

Catabolite repression by glucose 6-phosphate, gluconate and lactose in *Escherichia coli*

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Summary

While catabolite repression by glucose has been studied extensively and is understood in large detail in *Enterobacteriaceae*, catabolite repression by carbohydrates that are not transported by the phosphotransferase system (PTS) has always remained an enigma. Examples of non-PTS carbohydrates that cause catabolite repression in *Escherichia coli* are gluconate, lactose and glucose 6-phosphate. In this article it is shown that enzyme IIA^{Glc} of the PTS is not involved in catabolite repression by these carbon sources. Carbon sources that caused strong catabolite repression of β -galactosidase lowered the concentration of both cAMP and the cAMP receptor protein (CRP). A strong correlation was found between the amounts of cAMP and CRP and the strength of the repression. The levels of cAMP and CRP were modulated in various ways. Neither overproduction of CRP nor an increased cAMP concentration could completely relieve the repression by glucose 6-phosphate, lactose and gluconate. Simultaneously increasing the cAMP and the CRP levels was lethal for the cells. In a mutant expressing a constant amount of cAMP-independent CRP* protein, catabolite repression was absent. The same was found in a mutant in which *lac* transcription is independent of cAMP/CRP. These results, combined with the fact that both the cAMP and the CRP levels are lowered by glucose 6-phosphate, lactose and gluconate, lead to the conclusion that the decreased cAMP and CRP levels are the cause of catabolite repression by these non-PTS carbon sources.

Introduction

In microorganisms, certain carbon sources are preferred

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to others. This phenomenon was studied extensively by J. Monod in the 1940s, who observed that *Escherichia coli*, when it is offered, for example, both lactose and glucose, preferentially uses glucose and starts to metabolize lactose only when the glucose is exhausted (Monod, 1942). Monod referred to this as 'diauxic growth', and found that many pairs of carbohydrates exhibit this behaviour. Later, the more general term 'catabolite repression' was introduced (Magasanik, 1961). Subsequently, this term was restricted to describe only the permanent and not the transient repression of genes (Magasanik, 1970). More recently, the term catabolite repression became used to describe the general phenomenon whereby the presence of certain compounds in the medium (e.g. carbon sources or nitrogen sources) can repress the expression of certain genes or operons (see Wanner *et al.*, 1978; Postma and Lengeler, 1985; Saier *et al.*, 1996). Most research has focussed on catabolite repression by carbohydrates, which can be defined as the inhibitory effect of a carbon source in the growth medium on the gene expression and/or the activity of proteins involved in catabolism of other carbon sources. 'Carbon catabolite repression' and the 'glucose effect' are still sometimes used as synonyms, which is confusing because carbon sources other than glucose can also cause catabolite repression. We will use the term 'glucose repression' only to indicate catabolite repression elicited by glucose. Catabolite repression has been observed in many different prokaryotes, but research has mainly focussed on repression in *E. coli*, a few other enteric bacteria, and some other organisms such as *Bacillus subtilis*.

Glucose-mediated catabolite repression in *E. coli* is now quite well understood (recently reviewed by Postma *et al.*, 1993; Kolb *et al.*, 1993; Saier *et al.*, 1996). The current model proposes that glucose repression is mediated by dephosphorylation of enzyme IIA^{Glc}, which is one of the enzymes involved in glucose transport. Glucose and many other carbohydrates are transported by the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS), a complex carbohydrate uptake system which, via several enzymes, sequentially transfers a phosphoryl group originating from phosphoenolpyruvate to a carbohydrate molecule, which is then transported concomitantly. All PTS proteins become phosphorylated and dephosphorylated during this process. When a PTS carbohydrate is transported, the phosphorylation state of the PTS enzymes changes. The phosphorylation state of enzyme IIA^{Glc}

regulates catabolite repression by PTS substrates both at the post-transcriptional level and at the transcriptional level. When enzyme IIA^{Glc} is in the dephosphorylated state, i.e. when carbohydrate is transported, it binds to, and thereby inactivates, the transport systems of a number of non-PTS carbon sources (reviewed by Postma *et al.*, 1993). Consequently, induction of the genes encoding metabolic pathways necessary for the metabolism of these carbon sources is prevented. For this reason, this process is called 'inducer exclusion'. The other mechanism by which dephosphorylation of enzyme IIA^{Glc} causes repression is by decreasing the concentration of cAMP in cells grown on glucose or other PTS carbohydrates. It has been proposed that phosphorylated IIA^{Glc} stimulates the cAMP biosynthetic enzyme, adenylate cyclase (reviewed by Postma *et al.*, 1993; Kolb *et al.*, 1993; Botsford and Harman, 1992). Cyclic AMP can bind to the cAMP-receptor protein (CRP) to form the cAMP-CRP complex, which is a regulatory factor for the transcription of at least 45 genes, including the *lac* operon (Botsford and Harman, 1992).

Regulation of the concentration of the cAMP-CRP complex has for a long time been thought to be mediated only by changes in the cAMP concentration. More recently, it was discovered that the concentration of CRP is also lowered in cells grown on glucose compared to the level in cells grown on succinate, and that this is an additional important factor contributing to catabolite repression by glucose (Ishizuka *et al.*, 1993). The mechanism for the glucose-mediated decrease in CRP is not yet fully understood, but has been shown to be a consequence of the complex regulation of the *crp* gene, which is both positively and negatively regulated by the cAMP-CRP complex, depending on the concentration of the complex (Ishizuka *et al.*, 1994; Hanamura and Aiba, 1992).

