA8306 GY2615	CA8307	CA8404 TIT304	BM615 BM616 BM617	BM618 BM619	BM6163 BM6164
smg	+	−	+	+	−	−	−	+
smg + cyclic AMP	−	−	−	+	−	−	−	−

+ = growth; − = no detectable growth

tivity in a *relA* background. To further substantiate this conclusion, we selected mutants from a *relA* $cya_{\Delta}crp^*$ strain which were resistant to smg glucose medium. The frequency of these mutants was about 10^{-5}. Approximately 30% of them showed altered phenotypes for carbohydrate utilization. Whereas the parental strain was able to use all the carbon sources tested (lactose, arabinose, maltose, glycerol), smg resistant mutants exhibited various patterns of carbon source utilization. Most could not grow on either source; some could only use one or a few of them. Mapping of ten such mutations gave a location at, or very near, the *crp* gene (Daniel and Danchin, unpublished observations).

Alteration in the Branched-Chain Amino Acid Pathways

Some of the previous observations about serine sensitivity had shown that a low cell concentration is critical in order to obtain a straightforward growth inhibition. In synthetic minimal medium, cells at densities higher than 10^8 per ml appeared not to be sensitive to serine (Fig. 2). The fact that filtrates provided by cells grown at high densities in M63 glucose medium were usually able to counteract the serine influence, was indicative of excretion of anti-serine substance(s).

We first looked to determine whether, among various smg resistant or sensitive mutants, differences could be found in the relieving potential of their culture filtrates. As shown in Table 5, a wild-type strain and its *relA* derivative exhibited low anti-serine activity. In contrast two *relA* strains harboring independent *crp** mutations gave filtrates extremely active in counteracting the serine effect. Conversely filtrates obtained from an adenyl cyclase deficient mutant appeared not to significantly relieve serine inhibition.

Since it was previously shown that exogenous isoleucine and its metabolic precursors efficiently prevent serine inhibition, we then tried to characterize the compound(s) present in the culture filtrate of the *crp** mutants with respect to isoleucine metabolism. Amino acid analysis showed no significant levels of amino acids except glutamate (10–20 μM for a cell density of 2×10^8 per ml). Further characterization revealed the presence of high concentrations of 2-ketobutyrate (2–4 μM for a cell density of 2×10^8 per ml). This was established by chromatography procedures (see Materials and Methods). Moreover after partial purification and concentration, the

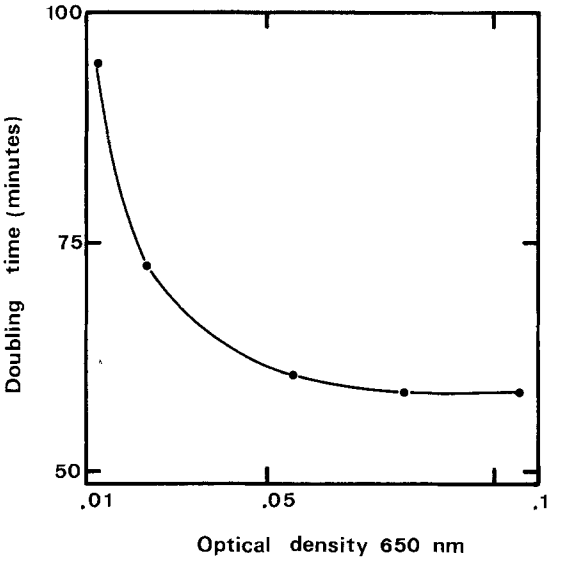

Fig. 2. Growth inhibition dependence on bacteria dilutions. Doubling time is plotted versus initial optical density of FB8r cultures in 63 glucose B_1 medium supplemented with 1 mM L-serine. In order to achieve the range of initial optical densities, increasing volumes of an exponential FB8r culture grown in 63 glucose B_1 medium were added to the test medium. 0.1 density at 650 nm corresponds to about 10^8 bacteria per ml. Doubling time in the absence of serine was 48 min

Table 5. Capacity of filtrates from strains FB8, FB8r and CA8404 to counteract serine inhibition

