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Nac-mediated repression of the *serA* promoter of *Escherichia coli*

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Summary

Escherichia coli **and related bacteria contain two paralogous PII-like proteins involved in nitrogen regulation, the *glnB* product, PII, and the *glnK* product, GlnK. Previous studies have shown that cells lacking both PII and GlnK have a severe growth defect on minimal media, resulting from elevated expression of the Ntr regulon. Here, we show that this growth defect is caused by activity of the *nac* product, Nac, a LysR-type transcription factor that is part of the Ntr regulon. Cells with elevated Ntr expression that also contain a null mutation in *nac* displayed growth rates on minimal medium similar to the wild type. When expressed from high-copy plasmids, Nac imparts a growth defect to wild-type cells in an expression level-dependent manner. Neither expression of Nac nor lack thereof significantly affected Ntr gene expression, suggesting that the activity of Nac at one or more promoters outside the Ntr regulon was responsible for its effects. The growth defect of cells lacking both PII and GlnK was also eliminated upon supplementation of minimal medium with serine or glycine for solid medium or with serine or glycine and glutamine for liquid medium. These observations suggest that high Nac expression results in a reduction in serine biosynthesis. β-Galactosidase activity expressed from a Mu *d1* insertion in *serA* was reduced approximately 10-fold in cells with high Nac expression. We hypothesize that one role of Nac is to limit serine biosynthesis as part of a cellular mechanism to reduce metabolism in a co-ordinated manner when cells become starved for nitrogen.**

Introduction

Most studies of nitrogen assimilation and its regulation have focused upon regulatory events that represent conditions of nitrogen sufficiency or conditions of moderate nitrogen limitation. Under such conditions, the main regulatory events are the regulation of glutamine synthetase levels and activity and, when the preferred nitrogen source ammonia is absent, the activation of genes whose products facilitate the scavenging of nitrogen atoms from various compounds containing reduced nitrogen (reviewed by Ninfa *et al*., 2000; see also Zimmer *et al*., 2000). This regulation of gene expression requires the NRI–NRII (NtrC–NtrB) two-component signal transduction system, encoded by two genes located downstream from the structural gene for glutamine synthetase, *glnA*. The phosphorylation state of the 'receiver' protein of the two-component system (NRI or NtrC) determines its ability to activate transcription and is controlled by interaction with the transmitter protein (NRII or NtrB). The default kinase activity of NRII is switched to phosphatase activity upon interaction with the PII signal transduction protein, resulting in the dephosphorylation of NRI and the cessation of gene activation. The availability of PII for this negative regulation is controlled, in turn, by signals of nitrogen status, such that PII is rendered ineffective for regulation of NRII in nitrogen-starved cells (reviewed by Ninfa and Atkinson, 2000). Ntr genes (genes activated by NRI~P) show different sensitivity to activation by NRI~P, owing to the strength of activator binding sites that constitute their transcriptional enhancer elements (Feng *et al*., 1995; Atkinson *et al*., personal communication). Thus, as the concentration of activator increases upon nitrogen limitation, Ntr genes should become activated in a fixed sequence.

Escherichia coli contains another PII-like protein, GlnK, that is 67% identical to PII (Allikmets *et al*., 1993). The expression of *glnK* is nitrogen regulated, such that high levels of *glnK* expression are only observed in nitrogen-limited cells (van Heeswijk *et al*., 1996; Atkinson and Ninfa, 1998; Atkinson *et al*., personal communication). The purified GlnK protein has been shown to interact with NRII *in vitro* in a manner similar to PII (Atkinson and Ninfa, 1999). However, GlnK has little effect upon the regulation of transcription of the *glnA* gene encoding glutamine synthetase in intact cells under the conditions examined (Atkinson and Ninfa, 1998). Nevertheless, GlnK has a role in nitrogen regulation. Although cells lacking either PII or GlnK do not show any obvious growth defect, cells lacking both PII and GlnK (strains BK) display a very dramatic growth defect on minimal medium, which is ameliorated by supplementation of the medium with a complex mixture of amino acids (casein hydrolysate). This growth defect

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is also prevented by mutations eliminating NRII or by genetic manipulations that prevent NRI~P from increasing its own concentration by activation of the nitrogen-regulated *glnA* promoter (Atkinson and Ninfa, 1998). Thus, it appears that the unregulated activity of NRII, leading to very high levels of NRI~P and very high levels of expression of Ntr genes, is responsible for the growth defect of cells lacking both PII and GlnK. That is, in cells lacking PII, GlnK is required to prevent inhibitory levels of Ntr gene expression.

Similarly, the Ntr regulon is very highly expressed in cells that lack GlnK and contain the *glnL2302* (*ntrB2302*) mutation altering NRII, and the cells display a similar growth defect to strains BK (Atkinson *et al*., personal communication). We will show elsewhere that the altered NRII2302 protein, containing the conversion A129T in the central domain of NRII, binds PII normally, but that the complex has about 10-fold lower phosphatase activity than does the NRII–PII complex (A. A. Pioszak and A. J. Ninfa, unpublished data). In strains containing the *glnL2302* mutation (hereafter referred to as strains L*), the low constitutive concentration of PII from the wild-type *glnB* locus is in-sufficient for the regulation of NRII2302 activity (Atkinson *et al*., personal communication).

Interestingly, when PII was expressed from the *glnK* promoter, the role of GlnK could be completely fulfilled by PII (Atkinson *et al*., personal communication). Strains BK containing a single-copy transgene in which the structural gene for PII was expressed from the *glnK* promoter, had elevated *glnA* expression but did not show the growth defect characteristic of strains BK. Similarly, the growth defect of cells lacking *glnK* and containing the *glnL2302* mutation (strains L*K) was eliminated upon introduction of the transgene in which PII was expressed from the *glnK* promoter (Atkinson *et al*., personal communication). These results indicated that the distinct functional roles of PII and GlnK, in as much as those roles were addressed, appeared to result solely from the promoters regulating the expression of these proteins (Atkinson *et al*., personal communication).

Here, we addressed the physiological basis for the growth defect of the double mutant *glnB glnK*, lacking both PII and GlnK (strains BK), and the double mutant *glnL2302 glnK* (strains L*K). We show that the combination of either glycine or serine and glutamine permitted strains BK to grow in liquid minimal medium, and that strains L*K were similar, but did not require glutamine. We observed that mutation of *nac*, an Ntr gene that encodes a LysR-type transcription factor, eliminated the growth defect of strains BK and L*K, without significantly affecting Ntr gene expression levels. Rather, the role of Nac in regulating *serA* expression (Zimmer *et al*., 2000; R. A Bender, personal communication) seemed to be involved, as Nac was found to repress *serA* expression 10-fold in cells lacking PII and GlnK. These results led us to hypothesize that one role of GlnK is to limit Ntr gene expression under conditions of nitrogen limitation, and that one role of Nac may be to co-ordinate the reduction in metabolism during nitrogen starvation by inhibiting an important anabolic pathway.

Results

High expression of Nac correlates with poor growth phenotypes of Ntr constitutive strains

Previous results have indicated that strains lacking both PII and GlnK, or strains that lack GlnK and contain the *glnL2302* (*ntrB2302*) mutation, have very high levels of Ntr gene expression and a severe growth defect on defined media that can be rescued by supplementation of the media with casein hydrolysate (Atkinson and Ninfa, 1998; Atkinson *et al*., personal communication). Studies of nitrogen regulation in *E. coli* and related bacteria have

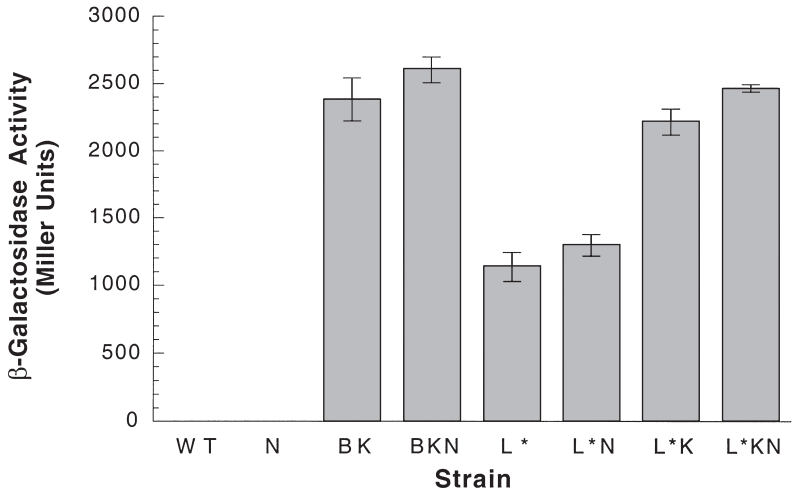

Fig. 1. Expression of a *nac–lacZYA* fusion in Ntr constitutive strains growing on nitrogen-rich media. Overnight cultures of wild-type and Ntr constitutive strains containing a single-copy *nacp–lacZYA* fusion were grown in 0.4% (w/v) glucose, 0.2% (w/v) ammonium sulphate, 0.1% (w/v) glycine and 0.004% each (w/v) tryptophan, isoleucine, leucine and valine and diluted 500-fold into like media. β-Galactosidase activity was assayed at mid-log phase, and the values reported are the average of triplicate samples. Strain aliases: WT, WT [*nacp–lacZYA*]; N, N_2 [*nacp–lacZYA*]; BK, BK_3 [*nacp–lacZYA*]; BKN, BK_3N_2 [*nacp–lac*ZYA]; L*, L* [*nacp–lacZYA*]; L*N, L* N_2 [*nacp–lacZYA*]; L*K, L*K_3 [*nacp–lacZYA*]; L*KN, L*K_3N_2 [*nacp–lacZYA*].