Although catabolite repression by glucose and other PTS carbohydrates is quite well understood, it should be realized that our knowledge of catabolite repression is still far from complete, even in *E. coli*. A number of studies have indicated that, in *E. coli*, cAMP/CRP-independent mechanisms may be involved in catabolite repression (reviewed by Magasanik and Neidhardt, 1987; Ullmann and Danchin, 1983; Saier *et al.*, 1996). Furthermore, it has been known for a long time that, in *E. coli*, carbon sources that are not transported by the PTS, and which therefore are not expected to change the phosphorylation state of enzyme IIA^{Glc}, can also cause very strong catabolite repression, which is often even stronger than that by glucose. Examples are glucose 6-phosphate (Perlman *et al.*, 1969) and gluconate (Okinaka and Dobrogosz, 1966). Surprisingly, the expression of the *lac* operon, induced with IPTG, is higher in cells grown on glycerol or succinate than in cells grown on lactose; therefore, lactose also represses the genes necessary for its own metabolism

(Jacob and Monod, 1961; Ishizuka *et al.*, 1993; Inada *et al.*, 1996).

Repression by glucose 6-phosphate in enteric bacteria has been studied to some extent. It has been found that glucose 6-phosphate still causes catabolite repression in a mutant lacking enzyme I of the PTS (Yang *et al.*, 1979), which suggests that the PTS is not involved in the repression. The role of cAMP in glucose 6-phosphate repression is not clear. It has been shown that the lactose operon is not repressed by glucose 6-phosphate in the *E. coli lacL8UV5* mutant (often called the *lacUV5* mutant), in which *lac* expression is independent of cAMP and CRP levels (Silverstone *et al.*, 1970). Although this result suggests that cAMP and/or CRP is involved in the repression, there is no agreement about the role of cAMP, because some authors showed that addition of cAMP to the culture medium largely overcomes catabolite repression by glucose 6-phosphate (Perlman *et al.*, 1969; Yang *et al.*, 1979), while others claimed the opposite (Goldenbaum and Dobrogosz, 1968).

Repression by gluconate and lactose has hardly been studied. It has been shown that gluconate causes strong catabolite repression of β -galactosidase synthesis (Okinaka and Dobrogosz, 1966), and that the cAMP level in gluconate-grown cells is low (Epstein *et al.*, 1975). Growth on lactose also leads to low cAMP levels in the cell (Inada *et al.*, 1996).

In this report, we analysed catabolite repression by glucose 6-phosphate, lactose and gluconate in different *E. coli* mutants. We modulated cAMP and CRP levels, and we measured cAMP and CRP levels in cells grown on different carbon sources. We have also shown that the non-PTS carbon sources cause a decrease in the cAMP and CRP levels. Neither the decrease in cAMP nor the decrease in CRP individually is sufficient to explain catabolite repression, but the combined decrease of cAMP and CRP levels can explain catabolite repression by these compounds.

Results

The PTS is not involved in catabolite repression by glucose 6-phosphate, lactose and gluconate

In order to investigate the possible role of the PTS in repression by glucose 6-phosphate, lactose and gluconate, we measured repression of β -galactosidase in a wild-type *E. coli* strain and an isogenic Crr⁻ mutant (which lacks enzyme IIA^{Glc}, the global regulator in glucose-mediated catabolite repression). The *lacZ* gene (encoding β -galactosidase) was induced with 0.4 mM IPTG. Since IPTG can enter the cell even in the absence of the lactose transporter (encoded by *lacY*), inducer exclusion does not play a role when IPTG is used as inducer.

Glucose 6-phosphate, lactose and gluconate caused

Table 1. Catabolite repression of β -galactosidase in different mutants.

Strain	Lactate	Lactate + glucose 6-phosphate	Lactate + gluconate	Lactate + glucose	Lactate + lactose
MG1655 (wild type)	12800	1260	4920	3350	2900
PPA484 (<i>crr-1</i>)	8000	460	1930	4880	1060
PPA549 (<i>lacL8UV5</i>)	8280	6980	7240	7080	6710
ATCC8739 (<i>cpdA</i>)	12620	1980	5580	4730	2720
MG1655 + 2.5 mM cAMP	14310	2160	8020	10130	4450
MG1655 + pFCRP7	15390	1860	7670	5540	4450
IT1106 (<i>crp*</i>)	6810	2000	3780	3780	2780
PPA530 (<i>cya crp</i>) + pHA7G141D	3060	2100 ^a	3240	2730	2650
PPA523 (del <i>lacZY</i>) + pIT296	1600	1660	1500	1210	ND ^b

Beta-galactosidase activities were measured as specified in the *Experimental procedures*, with IPTG as inducer of the *lacZ* gene. Activities are expressed in Miller units. At least three samples were taken and values are the average value of at least two independent experiments. The SEM was less than 10%.

a. The activity became lower at higher cell density (see the text).

b. ND, not determined.

approximately 10-fold, fourfold and threefold repression, respectively, in a wild-type strain. Strong repression by lactose, gluconate and glucose 6-phosphate was still observed in the Crr^- mutant (Table 1): in fact, the repression is relatively stronger in the Crr^- mutant. Thus, we conclude that enzyme IIA^{Glc} is not the mediator of catabolite repression by these carbon sources. Only in the case of glucose was repression in the Crr^- mutant less strong than in the wild-type strain, showing, as expected, that enzyme IIA^{Glc} is involved in glucose repression. The residual repression in the Crr^- mutant may be caused by a glucose-mediated reduction in the CRP level, or even by an enzyme IIA^{Glc}-independent decrease in the cAMP concentration, because it has been shown that the total cAMP production is still downregulated by glucose in a Crr^- mutant (Dumay *et al.*, 1996).

Re-examination of the role of cAMP in catabolite repression by glucose 6-phosphate, lactose and gluconate

The possibility that a decrease in the cAMP concentration is the mediator of catabolite repression by glucose 6-phosphate, lactose and gluconate has been investigated to some extent. The most important observation has been that there is no repression of β -galactosidase synthesis by glucose 6-phosphate in the *lacL8UV5* mutant (Silverstone *et al.*, 1970).

