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Interplay between CRP-cAMP and PII-Ntr systems forms novel regulatory network between carbon metabolism and nitrogen assimilation in *Escherichia coli*

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ABSTRACT

In *Escherichia coli*, utilization of carbon sources is regulated by the phosphoenolpyruvate-dependent phosphotransferase system (PTS), which modulates the intracellular levels of cAMP. The cAMP receptor protein (CRP) controls the transcription of many catabolic genes. The availability of nitrogen is sensed by the PII protein at the level of intracellular glutamine. Glutamine is transported mainly by GlnHPQ, and synthesized by glutamine synthetase (GS) encoded by *glnA*. Previous studies suggest that CRP affects nitrogen assimilation. Here we showed that at least two mechanisms are involved. First, CRP activates *glnH*p1 via synergistic binding with sigma 70 RNA polymerase ($E\sigma^{70}$) and represses *glnH*p2. As a consequence, in the presence of glutamine, the overall enhancement of *glnHPQ* expression alters GlnB signalling and de-activates *glnA*p2. Second, *in vitro* studies show that CRP can be recruited by sigma 54 holoenzyme ($E\sigma^{54}$) to a site centred at −51.5 upstream of *glnA*p2. CRP-induced DNA-bending prevents the nitrogen regulation protein C (NtrC) activator from approaching the activator-accessible face of the promoter-bound $E\sigma^{54}$ closed complex, and inhibits *glnA*p2. Therefore, as the major transcriptional effector of the 'glucose effect', CRP affects both the signal transduction pathway and the overall geometry of the transcriptional machinery of components of the nitrogen regulon.

In *Escherichia coli* and related bacteria, the presence of glucose in the growth medium prevents the utilization of other carbohydrates, the so-called classic 'glucose effect' (1). Preferential utilization of different carbon sources is regulated by the phosphoenolpyruvate-dependent phosphotransferase system (PTS) which modulates the intracellular levels of cAMP (2). cAMP levels are high when cells are grown on non-PTS carbon sources such as glycerol. When grown on carbohydrates such as glucose, cAMP levels are low (2,3). The activity of CRP can only be triggered by binding to cAMP (3), and therefore, in this article in order to simplify the text, the liganded CRP will be further designated as CRP, not CRP-cAMP. CRP contains three transcription activation regions and is considered as a proximal activator interacting over short distances with the major form of σ^{70}-RNA polymerase ($E\sigma^{70}$), often as the promoters of sugar catabolic genes. As a CRP point mutation defective in its activation region I (ARI), CRP^{H159L} is unable to interact with the αCTD of $E\sigma^{70}$, but is still able to bind and bend DNA in a manner similar to its wild-type counterpart (4). Location of CRP-binding sites upstream of CRP-dependent promoters, their affinity for CRP and the intrinsic promoter strength determine the extent of activation (3). Detailed three-dimensional models of CRP–$E\sigma^{70}$–promoter complexes constructed by Lawson *et al*. (5) have yielded insights into how CRP binds DNA and activates transcription.

Assimilation of ammonia and acquisition of various nitrogen sources is tightly controlled in *E. coli*. A key operon, *glnALG*, encodes a key enzyme of nitrogen assimilation, glutamine synthetase (GS), and two regulatory proteins that control expression of the Ntr regulon,

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NtrB (also called NRII) and NtrC (also called NRI) (6). NtrC is phosphorylated by NtrB to form a hexamer (7,8) of NtrC-phosphate (NtrC-P) under nitrogen-deficient growth conditions. The expression of the *glnALG* operon is controlled by tandem promoters (*glnA*p1 and *glnA*p2). *glnA*p1, located at 115 bp upstream of *glnA*p2, is a σ^{70}-dependent weak promoter. Its transcription can be activated by CRP and blocked by NtrC-P (9). *glnA*p2 is transcribed by another form of RNA polymerase ($E\sigma^{54}$) and is activated by NtrC-P (6,10,11). Taken together, *glnA*p2 is responsible for increasing the levels of *glnA* transcription under nitrogen-deficient growth conditions (11).

Glutamine transport in *E. coli* has been shown to occur through a well-characterized high-affinity-binding-protein-dependent system (GlnHPQ) and poorly characterized low-affinity system(s). The g*lnHPQ* operon is under the control of tandem promoters (*glnH*p1p2). *glnH*p1 is σ^{70}-dependent, while *glnH*p2 is σ^{54}- and NtrC-P-dependent (12,13).

The intracellular glutamine status is integrated and transduced by the PII signalling protein. *Escherichia coli* possesses two closely related PII paralogues, GlnB and GlnK. GlnB is produced constitutively, and it regulates the NtrB/NtrC two-component system (14,15). Its non-modified form can bind NtrB, which dephosphorylates the response regulator NtrC, thus switching off transcription of *glnA*p2 (16–18), and of other NtrC-dependent promoters including *glnH*p2 and the *glnK* promoter (19). Low intracellular glutamine levels, signalling nitrogen deficiency, leads to uridylylation of GlnB (20). The modified complex GlnB–UMP is not able to combine with NtrB. In this state, by phosphoryl transfer, NtrC is phosphorylated by NtrB and so activates transcription (17).

For *glnA*p2, NtrC-P binds to enhancer-like sequences centred at −108 and −140 and forms an oligomer (7) to activate transcription via direct interaction with promoter bound $E\sigma^{54}$. These NtrC-P binding sites are needed for activation at low intracellular NtrC-P concentration, while high concentrations of NtrC-P can stimulate transcription at *glnA*p2 without specific binding sites (6,21). In both cases, the NtrC-P must approach promoter bound $E\sigma^{54}$ from the opposite side of the DNA to which $E\sigma^{54}$ is bound (22).

Previously, we have shown that CRP down-regulates *glnA*p2 in *E. coli* (9). The latest studies suggest that this down-regulation may work through different mechanisms for different σ^{54}-dependent promoters. In the case of *glnA*p2, the effect of carbon source on NtrC-dependent gene expression can be mediated through CRP-dependent glutamine uptake, thereby affecting the glutamine-sensitive UTase–PII signalling system (16). However, the mechanisms behind the CRP-dependent glutamine uptake remain unknown.