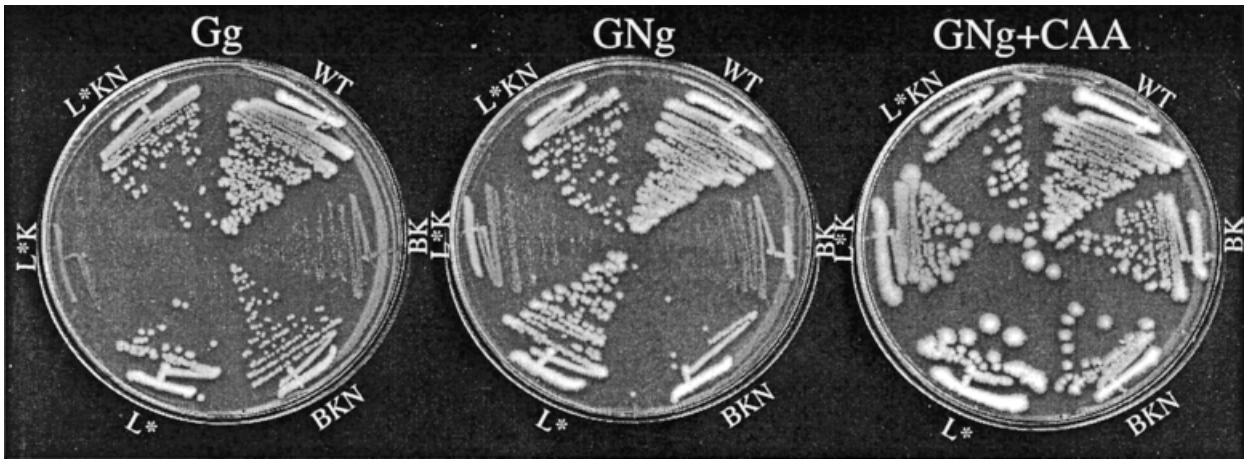

Fig. 2. Effect of *nac* deletion on Ntr constitutive strain growth on solid media. Single colony isolates from LB media supplemented with 0.2% (w/v) glutamine were streaked for single colonies on plates of the indicated composition and incubated at 37°C for 42 h. G, 0.4% (w/v) glucose; N, 0.2% (w/v) ammonium sulphate; g, 0.2% (w/v) glutamine; CAA, 0.1% (w/v) casein hydrolysate. Strain aliases: WT, YMC10; BK, BK_3; BKN, BK_3N_2; L*K, L^*K_3; L*KN, $L^*K_3N_2$.

identified the *nac* gene as an Ntr gene whose product is a LysR-type transcription factor (reviewed by Bender, 1991). The expression levels of *nac* were assessed in wild-type and Ntr constitutive strains by measuring the activity of β-galactosidase expressed from a *nac* promoter fusion to *lacZYA*, present on the chromosome in single copy (Fig. 1). When grown in nitrogen-rich media, wild-type cells had no detectable expression from the *nac* promoter, nor did cells that were wild type except that they contained a null mutation in *nac* (designated N in Fig. 1). In contrast, strain BK displayed a high level of *nac* promoter expression. Strain L* expressed the *nac* promoter fusion at about half the level obtained with strain BK (Fig. 1), approximately as high as that seen in wild-type cells starved for nitrogen by ammonia run-out (data not shown). Deletion of GlnK in the *glnL2302* background (strain L*K) resulted in a level of *nac* promoter expression nearly equal to that obtained in strain BK. These results corresponded to the known growth properties of the strains; strain L* displays a very subtle growth defect, and strains BK and L*K display a severe growth defect (Atkinson and Ninfa, 1998; Zimmer *et al*., 2000; Atkinson *et al*., personal communication).

Mutations in nac *suppress the growth defect of strains BK and L*K*

Nac has various known targets in *E. coli*, including *gltBD* (T. J. Goss *et al*., personal communication) and *gdh* (Schwacha & Bender 1993; Camarena *et al*., 1998; Muse and Bender, 1998), both of which it represses, and *codBA* (Muse, 1996), which it activates. Recent genome-wide expression studies using strains identical to strains L* and L*N (*glnL2302nac*) revealed a number of additional genes regulated by Nac, including *serA*, the expression of which was reduced by Nac (Zimmer *et al*., 2000). Thus, Nac was an excellent candidate for the Ntr gene mediating the growth defect in strains BK. We tested this possibility and observed that mutation of *nac* restored to strains L*K and BK the ability to grow on defined minimal medium (Fig. 2). The restoration of growth afforded by mutation of *nac* seemed to be nearly complete regardless of whether ammonia was present in the medium (Fig. 2). It should be noted that two different *nac* null alleles were tested in combination with three different *glnK* null alleles (strains BK_1N_2, BK_2N_1, BK_3N_1 and BK_3N_2; see Table 1), and the results were the same as those shown in Fig. 2. Mutation of *nac* did not have a significant effect on expression from the *nac* promoter in the BK, L*K or L* backgrounds in cells grown under nitrogen-rich conditions (Fig. 1).

We also identified a mutation in *nac* as a spontaneous suppressor of the poor growth defect of strain L*K. Starting with strain L^*K_2 [*glnKp-lacZYA*] containing multicopy plasmid p3Y15, which encodes NRII2302, cells were grown in medium supplemented with casein hydrolysate, and spontaneous mutations permitting growth on solid defined glucose–ammonia–glutamine medium were selected. [In retrospect, the cells were unable to grow for the lack of serine and glycine; see below.] Two classes of suppressors were obtained, one that lowered Ntr gene expression and one that did not, as assessed by examining the expression of the *glnKp–lacZYA* fusion on indicator medium containing Xgal. This was not unexpected, as earlier studies had shown that the poor growth phenotype of strain BK was suppressed by mutation of *glnG* (*ntrC*), which results in reduced Ntr gene expression (Atkinson and Ninfa, 1998). Only one suppressor in the class that did not lower Ntr gene expression was

Table 1. Strains and plasmids used in this study.