We have repeated the β -galactosidase measurements in a *lacL8UV5* mutant (PPA549) that is isogenic with our wild-type strain MG1655. All carbon sources tested caused only very weak repression (Table 1). The absence of repression in the *lacL8UV5* mutant strongly suggests that the cAMP–CRP complex is involved in regulation of catabolite repression by these carbon sources.

Another indication for the involvement of cAMP in repression by glucose 6-phosphate, lactose and gluconate is the observation reported by different groups that the

cAMP level in cells grown on these carbon sources is low. In fact, the cAMP level in glucose 6-phosphate-grown cells is even lower than that in glucose-grown cells (Epstein *et al.*, 1975; Joseph *et al.*, 1982). However, the reported cAMP levels differ significantly in these studies and it is not clear whether this is because of a difference between the procedures used to extract and measure cAMP or because of the use of different strains. To resolve this question, we determined the cAMP levels in our wild-type strain. Filtration, centrifugation and the fixation of the intracellular cAMP in cold (-40°C) 50% methanol were compared as methods for harvesting the cells. Filtration gave the most reliable results. The data in Table 2 and Fig. 1 show that the cAMP levels are the lowest in cells grown on carbon sources that cause stronger repression. The cAMP levels we have measured are between 0.4 and 3.1 μM , which is comparable to the values reported by Epstein *et al.* (1975), but much lower than those reported by Joseph *et al.* (1982). In both reports, the cAMP level in glucose 6-phosphate-grown cells was lower than that in glucose-grown cells, which agrees with our finding. We have checked that the cAMP level is also decreased by glucose 6-phosphate in a Crr^- mutant. Because the cAMP level in Crr^- mutants is much lower than that in the wild-type strain, it was not possible to determine the

Table 2. cAMP and CRP levels in cells of strain MG1655 grown on different carbon sources.

Carbon source	cAMP (μM)	CRP (μM)
Lactate	3.30 \pm 0.10	7.8 \pm 0.7
Lactate + glucose 6-phosphate	0.40 \pm 0.02	4.0 \pm 0.2
Lactate + gluconate	1.04 \pm 0.04	5.4 \pm 0.1
Lactate + glucose	0.72 \pm 0.03	4.9 \pm 0.4
Lactate + lactose	0.70 \pm 0.05	3.6 \pm 0.3

cAMP and CRP levels in the cell were measured as described in the *Experimental procedures*. Values for cAMP represent the average value (\pm SEM) of at least three independent triplicate determinations. Values for CRP represent the average of five independent determinations (\pm SEM).

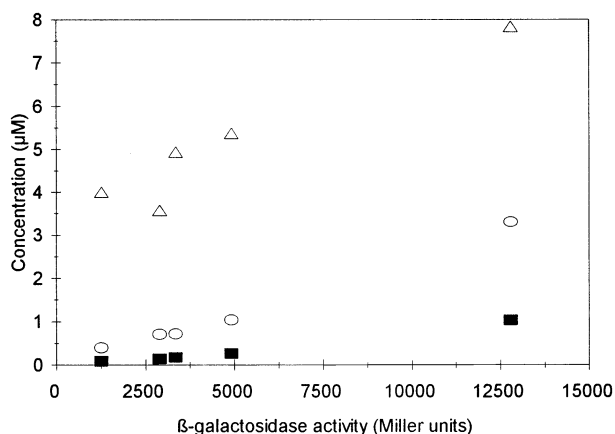


Fig. 1. The total concentration of cAMP (○) and CRP dimer (△) (Table 2) and the calculated concentration of cAMP-CRP complex (■) in MG1655 cells grown on different carbon sources, plotted against the corresponding β -galactosidase activities of the cells (Table 1). The calculated concentration of the complex is based on an equilibrium association constant of $6.7 \times 10^4 \text{ M}^{-1}$ for cAMP binding to the CRP dimer (Cheng *et al.*, 1993; Heyduk and Lee, 1989).

intracellular cAMP level accurately, but the extracellular level can be determined easily, because more than 95% of the cAMP in a culture of exponentially growing cells is found outside the cells (B. M. Hogema, unpublished; Epstein *et al.*, 1975). The extracellular cAMP levels were 25 nM and 8 nM in mid-log-phase cultures ($OD_{600} = 0.5$) of the Crr^- mutant PPA484 grown on lactate and glucose 6-phosphate, respectively. Assuming a similar decrease in the intracellular cAMP concentration, this could explain the fact that there is still catabolite repression in the Crr^- mutant, and this provides further evidence that catabolite repression by glucose 6-phosphate is not regulated by enzyme IIA^{Glc}, but is nevertheless cAMP dependent.

If a lowered cAMP concentration is involved in catabolite repression by glucose 6-phosphate, lactose and gluconate, it can be expected that addition of cAMP to cells growing on these carbon sources relieves the repression to a large extent, as is the case with glucose-mediated repression (Ishizuka *et al.*, 1993; Perlman *et al.*, 1969). Repression by different carbon sources in our wild-type strain was measured in the presence of 2.5 mM cAMP (Table 1). We confirmed that this concentration of cAMP strongly reduced glucose repression. Gluconate repression decreased slightly from 62% to 44% of the unrepressed activity (Table 1). In the case of glucose 6-phosphate and lactose, the effect of cAMP addition on the β -galactosidase activity was much smaller. Although cAMP addition increased the β -galactosidase activity from 1260 to 2160 Miller units in cells grown on glucose 6-phosphate, the repression only decreased from 90% to 85% of the unrepressed activity. Lactose repression decreased from 77% to 68% by the addition of cAMP.

To exclude the possibility that breakdown of cAMP was stimulated by lactose, gluconate and glucose 6-phosphate, the extracellular cAMP levels in the cultures grown in the presence of 2.5 mM cAMP were determined under conditions similar to those used for the induction studies. More than 90% of the added cAMP was recovered (data not shown), indicating that cAMP breakdown was not the reason that the repression was not relieved by adding 2.5 mM cAMP. We also measured catabolite repression in a *cpdA* mutant that lacks cAMP phosphodiesterase. Catabolite repression in this mutant was not significantly different from that in the wild-type strain (Table 1), again showing that cAMP breakdown is not involved in regulation of catabolite repression.