Filtrate preparations and test conditions were as described in Materials and Methods. Strain FB8r was used for the sensitivity test. Numbers represent the ratio of the doubling times in the presence and the absence of serine, with glucose as the carbon source

	No Filtrate	Filtrate		
		no dilution	2-fold dilution	4-fold dilution
Filtrate from:				
strain FB8r	1.8	1.3	1.8	–
strain FB8	1.8	1.2	1.8	–
strain CA8404	2.2	1.1	1.1	1.1

Table 6. Excretion of 2-ketoacid from exogenous threonine by strain CA8404

For filtrate preparations and DNP-hydrazone extraction, see Materials and Methods. ^{14}C L-threonine 0.25 mCi/10 μM was added to the M63 glucose B_1 medium during the whole culture time from 7×10^7 cells per ml to about 3×10^8 cells per ml

	Percentage of radioactivity recovered	
	Growth in glucose	Growth in glucose +isoleucine 1 mM
Filtrate	53	68
Toluene extracted DNP hydrazone material from filtrate	20	< 1

excreted product was able to replace isoleucine or 2-ketobutyrate for growth of an *ilvA* strain, but failed to support growth of an *ilvC* or *ilvD* strain (in the presence of valine).

In *Escherichia coli* under aerobic conditions, the main source of 2-ketobutyric acid is threonine, which is converted into the ketoacid by threonine deaminase encoded by the *ilvA* gene. Indeed addition of a low concentration of ^{14}C L-threonine (or ^{14}C L-homoserine, a precursor of threonine) resulted in the production of ^{14}C 2-ketobutyrate in the culture filtrate of the *rel*A *cya*$_{\Delta}$*crp** mutant (Table 6). In addition when this strain was grown in the presence of an excess of isoleucine – which is known to inhibit threonine deaminase activity (see Iaccarino et al., 1978) – exogenous ^{14}C L-threonine no longer led to ^{14}C 2-ketobutyrate production. It appears therefore that *rel*A *cya*$_{\Delta}$*crp** strains excrete 2-ketobutyrate through the activity of the *ilvA* gene product, normally sensitive to retroinhibition by isoleucine.

The *crp** mutations had been isolated after nitrosoguanidine mutagenesis which had also introduced a *rpsL* mutation at the same time. We wondered whether the excretion phenomenon described above might not have been due to this or another secondary mutation. Therefore transduction of the *crp** allele was performed into the parental strain CA8306 (which was shown not to excrete 2-ketobutyrate). Several transductants were investigated for their 2-ketobutyrate excretion. Results found with one *rpsL*$^+$ and one *rpsL* are given in Table 7: both transductants clearly behaved as the original *crp** strain.

Another result further confirmed the relationship between the modified cyclic AMP receptor protein and 2-ketobutyrate excretion. As already stated, spontaneous mutants derived form a *rel*A *cya*$_{\Delta}$*crp** strain which would grow on smg glucose plates, were found to map in the *crp* gene and yield a phenotype partially or completely identical to *crp*$^-$ mutants. The *crp*$^-$ mutants thus obtained would lose at the same time the ability to excrete 2-ketobutyrate (Table 7).

Finally we tested the effect of the *rel*A$^+$ allele on 2-ketobutyrate excretion by strains harboring a *crp** mutation. The same epistatic relationship between the *rel*A$^+$ and the *crp** alleles was seen with respect to the 2-ketoacid excretion as the one observed with respect to serine sensitivity (not shown).

Alteration of the Coordinated Expression of the ilvA and E Gene Products

When threonine deaminase and isoleucine transaminase activities were measured in crude extracts from strain CA8404 and compared with the activities found with the parental strain CA8306 or strain FB8r, an interesting finding was made. Although isoleucine transaminase activity was quite similar in all these strains, threonine deaminase activity from strain CA8404 increased to about 1.5 to 2 times the activity measured in the two other strains (Table 8). Since transcription in the *ilv EDA* operon is thought to proceed from *ilvE* (coding for isoleucine transaminase) to *ilvA* (coding for threonine deaminase) (Smith et al., 1976) the abnormal result found with strain CA8404 is not readily explained. Umbarger and coworkers (1973, 1974) noticed, by using an *Escherichia coli* ML or K 12 strain auxotrophic for isoleucine, valine and leucine, that isoleucine starvation – but not valine or leucine starvation – elicited a high ratio of threonine deaminase to dihydroxyacid dehydratase, an enzyme coded by the *ilvD* gene. Although strain CA8404 is not auxotrophic for isoleucine, we tested whether the high ratio of threonine deaminase to isoleucine transaminase found in that strain was the result of a partial isoleucine starvation for unknown reasons. As shown in Table 8, this does not seem to be the case since the activity ratios were similar whether the strain was grown in the presence or in the absence of 1 mM L-isoleucine.