Strain[a]	Relevant genotype	Source or construction
WT (YMC10)	Wild type	Backman *et al*. (1981)
B (RB9060)	Δ*glnB*	Bueno *et al*. (1985)
K_1 (MAK)	Δ*mdl-amtB*::chl^r	Atkinson and Ninfa (1998)
K_2 (MAKc)	Δ*mdl-amtB*::kan^r	Atkinson and Ninfa (1998)
K_3	ΩGm^r . . . Δ*glnK1*	YMC10 × WCH30 P1vir
WCH30	ΩGm^r . . . Δ*glnK1*	Arcondeguy *et al*., 1999)
BK_1 (BK)	Δ*glnB* Δ*mdl-amtB*::kan^r	Atkinson and Ninfa (1998)
BK_2 (BKc)	Δgl*n*B Δ*mdl-amtB*::chl^r	Atkinson and Ninfa (1998)
BK_3	Δ*glnB* ΩGm^r . . . Δ*glnK1*	RB9060 × K3 P1vir
L* (YMC15)	*glnL2302*	Chen *et al*. (1982)
L*K_3	*glnL2302* ΩGm^r . . . Δ*glnK1*	YMC15 × WCH30 P1vir
N_1	*nac-28*	YMC10 × EB3365 P1vir
N_2	*nac*::chl^r	YMC10 × K9193Nc P1vir
EB3365	*nac-28*	Muse and Bender (1998)
K9193Nc	(*recC ptr recB recD*)::*Plac-bet exo kan sbcB15 sbcC201 nac*::chl^r	KM20 × pNac::cam2-1
KM20	(*recC ptr recB recD*)::*Plac-bet exo kan sbcB15 sbcC201*	Murphy (1998)
BN_1	Δ*glnB nac-28*	B × N_1 P1vir
BN_2	Δ*glnB nac*::chl^r	RB9060 × N2 P1vir
BK_3N_1	Δ*glnB* Gm^r . . . Δ*glnK1 nac-28*	BN_1 × K_3 P1vir
BK_3N_2	Δ*glnB* Gm^r . . . Δ*glnK1 nac*::chl^r	BN_2 × K_3 P1vir
BK_2N_1	Δ*glnB* Δ*mdl-amtB*::chl^r *nac-28*	BN_1 × K_2 P1vir
BK_1N_2	Δ*glnB* Δ*mdl-amtB*::kan^r *nac*::chl^r	BN_2 × K_1 P1vir
L*N_2	*glnL2302 nac*::chl^r	L* × N_2 P1vir
L*K_3N_2	*glnL2302* ΩGm^r . . . Δ*glnK1 nac*::chl^r	L*N_2 × K_3 P1vir
L*K_2N_1	*glnL2302* Δ*mdl-amtB*::chl^r *nac-28*	L*K_2 × N_1 P1vir
TE2680	*recD1903*::Tn*10 trpDC700*::*putPA1303* [kan^s chl^r *lac*]	Elliot (1992)
TE2680-Np^f	*recD1903*::Tn*10 trpDC700*::*putPA1303* [kan^r chl^s *nacp–lac*]	TE2680 × *Pst*I/pNacpLacZ
WT [*glnKp–lacZYA*] (YMC10^f)	*trpDC700*::*putPA1303* [kan^r chl^s *glnKp–lac*]	Atkinson and Ninfa (1998)
WT [*nacp–lacZYA*]	*trpDC700*::*putPA1303* [kan^r chl^s *nacp–lac*]	WT × TE2680-Np^f P1vir
B [*nacp–lacZYA*]	Δ*glnB trpDC700*::*putPA1303* [kan^r chl^s *nacp–lac*]	B × TE2680-Np^f P1vir
BN_2 [*nacp–lacZYA*]	Δ*glnB nac*::chl^r *trpDC700*::*putPA1303* [kan^r chl^s *nacp–lac*]	B [*nacp-lacZYA*] × N_2 P1vir
BK_3 [*nacp–lacZYA*]	Δ*glnB* ΩGm^r . . . Δ*glnK1 trpDC700*::*putPA1303* [kan^r chl^s *nacp–lac*]	B [*nacp–lacZYA*] × K_3 P1vir
BK_3N_2 [*nacp–lacZYA*]	Δ*glnB* ΩGm^r . . . Δ*glnK1 nac*::chl^r *trpDC700*::*putPA1303* [kan^r chl^s *nacp–lac*]	BN_2 [*nacp–lacZYA*] × K_3 P1vir
L* [*nacp–lacZYA*]	*glnL2302 trpDC700*::*putPA1303* [kan^r chl^s *nacp–lac*]	L* × TE2680-Np^f P1vir
L*N_2 [*nacp–lacZYA*]	*glnL2302 nac*::cam^r *trpDC700*::*putPA1303* [kan^r chl^s *nacp–lac*]	L* [*nacp–lacZYA*] × N_2 P1vir
L*K_3 [*nacp–lacZYA*]	*glnL2302* ΩGm^r . . . Δ*glnK1 trpDC700*::*putPA1303* [kan^r chl^s *nacp–lac*]	L* [*nacp–lacZYA*] × K_3 P1vir
L*K_3N_2 [*nacp–lacZYA*]	*glnL2302* ΩGm^r . . . Δ*glnK1 nac*::cam^r *trpDC700*::*putPA1303* [kan^r chl^s *nacp–lac*]	L*N_2 [*nacp–lacZYA*] × K_3 P1vir
TAB9705	*serA*::*mu d1*	YMC10 × W3110 *serA* P1vir
TAB9702	*nac*::chl^r *serA*::*mu d1*	N_2 × W3110 *serA* P1vir
TAB9706	Δ*glnB* ΩGm^r . . . Δ*glnK1 serA*::*mu d1*	BK_3 × W3110 *serA* P1vir
TAB9704	Δ*glnB* ΩGm^r . . . Δ*glnK1 nac*::chl^r *serA*::*mu d1*	BK_3N_2 × W3110 *serA* P1vir
W3110 *serA*	W3110 background, *serA*::*mu d1*	R. Matthews; *Experimental procedures*
EC2541	Δ*cbl*::*kan*	van der Ploeg *et al*. (1997)
Plasmid	**Relevant features and construction**	**Source**
pUC18	amp^r cloning vector	Invitrogen
pRS551	lac fusion vector	Simons *et al*. (1987)
pNac::cam2-1	amp^r, *nac*::*cam* in pUC18	See *Experimental procedures*
pNacpLacZ	amp^r, *nac* promoter fused at +1 to *lac* operon	See *Experimental procedures*
pglnBpN	amp^r, *nac* coding region cloned into the *Nde*I–*Hin*dIII sites of pglnBpK1	
pglnKpN	amp^r, *nac* coding region cloned into the *Nde*I–*Hin*dIII site of pglnKpB1	
p3Y15	amp^r, *glnL2302* cloned into pBR322	Atkinson and Ninfa (1992)

a. Parenthesis contain the strain name used in a previous publication.

obtained, and the suppressing mutation in this strain was linked to *cbl* (van der Ploeg *et al*., 1997), the gene immediately downstream of *nac* (*Experimental procedures*). DNA sequencing of the *nac* gene from this strain revealed the presence of a frameshift mutation in codon 36 that results in a novel stop codon at the new codon 43, probably rendering the gene null (Fig. 3).

Nac does not affect Ntr expression levels

One possible explanation for the effect of the *nac* mutation is that, like the mutation of *glnL* (*ntrB*), it resulted in reduced expression of Ntr genes by lowering levels of NRI~P (NtrC~P). As a modest decrease in Ntr gene expression may not be evident when *glnKp–lacZYA*

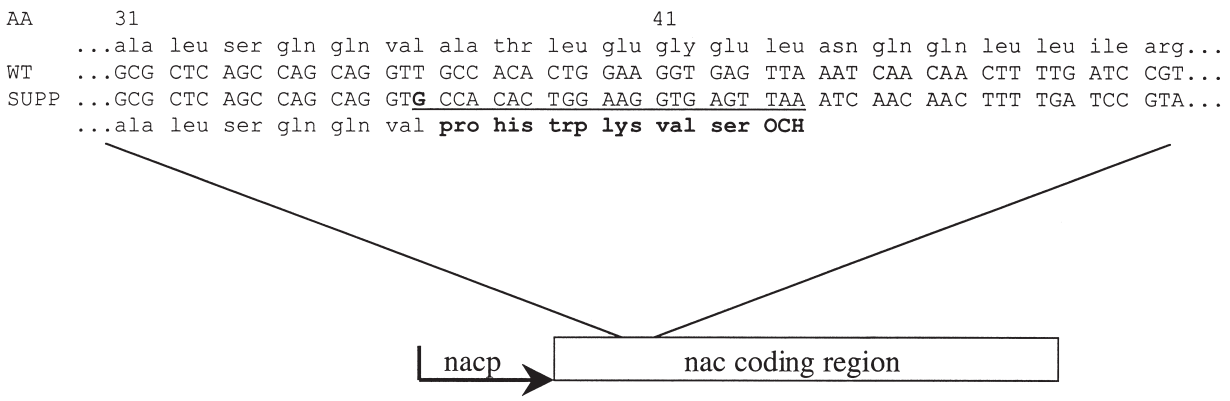

Fig. 3. Spontaneous *nac* suppressor. The nucleotide sequence and predicted translation for a spontaneous *nac* suppressor and its wild-type parent are shown. The bold G indicates the first nucleotide after the T deletion. Underlined codons encode amino acids that differ from wild type, which are shown in bold.

fusion expression is assessed on indicator plates containing Xgal, we measured the expression of this fusion in cells grown in liquid medium. Early in our studies, we noted that strains BK showed a dramatic decrease in growth rate when grown with casein hydrolysate at about OD = 0.1, unless the growth medium was supplemented with casein hydrolysate at 0.5% (w/v), that is with a five-fold higher level of this supplement than was used previously (Atkinson and Ninfa, 1998). Under these conditions, the various strains used in this study displayed identical rates of growth throughout their growth curves, which were superimposable (data not shown). Under the conditions described above (0.5% casein hydrolysate), the expression of the *glnKp–lacZYA* fusion was not altered by mutation of *nac* (data not shown). Expression of this fusion was nearly undetectable in the wild-type background and in a strain containing only the *nac* mutation (strain N), and was high in strains BK and BKN. These data are consistent with the results for the *nac* promoter fusion shown in Fig. 1.

Inappropriate expression of Nac results in a growth defect that is offset by casein hydrolysate

To determine whether elevated Nac expression alone was sufficient to cause a growth defect in cells containing PII and GlnK, we constructed multicopy plasmids in which *nac* gene transcription was under the control of the constitutive *glnB* promoter and the nitrogen-regulated *glnK* promoter (Fig. 4). The *glnB* promoter is a constitutive promoter of moderate strength. When expressed from the *glnB* promoter on a plasmid, Nac had no effect on the rate of cell growth in the presence of casein hydrolysate, but

A

	GNgtA + CAA Doubling Time (min)	GNgtA Doubling Time (min)	GgtA Doubling Time (min)
WT [pUC18]	58 ±2	88 ±3	92±4
WT [pBpNac]	55 ±2	173 ±20	170 ±15
WT [pKpNac]	55 ±2	88 ±4	622 ±117[a]

[a] These strains grew very poorly and spontaneous suppressors were common in these cultures.

