Regulation of glutamate dehydrogenase expression in *Pseudomonas putida* results from its direct repression by NtrC under nitrogen-limiting conditions.

Running title: NtrC directly represses *gdhA* expression in *Pseudomonas putida*

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*Keywords: Pseudomonas putida, NtrC repression, two-component system, gdhA, nitrogen-mediated regulation*
Abstract

Nitrogen-regulated genes in enterobacteria are positively controlled by the transcriptional activator of σ^N-dependent promoters NtrC, either directly or indirectly, through the dual regulator Nac. Similar to enterobacteria, \( gdhA \) encoding glutamate dehydrogenase from \( Pseudomonas putida \) is one of the few genes that is induced by excess nitrogen. In \( P. putida \), the binding of NtrC to the \( gdhA \) promoter region and \textit{in vitro} transcription suggest that, unlike its enterobacterial homologue that is repressed by Nac, \( gdhA \) is directly repressed by NtrC. Footprinting analyses demonstrated that NtrC binds to four distinct sites in the \( gdhA \) promoter. NtrC dimers bind cooperatively, and those bound closer to the promoter interact with the dimers bound further upstream, thus producing a proposed repressor loop in the DNA. The formation of the higher order complex and the repressor loop appears to be important for repression but not absolutely essential. Both the phosphorylated and the non-phosphorylated forms of NtrC efficiently repressed \( gdhA \) transcription \textit{in vitro} and \textit{in vivo}. Therefore, NtrC repression of \( gdhA \) under nitrogen-limiting conditions does not depend on the phosphorylation of the regulator; rather, it relies on an increase in the repressor concentration under these conditions.
Introduction

Nitrogen-mediated regulation in bacteria is a complex regulatory network involving a number of signal transduction and effector proteins. This global regulation has been intensively studied in enterobacteria. Briefly, nitrogen availability regulates the function of the *glnD* gene product. GlnD uridylylates (under nitrogen-limiting conditions) or deuridylylates (under nitrogen-sufficient conditions) the P$_{II}$ proteins encoded by *glnB* and *glnK*. Both P$_{II}$ proteins have similar and partially overlapping functions. P$_{II}$ proteins control ammonium assimilation by modifying the activity of glutamine synthetase via adenylylation by the adenylyl transferase GlnE; the function of other target proteins such as the NifL/NifA regulatory system, which in turn regulate the expression of nitrogen fixation genes in *Klebsiella oxytoca* or *Azotobacter vinelandii*; or the DraT/DraG system, which controls nitrogenase activity in other nitrogen fixing α-proteobacteria (Dixon and Kahn 2004; Forchhammer 2008). P$_{II}$ proteins also control the transcription of many nitrogen-regulated genes by regulating the function of the two-component regulatory NtrB/NtrC system. When nitrogen is limiting, the sensor NtrB phosphorylates the transcriptional activator NtrC. Phosphorylation is essential for transcriptional activation by NtrC. However, when nitrogen is in excess, non-uridylylated P$_{II}$ inhibits the kinase activity and stimulates the phosphatase activity of NtrB, which results in most of the NtrC being non-phosphorylated and thus inactive (reviewed in (Reitzer 2003; Leigh and Dodsworth 2007).

Most of the nitrogen-regulated operons contain promoter regions recognised by the alternative form of the RNA-polymerase holoenzyme containing the sigma factor σ$^{N}$. Transcription from σ$^{N}$-dependent promoters strictly requires an activation process dependent upon ATP hydrolysis carried out by an enhancer-binding protein, which in the case of nitrogen-regulated promoters is the phosphorylated form of NtrC (Zhang et al. 2002; Wigneshweraraj et al. 2008). Thus, when nitrogen is limiting, phosphorylated NtrC (NtrC~P)
activates transcription of a large number of operons that encode genes that are involved in providing nitrogen to the cell. However, transcription of a subset of nitrogen-regulated operons is dependent upon $\sigma^{70}$ instead of $\sigma^N$. Transcription from these promoters is regulated by nitrogen indirectly through the LysR-type dual regulator Nac (Best and Bender 1990; Bender 1991; Collins, Gutman, and Laman 1993). Nac is constitutively active and, therefore, its function is not regulated by nitrogen (Schwacha and Bender 1993). However, Nac-controlled genes are regulated by nitrogen availability because NtrC~P activates transcription of nac (Bender 1991). Therefore, under nitrogen-limiting conditions, Nac represents a second level of regulation that couples transcription of a number of nitrogen-regulated $\sigma^{70}$-dependent catabolic operons with the $\sigma^N$-dependent transcription of the Ntr system. Recent analyses have indicated that the Nac regulon is larger than initially believed since Nac is able to bind to 84 promoter regions of the Klebsiella pneumoniae genome in vitro (Frisch and Bender 2010).