In this study, we establish that CRP influences nitrogen regulation in *E. coli* by at least two mechanisms. First, with glutamine as the nitrogen source, *glnH*p1 is activated by CRP *in vivo* and *in vitro*. Through *glnHPQ*-dependent GlnB signalling, CRP acts to decrease the amount of the phosphorylated NtrC activator, which in turn causes a decrease in *glnA*p2 expression. Second, *in vitro* CRP is recruited to a sequence upstream of the core *glnA*p2 promoter through direct interaction between the ARI of CRP and the αCTD of $E\sigma^{54}$ resulting in a reduced activity of *glnA*p2. The latter direct down-regulatory effect is evident *in vivo* when a limiting amount of ammonium is used as the nitrogen source.

Table 1. Bacterial strains and plasmids used in this work

Strain/plasmid	Relevant characteristics	Source/reference
***E. coli* strains**		
TP2339-1	F$^-$, *xyl*, *cya*, *crp-39*, *lacΔX74*, *argH1*, *glp*	Tian *et al.* (9)
BD4000	F$^-$, *xyl*, *cya*, *crp-39*, *lacΔX74*, *argH1*, *glp*, *glnHPQ*	This article
Plasmids		
pKU101	*glnAp2mCRP::lacZYA* fusion in pGD926	Tian *et al.* (9)
pKU101H	entire *glnHPQ* operon inserted into pKU101	This article
pKU550	*glnHp1p2::lacZYA* fusion in pGD926	This article
pLG339CRP	pLG339 carrying *crp* under the control of the *crp* promoter	Kolb. A (23)
pLG339CRP^{H159L}	H159L mutant in pLG339CRP	Busby. S (28)
pLG339ΔRS	pLG339 with EcoRI/SalI internal deletion	Busby. S (28)

*m*CRP, mutated CRP-binding site; *::*, novel joint; pLG339ΔRS is the *crp* deleted version of plasmid pLG339CRP, as the negative control for CRP and its ARI mutant CRP^{H159L}.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. The entire *glnHPQ* operon in TP2339-1 was replaced by *bla* gene using pKO3 system (24). This results in strain BD4000.

For construction of plasmid pKU550, the entire *glnH*p1p2 region together with the first 7 codons of the *glnHPQ* open reading frame (ORF) (from −289 to +63 of *glnH*p2) was amplified as a 364-bp DNA fragment by PCR using *E. coli* strain MG1655 chromosome DNA as template and 5′-CCCAAGCTTGCAGCCCACCTCA ACGCAC-3′ (p#1) and 5′-CGGGATCCACTTTTAAT ACAGACTTCATAG-3′ (p#2) as primers. The 364-bp DNA fragment was digested with HindIII and BamHI, cloned into pUC18 and verified by DNA sequencing. The 364-bp HindIII–BamHI DNA fragment was cloned into pGD926, and the *glnA* ORF was fused in-frame with the eigth codon of the *lacZ* reporter gene. This results in plasmid pKU550.

The entire *glnHPQ* operon was amplified by PCR using p#1 and 5′-CCCAAGCTTAATAAGACACATTGC CTG-3′ (p#3) as primers and *E. coli* strain MG1655 chromosome DNA as template. The PCR product was restricted with HindIII, cloned into pUC18 and verified by DNA sequencing. The HindIII DNA fragment was cloned

into the *glnA*p2 reporter plasmid pKU101. This results in plasmid pKU101H, which complements strain BD4100 for *glnHPQ*.

A CRP consensus-binding site was introduced into position −50.5 nucleotides upstream of the *glnA*p2 by Takara Biotechnology (Dalian) and the promoter was designed as CC-50.5 according to the location of the centre of the CRP consensus-binding site (22).

Growth media and enzyme assays

M63 minimal medium was prepared as previously described (25) with glycerol (0.4% w/v) as the sole carbon source, and either glutamine (0.2% w/v) or 0.2 mM ammonium as nitrogen source, in the absence/presence of exogenous cAMP (2 mM), as indicated. Cells were grown at 30°C under aerobic conditions. The growth of the cells was measured spectrophotometrically by following the optical density of the culture (OD_{600}). The cultures that were used for measurements were initiated by diluting a cell suspension from an overnight-grown culture in the appropriate medium to an initial OD_{600} of 0.04–0.05. When the day-culture reached an OD_{600} between 0.8 and 1.0, β-galactosidase assays were performed as described before (25).

Primer extension and *in vivo* $KMnO_4$ footprinting experiments

As described before (9), two primers (5′-CCCAGTCAC GACGTTGTAAAACG-3′ (p#4) and 5′-CAACCGTT CCGCCAGTTTGCGT-3′ (p#5)) hybridize with the structural gene of *lacZ* (p#4, for probing transcripts from *glnH*p1p2) and *tetR* (p#5, for the control) on pGD926-derived plasmids, respectively, in primer extension experiments.

Protein purification, *in vitro* transcription assays and DNase I footprinting

As described before (22).

Real time RT-PCR assays

Total RNA was isolated from 5 ml *E. coli* cells (TP2339-1 with pKU101 and pLG339CRP/pLG339CRP^{H159L}) at OD_{600} 0.8 using RNeasy® Mini Kit (QIAGEN). About 1.5 μg of total DNase I-treated RNA were reverse-transcribed using SuperScriptII™ RT (Invitrogen) according to the manufacturer's instructions. A real-time PCR was performed with each pair of primers (5′-CGGATACCGCCTTCGTTC-3′ (p#6) and 5′-ACC GCTCTTCACAGCAACC-3′ (p#7) that were designed for quantifying transcripts from *glnH*p1p2; 5′-CCAGAATGGTGCATCTTCAGG-3′ (p#8) and 5′-TCAGCTCTTTAGCGATGGCAG-3′ (p#9) were designed for quantifying transcripts from *glnH*p1; 5′-ACGGCCCCAACAGTGAAGTA-3′ (p#10) and 5′-ACGGTCTGACGACACGCAA-3′ (p#11) were designed for quantifying transcripts of *tetR* from pKU101 as internal control) on a MJ OPTICON® 2 using SYBR Green PCR Master Mix (Applied Biosystems). Data analyses for a relative quantification of gene expression were performed by the comparative Ct (threshold cycle) method according to the manufacturer's instructions. The parameter Ct is defined as the cycle number at which intensity of fluorescence (which is proportional to the quantity of DNA present in the tube during the exponential phase of the PCR) passes a fixed threshold value. The relative amount of target in two preparations is $AE^{(Ct\ \text{condition A internal control}-Ct\ \text{condition A sample})-(Ct\ \text{condition B internal control}-Ct\ \text{condition B sample})}$, where conditions A and B were in the presence/absence of exogenous cAMP (2 mM), respectively, sample is *glnH*p1 or *glnH*p1p2 product, internal control is *tetR*, AE = 1.8. Experiments were performed three times.

