The nitrogen assimilation control (Nac) protein represses \( \text{asnC} \) and \( \text{asnA} \) transcription in \( \text{Escherichia coli} \)

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Abstract

In this work, we show that the expression of the \( \text{asnA} \) and \( \text{asnC} \) genes is regulated by the availability of ammonium in the growth medium. Our results suggest that, under nitrogen-limiting growth conditions, the nitrogen assimilation control (Nac) protein is involved in the repression of the \( \text{asnC} \) gene, whose product is required to activate the transcription of \( \text{asnA} \). We also show that asparagine negatively affects the expression of \( \text{asnA} \), independently of the presence of Nac. These results allow us to conclude that \( \text{asnA} \) transcription is regulated by two different mechanisms that respond to different effectors: nitrogen and asparagine availability. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: Nitrogen assimilation control; \( \text{asnA} \); \( \text{asnC} \); Asparagine synthesis; Nitrogen control; Ntr system

1. Introduction

In enteric bacteria, nitrogen assimilation is controlled by a two-component system consisting of the NRI and NRII proteins. NRI, the response regulator, is phosphorylated by NRII in response to signals of nitrogen deficiency. NRI-P activates the \( \sigma^{54} \)-dependent expression of a variety of genes involved in the utilization of organic and inorganic nitrogen sources [1,2]. In \( \text{Klebsiella aerogenes} \), a subset of nitrogen-regulated (Ntr) genes is expressed under the control of \( \epsilon^{70} \) promoters, and the regulation of these genes is mediated by the nitrogen assimilation control protein, Nac [3]. Transcription of the \( \text{nac} \) gene is dependent on the \( \text{E}^{54} \) holoenzyme and NRI-P [4]. In \( \text{K. aerogenes} \), Nac activates the transcription of \text{hatUH}, \text{putP}, \text{ureDABCEFG}, and \text{dadAB} [5,6], whereas represses \text{gdhA}, \text{gltBDF}, and \text{nac} itself [5,7,8].

It has been demonstrated that \text{gdhA} is repressed by Nac in \( \text{Escherichia coli} \) [9,10], although this repression seems less straightforward than in \( \text{K. aerogenes} \). For instance, in \( \text{E. coli} \), glutamate dehydrogenase (GDH) activity is repressed in some nitrogen-limiting growth conditions, like arginine or glutamate but is elevated when cells are cultured in glutamine, or in strains carrying a \text{glnLc} mutation [10]. Recently, it has been demonstrated using micro-arrays that Nac activates the transcription of several genes and operons encoding transporter proteins, such as \text{co-dAB}, \text{magC}, and \text{gabDTPC}, which provide the cell with organic nitrogen-containing compounds [11]. To gather additional information about the role of Nac in the nitrogen metabolism of \( \text{E. coli} \), we explored its possible role in the control of the synthesis of asparagine.

Information about the regulation of asparagine biosynthesis in enteric bacteria is scarce. For \( \text{E. coli} \) and \( \text{K. aerogenes} \), it is known that asparagine is synthesized by the action of two different enzymes: \text{AsnA}, which uses aspartic acid and ammonium to produce asparagine, and \text{AsnB}, which uses aspartic acid and glutamine as substrates [1]. It has been reported that the expression of the \text{asnA} gene in \( \text{E. coli} \) is dependent on the activator protein, AsnC. Asparagine is able to suppress AsnC-dependent activation of \text{asnA} through an unknown mechanism [12,13]. There is no information about the regulation of these enzymes in response to nitrogen availability for \( \text{E. coli} \). However, low AsnA activity has been reported in \( \text{K. aerogenes} \) when the cells are grown in N-limiting conditions, although a higher level of activity was detected when the
gene coding for NRI (ntrC, also called glnG) was inactivated [14].

In this work, we present evidence that transcription of \textit{asnA} and \textit{asnC} in \textit{E. coli} is controlled by the availability of nitrogen in the culture medium, and we show that this control is mediated by the Ntr system through the Nac protein. Our results suggest that under N-limiting growth conditions, the Nac protein represses \textit{asnC} transcription. Consequently, the expression of \textit{asnA} is severely reduced.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth media