An analysis of the global transcriptome in E. coli revealed that most of the nitrogen-regulated operons were transcribed only under nitrogen-limiting conditions and were therefore repressed by excess nitrogen (Zimmer et al. 2000). However, a small subset of genes showed the opposite regulatory pattern; their expression was induced under conditions of nitrogen excess. Although regulated by the global Ntr system, these genes appear to be repressed by Nac (Zimmer et al. 2000). The transcription of at least four operons has been reported to be repressed by Nac in enterobacteria (Camarena et al. 1998; Blauwkamp and Ninfa 2002; Goss, Janes, and Bender 2002; Poggio et al. 2002; Rosario and Bender 2005), indicating that the expression of these nitrogen-induced genes is controlled by a second tier of regulation. Unlike most NtrC-controlled genes, the genes induced by nitrogen are not involved in scavenging nitrogen from alternative sources; rather, they are involved in amino acid biosynthesis. gdhA, which encodes glutamate dehydrogenase, is one of these genes. Although
the enzyme is able to catalyse the assimilation of ammonium to yield glutamate and may be important to provide glutamate under specific conditions (Helling 1994; Helling 1998), to do so, it requires a high ammonium concentration. Under nitrogen-limiting conditions, the reaction usually takes place in the reverse direction. The physiological role of glutamate dehydrogenase is not nitrogen provision since, in the absence of functional glutamate synthase, the glutamate biosynthesis rate of the glutamate dehydrogenase pathway alone restricts growth under nitrogen-limited conditions (Goss, Perez-Matos, and Bender 2001).

The genus *Pseudomonas* includes a number of species that are plant or animal pathogens (including humans) and many other strains that are of great environmental relevance. In spite of the relevance of this genus, the nitrogen-mediated regulatory and signalling cascade in pseudomonads is poorly characterised. Nevertheless, a number of studies have demonstrated that nitrogen control in *Pseudomonas* may share many features with the regulatory system in enterobacteria. The *ntrB*, *ntrC*, and *rpoN* genes are found in the genomes of different *Pseudomonas* species, and mutants lacking $\sigma^N$ showed impairment in the utilisation of a number of nitrogen sources (Kohler et al. 1989; Totten, Lara, and Lory 1990). In addition, mutational analyses have indicated that the *ntrC* orthologue in *Pseudomonas* is the master nitrogen-mediated regulator that controls a number of operons involved in the uptake and/or assimilation of alternative nitrogen sources when the preferred nitrogen compounds are scarce, including its own operon, *glnAntrBC* (Hervas, Canosa, and Santero 2008); the utilisation of cyanuric acid as a nitrogen source (Garcia-Gonzalez et al. 2005); or nitrogen fixation in *Pseudomonas stutzeri* (Desnoues et al. 2003). Nevertheless, nitrogen-mediated regulation in *Pseudomonas* has a number of particular features that makes it different from that in enterobacteria. The function of the Ntr system is controlled by only one P$_\Pi$ protein, which is encoded by *glnK*, whose expression is activated by NtrC under nitrogen-limiting conditions (Hervas et al. 2009). The *Pseudomonas* genomes that have been sequenced
indicate that there is no nac orthologue in this genus. The *Pseudomonas putida* nitrogen-regulated genes that are orthologues to those activated by Nac in enterobacteria have $\sigma^N$-dependent promoters that are directly activated by NtrC, which avoids the requirement for an adapter to co-regulate $\sigma^N$- and $\sigma^{70}$-dependent genes (Hervas et al. 2009). Therefore, it appears that the nitrogen-mediated regulatory network in *P. putida* is a simplified version of the network that operates in enterobacteria with only one $\sigma^H$ protein and no cascade regulation by the Nac adapter.