Determination of the modification status of GlnB

The modification status of GlnB was determined by non-denaturing polyacrylamide gel electrophoresis followed by immunodetection of GlnB using GlnB-specific antibodies as described before (26). The cell pellet was resuspended in 50 μl of ice-cold buffer (50 mM Tris-HCl pH7.4; 5 mM EDTA, 1 mM DTT and 1 mM benzamidin), added with glass beads (0.11 mm) to about the same volume as the cell pellet, and broken in a FastPrep (Thermo). Cell debris and glass beads were removed by centrifugation and the protein concentration in the supernatant was estimated as described before (27). A total of 2.5 μg protein was loaded per lane for non-denaturing PAGE. The proteins were blotted onto nitrocellulose membranes (Hybond-C Extra, Amersham) and the blots were probed with specific anti-GlnB (provided by K. Forchhammer), which were subsequently visualized using peroxidase-conjugated secondary antibodies (Promega) and DAB (Pierce) diluted 1:10 in stable peroxide substrate buffer (Pierce) as substrate.

RESULTS

CRP activates the transcription of *glnH*p1 *in vitro* through its synergistic binding with $E\sigma^{70}$

Previous studies suggested that glutamine uptake is increased by the presence of CRP and increased extracellular glutamine elicits a nitrogen-excess signal (16). The *glnHPQ* operon encodes the high-affinity glutamine uptake system, and is under the control of σ^{70}-dependent *glnH*p1 and σ^{54}- and NtrC-P-dependent *glnH*p2 (12,13). Under *in vivo* conditions, *glnH*p2 is repressed by CRP (Li *et al.*, unpublished data, see also Figure 1). However, in a macroarray assay, we have found that the overall transcription of *glnHPQ* is activated by the presence of exogenous cAMP (2 mM, data not shown). Therefore whether *glnH*p1 transcription and the overall transcription of the *glnHPQ* operon are activated by CRP in ARI-dependent manner was studied.

Sequence analysis indicates that no obvious CRP-binding site can be found upstream of *glnH*p1, and DNA fragment containing *glnH*p1 cannot be retarded by CRP alone in gel shift assays (data not shown). In order to investigate the biochemical behaviour of CRP and $E\sigma^{70}$ on *glnH*p1, DNase I footprinting assays were carried out *in vitro*. The results show that in the absence of $E\sigma^{70}$, CRP cannot occupy the upstream regulatory sequences

Figure 1. Effect of CRP and its ARI mutant CRP^{H159L} on the *glnH*p1p2 expression in the *E. coli cya crp* double mutant TP2339-1 detected by primer extension analysis. Lane 5 is G marker ladder. Cells were grown in M63 minimal medium with glycerol (0.4% w/v) as the sole carbon source, and glutamine (0.2% w/v) as nitrogen source, in the absence/presence of exogenous cAMP (2 mM).

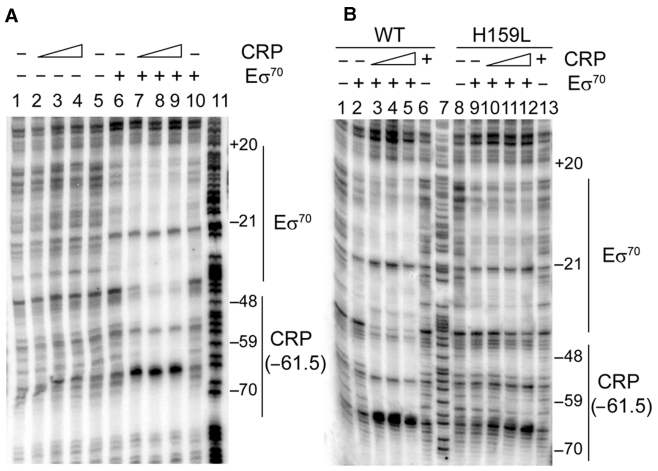

Figure 2. DNase I footprints of $E\sigma^{70}$ in the presence or absence of CRP and CRP^{H159L} on *glnH*p1 (non template strand). (**A**) Titration with increasing concentrations of CRP (lanes 2 and 7, 30 nM; lanes 3 and 8, 100 nM; lanes 4 and 9, 300 nM) were performed in the absence (2–4) or presence of 50 nM $E\sigma^{70}$ (lanes 7–9). Lane 11 is A + G marker ladder. The protected regions were monitored by adding increasing concentrations of CRP in the presence or absence of $E\sigma^{70}$. (**B**) Titration with increasing concentrations of CRP (lane 3, 33 nM; lane 4, 100 nM; lane 5, 300 nM) and CRP^{H159L} (lane 10, 33 nM; lane 11, 100 nM; lane 12, 300 nM) were performed in the presence of 25 nM $E\sigma^{70}$ (lanes 3–5 and 10–12). Lane 7 is A + G marker ladder. The limits of protected regions are indicated. Note that wild-type CRP is recruited to a site centred at −61.5 by $E\sigma^{70}$-RNA polymerase, which suggests a higher affinity for DNA binding of $E\sigma^{70}$ than that of CRP.

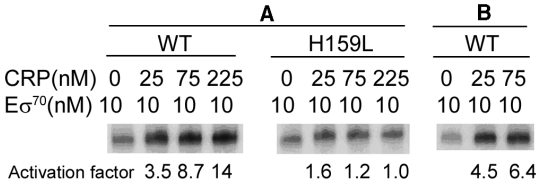

Figure 3. Effect of CRP and its ARI mutant CRP^{H159L} on the *glnH*p1 expression *in vitro*. (**A**) multiple-round run-off assay; (**B**) single-round run-off assay.

of *glnH*p1 even at high concentrations (lanes 1–5 of Figure 2A). Also, in the absence of CRP, only weak occupancy of $E\sigma^{70}$ at the core promoter of *glnH*p1 is evident (lanes 6 and 10 of Figure 2A; lanes 2 and 9 of Figure 2B). In contrast, when wild-type CRP is present together with $E\sigma^{70}$, the binding of $E\sigma^{70}$ is strongly increased (Figure 2A and B). Also, wild-type CRP is recruited by $E\sigma^{70}$ to a site centred at −61.5, organized as Class I σ^{70}-promoters (28). Moreover, the above synergistic binding is abolished when wild-type CRP is replaced by its ARI mutant CRP^{H159L} (Figure 2B).