Table 7. Excretion of 2-ketoacid from exogenous threonine by different *crp*$^-$ and *crp** strains.

Procedure was as given in legend of Table 6. Numbers represent percentage of radioactivity recovered

	BM617 (*cya*$_\Delta$, *crp** *rel* A)	BM618 (*cya*$_\Delta$, *crp**, *rps L* *rel* A)	BM617-a (*cya*$_\Delta$, *crp*$^-$ *rel* A)	BM618-a (*cya*$_\Delta$, *crp*$^-$, *rps L* *rel* A)
Filtrate	62	79	44	45
Toluene extracted DNP hydrazone material from filtrates	23	43	< 1	< 1

Table 8. Threonine deaminase and isoleucine transaminase activities in crude extracts from strains CA8404, CA8306 and FB8r.

Activities are given in nmoles per mg of proteins and represent the average measurement of two independent crude extracts

	TD	IT	$IT \times \left(\frac{1}{IT}\right)_{FB8r}$	$\frac{TD}{IT} \times \left(\frac{IT}{TD}\right)_{FB8r}$
FB8r	60	39.6	1	1
CA8404	105	37.6	0.95	1.75
CA8306	54	33.9	0 85	1.05

TD = threonine deaminase activity; IT = isoleucine transaminase activity

Discussion

In previous papers (see Uzan and Danchin, 1976), it has been shown that growth of *rel*A strains is more sensitive to serine than their stringent counterparts. This behavior results – at least in part – from a lack of full derepressibility of the *ilv* operons in the *rel*A background (Uzan and Danchin, 1978). Here we demonstrate that cyclic AMP and its receptor protein (CAP) also modulate the pattern of response to serine: mutations abolishing either the adenylcyclase activity or the CAP function in a *rel*A background lead to resistance to growth inhibition by serine. In other words, when considering the sensitivity/resistance phenotype, one finds that the effect of the *cya* or *crp*$^-$ allele is epistatic on the *rel*A allele; however the *rel*A$^+$ allele is epistatic on the cyclic AMP-insensitive *crp** allele. This observation clearly implies some kind of antagonistic relationship between two basic regulatory mechanisms, one involved in the modulation of the catabolism level, the other monitoring the anabolism level of the cell.

It is noteworthy that cyclic AMP significantly enhances the serine-induced growth inhibition of a *rel*A strain in a glucose medium; however the serine growth inhibition effect obtained when different carbon sources are used is not correlated with the catabolite repression state of the cell, as measured by the rates of induced β-galactosidase synthesis. This suggests either the existence of some other modulators than ppGpp and the cyclic AMP-CAP complex of the serine sensitivity phenotype or, as already stated by Ullmann, an absence of a strict correlation between the cyclic AMP-CAP complex levels and the catabolite repression state (Ullmann, 1974; Ullmann et al., 1976; Wanner et al., 1978; Dessein et al., 1978a and b), or both.