B

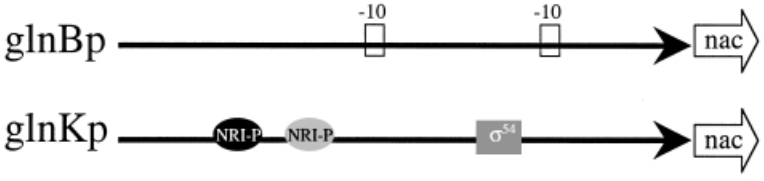

Fig. 4. Effects of Nac expression in WT cells in defined liquid media.
A. Wild-type cells were transformed with the plasmid indicated. Overnight cultures were grown in the media indicated and diluted 1:200 into like media, and the doubling time is reported. The doubling times were calculated by periodically measuring the optical density of the cultures at 600 nm using a Beckman DU65 spectrophotometer. The values reported are the average of duplicate samples in each of two independent experiments. G, 0.4% (w/v) glucose; N, 0.2% (w/v) ammonium sulphate; g, 0.2% (w/v) glutamine; t, 0.004% (w/v) tryptophan; CAA, 0.1% (w/v) casein hydrolysate; A, 100 μg ml^{-1} ampicillin. WT, YMC10ϕ.
B. The *glnB* promoter and *glnK* promoter drive expression of the *nac* coding region in these constructs. The lack of NRI~P binding sites in the *glnB* promoter and the high-affinity (dark oval) and low-affinity (light-oval) NRI~P binding sites of the *glnK* promoter are shown. Both promoters were fused to the *nac* coding region and used to express Nac from a plasmid in WT (*glnKp–acZYA*).

caused a modest inhibition of cell growth on medium lacking glycine or serine whether the medium was nitrogen rich or relatively nitrogen poor. The *glnK* promoter, on the other hand, is tightly regulated by ammonia (Atkinson and Ninfa, 1998; Atkinson *et al*., 2002). When expressed from this promoter on a multicopy plasmid, Nac resulted in a severe growth defect in wild-type cells grown under nitrogen-limiting conditions, but had little effect on the growth of cells grown under nitrogen-excess conditions. These experiments suggest that Nac was directly responsible for inhibiting the growth of the cells and could do so even in the presence of GlnK and PII.

Identification of the active ingredients of casein hydrolysate

As strains BK and L*K grew poorly on defined medium containing glucose as the carbon source and both ammonia and glutamine as nitrogen sources, but could grow when this medium was supplemented with 0.1% casein hydrolysate, it seemed clear that some combination of amino acids was responsible for permitting growth. Furthermore, our preliminary studies showed that the rate of growth of strains BK and L*K on minimal medium supplemented with casein hydrolysate slowed as the cultures became turbid, and that the OD at which growth rate decreased was dependent on the concentration of casein hydrolysate in the medium. This behaviour suggested that one or more essential components of casein hydrolysate were being depleted during cell growth.

To identify the essential compounds in casein hydrolysate, strains BK and L*K were grown overnight in medium supplemented with casein hydrolysate and then subcultured at 1% density into defined medium lacking casein hydrolysate. In such experiments, the carry-over of casein hydrolysate permits growth of the cells to OD 0.03–0.05, after which a slow rate of growth was obtained, relative to the wild type (Fig. 5A). We conducted numerous experiments in which single amino acids and combinations of amino acids were present in the defined

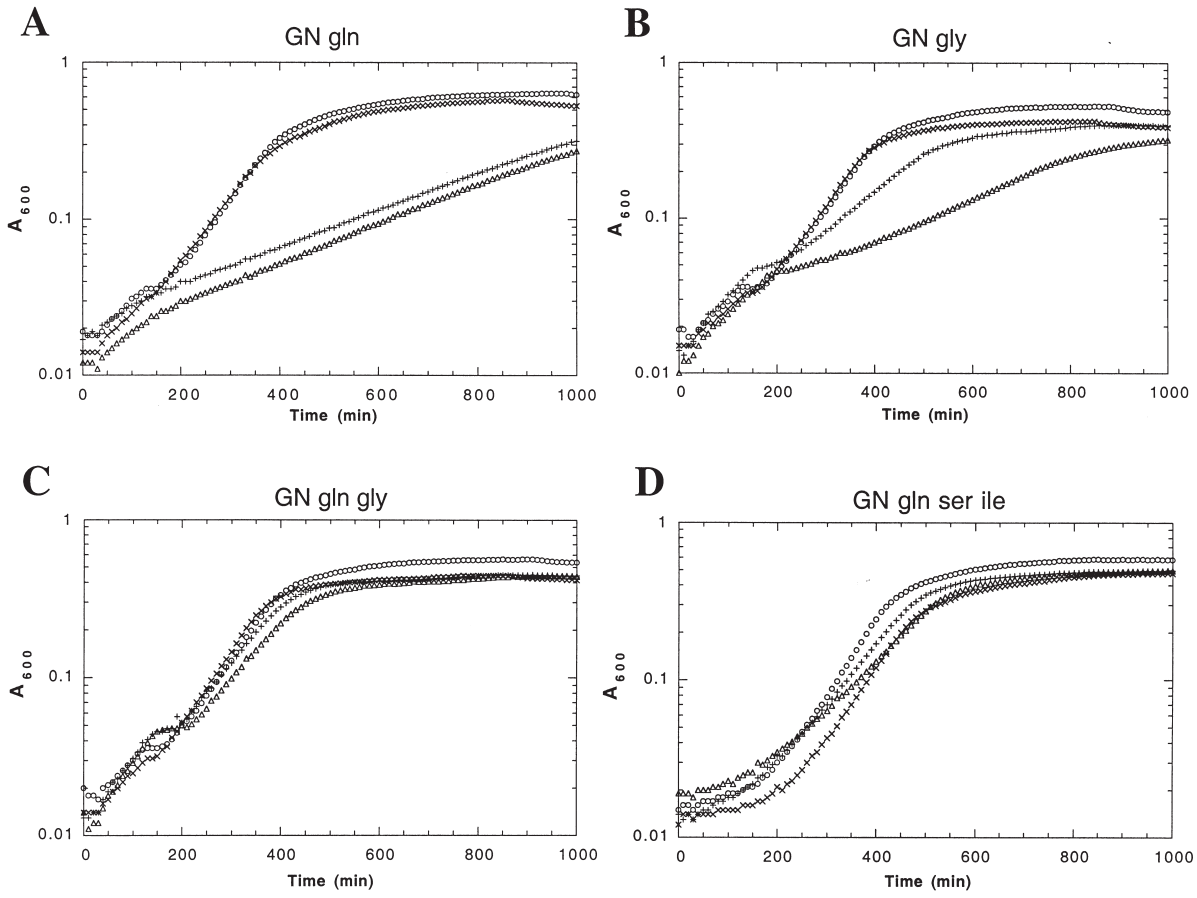

Fig. 5. Amino acid requirements of Ntr constitutive strains in defined liquid media. Overnight cultures grown in 0.4% (w/v) glucose, 0.2% (w/v) ammonium sulphate and 0.5% (w/v) casein hydrolysate were diluted 1:100 into media of the indicated composition. G, 0.4% (w/v) glucose; N, 0.2% (w/v) ammonium sulphate; gln, 0.1% (w/v) glutamine; gly, 0.1% (w/v) glycine; ser, 0.1% (w/v) serine; ile, 0.1% (w/v) isoleucine. WT, YMC10 (open circles); L* (cross); BK, BK_3 (open triangles); L*K, $L*K_3$ (+).

medium, using an automated system for following the growth of 96 cultures simultaneously (*Experimental procedures*). By testing various combinations of amino acids, we believe we have identified the minimal components required for good growth by strains BK and L*K. Specifically, in liquid medium, either glycine or serine must be present, along with glutamine (Fig. 5). Glycine alone was observed to improve the growth of strain L*K significantly, while providing only modest improvement in strain BK. The combination of both glycine and glutamine provided nearly wild-type rates of growth for both strain BK and strain L*K. Serine could replace glycine for both strain BK and strain L*K, with the caveat that, when serine was used, isoleucine had to be present to offset serine toxicity (Stauffer, 1996).

To ensure that the restoration of growth by amino acids was physiologically relevant, and not caused by various artifacts such as stimulation of the ability to use the 1% carry-over of casein hydrolysate in the single-step growth curves, we examined the conditions required for colony formation and sustained growth on solid media. We observed that the combinations of supplements described for liquid cultures also permitted strains L*K and BK to grow on defined solid medium and that the strains could be carried through multiple passages on defined solid media with these supplements (Fig. 6; data not shown). However, the amino acid requirements on solid defined media differed slightly from those of liquid cultures in that glutamine was not required on solid defined media for growth of strains L*K and BK (Fig. 6). Furthermore, isoleucine, leucine and valine were required at 0.005% in solid media to prevent a subtle growth inhibition, presumably caused by conversion of glycine to serine by serine hydroxymethyl transferase and subsequent serine toxicity (Fig. 6; data not shown). Leucine and valine were required to offset the repression of branched-chain amino acid biosynthesis caused by including isoleucine in the media. Apparently, in our experiments using liquid medium (Fig. 5), sufficient concentrations of leucine and valine were present as a result of the carry-over from the inoculum to satisfy this requirement.