In order to investigate whether CRP can activate the transcription of *glnH*p1 in an ARI-dependent manner *in vitro*, run-off and multiple-round transcriptional assays were carried out with different concentrations of CRP or its ARI mutant CRP^{H159L}. Results showed that, regardless of the concentration of $E\sigma^{70}$, wild-type CRP, but not its ARI mutant CRP^{H159L}, can activate *glnH*p1 by a factor up to 6- and 14-fold in single- and multiple-round transcriptional assays, respectively. The activation factor increased when the concentration of CRP was increased (Figure 3).

Taken together, we have identified a naturally occurring case, where $E\sigma^{70}$ RNA polymerase binds very weakly to *glnH*p1. Binding of $E\sigma^{70}$ to *glnH*p1 recruits CRP to a degenerate site at −61.5 ('ttgTATCCacatcaTCACA caa', which has a score of 1.44 bits, according to methods described before ((29), in which 17 bits was used as the cutoff score for strong CRP-binding sites; and 10 bits as the cutoff score for weak CRP-binding sites)), and the synergistic binding between CRP and $E\sigma^{70}$ clearly helps both partners in a reciprocal manner, which results in transcriptional activation of *glnH*p1.

The overall transcription of the *glnHPQ* operon is activated by CRP *in vivo*

To investigate the effect of CRP on the expression of *glnHPQ in vivo*, pKU550 carrying *glnH*p1p2 fused with the *lacZ* reporter gene was transformed into *cya crp* double mutant TP2339-1 containing either pLG339CRP or $pLG339CRP^{H159L}$. The cells were grown in

Table 2. Effect of CRP and its ARI mutant CRP^{H159L} on the expression of *glnH*p1p2 and *glnA*p2 in the *E. coli cya crp* double mutant TP2339-1 and *cya crp glnHPQ* triple mutant BD4000

	Strain	CRP plasmid	Promoter (plasmid)	β-gal	
				−cAMP	+cAMP
a	TP2339-1	pLG339ΔRS	*glnH*p1p2(pKU550)	6533 ± 350	6245 ± 347
	TP2339-1	pLG339CRP	*glnH*p1p2(pKU550)	6378 ± 287	20190 ± 1851
	TP2339-1	pLG339CRP^{H159L}	*glnH*p1p2(pKU550)	6829 ± 393	6416 ± 334
b	TP2339-1	pLG339ΔRS	*glnA*p2(pKU101)	6650 ± 276	6188 ± 265
	TP2339-1	pLG339CRP	*glnA*p2(pKU101)	6588 ± 355	207 ± 43
	TP2339-1	pLG339CRP^{H159L}	*glnA*p2(pKU101)	6423 ± 287	5582 ± 255
	BD4000	pLG339ΔRS	*glnA*p2(pKU101)	6478 ± 312	6033 ± 283
	BD4000	pLG339CRP	*glnA*p2(pKU101)	6431 ± 305	3509 ± 167
	BD4000	pLG339CRP^{H159L}	*glnA*p2(pKU101)	6249 ± 203	5163 ± 179
	BD4000	pLG339ΔRS	*glnA*p2(pKU101H)	912 ± 124	712 ± 76
	BD4000	pLG339CRP	*glnA*p2(pKU101H)	896 ± 130	20 ± 17
	BD4000	pLG339CRP^{H159L}	*glnA*p2(pKU101H)	869 ± 117	578 ± 50
c	TP2339-1	pLG339ΔRS	*glnA*p2(pKU101)	7106 ± 312	6779 ± 259
	TP2339-1	pLG339CRP	*glnA*p2(pKU101)	6907 ± 296	3542 ± 229
	TP2339-1	pLG339CRP^{H159L}	*glnA*p2(pKU101)	6891 ± 355	6682 ± 203
	BD4000	pLG339ΔRS	*glnA*p2(pKU101)	6485 ± 278	6135 ± 264
	BD4000	pLG339CRP	*glnA*p2(pKU101)	6431 ± 257	3509 ± 305
	BD4000	pLG339CRP^{H159L}	*glnA*p2(pKU101)	6249 ± 219	5163 ± 249
	Strain	CRP plasmid	Promoter	Relative expression	
				− cAMP	+ cAMP
d	TP2339-1	pLG339ΔRS	*glnH*p1	1	1.8 ± 0.7
	TP2339-1	pLG339CRP	*glnH*p1	1	56.6 ± 2.1
	TP2339-1	pLG339CRP^{H159L}	*glnH*p1	1	3.0 ± 1.5
	TP2339-1	pLG339ΔRS	*glnH*p1p2	1	1.0 ± 0.3
	TP2339-1	pLG339CRP	*glnH*p1p2	1	3.1 ± 0.5
	TP2339-1	pLG339CRP^{H159L}	*glnH*p1p2	1	0.9 ± 0.2

All strains were grown in M63 minimal medium with glycerol (0.4% w/v) as the sole carbon source, and 0.2% w/v glutamine as nitrogen source (except c, 0.2 mM ammonium as nitrogen source), in the absence/presence of exogenous cAMP (2 mM). pLG339ΔRS is the negative control for CRP and its ARI mutant CRP^{H159L} (see Table 1). a–c: Promoter expression is measured by β-galactosidase assays. d: level of the *glnH*p1 and *glnH*p1p2 expression from chromosome measured by real time RT-PCR in TP2339-1.

M63 medium in the absence or presence of exogenous cAMP. The results showed that in the presence of exogenous cAMP, *glnH*p1p2 can be activated 3-fold by wild-type CRP. This activation was abolished when ARI mutant CRP^{H159L} replaced wild-type CRP (Table 2a).

In order to verify the CRP effect on *glnH*p1 and *glnH*p2 respectively, two parallel experiments were carried out. First, *in vivo* primer extension assays were carried out on both promoters from plasmid pKU550 (Figure 1). Second, a more quantifiable real time RT-PCR was carried out to verify the CRP effect on both *glnH*p1 and *glnH*p1p2 transcribed directly from chromosome (Table 2d). In both cases, the results showed that wild-type CRP activates *glnH*p1, but represses *glnH*p2. In contrast, CRP^{H159L} does not affect the expression of *glnH*p1 or *glnH*p2. Quantitatively, the results obtained from real time RT-PCR correlate well with the results obtained from β-galactosidase assays (compare Table 2d with 2a). Therefore, it seems that the CRP effect on *glnH*p1p2 is somewhat similar to that obtained with *glnA*p1p2, where CRP also activates σ^{70}-dependent *glnA*p1, and represses σ^{54}-dependent *glnA*p2 (9). However, they are different in at least two ways. First, the overall output is different. In this case, CRP causes a 3–4-fold decrease of overall expression for *glnA* (9), but a 3-fold increase for *glnH* (Table 2a and d). Second, it is known that a classical CRP-binding site is located upstream of the *glnA*p1 promoter (10,30), essential for CRP-dependent activation of *glnA*p1 (9). In contrast, no obvious CRP-binding site can be found upstream of *glnH*p1. CRP activates the transcription of *glnH*p1 *in vitro* through its synergistic binding with $E\sigma^{70}$ (Figure 2).