It is possible that the amount of cAMP that enters the cells or remains in the cells when exogenous cAMP is added is too low to overcome repression. Accurate measurement of the actual cAMP concentration in the cell is not possible when the extracellular concentration is very high. To be able to manipulate the cAMP level in the cells in such a way that the actual cAMP concentration can be monitored, we constructed an inducible expression vector (pBCP540) containing the adenylate cyclase (*cya*) gene cloned behind the *L*-arabinose-inducible promoter of pBAD18. Repression of β -galactosidase was measured in wild-type cells transformed with pBCP540. The *L*-arabinose concentration was adjusted in such a way that the cAMP concentration was similar to or higher than that in wild-type cells (without plasmid) grown on lactate. In the case of glucose 6-phosphate, this was not possible, but the concentration was significantly increased. As before, repression by glucose and gluconate was partially relieved, while the effect of the higher intracellular cAMP concentration on glucose 6-phosphate-mediated repression was small (Table 3).

Table 3. Beta-galactosidase activity and cAMP levels in cells over-producing adenylate cyclase from the plasmid pBCP540.

Carbon source	<i>L</i> -arabinose concentration (μM)	Beta-galactosidase activity	cAMP concentration (μM)
Lactate	0	14400	7.5 ± 2.0
Lactate + glucose 6-phosphate	75	1710	1.7 ± 0.4
Lactate + gluconate	10	6200	3.8 ± 1.1
Lactate + glucose	10	4950	5.0 ± 1.6

MG1655 transformed with pBCP540 was grown on the indicated carbon sources in the presence of the indicated concentration of arabinose. Beta-galactosidase and intracellular cAMP concentrations were determined as described in the *Experimental procedures*. Beta-galactosidase activities are expressed in Miller units. To determine β -galactosidase activity, at least three samples were taken, and values are the average value of at least two independent experiments. The SEM was less than 10%.

The role of cAMP in catabolite repression by glucose 6-phosphate, lactose and gluconate was examined further by measuring repression in a $Cya^- Crp^*$ mutant (containing a Crp^* protein that is active in the absence of cAMP). In this mutant, repression by all substrates tested was still strong, albeit less strong than in the wild-type strain (Table 1). As this mutant does not contain cAMP, the mechanism of catabolite repression by these carbon sources cannot be dependent on cAMP under these conditions. In the case of glucose, it has been shown that repression is caused by a decrease in the amount of the Crp^* protein (Tagami *et al.*, 1995).

Glucose 6-phosphate, lactose and gluconate lower the level of CRP

The near-absence of repression in the *lacL8UV5* mutant (Table 1) and the decrease in cAMP levels in cells grown on the different non-PTS carbon sources strongly suggest that a decrease in the concentration of the cAMP–CRP complex is the mediator of catabolite repression. The incomplete relief of repression by the addition of cAMP, and the occurrence of catabolite repression by glucose 6-phosphate, lactose and gluconate in IT1106, a $Cya^- Crp^*$ mutant (Table 1) on the other hand suggest that cAMP is not the sole mediator of catabolite repression by these compounds. We therefore determined, by means of immunoblotting, the amount of CRP in cells grown in minimal medium with different carbon sources. The result of a typical experiment is shown in Fig. 2, and the results are summarized in Table 2. As has been shown previously by Ishizuka *et al.* (1993), glucose lowers the level of CRP compared to that in cells grown on a non-repressing carbon source. The reduction in CRP level was somewhat smaller than the two- to threefold reduction reported previously, and may be caused by the use of a different non-repressing carbon source (lactate instead of succinate) or may reflect a difference between the

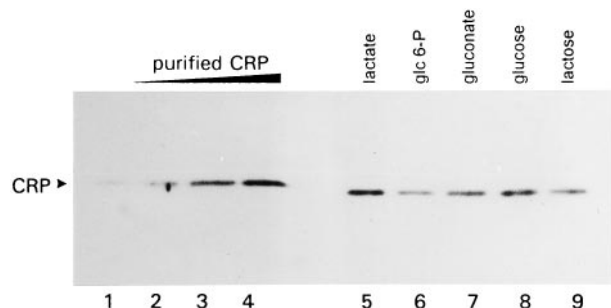


Fig. 2. Quantitative determination of CRP in wild-type cells by Western blotting. Cells were grown on different carbon sources and treated as described in the *Experimental procedures*. Lanes 1–4, Western blot of purified CRP protein (0.65 ng, 1.3 ng, 2.7 ng and 5.3 ng, respectively). Lanes 5–9, Western blot of cell extracts from cells grown on the different carbon sources indicated.

strains used. Gluconate lowered the CRP level to the same extent as glucose, while the CRP level in glucose 6-phosphate-grown cells was 20% lower than that in glucose-grown cells. The CRP level in cells grown on lactose was slightly lower still.

The decrease in CRP level is not the only cause of catabolite repression by glucose 6-phosphate, lactose and gluconate

To check whether the reduced CRP levels in cells grown on glucose 6-phosphate, lactose or gluconate were the cause of catabolite repression by these compounds, catabolite repression by different carbon sources was measured in cells overproducing CRP from the pFCRP7 plasmid which contains the *crp* gene behind the constitutive *bla* promoter (Table 1). Gluconate repression was partly relieved, but, surprisingly, an increase in the CRP level did not relieve catabolite repression by lactose and glucose 6-phosphate. It was confirmed by immunoblotting that during growth on all carbon sources tested, the CRP level in the cells containing the *crp* plasmid was higher than that in wild-type cells during growth on lactate (data not shown). The strong repression in glucose 6-phosphate-grown cells containing the *crp* plasmid could therefore not be caused by reduced CRP levels, and the reduction in the CRP level can therefore not be the only cause of repression by glucose 6-phosphate and lactose.