As in enterobacteria, an analysis of the global transcriptome of *P. putida* revealed that, although most of the nitrogen-regulated operons were repressed by nitrogen, a few were induced in the presence of ammonium. Nitrogen-induced genes of *P. putida* include *gdhA* and others that are involved in different steps of carbon utilisation pathways that converge on pyruvate (Hervas, Canosa, and Santero 2008). In the present work, we characterise the nitrogen-mediated regulation of *gdhA*, which encodes glutamate dehydrogenase, both in vivo and in vitro, and show that the *gdhA* promoter is actively repressed by NtrC under nitrogen-limiting conditions. Therefore, we show that the *P. putida* global nitrogen-mediated regulator NtrC can regulate its target genes through either positive or negative control.

**Results**

**Nitrogen-mediated regulation and transcriptional start site of *gdhA***.

To study the expression level of *gdhA* and its dependence on nitrogen availability and NtrC in detail, a transcriptional fusion to the *trp-lacZ* reporter gene was constructed in the plasmid pMPO323. As shown in Fig. 1, the expression level of the reporter in the wild-type strain (KT2442) under nitrogen-limiting conditions (serine) was low, approximately twice the level of the control vector pMPO234 that lacked the *gdhA* promoter. However, the expression level of the reporter increased tenfold in the presence of ammonium, demonstrating that, in
contrast to most nitrogen-regulated genes, \textit{gdhA} is induced by excess nitrogen. To confirm that the regulated expression of \textit{gdhA} was dependent on the global nitrogen-mediated regulator NtrC, the same plasmids were transferred to the isogenic ΔntrC strain MPO201. In this strain, \textit{gdhA} expression was also high in the presence of ammonium. Unlike in the wild-type strain, \textit{gdhA} transcription under nitrogen-limingiting conditions was also very high; 80% of the level when nitrogen was in excess. This indicates that this strain cannot repress \textit{gdhA} transcription in response to the nitrogen-limiting conditions. Therefore, the global regulator NtrC controls, either directly or indirectly, \textit{gdhA} expression in response to nitrogen availability.

The mapping of the transcription initiation site was carried out in the wild-type strain KT2442 to identify the regulatory region of the \textit{gdhA} promoter. As shown in Fig. 2A, signals corresponding to two contiguous initiation sites were detected under nitrogen-limiting conditions, and these signals were more evident with excess nitrogen. These initiation sites are located 73 bp upstream from the initiation codon of \textit{gdhA} (Fig. 2B). However, an inspection of the upstream sequence did not allow unambiguous identification of the -10 and -35 promoter regions. According to the spacing of these promoter elements in this species, the -10 region should fall within a poly-A string 7 bp long, and the putative -35 sequence does not resemble the consensus established for this element in \textit{P. putida} (TTGACC) (Domínguez-Cuevas and Marqués 2004). Nevertheless, using an algorithm for the \textit{P. putida} NtrC consensus sequence (Hervas, Canosa, and Santero 2008), we were able to identify three sequences that resembled the NtrC binding site. The first one (designated site I) was located downstream of the transcription initiation site (+3 to +20), the second one (site II) appears to overlap the -10 and -35 promoter regions (-29 to -13), and the third one (site III) spanned positions -104 to -87 upstream of the start site (Fig. 2B).
NtrC binds to four boxes surrounding the *gdhA* promoter region.