The CRP effect is maximized through *glnHPQ*-mediated glutamine uptake

The above results, together with the results obtained from Maheswaran and Forchhammer (16), suggest that CRP increases expression of the *glnHPQ* operon, which in turn, increases glutamine uptake. It has been proposed that, as a consequence, the intracellular glutamine level is increased, and so elicits a nitrogen-excess signal. In this case, the GlnB signalling process leads to the dephosphorylation of NtrC, and subsequent de-activation of NtrC-P-dependent promoters such as *glnA*p2 and *glnH*p2 (16,31).

To further investigate the above proposal, the *glnHPQ* operon was deleted from *cya* and *crp* double mutant TP2339-1 to give a triple mutant strain designated BD4000. The expression of *glnA*p2 (pKU101) was

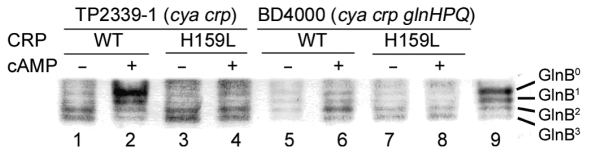

Figure 4. Effect of CRP and its ARI mutant CRP^{H159L} on the uridylylation status of GlnB in the *E. coli cya crp* double mutant TP2339-1 and *cya crp glnHPQ* triple mutant BD4000. All strains (lanes 1–8) were grown in M63 minimal medium with glycerol (0.4% w/v) as the sole carbon source, and glutamine (0.2% w/v) as nitrogen source, in the absence/presence of exogenous cAMP (2 mM). TP2339-1 was grown in nitrogen-sufficient medium with 40 mM ammonium as control (lane 9).

measured in the carbon poor medium in the presence of glutamine, in the presence/absence of cAMP. The results showed that the repression effect of wild-type CRP on the expression of *glnA*p2 is reduced from 30-fold to 2-fold upon deletion of *glnHPQ*. As control, the ARI mutant CRP^{H159L} has no such repression effect (Table 2b). The *glnHPQ* mutant could still grow in the carbon-poor medium in the presence of glutamine, where glutamine uptake might be carried out by the low-affinity systems (32) (also see Figure 4).

To complement BD4000 for the *glnHPQ* operon, and minimize the artificial effects from genetic manipulation, a new *glnA*p2 reporter low copy number plasmid carrying the entire *glnHPQ* operon was constructed (designed as pKU101H), and used for the following assays. In this case, the CRP/cAMP effect on the expression of *glnA*p2 was investigated. Results indicated that when BD4000 was complemented by pKU101H, the CRP-mediated repression effect could be restored from 2-fold to 50-fold by wild-type CRP, while its ARI mutant CRP^{H159L} had no such effect (Table 2b). We also noticed that such restoration could only be observed when cells grown in M63-minimum medium supplemented with glutamine, but not with ammonium, as the sole nitrogen source, indicating that the restoration effect requires physical transport of glutamine into the cells, rather than the sole enhancement of *glnHPQ* expression (Table 2c).

Maheswaran and Forchhammer (16) showed that deuridylylation of GlnB is a direct consequence of glutamine addition to cAMP-treated cells, which agrees with their proposal that cAMP increases the intracellular glutamine level when glutamine is used as the sole nitrogen source. In this study, similar assays were done with both TP2339-1 and BD4000 strains (Figure 4). The cells were grown in the presence of glutamine as the sole nitrogen source except the control (lane 9). As shown in the figure, in the absence of wild-type CRP (lanes 1, 3 and 4) or in the absence of GlnHPQ (lanes 5–8), GlnB is in its highly uridylylated state. By contrast, more than 80% of GlnB is in its deuridylylated state in the presence of both wild-type CRP and GlnHPQ (lane 2). As control, we showed that GlnB is mostly deuridylylated in the presence of 40 mM ammonium (lane 9). Taken together, the above results show that the GlnHPQ-mediated GlnB signalling is regulated by CRP. The intracellular concentration of glutamine is increased to a nitrogen-excess level by the GlnHPQ transport system in the presence of CRP and GlnB is deuridylylated in this case. As a consequence, NtrC is dephosphorylated and the expression of *glnA*p2 is decreased in the presence of CRP.

It is interesting to note that when the *glnHPQ* operon is deleted, the carbon source/cAMP effect remains, although it is reduced dramatically (Table 2b). This result indicates that there is another repression mechanism, which might not be related to the glutamine uptake. To further investigate this possibility, ammonium was used to replace the glutamine as the sole nitrogen source. Results showed that wild-type CRP, but not its ARI mutant CRP^{H159L}, still represses *glnA*p2 by a factor of 2-fold in both TP2339-1 and BD4000 (Table 2c). This result supports the notion that alternative mechanism(s) may exist for CRP-mediated repression on *glnA*p2.

CRP repressed the expression of *glnA*p2 directly *in vitro*

To investigate whether CRP can repress *glnA*p2 directly, multiple-round transcriptional assays were carried out with different concentrations of CRP or its ARI mutant CRP^{H159L}. Results showed that wild-type CRP can repress the transcriptional activity of *glnA*p2 by a factor of 2-fold. Moreover, when wild-type CRP was replaced by its ARI mutant CRP^{H159L}, the repression effect was abolished (Figure 5). Also, this repression effect can be increased to 10-fold, when $NtrC^{S160F}$ (a constitutive active form of NtrC with a lower activity compared to NtrC-P) was used to replace wild-type NtrC-P (Figure 5), indicating that repression would be greater when a partially active form of NtrC exists in the cell (33).

To investigate the mechanism of the ARI-dependent repression effect on *glnA*p2, DNase I footprinting assays were carried out *in vitro*. In the absence of $E\sigma^{54}$, CRP or its ARI mutant CRP^{H159L} cannot occupy the upstream sequence of *glnA*p2 (lanes 9 and 10 of Figure 6). On the other hand, in the absence of CRP, full occupancy of $E\sigma^{54}$ on the core promoter of *glnA*p2 was observed (lane 13 of Figure 6). When CRP is present together with $E\sigma^{54}$, two additional protection regions were observed upstream of the binding site of $E\sigma^{54}$. In contrast, when ARI mutant CRP^{H159L} was used in the assay, these additional protections were not evident (compare lanes 11 and 12 of Figure 6).