Catabolite repression by glucose 6-phosphate, lactose and gluconate is caused by the combination of a decrease in cAMP and CRP concentration

While many lines of evidence suggest that catabolite repression by glucose 6-phosphate, lactose and gluconate is cAMP/CRP dependent, neither addition of cAMP nor overproduction of CRP relieved the repression completely, especially in the case of lactose and glucose 6-phosphate. Because *cya* expression is reduced when CRP is overproduced (Aiba, 1985; Mori and Aiba, 1985) and adenylate cyclase activity is negatively regulated by CRP (reviewed by Botsford and Harman, 1992), overproduction of CRP could lead to lower cAMP levels, and consequently to a smaller increase in the concentration of the active cAMP/CRP complex. If this explanation is correct, overproduction of CRP in combination with cAMP addition should result in relief of catabolite repression. These experiments were without success, however, because the growth of cells containing pFCRP7 was severely inhibited by the addition of cAMP. This was also the case for cells that contained other *crp*-encoding plasmids (data not shown). At the maximal cAMP concentration that still allowed growth on glucose 6-phosphate (approx. 100 μ M), we observed a small increase (30%) in β -galactosidase activity.

Because these experiments did not answer the question posed above, we measured repression in a *Cya*⁻ *Crp*⁻ mutant that contained the plasmid pHA7G141D which constitutively expresses Crp* (Aiba *et al.*, 1985). As cAMP is not synthesized and the amount of CRP* protein is constant, there should be no catabolite repression if catabolite repression is only regulated by cAMP and CRP. We found that glucose and lactose caused only 11% and 13% repression in this strain, respectively, while gluconate caused no repression at all (Table 1). From this we conclude that, although neither cAMP addition nor CRP overproduction lead to complete relief of glucose-, lactose- and gluconate-mediated repression, the combined decrease of both the cAMP and the CRP concentrations is the cause of catabolite repression by these carbohydrates. With glucose 6-phosphate, the results were more difficult to interpret, because the growth rate of the strain containing the plasmid pHA7G141D decreased strongly before the cells entered the mid-log phase. At lower cell densities, the β -galactosidase activity was only 31% lower than that in lactate-grown cells, indicating that catabolite repression by glucose 6-phosphate was also largely relieved in this strain. We conclude that the combined decrease in cAMP and CRP is responsible for the repression by glucose 6-phosphate.

The decrease in cAMP concentration caused by glucose 6-phosphate and gluconate is caused by decreased adenylate cyclase activity

The decrease in cAMP concentration caused by growth on gluconate or glucose 6-phosphate is probably the result of reduced adenylate cyclase activity (Gstrein-Reider and Schweiger, 1982; Epstein *et al.*, 1975). Reduced adenylate cyclase activity could be caused either by a lowered amount or a lowered activity of adenylate cyclase. A plasmid containing a *cya::lacZ* protein fusion was used to distinguish between these two possibilities. Table 1 shows that the β -galactosidase activity of the fusion protein was approximately the same in cells grown on lactate, glucose 6-phosphate or gluconate; only in glucose-grown cells was the activity slightly lower, indicating that the regulation of the cAMP level in the cells is probably caused by a change in adenylate cyclase activity rather than in the amount of the enzyme.

Discussion

The involvement of the phosphorylation state of IIA^{Glc} of the PTS in PTS-mediated catabolite repression has been clearly established (for a review, see Postma *et al.*, 1993). Although there is no mechanism known that could account for a change in the phosphorylation state of enzyme IIA^{Glc} when non-PTS sugars are transported, it is possible that enzyme IIA^{Glc} is also controlling catabolite repression by

non-PTS substrates. In a recent paper by Dumay *et al.* (1996), it was suggested that enzyme IIA^{Glc} is indeed controlling the downregulation of the cAMP level in cells grown on glucose 6-phosphate. We have investigated the possible role of enzyme IIA^{Glc} by measuring catabolite repression by different carbon sources in a *Crr*⁻ mutant (lacking enzyme IIA^{Glc}). It is concluded that enzyme IIA^{Glc} is not involved in repression by these compounds. This conclusion agrees with what is reported by Yang *et al.* (1979), who showed that glucose 6-phosphate and gluconate still cause catabolite repression in a mutant lacking enzyme I of the PTS, i.e. a condition in which enzyme IIA^{Glc} is always in the dephosphorylated state.

A possible involvement of cAMP in catabolite repression by glucose 6-phosphate had already been suggested on the basis of a low cAMP level in glucose 6-phosphate-grown cells (Epstein *et al.*, 1975; Joseph *et al.*, 1982) and by the absence of repression in the *E. coli lacL8UV5* mutant (usually called *lacUV5*) (Table 1; Silverstone *et al.*, 1970), a strain in which the *lac* promoter is independent of cAMP and CRP. Some reservations have to be made, however. The *lacL8UV5* mutant contains two mutations in the *lac* promoter region. One of these mutations, the *UV5* mutation, is a change in the -10 region of the promoter, which results in the optimal box (Reznikoff and Abelson, 1978). Apart from disturbing cAMP/CRP-dependent regulation (Rockwell and Gottesman, 1991), other regulatory processes could also be changed by this mutation. Because the *lacL8* mutant, which only has a mutation in the CRP-binding site of the promoter, is still repressed approx. fivefold by glucose 6-phosphate (Silverstone *et al.*, 1970) and twofold by glucose (Wanner *et al.*, 1978), it cannot be concluded that no factors other than cAMP and CRP are involved in catabolite repression by glucose 6-phosphate, gluconate and lactose only on the basis of the fact that repression by these carbohydrates is absent in the *lacL8UV5* mutant. However, it is a strong indication that these factors are involved in the repression.