The bacterial strains and plasmids used in this work are described in Table 1. Bacterial cells were grown aerobically at 37°C in NN-minimal medium [15]. Amino acids were used at 50 μg ml⁻¹. Glucose was used at 0.2%. 15 mM NH₄Cl or 0.5 mM NH₄Cl were used as N-excess or N-limiting conditions. 3.8 mM L-asparagine was used as sole N-source. pKC1 plasmid was constructed using the pKK223-3 plasmid (Pharmacia), in which the \textit{Bam}HI fragment carrying the \textit{tac} promoter was replaced by a fragment carrying the \textit{lacIq}lacOP region. The \textit{asnC} gene without its regulatory region was amplified by PCR, using the oligonucleotides 5'-CGCTGCAGTAAGAAAATCTATGGAAAATT-3' (carrying a PstI recognition site at the 5'-end), and 5'-CCCAAGCTTTCAGGGCTTGATGGTACGCAT-3' (carrying a HiII site). The amplification product was sequenced and cloned in the plasmid described above.

2.2. Construction of the \textit{asnA::uidA-aadA} allele

A 1.9 kb DNA fragment containing the \textit{asnA} and \textit{asnC} genes was amplified by PCR using the oligonucleotides 5'-CGCTGCAGTAAGAAAATCTATGGAAAATT-3' (carrying a PstI recognition site at the 5'-end), and 5'-CCCAAGCTTTCAGGGCTTGATGGTACGCAT-3' (carrying a HiII site). The amplification product was sequenced and cloned in the plasmid described above.

Table 1

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MX614</td>
<td>F' Δ(lac-pro) galE ilv-680 thi-1</td>
<td>[15]</td>
</tr>
<tr>
<td>MX302</td>
<td>glnG74::Tn5 derivative of MX614</td>
<td>[15]</td>
</tr>
<tr>
<td>MX4002</td>
<td>nac::kan derivative of MX614</td>
<td>[10]</td>
</tr>
<tr>
<td>MX4017</td>
<td>\textit{asnA::uidA-aadA} derivative of MX614</td>
<td>this work</td>
</tr>
<tr>
<td>MX4018</td>
<td>\textit{asnA::uidA-aadA} nac::kan</td>
<td>this work</td>
</tr>
<tr>
<td>MX4019</td>
<td>\textit{asnA::uidA-aadA} glnG74::Tn5</td>
<td>this work</td>
</tr>
<tr>
<td>Plasmids</td>
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<tr>
<td>pACR5</td>
<td>glnA⁺ Ap⁺ derivative of pBR322</td>
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<tr>
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<td>cloning vector, Ap⁺, pUC derivative</td>
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<tr>
<td>pWM5</td>
<td>pUC derivative carrying the \textit{uidA-aadA} cassette</td>
<td>[22]</td>
</tr>
<tr>
<td>pKO3</td>
<td>suicide vector used for gene replacement in Gram-negative bacteria, sucB Cm⁺</td>
<td>[23]</td>
</tr>
<tr>
<td>pKC1</td>
<td>plasmid carrying the \textit{asnC} gene under control of the \textit{lac} promoter, also carries the \textit{lacP} gene</td>
<td>this work</td>
</tr>
</tbody>
</table>

2.3. Enzyme assays

β-Glucuronidase (GUS) was measured as described [17]. Specific enzyme activity in cell extracts was expressed as picomoles of methylumbelliferone per min per mg of protein. Protein content was determined using the Bio-Rad assay kit, using bovine serum albumin as the standard.

2.4. RNA extraction and Northern blots

RNA extraction and Northern blot were performed as described [18].

2.5. Mobility shift retardation assay

It was carried out as described previously [9].

3. Results

3.1. \textit{asnA} expression is regulated by nitrogen availability

To ascertain whether \textit{asnA} transcription is affected by
nitrogen availability in *E. coli*, we first isolated a chromosomal fusion of *asnA* to the promoterless *uidA* gene. The fusion product *asnA::uidA-aadA* was transferred into the chromosome by replacement of the wild-type *asnA* allele. The successful double recombination event was confirmed by Southern blot (data not shown), and the strain carrying the *asnA::uidA-aadA* allele was named MX4017. This strain was able to grow in all the N-sources tested, and did not show any apparent phenotype, as it was previously observed for different *asnA* mutants [19,20]. The amount of β-glucuronidase synthesized in MX4017 cells grown in different N-sources was determined. The highest activity was detected when 15 mM NH₄Cl (N-excess) was used as the nitrogen source, whereas a strong reduction was observed when the cells were grown in 0.5 mM NH₄Cl (N-limiting) or 3.8 mM L-asparagine (Fig. 1B).