The two additional protection regions are from −42 to −61 and from −63 to −71. According to the location and size of the protected regions, it is possible that CRP binds and bends DNA from −42 to −61 and αCTD of $E\sigma^{54}$ binds DNA upstream. The binding and bending centre for CRP is at −51.5. Previous research by Huo *et al.* (22) had shown that when CRP was located at −50.5 on *glnA*p2, CRP and $E\sigma^{54}$ lie on the same face of DNA helix. Presumably the DNA bending induced by CRP, places NtrC-P far from the activator-accessible face of the closed complex and in turn represses the expression of *glnA*p2. The contact between αCTD and the upstream DNA region supports the view that DNA bending exists. Taken together, CRP was recruited by $E\sigma^{54}$ in an

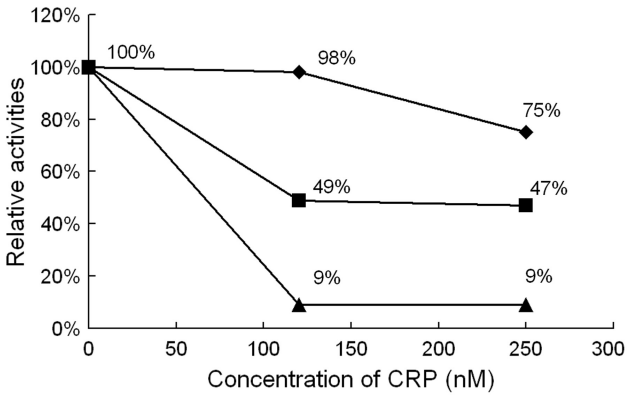

Figure 5. Effect of CRP and its ARI mutant CRP^{H159L} on the *glnA*p2 expression *in vitro*. Wild-type CRP and $NtrC^{S160F}$ (▲) CRP and NtrC-P (■), and CRP^{H159L} and NtrC-P (◆), were used in the multiple-round transcriptional assays. The activity of *glnA*p2 with $E\sigma^{54}$+NtrC-P/$NtrC^{S160F}$ in the absence of CRP or CRP^{H159L} was taken as 100%.

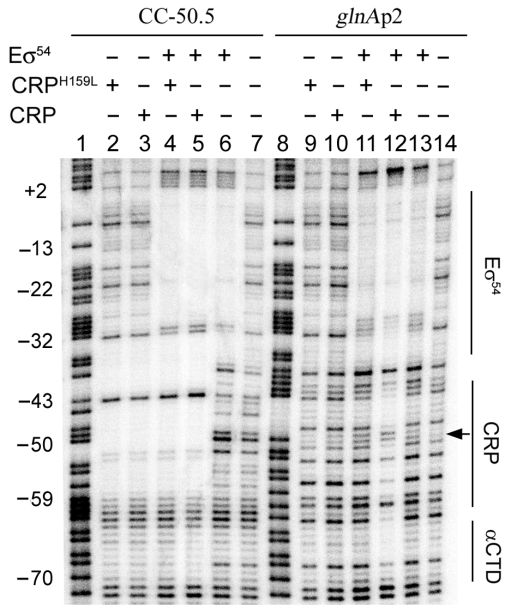

Figure 6. DNase I footprints of $E\sigma^{54}$ in the presence or absence of CRP and CRP^{H159L} on CC-50.5 (A CRP consensus-binding site was introduced into position −50.5 nucleotides upstream of the *glnA*p2) or wild-type *glnA*p2. CRP, CRP^{H159L} and $E\sigma^{54}$, when added, were present at a final concentration of 50 nM. Lanes 1 and 8 are A+G marker ladders. The limits of protected regions are indicated. On *glnA*p2, only wild-type CRP can bind co-operatively with $E\sigma^{54}$ and the protection region is comparable to the one on CC-50.5. The locations of specific protected or hypersensitive bands are different on the two templates due to the difference of the sequence of CRP-binding sites. The hypersensitive band due to the CRP-binding on *glnA*p2 is marked by arrow. The protection of αCTD binding, which is co-operative only with wild-type CRP, is similar on both *glnA*p2 and CC-50.5 (lanes 5 and 12). These results strongly support that the relative orientation between CRP and $E\sigma^{54}$ is similar on *glnA*p2 and CC-50.5.

ARI-dependent manner and repressed NtrC-P mediated activation of *glnA*p2 directly.

DISCUSSION

In this study, we have shown that there are two novel mechanisms for CRP to mediate nitrogen regulation. In one mechanism CRP can affect the PII-mediated signal transduction pathway, hence causing deactivation of σ^{54}-dependent *gln* promoters (for instance, *glnA*p2). Specifically, *in vitro* experiments show that *glnH*p1 promoter is a Class I-like CRP-dependent promoter. Through ARI–αCTD interaction, CRP and $E\sigma^{70}$ bind synergistically to *glnH*p1 to form the closed complex (Figure 2). The synergy between CRP and $E\sigma^{70}$ activates *glnH*p1 (Table 2d). Despite *glnH*p2 repression (Figure 1), the overall enhanced *glnHPQ* expression (~3-fold, see Table 2a and d) leads to increased glutamine uptake, leading to PII-UMP deuridylylation (Figure 4) and shuts down the Ntr response in consequence. The other mechanism is that CRP can change the topological arrangement of $E\sigma^{54}$, NtrC-phosphate and *glnA*p2 promoter, thus directly repressing the *glnA*p2 promoter (Figure 5). Specifically, CRP can be recruited to the *glnA*p2 promoter at position −51.5 through ARI–αCTD interactions. This recruitment leads to DNA bending by CRP at −51.5, and hence inhibits the *glnA*p2 promoter activity (Figure 6). Taken together, our study reveals the mechanisms of CRP-mediated nitrogen regulation and presents two novel regulatory linkages between carbon metabolism and nitrogen assimilation in *E. coli*. The signal transduction pathway shown in our study illuminates the regulatory link between PII and carbon regulation (31). Although glutamine can be used as a sole carbon source supporting slow growth (34), it is surprising to find that the transport of glutamine is controlled by both global-carbon and global-nitrogen signal transduction pathways.