Although Nac has been shown to repress both glutamate dehydrogenase and glutamate synthase, no combination of ammonia, glutamine or glutamate was able to improve the growth of strains L*K and BK as well as supplementation of the medium with glycine (Fig. 7). Furthermore, expression of glutamate synthase from a multicopy plasmid did not improve the growth of strains L*K and BK (data not shown).

Nac-mediated repression of serA

The serine or glycine requirement of strains with high Nac levels in combination with the approximately three regulation of *serA* by Nac observed by Zimmer *et al*. (2000) strongly suggested that Nac directly repressed *serA* transcription. As Nac levels are much higher in strains BK than in L*, repression of *serA* was also expected to be much greater. A Mu *d1* insertion in *serA* was used to assess the degree of Nac-mediated repression of *serA* in wild-type and BK backgrounds (*Experimental procedures*). As shown in Fig. 8, Nac exerts ≈ 10-fold repression of *serA* in strain BK when grown on nitrogen-rich media containing serine and glycine, as measured by β-galactosidase activity expressed from the Mu *d1* insertion in *serA*.

Discussion

Our experiments demonstrate an unexpected link between the regulation of nitrogen assimilation and the regulation of central metabolism in *E. coli*. Specifically, we showed that the nitrogen-regulated *nac* gene, encoding a LysR-type transcription factor, mediates repression of the *serA* promoter in mutant forms of *E. coli* that express the Ntr regulon strongly. Our studies did not address whether this repression is directly by Nac binding to the *serA* promoter or caused by Nac-mediated activation of a *serA* repressor. However, the former possibility seems more likely, as we note that two overlapping consensus Nac binding sites (ATA-N_9-TAT and ATA-N_9-GAT; Muse, 1996) can be recognized in the *serA* promoter region, overlapping the –35 and spacer region of the *serA* P1 promoter (Yang *et al*., 2002).

As mutation of *nac* almost completely restored wild-type growth properties to strains that constitutively express the Ntr regulon, and did not have a significant effect on the expression of Ntr genes, it seems that *nac* is the only Ntr gene involved in the growth defect of cells constitutively expressing the Ntr regulon. This point is strengthened by the observation that expression of Nac from multicopy plasmids resulted in a growth defect even in cells that were not expressing the Ntr regulon strongly.

As already noted, Nac was known to repress *gltBD* and *gdh*, and activate *codBA* (T. J. Goss *et al*., personal communication; Muse, 1996; Camarena *et al*., 1998; Muse and Bender, 1998). Recent genome-wide expression studies using strains identical to L* and L*N suggested that Nac reduced the expression of *serA* (Zimmer *et al*., 2000). By examination of the compounds needed to offset the growth defect of strains constitutively expressing the Ntr regulon, we conclude that Ntr constitutive strains are defective in the biosynthesis of serine and, in the case of strains BK grown in liquid medium, are also defective in glutamine biosynthesis. The glutamine defect in strains

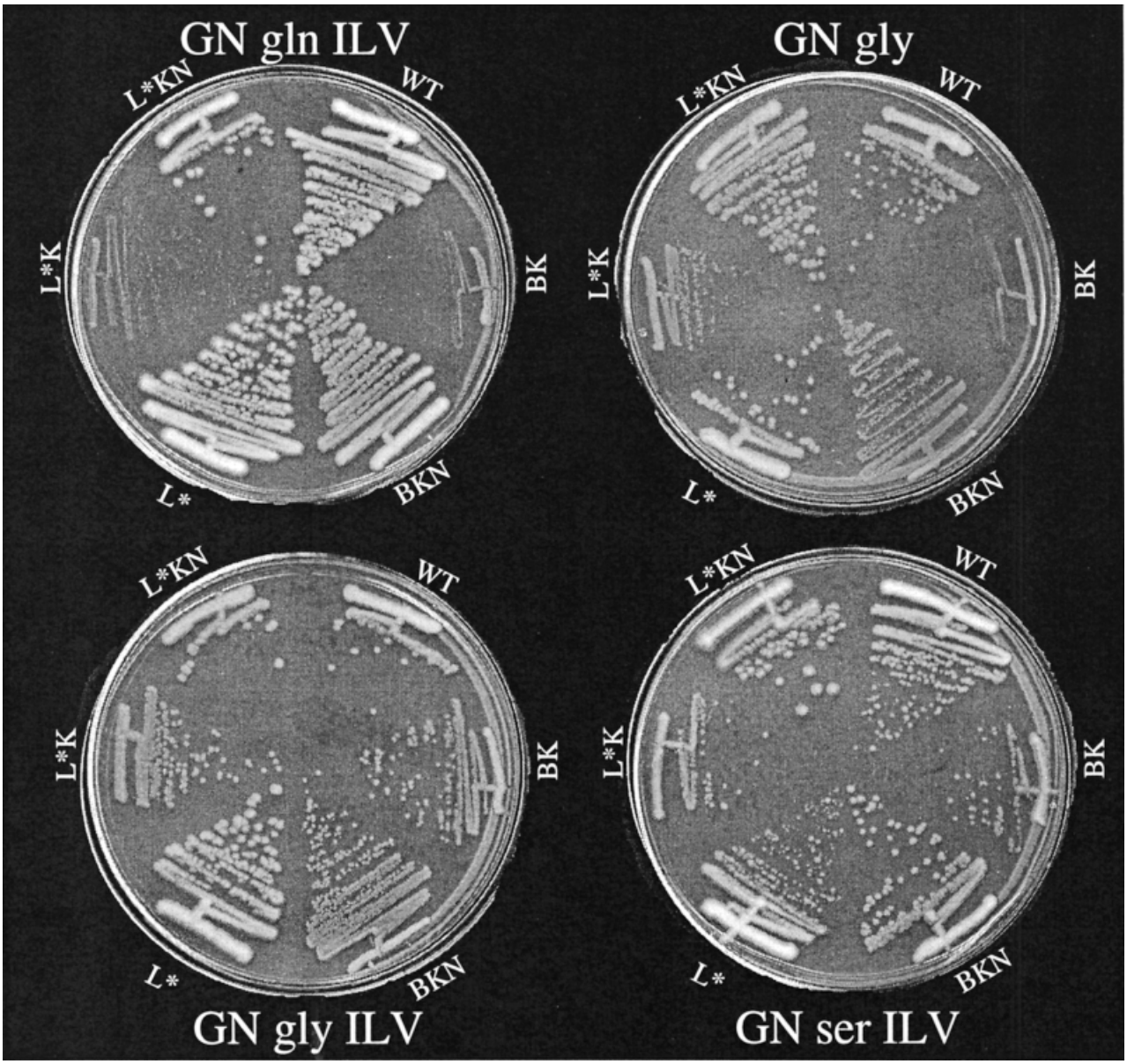

Fig. 6. Amino acid requirements of Ntr constitutive strains on defined solid media. Single colony isolates from LB media supplemented with 0.2% (w/v) glutamine were streaked for single colonies on plates of the indicated composition and incubated at 37°C for 33 h. G, 0.4% (w/v) glucose; N, 0.2% (w/v) ammonium sulphate; gln, 0.1% (w/v) glutamine; gly, 0.1% (w/v) glycine; ser, 0.1% (w/v) serine; ile, 0.1% (w/v) isoleucine. Strain aliases: WT, YMC10; BK, BK_3; BKN, BK_3N_2; L*K, L^*K_3; L*KN, $L^*K_3N_2$.

BK probably reflects the overadenylylation of glutamine synthetase that occurs in cells lacking PII and GlnK (Atkinson and Ninfa, 1998). Strains L*K, which contain PII, are probably able to deadenylylate glutamine synthetase. The defect in serine biosynthesis, which could be offset by the inclusion of either serine or glycine in the medium, probably reflects regulation of the *serA* promoter by Nac. Relatively weak repression of this promoter by Nac was observed previously in a strain identical to L* (Zimmer *et al*., 2000), in which *nac* expression was not as high as in Ntr constitutive strains, such as strains BK and L*K (Fig. 1). In the Ntr constitutive strains, Nac was expressed about twice as strongly as in strain L* and provided ≈ 10-fold repression of *serA* expression (Fig. 8). We cannot exclude at this time that Nac action at additional targets, other than *serA*, contributes to the growth defect in Ntr constitutive strains. The growth of Ntr constitutive strains in the presence of serine or glycine is not quite as vigorous as the growth of derivatives lacking Nac in the absence of serine or glycine, which may indicate the involvement of another Nac target. However, *gdh* and *gltBD* do not seem to be involved, as glutamate did not