We have shown that the cAMP levels are low in cells grown on glucose 6-phosphate, gluconate and lactose compared to the level in lactate-grown cells (Table 2). These results confirm data reported by Epstein *et al.* (1975) and Joseph *et al.* (1982). The CRP levels in cells grown on these repressing carbon sources are also strongly reduced compared to the levels in cells grown on a non-repressing carbon source (Table 2). In general, low concentrations of cAMP correlate with low levels of CRP, so a decrease in the CRP levels could contribute to catabolite repression by the different carbon sources. The amount of cAMP-CRP complex in cells grown on different carbon sources was calculated and plotted against the β -galactosidase activity (Fig. 1), assuming that the dissociation constant of the cAMP-CRP complex is 15 μ M (Cheng *et al.*, 1993; Heyduk and Lee, 1989). A strong correlation

is found between the concentration of the cAMP–CRP complex and the β -galactosidase activity. The combined decrease in cAMP and CRP in cells grown on repressing carbon sources offers an attractive explanation for the observed repression. The fact that repression by glucose, lactose and gluconate is relieved when the amount of cAMP/CRP is deregulated in a $Cya^- Crp^-$ double mutant in which a *crp** gene is constitutively expressed from a plasmid (Table 1) provides additional evidence that catabolite repression is only mediated by cAMP/CRP. Glucose 6-phosphate decreased the β -galactosidase expression in this strain by only 31% at low cell densities. This decrease may be caused by cAMP/CRP-independent catabolite repression, but probably results from the fact that glucose 6-phosphate is toxic to this strain. At higher cell densities, the β -galactosidase activity decreased by more than 50%, but the growth rate also decreased strongly, possibly due to the formation of methyl glyoxal (Ackerman *et al.*, 1974; Kadner *et al.*, 1992).

If catabolite repression is only cAMP/CRP dependent, it is expected that addition of extracellular cAMP or overexpression of CRP relieves catabolite repression. With glucose and gluconate, repression was relieved to a large extent, as had been shown previously for glucose (Ishizuka *et al.*, 1993). In the case of lactose and glucose 6-phosphate, we found, to our surprise, that neither overproduction of CRP nor addition of cAMP could overcome catabolite repression. Similarly, raising the cAMP level by introducing a plasmid containing the adenylate cyclase gene behind an inducible promoter only had a small effect on glucose 6-phosphate repression. In the literature, different effects of cAMP addition on glucose 6-phosphate repression have been described, ranging from a nearly complete relief of repression (Perlman *et al.*, 1969; Yang *et al.*, 1979) to a mild increase in β -galactosidase activity without a strong effect on the repression (Goldenbaum and Dobrogosz, 1968), which is similar to the result we have obtained (Table 1). It is possible that the concentration of extracellular cAMP necessary to overcome repression by glucose 6-phosphate is strain dependent, as is the case with glucose repression (Perlman *et al.*, 1969). However, the growth of the (wild-type) strain MG1655 used in our studies, was severely inhibited by cAMP concentrations higher than 2.5 mM. Thus, we were not able to relieve the repression by adding more cAMP.

It may seem paradoxical that most results suggest that catabolite repression by glucose 6-phosphate, gluconate and lactose is cAMP/CRP dependent, but that we were unable to relieve the repression in a wild-type strain. We believe that the combination of low cAMP and low CRP levels in cells grown on lactose and glucose 6-phosphate can explain this apparent paradox. It should be noted that during growth on glucose 6-phosphate and lactose, the CRP levels are significantly lower than those in

glucose-grown cells. When the CRP level is lower, the absolute effect of an increase in the intracellular concentration of cAMP on the concentration of the cAMP–CRP complex will be smaller. Moreover, addition of cAMP may lower the CRP level even further, because the *crp* promoter is negatively regulated by the cAMP–CRP complex when the levels of this complex are low (Hanamura and Aiba, 1992; Isizuka *et al.*, 1994). If CRP is overproduced, on the other hand, the cAMP levels, which are already very low in cells grown on glucose 6-phosphate or lactose, may be too low to lead to an increase in the concentration of the complex and relieve repression. Furthermore, the cAMP level is probably further reduced by the increase in the CRP level, because both *cya* transcription and adenylate cyclase activity are negatively regulated by the cAMP–CRP complex (Aiba, 1985; Botsford and Harman, 1992). If this explanation is correct, repression cannot be relieved by either adding cAMP or overproducing CRP when the levels of both cAMP and CRP are low. The CRP level in cells grown on glucose is strain dependent (Ishizuka *et al.*, 1993). If the same is true with glucose 6-phosphate, the effect of adding cAMP in strains with a low CRP level will be smaller than in strains with a higher CRP level. This could explain why some groups have found that cAMP addition relieves catabolite repression by glucose 6-phosphate (Perlman *et al.*, 1969; Yang *et al.*, 1979), while we and others only found a small effect on the repression (Table 1; Goldenbaum and Dobrogosz, 1968).

How can glucose 6-phosphate, lactose and gluconate lower the cAMP and CRP levels? The most likely explanation for the decrease in the cAMP level is a decrease in the cAMP synthesis rate. Increased cAMP excretion cannot be the cause, because extracellular and intracellular levels of cAMP are lowered to the same extent by these carbon sources (B. M. Hogema, unpublished; Epstein *et al.*, 1975), and cAMP breakdown by phosphodiesterase is unlikely to play a role in catabolite repression (Table 1; Botsford, 1981). Using a *cya::lacZ* fusion protein with a functional *cya* gene, we demonstrated that the carbon source does not have a strong influence on the amount of fusion protein produced (Table 1). We conclude that it is not the amount but the activity of adenylate cyclase that is regulated by glucose 6-phosphate and gluconate. Other experiments showed that the activity of adenylate cyclase in washed cell suspensions in the absence of a carbon source is inversely related to the intracellular cAMP concentration (Joseph *et al.*, 1982). In this case, the adenylate cyclase activity probably reflects the amount of enzyme rather than the activity during growth. The high adenylate cyclase activity in glucose 6-phosphate-grown cells therefore suggests that the amount of adenylate cyclase is high in cells grown on glucose 6-phosphate or glucose, and is much lower in cells grown on a non-repressing carbon