The dynamic range of expression of *glnHPQ* from the 'off' state to the 'on' state is greater when it has one σ^{54}-dependent promoter and one σ^{70} promoter. The σ^{54}-dependent promoter may be able to give higher levels of transcription than the σ^{70}-dependent one could, but still is repressible by carbon status through action of CRP. In the presence of glutamine, the product of these two tandem promoters provides a novel feed-back to the *glnA*p2 expression, through PII-NtrBC in the genetic cascade controlling nitrogen assimilation, other than GlnK-AmtB (35). The complicated regulatory mechanisms described above are involved in a related physiological pathway. To manage the nitrogen deficiency, *E. coli* uses NtrC/Nac system to scavenge for nitrogen-containing compounds as a first line of defense against nitrogen starvation (36). To manage the carbon deficiency, *E. coli* uses CRP to activate various carbon source transporter systems (37–39) (You *et al.* unpublished data). Results obtained from this study indicate that CRP also accelerates transport of glutamine, even in the absence of glutamine in the medium, and represses the synthesis of glutamine to efficiently balance the carbon metabolism and nitrogen assimilation. When the relevant key signals in the promoter sequences are searched

for *in silico*, the CRP inhibitory effect on glutamine biosynthesis appears to be also encountered in other enterobacteria (such as *Klebsiella pneumoniae*, *Shigella flexneri*, *Enterobacter gergoviae*, *Erwinia carotovora* and *Salmonella enterica* serovar Typhimurium), although the details of the regulation might differ. Interestingly in *S. typhimurium*, a *bona fide* CRP-binding site ('caaTGTGAaagttgGCACAgat', which has a score of 10.99 bits, according to methods described before (29)) is found centred at −27.5 upstream of *glnA*p2. This suggests that competition may occur between CRP and $E\sigma^{54}$ for binding to the promoter region. As there is no Nac protein found yet in *S. typhimurium* (40), it would be interesting to know if a tighter regulatory linkage exists.

As a global regulator, CRP is estimated to interact at ≈200 regulatory regions in *E. coli*. Zheng *et al*. (41) had revealed 152 hitherto unknown CRP regulons involved in energy production, amino acid metabolism, nucleotide metabolism and ion transport systems by run-off transcription/microarray analysis (ROMA). Moreover, Grainger *et al*. (42) had shown that CRP also interacts with thousands of weaker sites across the whole *E. coli* chromosome other than CRP-binding sites at known CRP regulated promoters by chromatin immunoprecipitation and high-density microarrays (ChIP-chip). We demonstrate that, physiologically, CRP tends to use weak affinity DNA-binding sites for transcriptional activation or repression by a regulated recruitment mechanism (43). It would be interesting to see if cross-talk between carbon metabolism and the other major areas of metabolism are mediated through mechanisms similar to those identified in our studies.

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REFERENCES

1. Monod, J. (1942) Recherches sur la croissance des cellules bactériennes. Ph.D. thesis, Actualités scientifiques etindustrielles, Hermann Paris.
2. Saier,M.H., Ramseier,T.M. and Reizer,J. (1996). In Neidhardt,F.C., Curtiss,R.,III, Ingraham,J.L., Lin,E.C.C., Low,K.B., Magasanik,B., Reznikoff,W.S., Riley,M., Schaechter,M. and Umbarger,H.E. (eds), *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn. Am. Soc. Microbiol., Washington, DC, Vol.1, pp. 1325–1343.
3. Kolb,A., Busby,S., Buc,H., Garges,S. and Adhya,S. (1993) Transcriptional regulation by cAMP and its receptor protein. *Annu. Rev. Biochem.*, **62**, 749–795.
4. Zhou,Y., Zhang,X. and Ebright,R.H. (1993) Identification of the activating region of catabolite gene activator protein (CAP): isolation and characterization of mutants of CAP specifically defective in transcription activation. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 6081–6085.
5. Lawson,C.L., Swigon,D., Murakami,K.S., Darst,S.A., Berman,H.M. and Ebright,R.H. (2004) Catabolite activator protein: DNA binding and transcription activation. *Curr. Opin. Struct. Biol.*, **14**, 10–20.
6. Reitzer,L.J. and Magasanik,B. (1986) Transcription of *glnA* in *E. coli* is stimulated by activator bound to sites far from the promoter. *Cell*, **45**, 785–792.
7. Wyman,C., Rombel,I., North,A.K., Bustamante,C. and Kustu,S. (1997) Unusual oligomerization required for activity of NtrC, a bacterial enhancer-binding protein. *Science*, **275**, 1658–1661.
8. De Carlo,S., Chen,B., Kondrashkina,E., Hoover,T.R., Nogales,E. and Nixon,B.T. (2006) The structural basis for regulated assembly of the transcriptional activator NtrC. *Genes Dev.*, **20**, 1485–1495.
9. Tian,Z.X., Li,Q.S., Buck,M., Kolb,A. and Wang,Y.P. (2001) The CRP-cAMP complex and downregulation of the *glnA*p2 promoter provides a novel regulatory linkage between carbon metabolism and nitrogen assimilation in *Escherichia coli*. *Mol. Microbiol.*, **41**, 911–924.
10. Reitzer,L.J. and Magasanik,B. (1985) Expression of *glnA* in *Escherichia coli* is regulated at tandem promoters. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 1979–1983.
11. Magasanik,B. (1996). In Neidhardt F.C., Curtiss R., III, Ingraham J.L., Lin E.C.C., Low K.B., Magasanik B., Reznikoff W.S., Riley M., Schaechter M., Umbarger H.E. (eds), *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn. Am. Soc. Microbiol., Washington, DC, Vol. 1, pp. 1344–1356.
12. Willis,R.C., Iwata,K.K. and Furlong,C.E. (1975) Regulation of glutamine transport in *Escherichia coli*. *J. Bacteriol.*, **122**, 1032–1037.
13. Claverie-Martin,F. and Magasanik,B. (1991) Role of the integration host factor in the regulation of the *glnH*p2 promoter of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 1631–1635.
14. Ninfa,A.J. and Atkinson,M.R. (2000) PII signal transduction proteins. *Trends Microbiol.*, **8**, 172–179.
15. Ninfa,A.J. and Jiang,P. (2005) PII signal transduction proteins: sensors of alpha-ketoglutarate that regulate nitrogen metabolism. *Curr. Opin. Microbiol.*, **8**, 168–173.
16. Maheswaran,M. and Forchhammer,K. (2003) Carbon-source-dependent nitrogen regulation in *Escherichia coli* is mediated through glutamine-dependent GlnB signalling. *Microbiology*, **149**, 2163–2172.
17. Jiang,P. and Ninfa,A.J. (1999) Regulation of autophosphorylation of *Escherichia coli* nitrogen regulator II by the PII signal transduction protein. *J. Bacteriol.*, **181**, 1906–1911.
18. Pioszak,A.A., Jiang,P. and Ninfa,A.J. (2000) The *Escherichia coli* PII signal transduction protein regulates the activities of the two-component system transmitter protein NRII by direct interaction with the kinase domain of the transmitter module. *Biochemistry*, **39**, 13450–13461.
19. Atkinson,M.R., Blauwkamp,T.A., Bondarenko,V., Studitsky,V. and Ninfa,A.J. (2002) Activation of the *glnA*, *glnK*, and *nac* promoters as *Escherichia coli* undergoes the transition from nitrogen excess growth to nitrogen starvation. *J. Bacteriol.*, **184**, 5358–5363.
20. Jiang,P., Peliska,J.A. and Ninfa,A.J. (1998) Enzymological characterization of the signal-transducing uridylyltransferase/uridylyl-removing enzyme (EC 2.7.7.59) of *Escherichia coli* and its interaction with the PII protein. *Biochemistry*, **37**, 12782–12794.