To rule out the possibility that the absence of the wild-type *asnA* allele was interfering with its proper transcriptional control, we carried out a Northern blot assay using total RNA extracted from wild-type cells grown in minimal medium supplemented with different N-sources. As shown in Fig. 1A, *asnA* transcripts were clearly detected in cells grown in 15 mM NH₄Cl (N-excess). In contrast, when the cells were grown in 0.5 mM NH₄Cl (N-limiting), *asnA* transcripts were barely detectable. When asparagine was used as the N-source, *asnA* mRNA was almost undetectable. From these results we conclude that *asnA* transcription is strongly restricted during nitrogen-limited growth. This fact may reflect the existence of a negative control exerted by the Ntr system, which is active under these growth conditions.

### 3.2. Nac protein represses *asnA* transcription

To obtain evidence of a possible role of the Ntr system in the control of *asnA* transcription, we transduced the *asnA::uidA-aadA* allele into the strains MX902 and MX4002. These strains carry the mutation *glnG74::Tn5* and *nac::kan*, respectively. The amount of β-glucuronidase produced by the double mutants was determined under N-excess and N-limiting growth conditions. As shown in Table 2, when the cells were grown in 0.5 mM NH₄Cl (N-limiting), a strong increase in the activity of the reporter gene was observed for both strains, compared with the activity determined for the MX4017 strain (*asnA::uidA-aadA*). This result suggests that the Ntr system controls *asnA* transcription through Nac in N-limiting growth conditions. Unexpectedly, a remarkable increase in the amount of β-glucuronidase was detected in MX4019 cells (*asnA::uidA-aadA glnG74::Tn5*) when 15 mM NH₄Cl was used as the N-source (Table 2). As MX4018 cells (*asnA::uidA-aadA nac::kan*) did not show this increment under this growth condition, we hypothesized that the low level of glutamine synthetase (GS) activity present in MX4019 cells may provoke an undersupply of glutamate to AsnB and as a consequence asparagine deficiency, which may stimulate *asnA* expression. To test this possibility, a plasmid (pACR5) carrying the wild-type *glnA* gene, which encodes for GS, was introduced into MX4019 cells. Because of the high copy number of this plasmid, the resultant cells produce a high level of GS activity even in the absence of NRI (data not shown). In agreement with our prediction, when MX4019 cells carrying pACR5 were grown in 15 mM NH₄Cl, the amount of β-glucuronidase was reduced to a level similar to that detected in MX4018 cells, whereas a high level of activity was still observed during growth in 0.5 mM NH₄Cl (Table 2).

A Northern blot assay using total mRNA from

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**Table 2**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>15 mM NH₄Cl</th>
<th>0.5 mM NH₄Cl</th>
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</thead>
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<td>5</td>
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<tr>
<td>MX4019</td>
<td><em>asnA::uidA-aadA glnG74::Tn5</em></td>
<td>524</td>
<td>619</td>
</tr>
<tr>
<td>MX4018</td>
<td><em>asnA::uidA-aadA nac::kan</em></td>
<td>38</td>
<td>637</td>
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<tr>
<td>MX4019/pACR5</td>
<td><em>glnA</em> plasmid</td>
<td>46</td>
<td>750</td>
</tr>
</tbody>
</table>

*Specific activities are given in picomoles of 4-methylumbelliferone produced per min per mg of protein. Each value is the mean obtained from three independent assays, which showed <25% variation.
MX4002 (nac::kan) cells confirmed that, in this strain, the expression of asnA was derepressed in N-limiting conditions (Fig. 2A). Therefore, we conclude that Nac is involved in the repression of asnA. However, when asparagine was used as the sole N-source, asnA repression was observed in the MX4002 strain, indicating that, even in the absence of Nac, asnA transcription is still controlled by asparagine (Fig. 2A).

### 3.3. The Nac protein controls asnA expression by repression of asnC

It is known that AsnC positively controls asnA expression, and negatively controls its own transcription [12,13]. However, so far no studies have reported whether other stimuli control the expression of asnC. Therefore, to investigate the expression of the asnC gene, we carried out a Northern blot using total mRNA from wild-type cells grown in N-excess or in N-limiting conditions. As shown in Fig. 2B, the asnC mRNA is abundant when the cells are grown in N-excess medium, but is scarce when they are grown in 0.5 mM NH₄Cl. This result suggests that asnC transcription may also be repressed by Nac. To test this possibility, a Northern blot using total RNA from MX4002 (nac::kan) cells was done. In this case, we observed that the asnC transcripts were abundant even in 0.5 mM NH₄Cl (N-limiting) (Fig. 2B), indicating that Nac represses asnC transcription.