As we have shown, 2-ketobutyrate can relieve serine-induced growth inhibition in *rel*A strains. Therefore it is quite striking that a mutant particularly sensitive to serine, the *rel*A *cya*$_\Delta$*crp** strain, excretes large quantities of 2-ketobutyrate into M63 glucose medium through the normal anabolic L-threonine deaminase activity (encoded by the A gene of the *ilvEDA* operon). Correlated with this fact is the finding that crude extracts from this strain show significantly increased L-threonine deaminase activity as compared to the parental *rel*A *cya*$_\Delta$ strain or to the *rel*A strain FB8r, under conditions where the three strains exhibit no differences in the isoleucine transaminase activity (encoded by the *E* gene of the *ilvEDA* operon). Thus the presence of the *crp** allele results in a decoordination between two enzymatic activities, one encoded by an operator-proximal gene, the other by an operator-distal gene on the same anabolic *ilv* operon. The molecular bases for this decoordination are not understood. Since an effect on the stability of L-threonine deaminase is unlikely, one might consider that the *crp** product exerts an antipolar effect on the operon, either directly or indirectly. In fact an antipolar effect of the cyclic AMP-CAP complex was recently described in the lactose and galactose operons by Ullmann et al. (1979). Alternatively, a secondary promoter sensitive to the *crp** product might explain our finding. Yet we believe this possibility is unlikely since we observe that the increased L-threonine deaminase activity remains sensitive to repression by isoleucine, valine and leucine added to the culture medium (data not shown).

Whatever its mechanism, this decoordination is nevertheless not sufficient for promoting 2-ketobutyrate excretion because the flow of intermediary metabolites derived from threonine should end up with isoleucine. Some block at the level of the acetolactate synthase enzymes must therefore be invoked suggesting that under growth conditions in the M63 glucose medium the *crp** gene product also exerts an inhibitory effect on the acetolactate synthase activities or syntheses, either directly or indirectly. Yet the presumed inhibition should be partial since strains carrying the *crp** allele do not become auxotrophic for isoleucine, or isoleucine and valine. Besides, the fact that the *rel*A$^+$ derivative of the *rel*A *cya*$_\Delta$*crp** strain does not excrete measurable amounts of 2-ketobutyrate under

the same growth conditions, strongly suggests that the expression of the acetolactate synthase enzymes is also related to the stringent control.

Studying two isogenic *rel*A$^+$ and *rel*A strains containing a mutation in the leucine biosynthetic pathway which allows derepression of the isoleucine and valine enzymes by starving the cells for leucine, Freundlich (1977) has recently reported that cyclic AMP could replace the *rel*A dependent requirement for derepression of acetolactate synthase. Since we did not assay the acetolactate synthase activities in strains harboring the *crp** mutation, we do not know whether the mutation, in a mineral unsupplemented glucose medium, gives rise to a derepression of these enzymes. If that were the case however, it would indicate that the *crp** gene product, whether directly or not, acts by partially blocking the acetolactate synthase activities, thus favoring accumulation of 2-ketobutyrate in a *rel*A background.

In any event we hypothesize a major role for the acetolactate synthases as targets of the regulation mediated by the cyclic AMP-CAP complex. Certainly the sophistication of the acetolactate synthases in *E. coli* K 12 – as described in the review of Iaccarino et al. (1978) – could accommodate such a hypothesis. Furthermore serine sensitivity of *rel*A strains seems also to be linked to regulatory patterns involving acetolactate synthase activities and resulting in a need for external isoleucine in order for growth to be resumed. This appears to be best demonstrated by the finding (Uzan and Danchin, 1978) that the *ilvO* mutation – which is known to derepress the cryptic acetolactate synthase coded by the *ilvG* gene – reverts a *rel*A strain to resistance to serine. Moreover an amber mutation in the ilvG gene restores the serine sensitivity of such an *ilvO rel*A strain.

Another striking features in *E. coli* K 12 is its well-known valine sensitivity, which results from the inhibitory effect of the acetolactate synthase activities by the amino acid. We thus believe that these enzymes are the targets of a complex regulatory system. Progress is presently being made toward the understanding of the physiological role of such a regulation.

Acknowledgments. We wish to thank A. Ullmann for fruitful discussions and the critical comment of the manuscript, G. Cohen for support, and S. Busby and B. Ashby for revising the manuscript.

This work was supported by grants from the Centre National de la Recherche Scientifique (Groupes de Recherche n° 30 et n° 18) and the Délégation Générale à la Recherche Scientifique et Technique.