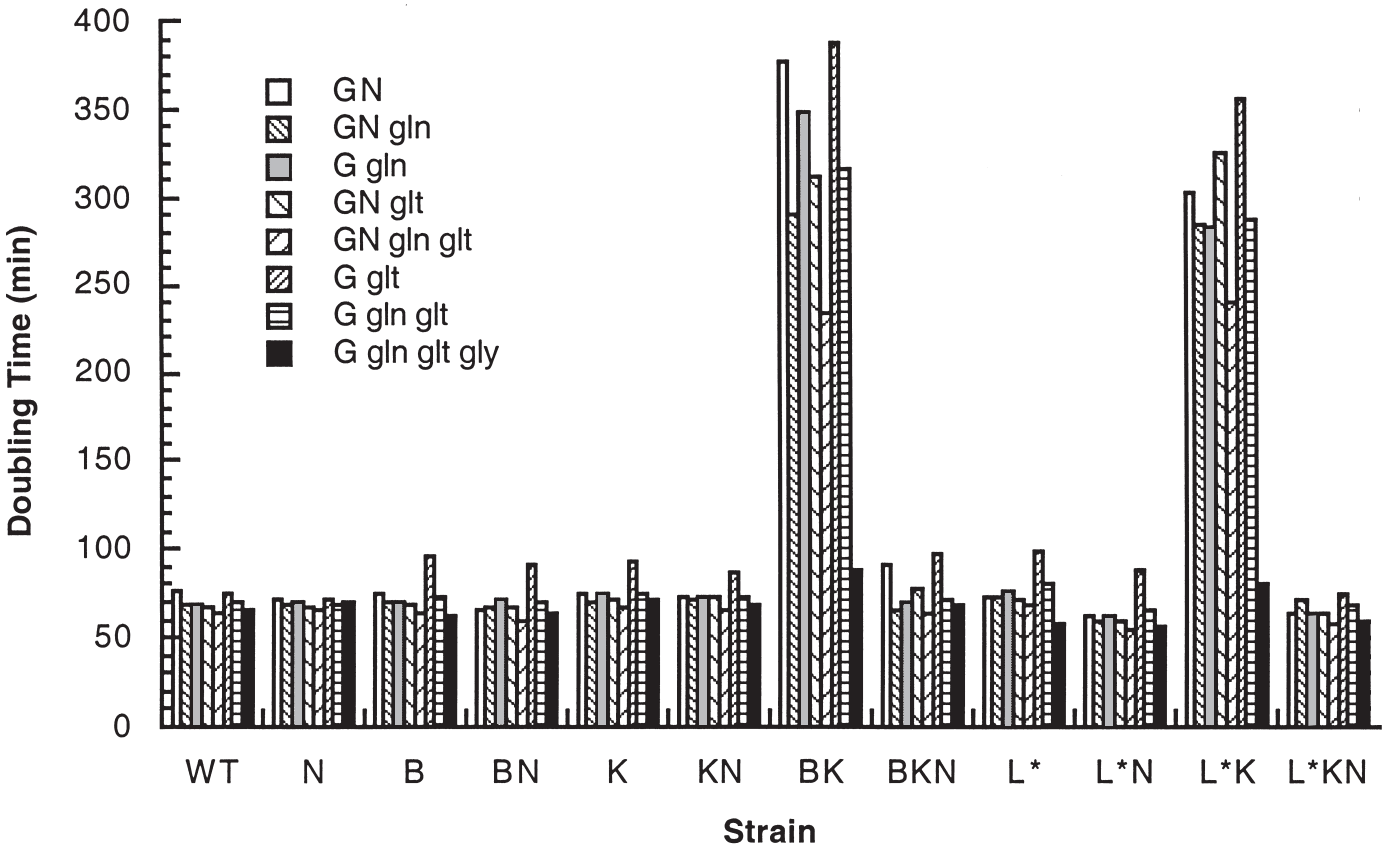

Fig. 7. Growth rates of Ntr constitutive strains in defined liquid media. Overnight cultures grown in 0.4% (w/v) glucose, 0.2% (w/v) ammonium sulphate and 0.5% (w/v) casein hydrolysate were diluted 1:100 into media of the indicated composition. G, 0.4% (w/v) glucose; N, 0.2% (w/v) ammonium sulphate; gln, 0.1% (w/v) glutamine; glt 0.1% (w/v) glutamate. Strain aliases: WT, YMC10; N, N_2; BN, BN_2; K, K_3; KN, K_3N_2; BK, BK_3; BKN, BK_3N_2; L*K, L*K_3; L*KN, L*K_3N_2.

affect the growth of cells (Fig. 5), and neither did expression of *gltBD* from a plasmid.

Repression of *serA* is likely to have broad physiological consequences, as the pathway below *serA* is the main route for the formation of glycine and for the synthesis of C1 units from glucose (Newman and Magasanik, 1963; Stauffer, 1996). Reduction in the ability to form C1 units is likely to affect growth, as both nucleotide biosynthesis and protein translation would be affected. Reduction in glycine biosynthesis may affect purine synthesis.

The regulation of the anabolic pathway leading to the formation of glycine and C1 units by Nac in Ntr constitutive strains may represent a non-physiological, adventitious regulation by Nac when it is expressed at levels never normally seen in cells. It may prove to be the case that, in wild-type cells, GlnK is never completely inactivated by the UTase/UR regardless of the environmental conditions and, thus, in nature Nac is never expressed at the levels reached in our experiments. However, in this case, we must question why the *nac* promoter, with its capacity to stop cell growth in the event of a GlnK deficiency, was not weakened by mutation limiting its maximum level of expression at some point in evolution. Indeed, one might question why a protein such as Nac, which has the capacity to inhibit cell growth, was selected and maintained in evolution and why the *serA* promoter (Yang *et al*., 2002) should retain two consensus Nac sites. We hypothesize that Nac is part of a physiologically important response to nitrogen starvation that results in the co-ordinated reduction in metabolism and the maintenance of metabolic potential. Here, using mutant cells that dramatically overexpress Nac, this role may have been magnified, permitting its facile genetic and physiological dissection. However, the apparent specificity of the action of highly expressed Nac (this work; Zimmer *et al*., 2000; R. A. Bender, personal communication) suggests to us that the relationship between Nac and serine biosynthesis is physiologically significant.

The ability of Nac to block serine biosynthesis may be a response to the decrease in the intracellular concentration of glutamine that occurs upon nitrogen starvation (Ikeda *et al*., 1996). A low intracellular glutamine concentration will severely affect the synthesis of purine nucleotides, particularly the first step in this pathway, the

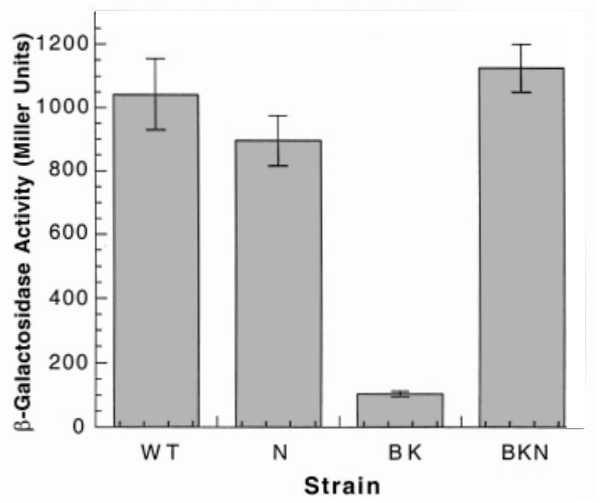

Fig. 8. Nac repression of *serA* expression. Freezer stocks made from single isolated colonies of the indicated strains were used to inoculate 2 ml W-salts cultures containing 0.2% (w/v) glucose, 0.2% (w/v) ammonium sulphate, 0.2% (w/v) glutamine, 0.1% (w/v) serine, 0.1% (w/v) glycine, 0.005% (w/v) isoleucine, 0.005% (w/v) leucine and 0.005% (w/v) valine. The cultures were grown at 30°C to an approximate OD_{600} of 0.2, at which point β-galactosidase activity was measure as described previously. Values reported are the average of three experiments. Strain aliases: WT, TAB9705; N, TAB9702; BK, TAB9706; BKN, TAB9704.

reaction of glutamine with P-ribose-PP to form phosphoribosylamine. Consequently, there will be an excess of serine, which provides glycine required for the next step and C1 units required for subsequent steps. Thus, when glutamine is limiting, it may be important to slow down the production of serine by having Nac reduce the expression of *serA*. The intracellular concentration of glutamine regulates the activity of the UTase/UR, which controls the PII and GlnK uridylylation state, regulating the activity of NRII and the expression of Nac.

Such a role for Nac in reducing metabolism in response to nitrogen limitation is consistent with the earlier observations that Nac represses both *gdh* and *gltBD*, encoding glutamate dehydrogenase and glutamate synthase respectively (T. J. Goss *et al*. personal communication, Camarena *et al*., 1998, Muse and Bender, 1998). These enzymes are the main routes for glutamate formation from 2-ketoglutarate and ammonia or glutamine. Thus, Nac may serve to co-ordinate glutamate and serine levels with the glutamine concentration. However, we are only beginning to understand the physiological role of Nac. Muse and Bender (1998) reported that strain L* is unable to use serine as the sole nitrogen source in the presence of glucose, but that strain L*N was able to use serine as sole nitrogen source. This observation remains unexplained.

If Nac provides a benefit to cells by enabling the co-ordinated reduction of metabolism in response to nitrogen starvation, then there should be some environmental conditions in which this advantage is readily discernible in otherwise wild-type cells. The advantage provided by Nac may be subtle and only revealed in experiments in which cells are repeatedly cycled between conditions of nitrogen excess and starvation, as in nature.