source. This result was also found with a *Salmonella typhimurium cya::lacZ* transcriptional fusion (Fandl *et al.*, 1990). These facts together show that the regulation of adenylate cyclase by different carbon sources is post-translational. The mechanism of this post-translational regulation is still not elucidated. Control of adenylate cyclase activity is probably exerted via the uptake systems of different (PTS and non-PTS) carbon sources. This control should be independent of subsequent metabolism, because non-metabolizable analogues of lactose, glucose and galactose are able to decrease the cAMP accumulation in starving cells to about the same extent as the metabolizable sugars, provided that the uptake systems are induced (Gstrein-Reider and Schweiger, 1982). Recently it was shown that the total amount of cAMP in cultures grown on glucose 6-phosphate is indeed dependent on the transport capacity. A correlation between cAMP levels and the doubling time was shown when the amount of UhpT protein was modulated using an IPTG-inducible *uhpT* expression vector (Dumay *et al.*, 1996). Using a similar expression vector, it was found that the activity of β -galactosidase was also high in cells grown slowly on glucose 6-phosphate (B. M. Hogema, unpublished). The non-metabolizable glucose 6-phosphate analogue, 2-deoxyglucose 6-phosphate, also lowered the cAMP level in cells grown on pyruvate, showing that also in the case of glucose 6-phosphate, metabolism is not required for the regulation of adenylate cyclase activity (Dumay *et al.*, 1996). In addition, 2-deoxyglucose

6-phosphate causes repression of β -galactosidase (Kana-zawa and Anraku, 1977), and glucose 6-phosphate causes catabolite repression in a mutant that is unable to metabolize glucose 6-phosphate (J. C. Arents, unpublished; Kana-zawa and Anraku, 1977), also showing that metabolism is not involved in catabolite repression.

The carbon source-dependent regulation of the CRP level is not fully understood. It has been shown that the decrease in CRP level during growth on glucose is caused by the dual regulation of the *crp* gene, which is regulated both positively and negatively by the cAMP–CRP complex (Ishizuka *et al.*, 1994). The concentration of the complex needed for stimulation of the transcription is higher than the concentration needed for inhibition (Hanamura and Aiba, 1992). It was suggested that the CRP decrease is triggered by a decrease in cAMP, leading to a lowered concentration of the cAMP–CRP complex, resulting in less stimulation of CRP transcription (Ishizuka *et al.*, 1994). It is possible that a similar mechanism causes the decrease of CRP in cells grown on gluconate, lactose and glucose 6-phosphate. The mechanism of the decrease in CRP concentration will be the subject of further studies.

Experimental procedures

Bacterial strains and plasmids

The *E. coli* strains and plasmids used in this study are listed in Table 4.

Table 4. List of *E. coli* strains and plasmids used in this study.

Strain/Plasmid	Relevant genotype/phenotype	Source/Reference
Strain		
ATCC8739	<i>cpdA</i>	ATCC
CA8050	<i>lacL8UV5 thi-1</i>	P. R. Jensen
CAG12080	<i>zah-281::Tn10</i>	Singer <i>et al.</i> (1989)
CAG18456	<i>cysG zhe-3084::Tn10</i>	Singer <i>et al.</i> (1989)
CAG18468	<i>nupC510::Tn10</i>	Singer <i>et al.</i> (1989)
DW2	<i>rpsL Δ(melB) Δ(lacZY)</i>	G. Leblanc
IT1106	<i>Δ(cya-854::Cm) crp*B1</i>	T. Inada
LM1	<i>crr-1 thi-1 his-1 argG6 metB galT rpsL manXYZ manI nagE</i>	J. Lengeler
MG1655	Wild type	B. Bachmann
PP47	<i>crp-1</i>	Ishizuka <i>et al.</i> (1994)
PPA237	<i>crr-1 nupC510::Tn10 thi-1 his-1 argG6 metB galT rpsL manXYZ manI nagE</i>	LM1 × P1.CAG18468
PPA418	<i>Δ(cyaA1400::Km)</i>	MG1655 × P1.SP850
PPA484	<i>crr-1 nupC510::Tn10</i>	MG1655 × P1.PPA237
PPA522	<i>rpsL Δ(melB) Δ(lacZY) zah-281::Tn10</i>	DW2 × P1.CAG12080
PPA523	<i>Δ(lacZY) zah-281::Tn10</i>	MG1655 × P1.PPA522
PPA529	<i>crp-1 zhe-3084::Tn10</i>	PP47 × P1.CAG18456
PPA530	<i>Δ(cyaA1400::Km) crp-1 zhe-3084::Tn10</i>	PPA418 × P1.PPA529
PPA549	<i>lacL8UV5</i>	PPA523 × P1.CA8050
SP850	<i>Δ(cyaA1400::Km)</i>	Shah and Peterkofsky (1991)
Plasmid		
pIT296	<i>cya-lacZ</i> fusion derived from pMC1403, Ap ^R	T. Inada
pFCRP7	Constitutive low level <i>crp</i> plasmid	Ishizuka <i>et al.</i> (1993)
pHA7G141D	Expresses <i>crp*</i> constitutively	Aiba <i>et al.</i> (1985)
pBCP540	Expression vector containing adenylate cyclase	This work

Cm, chloramphenicol-resistance gene; Km, kanamycin-resistance gene; Ap^R, ampicillin resistant; P1, phage P1.