References

Aföldi, L., Kerekes, E.: Neutralization of the amino acid sensitivity of RC^{rel} *E coli* Biochim. Biophys. Acta **91**, 155–157 (1964)

Castellazzi, M., George, J., Buttin, G.: Prophage induction and cell division in *E. coli;* I – Further characterization of the thermosensitive mutation *tif*-1 whose expression mimics the effect of UV irradiation. Mol. Gen. Genet. **119**, 139–152 (1972)

Danchin, A.: A new technique for selection of sensitive and auxotrophic mutants of *E. coli:* isolation of a strain sensitive to an excess of one carbon metabolites. Mol. Gen. Genet **150**, 293–299 (1977)

Danchin, A., Dondon, L.: Serine sensitivity of *Escherichia coli* K12: A serine resistant, thermosensitive mutant, altered in carbohydrate permeation, cAMP intracellular level, ppGpp turnover, with extreme sensitivity to 2-ketobutyrate. Mol. Gen. Genet. (submitted)

Dessein, A., Schwartz, M., Ullmann, A.: Catabolite repression in *E. coli* mutants lacking cyclic AMP. Mol. Gen. Genet. **162**, 83–87 (1978)

Dessein, A., Tillier, F., Ullmann, A.: Catabolite modulator factor: physiological properties and in vivo effects. Mol. Gen. Genet. **162**, 89–94 (1978)

Freundlich, M.: Cyclic AMP can replace the relA$^-$ dependent requirement for derepression of acetohydroxy acid synthase in *E. coli* K 12. Cell **12**, 1121–1126 (1977)

Guardiola, J.: Requirement of the newly recognized *ilvJ* gene for the expression of a valine transaminase activity in *E. coli* K 12. Molec. Gen. Genet. **158**, 157–164 (1977)

Iaccarino, M., Guardiola, J., De Felice, M., Favre, R.: Regulation of isoleucine and valine biosynthesis Curr. Top. Cell. Regul. **14**, 29–73 (1978)

Magasanik, B.: Catabolite repression. Cold Spring Harbor Symp. Quant. Biol. **26**, 249 (1961)

Miller, J H.: Experiment in molecular genetics. New York. Cold Spring Harbor Laboratory 1974

Pardee A.B., Jacob, F., Monod, J.: The genetic control and cytoplasmic expression of "inductibility" in the synthesis of β-galactosidase by *E. coli* J. Mol. Biol **1**, 165–178 (1959)

Sabourin, D., Beckwith, J.: Deletion of *E. coli crp* genes. J. Bacteriol. **122**, 338–340 (1975)

Smith, J.M., Smolin, D.E., Umbarger, H.E.: Polarity and the regulation of the *ilv* gene cluster in *E. coli* strain K 12. Mol. Gen. Genet. **148**, 111–124 (1976)

Ullmann, A.: Are cyclic AMP effects related to real physiological phenomena? Biochem. Biophys. Res. Commun. **57**, 348–352 (1974)

Ullmann, A., Joseph, E., Danchin, A.: Cyclic AMP as a modulator of polarity in polycistronic transcriptional units. Proc. Natl. Acad. Sci. U.S.A. **76**, 3194–3197 (1979)

Ullmann, A., Tillier, F , Monod, J.: Catabolite modulator factor. A possible mediator of catabolite repression in bacteria. Proc. Natl. Acad Sci. U.S.A. **73**, 3476–3479 (1976)

Uzan, M., Danchin, A.: A rapid test for the *relA*$^-$ mutation in *E. coli* . Biochem. Biophys. Res. Commun. **69**, 751–758 (1976)

Uzan, M., Danchin, A., Correlation between the serine sensitivity and the derepressibility of the *ilv* genes in *E. coli relA*$^-$ mutants. Mol. Gen. Genet. **165**, 21–30 (1978)

Wanner, B., Kodaira, R., Neidhardt, F.: Regulation of *lac* operon expression: Reappraisal of the theory of catabolite repression. J Bacteriol. **136**, 947–954 (1978)

Wasmuth, J., Umbarger, H.E.: Effect of isoleucine, valine, or leucine starvation on the potential for formation of the branched-chain amino acid biosynthetic enzymes. J. Bacteriol. **116**, 548–561 (1973)

Communicated by G.O'Donovan

Received June 1, 1979