The regulation of the *serA* promoter is apparently complex. Recent results by Newman and colleagues have shown that the *serA* control region contains two promoters, an upstream P2 promoter and a downstream P1 promoter (Yang *et al*., 2002). The upstream P2 promoter, overlapping the Lrp binding sites, was repressed up to 10-fold by Lrp, and the downstream P1 promoter was activated up to 10-fold by Lrp (Yang *et al*., 2002). The net effect of these interactions is that, in intact cells, *serA* transcription was regulated about twofold by Lrp. These workers also report that the upstream P1 promoter was activated, directly or indirectly, by cAMP-CAP, with about a twofold effect (Yang *et al*., 2002). The *serA* control region contains two consensus Nac binding sites that overlap the –35 region and spacer region of the downstream P1 promoter. From this position, Nac could antagonize the activation of the P1 promoter by Lrp, or affect both *serA* promoters. Interestingly, Lrp and Nac also act antagonistically at the *gltBD* promoter, which, like *serA*, is activated by Lrp and repressed by Nac (Ernsting *et al*., 1993; R. A. Bender and R. A. Muse, personal communication). This may indicate an important physiological relationship between these two transcription factors.

Our results also clarify the nature of Ntr strains previously thought to be 'constitutive'. It appears that existing mutations resulting in elevated expression of Ntr genes are not truly 'constitutive', in the strictest sense of this word, meaning the absence of regulation. This is because the existing mutations were selected as permitting cell growth on minimal medium and, typically, only the level of glutamine synthetase or *glnA* expression was characterized in the mutant hunts (e.g. Atkinson and Ninfa, 1992). As *glnA* expression is exquisitely sensitive to NRI~P and reaches its maximal level well before the cellular concentration of NRI~P reaches the point at which a high level of *nac* expression occurs, 'constitutive' mutations selected on minimal medium are expected (in retrospect) as fixing NRI~P levels such that *glnA* is fully expressed, but *nac* is not. Indeed, by examination of old laboratory notebooks, we can see that, in earlier mutant searches for constitutive expression of the Ntr regulon in this laboratory (such as by selecting spontaneous suppressors of a *glnD* mutation on glucose–arginine medium), mutations that resulted in the poor growth of cells were obtained but discarded (M. R. Atkinson and A. J. Ninfa, unpublished data). Thus, it should be possible to isolate mutations that render NRII (NtrB) resistant to control by both PII and GlnK, and we predict that such mutations will result in the growth phenotype associated with the

absence of both PII and GlnK, as Nac would be highly expressed and repress *serA* in such cells.

Experimental procedures

Bacterial strains, plasmids and media

Bacterial strains and plasmids used in this study are shown in Table 1. Minimal media was W-salts (Pahel *et al*., 1978) supplemented with 0.005% thymine and the following as indicated: 0.4% glucose (G), 0.2% $(NH4)_2SO_4$ (N), 0.2% glutamine (g), 0.2% sodium glutamate, pH 7.1 (glt), 0.005% tryptophan (t) and casamino acids (CAA) (Difco). Ampicillin (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}), tetracycline (20 μg ml^{-1}), gentamicin (10 μg ml^{-1} on W-salts, 50 μg ml^{-1} on LB), spectinomycin (50 μg ml^{-1}) and streptomycin (25 μg ml^{-1}) were used when necessary. Media used in liquid cultures were filter sterilized before use, and media used in plates were autoclaved and combined with 1.5% (w/v) Bacto agar (Difco). Generalized transduction was performed as described using P1vir (Silhavy *et al*., 1984). Transformation with plasmid DNA was performed as described previously (Maniatis *et al*., 1982). Transformation with linear DNA to obtain recombination onto the chromosome was performed by electroporation of the recipient strain with ≈ 0.5 μg of gel-purified linear DNA in water using a Bio-Rad Gene Pulser apparatus. Transformants were selected on rich LB + glutamine medium supplemented with the appropriate drug. Generalized P1vir transduction was used to move the antibiotic marker and linked mutations to various strains.

Determination of growth rate

Growth rates were determined by monitoring OD_{600} in a Molecular Devices SpectraMax 250 96-well plate reader while shaking the samples at 37°C after 1:100 dilution of a GN + 0.5% CAA overnight culture into 200 μl fresh of media of the indicated composition, unless otherwise noted.

β-Galactosidase assay

β-Galactosidase activity was measured by the Miller assay and expressed as Miller units, using SDS and chloroform to disrupt the cells, as described previously (Miller, 1992).

Construction of a chloramphenicol resistance cassette insertion in nac

The *nac* region of YMC10 was polymerase chain reaction (PCR) amplified using primers NacCodeds2 (CGCGGATCC AA-GCTTCACGGTAAACTCCGGGC) and NacpLacZus2 (CCGGAATTCGCTTTCAATCTTA-TTGG), containing overhanging *Hin*dIII and EcoRI restriction sites respectively. This PCR product was cloned into the EcoRI–*Hin*dIII sites of pUC18 to make plasmid pNac2. Plasmid pNac::cam2, containing a chloramphenicol resistance cassette insertion at codon 32 of *nac*, was generated by cloning the *Bst*UI fragment of pBR325, containing the chloramphenicol resistance cassette, into the blunted *Blp*I site of pNac2. Strain K9193Nc1 was generated by electroporating K9193 with gel-purified, *Nde*I-digested pNac::cam2 and selecting cam^r recombinants. The *nac*::*cam*^{r} allele was moved into other strains by generalized P1vir-mediated transduction. The correct recombination of pNac::cam2 at the *nac* locus was confirmed by mapping the chloramphenicol resistance of N_2 with the *nac28* allele.

Construction of glnBp–nac *and* glnKp–nac

The *nac* coding region was PCR amplified with primers NacCodeus2 (GGAATTCCATATGAACTTCAGACGCCTG AA), which introduces an *Nde*I restriction site at the start codon, and NacCodeds2 (CGCGGATCCAAGCTTCACGGT AAACTCCGGGC). This PCR product was cloned into the *Nde*I–*Hin*dIII sites of pglnKpB1 (Atkinson *et al*., personal communication) to make pglnKpN and into the *Nde*I–*Hin*dIII site of pGlnBpK1 (Atkinson *et al*., personal communication) to make pglnBpN. All constructions were confirmed by sequencing.

Construction of lacZYA *fusion to the* nac *promoter*

The *nac* promoter region of –225 to –2 relative to the start codon was PCR amplified from YMC10 with primers NacpLacZus2 (CCGGAATTCGCTTTCAATCTTATTGG) and NacpLacZds2 (CGCGGATCCTGCCGCCATTACTTACA). The PCR product was cloned into the *Bam*HI–*Eco*RI site of plasmid pRS551 (Simons *et al*., 1987) to form a transcriptional fusion to *lacZYA* bracketed by transcriptional termination sequences with flanking sequence homology to the *trp* genes, pNacLacZ. The fusion was recombined onto the *E. coli* chromosome into the *trp* operon by linearizing the DNA with *Nde*I, followed by electroporation into TE2680 with recombinants selected for kanamycin resistance, as described previously (Elliot, 1992). Recombinants were confirmed to have a new tryptophan auxotrophic requirement and chloramphenicol sensitivity, indicating correct recombination into the *trp* locus.

Suppressor analysis

Spontaneous suppressors of the growth defect of strain L*K (*glnKp–lacZYA*) were isolated on GNg media. A multicopy plasmid containing the *glnL2302* allele, p3Y15, was maintained in strain L*K (*glnKp–lacZYA*) throughout the selection to reduce the number of suppressors mapping to *glnL*. Robustly growing colonies were repurified through two passages on GNg media and then screened for Ntr induction on GNg media containing Xgal. A single suppressor strain, TAB6757, not showing lower expression of the *glnK* promoter fusion relative to its parent, was chosen for further analysis. Over 70 suppressors in which *glnK* promoter expression was at basal levels were also obtained.

In order to map the mutation in TAB6757, we made strain TAB6 containing a kan insertion in the *cbl* locus directly downstream of the *nac* gene in the BK_3 background. Strain TAB6 was generated by crossing P1vir grown on EC2541 (generously provided by M. Hryniewicz) into BK_3 and selecting kan^r colonies that, upon screening, retained poor growth phenotype of BK strains on GNg media (the *nac* promoter of EC2541 was shown to have a mutation in the σ^{54} binding

site). The suppressor mutation in strain TAB6757 was found to be linked to the kan insertion in *cbl* in TAB6. Subsequent sequencing of the *nac* gene in strain TAB6757 revealed a nonsense mutation that is predicted to result in premature termination of Nac at amino acid 42 (Fig. 4).