21. Schneider,B.L., Shiau,S.-P. and Reitzer,L.J. (1991) Role of multiple environmental stimuli in control of transcription from nitrogen-regulated promoter in *Escherichia coli* with weak or no activator-binding sites. *J. Bacteriol.*, **173**, 6355–6363.
22. Huo,Y.X., Tian,Z.X., Rappas,M., Wen,J., Chen,Y.C., You,C.H., Zhang,X., Buck,M., Wang,Y.P. *et al.* (2006) Protein-induced DNA bending clarifies the architectural organization of the sigma54-dependent *glnA*p2 promoter. *Mol. Microbiol.*, **59**, 168–180.
23. Bell,A., Gaston,K., Williams,R., Chapman,K., Kolb,A., Buc,H., Minchin,S., Williams,J. and Busby,S. (1990) Mutations that alter the ability of the *Escherichia coli* cyclic AMP receptor protein to activate transcription. *Nucleic Acids Res.*, **18**, 7243–7250.
24. Link,A.J., Philips,D. and Church,G.M. (1997) Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J. Bacteriol.*, **179**, 6228–6237.
25. Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, New York.
26. Forchhammer,K., Hedler,A., Strobel,H. and Weiss,V. (1999) Heterotrimerization of PII-like signalling proteins: implications for PII-mediated signal transduction systems. *Mol. Microbiol.*, **33**, 338–349.
27. Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.
28. Busby,S. and Ebright,R.H. (1999) Transcription activation by catabolite activator protein (CAP). *J. Mol. Biol.*, **293**, 199–213.
29. Tan,K., Moreno-Hagelsieb,G., Collado-Vides,J. and Stormo,G.D. (2001) A comparative genomics approach to prediction of new members of regulons. *Genome Res.*, **11**, 566–584.
30. MacNeil,T., MacNeil,D. and Tyler,B. (1982) Fine-structure deletion map and complementation analysis of the *glnA-glnL-glnG* region in *Escherichia coli*. *J. Bacteriol.*, **150**, 1302–1313.
31. Commichau,F.M., Forchhammer,K. and Stulke,J. (2006) Regulatory links between carbon and nitrogen metabolism. *Curr. Opin. Microbiol.*, **9**, 167–172.
32. Reizer, L.J. (1996) In: Neidhardt, F. C., Curtiss, R., III, Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M. and Umbarger, H. E. (eds.), *Escherichia coli and Salmonella: Cellular and Molecular Biology*. 2nd edn. Am. Soc. Microbiol., Washington, DC, Vol. 1, pp. 380–390.
33. Rombel,I., North,A., Hwang,I., Wyman,C. and Kustu,S. (1998) The bacterial enhancer-binding NtrC as a molecular machine. *Cold Spring Harb. Symp. Quant. Biol.*, **63**, 157–166.
34. Mcfall E. and Newman E.B. (1996) In: Neidhardt, F. C., Curtiss, R., III, Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M. and Umbarger, H. E. (eds.), *Escherichia coli and Salmonella: Cellular and Molecular Biology*. 2nd edn. Am. Soc. Microbiol., Washington, DC, Vol. 1, pp. 358-379.
35. Javelle,A. and Merrick,M. (2005) Complex formation between AmtB and GlnK: an ancestral role in prokaryotic nitrogen control. *Biochem. Soc. Trans.*, **33**, 170–172.
36. Zimmer,D.P., Soupene,E., Lee,H.L., Wendisch,V.F., Khodursky,A.B., Peter,B.J., Bender,R.A. and Kustu,S. (2000) Nitrogen regulatory protein C-controlled genes of *Escherichia coli*: scavenging as a defense against nitrogen limitation. *Proc. Natl. Acad. Sci. U.S.A.*, **97**, 14674–14679.
37. Ferenci,T. (1996) Adaptation to life at micromolar nutrient levels: the regulation of *Escherichia coli* glucose transport by endoinduction and cAMP. *FEMS Microbiol. Rev.*, **18**, 301–317.
38. Hua,Q., Yang,C., Oshima,T., Mori,H. and Shimizu,K. (2004) Analysis of gene expression in *Escherichia coli* in response to changes of growth-limiting nutrient in chemostat cultures. *Appl. Environ. Microbiol.*, **70**, 2354–2366.
39. Lendenmann,U. and Egli,T. (1995) Is *Escherichia coli* growing in glucose-limited chemostat culture able to utilize other sugars without lag. *Microbiology*, **141**, 71–78.
40. Muse,W.B. and Bender,R.A. (1998) The nac (nitrogen assimilation control) gene from *Escherichia coli*. *J. Bacteriol.*, **180**, 1166–1173.
41. Zheng,D.L., Constantinidou,C., Hobman,J.L. and Minchin,S.D. (2004) Identification of the CRP regulon using *in vitro* and *in vivo* transcriptional profiling. *Nucleic Acids Res.*, **32**, 5874–5893.
42. Grainger,D.C., Hurd,D., Harrison,M., Holdstock,J. and Busby,S.J. (2005) Studies of the distribution of *Escherichia coli* cAMP-receptor protein and RNA polymerase along the *E. coli* chromosome. *Proc. Natl. Acad. Sci. U.S.A.*, **102**, 17693–17698.
43. Ptashne,M. and Gann,A. (1997) Transcriptional activation by recruitment. *Nature*, **386**, 569–577.