Media and growth conditions

Cells were grown in 50 ml Erlenmeyer flasks at 37°C on a rotary shaker. Minimal medium A (pH 7) contained 1 g (NH₄)₂SO₄, 10.5 g K₂HPO₄, 4.5 g KH₂PO₄ and 0.1 g MgSO₄·7H₂O per litre of demineralized water. Cells were first pre-grown in rich medium (A medium containing 1% tryptone) for 8–16 h and then diluted 1:80 in 10 ml of A medium containing 0.4% (w/v) D,L-lactate as the carbon source. After overnight growth, the cells were still in the logarithmic phase and were diluted to the desired optical density (monitored at 600 nm) in A medium containing 0.4% lactate and 0.2% of the other indicated carbon sources. The optical density to which cells were diluted was usually 0.025 if lactate was the sole carbon source, and 0.005 when the cells were grown on a carbon source that supported fast growth. When appropriate, 20 µg ml⁻¹ of necessary amino acids were added. When cells contained plasmids, ampicillin was added at 100 µg ml⁻¹.

Construction of pBCP540

To clone the *E. coli* adenylate cyclase gene behind the L-arabinose-inducible promoter from pBAD18 (Guzman *et al.*, 1995), the *cya* gene was first cloned at the optimal distance from a ribosome-binding site (RBS) in plasmid pT7.7 (Tabor and Richardson, 1985). The 4.4 kb *Nde*I–*Sal*I fragment from pPR100 (Amin and Peterkofsky, 1992) containing the *cya* gene, was inserted in pT7.7 digested with *Nde*I and *Sal*I, resulting in pBCP530. pBCP530 was digested with *Xba*I, treated with Klenow fragment of DNA polymerase I, digested with *Sal*I, and the 4.4 kb *Xba*I–*Sal*I fragment containing the *cya* gene (with RBS) was cloned behind the *ara* promoter of pBAD18 (provided by M. Ehrmann; Guzman *et al.*, 1995) that had been digested with *Sma*I and *Sal*I, resulting in pBCP540.

Beta-galactosidase assay

Beta-galactosidase activity was determined in permeabilized cells using the improved method of Giacomini *et al.* (1992). Cells were grown as described above with 400 µM IPTG (maximal induction). As the optical density after dilution increased more than 20-fold (see *Media and growth conditions*), this ensured at least four doublings. The β-galactosidase activity was constant at OD₆₀₀ levels of between 0.1 and 0.6. During growth at least three samples were taken and analysed while the cells were in this growth phase. The average value of at least two independent experiments was taken. The standard error of the mean (SEM) was always less than 10%. When cells were grown on lactate as the sole carbon source, the β-galactosidase activity was slightly dependent on the growth phase. A maximum level was reached in the early log phase (OD₆₀₀ ≈ 0.25). We therefore analysed samples while the cells were at OD₆₀₀ levels of between 0.2 and 0.3 when the cells were grown on lactate as the sole carbon source.

cAMP measurement

To determine the intracellular cAMP levels as accurately as possible, we compared several methods of harvesting the cells, namely centrifugation, filtration and quenching in cold

(–40°C) methanol. We used perchloric acid and boiling to prepare cell extracts after harvesting. The most reliable method was a combination of filtration and boiling. Cells were grown to an OD₆₀₀ of 0.5 in A medium containing the indicated carbon source. Three ml of culture was then filtered through a Whatman GF/F glass fibre filter, and immediately washed with 3 ml of ice cold A medium. The cells were extracted by placing the filter in 2.5 ml of 0.2 M hot formic acid in a glass vial which was pre-heated for 1 min in a boiling-water bath. The entire filtration procedure was completed in less than 15 s. The cells were boiled for 8 min. Before use, the glass vials were boiled with 0.2 M formic acid for 10 min and then dried; this lowered the background in cells unable to synthesize cAMP (IT1106) from an apparent intracellular cAMP concentration of 1 µM to less than 0.1 µM. After boiling the cells, the extract was centrifuged for 5 min in an Eppendorf micro-centrifuge to remove cell debris and glass particles. Next, 1.5 ml of the supernatant was freeze-dried, dissolved in 95 µl of water, and the pH was adjusted to 7 with 1 M NaOH. After addition of 2.5 µl of 2 M potassium phosphate buffer pH 7, the samples were analysed by means of a radioactive immunoassay (Tovey *et al.*, 1974; Gilman, 1970). All samples were analysed three or four times; different amounts of sample and an internal standard were used. Average values of at least three independent experiments were calculated. The following conversion factors were used to calculate the intracellular cAMP concentrations: an OD₆₀₀ of 1 corresponds to 350 µg ml⁻¹ dry weight, and 1 mg of dry weight corresponds to an internal volume of 2.48 µl (Winkler and Wilson, 1966; Zwaig *et al.*, 1970). For determination of the extracellular cAMP concentration, the culture was centrifuged for 2 min and the supernatant was analysed as obtained, after two- to fivefold dilution in 50 mM potassium phosphate buffer.

Determination of CRP

Western blotting was performed as described (Ishizuka *et al.*, 1993). Culture samples equivalent to 1 ml with an OD₆₀₀ of 0.5 were centrifuged and the pellets were resuspended in 500 µl of loading buffer and heated at 95°C for 10 min. Next, 10 µl of this solution was loaded onto SDS–polyacrylamide gels, and, after blotting onto nitrocellulose, the blots were treated as described previously (Ishizuka *et al.*, 1993), using polyclonal anti-CRP antibody and a horseradish peroxidase-conjugated secondary antibody. The absolute amount of CRP in cell-free extracts was determined by comparing the extracts with purified CRP, which was purified by the procedure of Eilen *et al.* (1978). For detection, the Amersham ECL kit was used according to the method specified by the manufacturer. Because the signal was dependent on the presence of cell-free extract, the values were corrected using a calibration curve made with purified CRP and different amounts of cell-free extract from a Crp⁻ mutant (PP47). The films were scanned with a Sharp JX-330 transmission scanner and the bands were quantified using Pharmacia Image System 1D software.

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