On the other hand, we observed that asnC transcription is not repressed when asparagine is included in the culture medium, in either MX614 or MX4002 cells (data not shown). This result is in agreement with previous evidence showing that asnC expression is not controlled by asparagine availability [12,13].

As a first approach to elucidate if the Nac protein represses only asnC, or asnA and asnC simultaneously, we cloned the asnC gene under control of the lac promoter in a medium copy number plasmid that also expresses the lacIq gene. The resultant plasmid, pKC1, was introduced to MX4017 cells and the level of β-glucuronidase was determined. It can be observed (Fig. 3) that MX4017/pKC1 cells grown in 0.5 mM NH₄Cl showed a high level of β-glucuronidase activity compared with that of MX4017 cells. A progressive induction of the activity was observed when 5, 10, and 25 μM IPTG was added in the culture medium. This result suggests that Nac only represses asnC transcription whereas the reduction in the expression of asnA is just probably due to the absence of AsnC.

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**Fig. 2.** A: Northern blot of asnA transcripts. Total RNA from MX614 (wild-type) or MX4002 (nac::kan) cells was probed with a 32P-asnA fragment. B: Northern blot of asnC transcripts. Total RNA from MX614 or MX4002 cells was probed with a 32P-asnC fragment obtained by PCR. The nitrogen sources used for growth were the same as indicated in Fig. 1.

**Fig. 3.** β-Glucuronidase activity expressed as percent of the specific activity found in MX4017 strain grown in 15 mM NH₄Cl. Bar number 1, MX4017 grown in 15 mM NH₄Cl; 2, MX4017 grown in 0.5 mM NH₄Cl; 3, MX4017/pKC1 grown in 15 mM NH₄Cl; 4 MX4017/pKC1 grown in 0.5 mM NH₄Cl; 5, 6, and 7, MX4017/pKC1 grown in 0.5 mM NH₄Cl with 5, 10, and 25 μM IPTG respectively. Each value is the mean obtained from three independent assays, which showed <25% variation.

**Fig. 4.** Gel mobility shift assay showing the interaction of Nac with the asnA-asnC region. A 273 bp DNA fragment containing the E. coli asnA-asnC region, was labeled with 32P and incubated with 0, 0.6, 1.8, and 3.5 pmol of purified K. aerogenes NAC protein, as described previously [9].
Analysis of the *asnC-asnA* regulatory region showed a consensus Nac binding sequence (ATA-N9-TAT) 13 bp upstream of the putative *asnC* start codon. In addition, two more sequences resembling the Nac binding consensus were located at −35 and −92; the site located at −35 partially overlaps the putative PrbN box for *asnC* [12]. Although the functionality of each of these sequences remains to be tested, they may be involved in *asnC* repression. According with this proposal, a gel mobility shift assay revealed that the Nac protein binds the *asnA-asnC* control region (Fig. 4), suggesting that Nac may recognize at least one of these sites.

4. Discussion

In this work, we show evidence supporting the idea that the Nac protein is involved in the negative control of the expression of the *asnA* and *asnC* genes. This is the first report showing that *asnC* expression is subject to Ntr control. Therefore, *asnC* seems to be part of the few transcriptional factors under control of the Ntr system. Our results indicate that the Nac protein directly represses *asnC* transcription and consequently the level of *asnA* expression is diminished. The proposal that Nac only represses *asnC* derives from the fact that the expression of *asnA* was induced in N-limiting growth conditions when AsnC was expressed from the *lac* promoter.

Physiologically the regulation of *asnA* expression by ammonium availability implies that AsnA synthesizes asparagine only under N-excess growth conditions, whereas under N-limiting conditions, ammonium is preferential assimilated by GS to yield glutamine, which in turn is used by AsnB to synthesize asparagine.

On the other hand, we showed that the Nac protein is not involved in the control of *asnA* transcription in response to asparagine availability. Therefore, in agreement with earlier observations, this control must be entirely mediated by AsnC [12,13].

The existence of two different mechanisms to control *asnA* expression allows the cell to modulate the amount of AsnA in response to asparagine and nitrogen availability. For instance, if the cells are growing under N-excess conditions, asparagine will be synthesized by AsnA and AsnB; if asparagine is introduced under this condition, AsnC will adjust the expression of *asnA* according to the asparagine requirement of the cells. In contrast, under N-limiting conditions, Nac will repress *asnC*, therefore reducing the level of AsnA, and asparagine will be only synthesized by AsnB.

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References