Overexpression of gltBD

Plasmid pCB503, generously provided by R. Bender, expresses the *gltBD* coding region of *E. coli* from a promoter within the vector, pGB2. This plasmid was transformed into strains BK and strains L* and shown not to affect the poor growth phenotype of these strains. To make sure that this plasmid was expressing *gltBD*, GOGAT activity was measured as described previously (Macaluso *et al*., 1990). Cells were grown at 37°C in 2× W-salts containing 0.4% (w/v) glucose, 0.2% (w/v) ammonium sulphate, 0.2% (w/v) glutamine, 0.005% thiamine and 25 μg ml^{-1} streptomycin and 50 μg ml^{-1} spectinomycin, harvested at $OD_{600} \approx 0.2$ and disrupted by sonication. Wild-type cells harbouring pCB503 were found to have approximately 2.5- to threefold more GOGAT activity than wild-type cells harbouring vector. Strain L* harbouring pCB503 was found to have GOGAT activity nearly identical to the wild-type strain when harbouring the same plasmid. This level of GOGAT activity in L* was a 7.5-fold increase over L* harbouring vector alone (L* has a threefold lower basal GOGAT activity than wild type).

Construction of strains with a Mu d1 *insertion in* serA

Strain W3110 *serA*, containing a *mu d1* insertion in the *serA* gene, was generously provided by R. Matthews. P1vir transduction was used to move the *mu d1* insertion allele into the noted strains, and the resulting ampicillin-resistant colonies were screened for serine auxotrophy on W-salts media containing 0.2% (w/v) glucose, 0.2% (w/v) ammonium sulphate, 0.2% (w/v) glutamine and 0.005% vitamin B1. Approximately 5% of the Amp^r colonies were found to be serine auxotrophs, with the remainder resulting from zygotic induction of the Mu during transduction. To determine whether the strains used to measure *serA* expression contained only a single copy of Mu *d1*, amp^r/ser$^-$ strains were then back-crossed to Ser$^+$ with P1vir grown on the wild type, by selection on W-salts media containing 0.2% (w/v) glucose, 0.2% (w/v) ammonium sulphate, 0.2% (w/v) glutamine and 0.005% vitamin B1. Ser$^+$ candidates were then screened for amp^s. All strains were shown to be Ser$^+$/amp^s after the back-cross, indicating that they contained single Mu *d1* insertions, except for strain BK in which single Mu *d1* insertions were not able to be isolated in several attempts. The contribution of the additional Mu *d1* insertions in the BK strains used to assess *serA* expression was assessed by measuring β-galactosidase activity of the ser$^+$amp^r back-cross product under conditions identical to those used to generate Fig. 8 and found to be too low to be detected.

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References

Arcondeguy, T., van Heeswijk, W.C., and Merrick, M. (1999) Studies on the roles of GlnK and GlnB in regulating *Klebsiella pneumoniae* NifL-dependent nitrogen control. *FEMS Microbiol Lett* **180:** 263–270.

Allikmets, R., Gerrard, B., Court, D., and Dean, M. (1993) Cloning and organization of the abc and mdl genes of *Escherichia coli*: relationship to eukaryotic multidrug resistance. *Gene* **136:** 231–236.

Atkinson, M.R., and Ninfa, A.J. (1992) Characterization of *Escherichia coli glnL* mutations affecting nitrogen regulation. *J Bacteriol* **174:** 4538–4548.

Atkinson, M.R., and Ninfa, A.J. (1998) Role of the GlnK signal transduction protein in the regulation of nitrogen assimilation in *Escherichia coli*. *Mol Microbiol* **29:** 431–447.

Atkinson, M.R., and Ninfa, A.J. (1999) Characterization of the GlnK protein of *Escherichia coli*. *Mol Microbiol* **32:** 301–313.

Backman, K., Chen, Y.-M., and Magasanik, B. (1981) Physical and genetic characterization of the glnA-glnG region of the *Escherichia coli* chromosome. *Proc Natl Acad Sci USA* **78:** 3743–3747.

Bender, R.A. (1991) The role of the NAC protein in the nitrogen regulation of *Klebsiella aerogenes*. *Mol Microbiol* **5:** 2575–2580.

Bueno, R., Pahel, G., and Magasanik, B. (1985) Role of glnB and glnD gene products in regulation of the glnALG operon of *Escherichia coli*. *J Bacteriol* **164:** 816–822.

Camarena, L., Poggio, S., Garcia, N., and Osorio, A. (1998) Transcriptional repression of gdhA in *Escherichia coli* is mediated by the Nac protein. *FEMS Microbiol Lett* **167:** 51–56.

Chen, Y.-M., Backman, K., and Magasanik, B. (1982) Characterization of a gene, glnL, the product of which is involved in the regulation of nitrogen utilization in *Escherichia coli*. *J Bacteriol* **150:** 214–220.

Elliot, T. (1992) A method for constructing single-copy lac fusions in *Salmonella typhimurium* and its application to the hemA-pfrA operon. *J Bacteriol* **174:** 245–253.

Ernsting, B.R., Denninger, J.W., Blumenthal, R.M., and Matthews, R.G. (1993) Regulation of the gltBDF operon of *Escherichia coli*: how is a leucine-insensitive operon regulated by the leucine-responsive regulatory protein? *J Bacteriol* **175:** 7160–7169.

Feng, J., Goss, T.J., Bender, R.A., and Ninfa, A.J. (1995) Activation of transcription initiation from the nac promoter of *Klebsiella aerogenes*. *J Bacteriol* **177:** 5523–5534.

van Heeswijk, W.C., Hoving, S., Molenaar, D., Stegeman, B., Kahn, D., and Westerhoff, H.V. (1996) An alternative PII protein in the regulation of glutamine synthetase in *Escherichia coli*. *Mol Microbiol* **21:** 133–146.

Ikeda, T.P., Shauger, A.E., and Kustu, S. (1996) *Salmonella typhimurium* apparently perceives external nitrogen limitation as internal glutamine limitation. *J Mol Biol* **259:** 589–607.

Macaluso, A., Best, E.A., and Bender, R.A. (1990) Role of the nac gene product in the nitrogen regulation of some NTR-regulated operons of *Klebsiella aerogenes*. *J Bacteriol* **172:** 7249–7255.

Maniatis, T.I., Fritsch, E.F., and Sambrook, J. (1982) *Molecular Cloning, A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 68 and 250.

Miller, J.H. (1992) *A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for* Escherichia coli *and Related Bacteria.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 72–74.

Murphy, K.C. (1998) Use of bacteriophage recombination functions to promote gene replacement in *Escherichia coli*. *J Bacteriol* **180:** 2063–2071.

Muse, R.A. (1996) *The* nac *Gene of* E. coli *and its Role in Nitrogen Regulation.* Thesis, Department of Biology, University of Michigan.

Muse, W.B., and Bender, R.A. (1998) The nac (nitrogen assimilation control) gene from *Escherichia coli*. *J Bacteriol* **180:** 1166–1173.

Newman, E.B., and Magasanik, B. (1963) The relation of serine-glycine metabolism to the formation of single-carbon units. *Biochim Biophys Acta* **78:** 437–448.

Ninfa, A.J., and Atkinson, M.R. (2000) PII signal transduction proteins. *Trends Microbiol* **8:** 172–179.

Ninfa, A.J., Jiang, P., Atkinson, M.R., and Peliska, J.A. (2000) Integration of antagonistic signals in the regulation of nitrogen assimilation in *Escherichia coli. Curr Topics Cell Regul* **36:** 31–75.

Pahel, G., Zelentz, A.D., and Tyler, B.M. (1978) gltB gene and regulation of nitrogen metabolism by glutamine synthetase in *Escherichia coli*. *J Bacteriol* **133:** 139–148.

van der Ploeg, J.R., Iwanicka-Nowicka, R., Kertesz, M.A., Leisinger, T., and Hryniewicz, M.M. (1997) Involvement of CysB and Cbl regulatory proteins in expression of the tauABCD operon and other sulfate starvation-inducible genes in *Escherichia coli*. *J Bacteriol* **179:** 7671–7678.

Schwacha, A., and Bender, R.A. (1993) The product of the *Klebsiella aerogenes* nac (nitrogen assimilation control) gene is sufficient for activation of the hut operons and repression of the gdh operon. *J Bacteriol* **175:** 2116–2124.

Silhavy, T.J., Berman, M.L., and Enquist, L.W. (1984) *Experiments with Gene Fusions*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 107–112.

Simons, R.W., Houman, F., and Kleckner, N. (1987) Improved single copy and multicopy lac-based cloning vectors for protein and operon fusions. *Gene* **53:** 85–96.

Stauffer, G.V. (1996) Biosynthesis of serine, glycine, and one-carbon units. In Escherichia coli *and* Salmonella*. Cellular and Molecular Biology*, 2nd edn. Neidhardt, F.C. (ed.). Washington, DC: American Society for Microbiology Press, pp. 506–513.

Yang, L., Lin, R.T., and Newman, E.B. (2002) Structure of the Lrp-regulated serA promoter of *Escherichia coli* K-12. *Mol Microbiol* **43:** 323–333.

Zimmer, D.P., Soupene, E., Lee, H.L., Wendisch, V.F., Khodursky, A.B., Peter, B.J., *et al.* (2000) Nitrogen regulatory protein C-controlled genes of *Escherichia coli*: scavenging as a defense against nitrogen limitation. *Proc Natl Acad Sci USA* **97:** 14674–14679.