To analyse the binding of NtrC to this region and to better determine its binding sites, probes containing the *gdhA* promoter region spanning from -146 to +76 were used for DNAse I protection assays (Fig. 3). An NtrC mutant that does not require phosphorylation to activate transcription (NtrC<sup>D55E,S161F</sup>) (Hervas et al. 2009) was used in this assay. When the bottom strand was labelled, a clear region spanning from +2 to +25 was protected. This region comprised the putative NtrC box I. Another region with protected and hypersensitive positions spanning from -11 to -31 was identified three turns of the helix upstream from the transcription start. This region comprised the predicted NtrC box II. While NtrC binding to box III could also be detected in the labelled bottom strand, the signals were very high in the gel and could not be conveniently resolved. However, NtrC binding to box III, the region spanning from -89 to -104, was very evident when the top strand was labelled. Additional protected positions and one hypersensitive position were also detected further upstream, up to -130, suggesting that NtrC binds to a fourth site that has not been previously predicted by sequence inspection. In this region, we identified a sequence loosely resembling the consensus NtrC binding site (see Fig. 2) that was located two turns of the helix upstream of box III; we designated this region box IV. The centres of boxes I and II are separated by 30 bp while those of boxes III and IV are separated by 22 bp.

In addition to the altered protection pattern in these NtrC boxes, five zones of hypersensitive positions in the region between boxes II and III were also identified in each DNA strand. These hypersensitive positions show a remarkable periodicity of approximately one turn of the helix (the distance between these positions ranged from 9 to 12 bp within each strand). This pattern of hypersensitive positions outside the NtrC binding sites clearly suggests formation of a DNA loop upon NtrC binding to the four sites. This loop is most likely
NtrC directly represses open complex formation at the gdhA promoter.

The gdhA promoter region spanning from –146 to +76 was cloned into the transcription vector pTE103 to generate that plasmid pMPO325 that was used as a template for in vitro transcription assays using E. coli σ70 holoenzyme. As shown in Fig. 4A (top), the E. coli σ70-RNA polymerase holoenzyme was able to use the gdhA promoter to initiate transcription. Two different transcription assays were performed: one allowing binding of NtrC prior to the addition of the RNA polymerase and the NTPs, and the other allowing formation of DNA-RNA-polymerase open complexes prior to the addition of NtrC and NTPs. Although we carried out multi-round in vitro transcription assays, if the σ70-RNA polymerase forms the open complexes prior to the addition of NtrC, transcription cannot be repressed (Fig. 4A top). However, if NtrC binds the promoter region before the formation of the open complexes, transcription is efficiently repressed if sufficient NtrC is added (Fig. 4A bottom). This result clearly indicates that NtrC directly represses the transcription of gdhA by a form of RNA polymerase containing σ70 and that the repression takes place prior to the formation of the open complex.

The role of the NtrC binding sites in the repression of gdhA transcription

The in vitro transcription assays and footprinting analyses indicate that NtrC is able to bind to four sites in the gdhA promoter and repress transcription. To analyse the importance of these binding sites in the NtrC-mediated repression of gdhA transcription, we analysed the in vitro transcription and binding ability of three probes containing point mutations that altered binding sites I or II or deleted sites III and IV. The mutations introduced into NtrC binding site I, located within the transcribed region, consisted of substitution of the most conserved
bases in both half-sites (Hervas, Canosa, and Santero 2008); therefore, the site was completely modified. However, since site II overlaps with the minimal promoter, and to avoid altering the -10 region, the mutations introduced into site II only affected the 5’ half-site, which was located between the -35 and -10 promoter regions.

As shown in Fig. 5, when sites III and IV were deleted, *gdhA* transcription was still detectable even at the highest NtrC concentration. Nevertheless, the transcription level was approximately 40% of that in the absence of NtrC. This result indicates that these NtrC binding sites are important, but not essential, for transcriptional repression, since their simultaneous elimination only partially affected transcription. Similarly, mutations in site II had an evident but only partial effect on the NtrC-mediated repression of *gdhA* transcription (40% of the total transcription was still detected at the highest NtrC concentration). The mutations in the transcribed region that altered NtrC binding site I had a negative effect on the general transcription level (Fig. 5): transcription in the absence of NtrC was threefold lower than transcription from the other templates. In spite of this, the addition of NtrC barely had an effect on the transcription level, which was only detectable after quantification (Fig. 5B). These data clearly indicate that mutation of site I has the most dramatic effect and that this site is essential for efficient NtrC-mediated repression.

The partial effect of mutations in site II on the NtrC-mediated repression of *gdhA* can be explained in two different ways. On one hand, the mutations could abolish NtrC binding, but efficient repression would still be exerted from the other sites. On the other hand, mutations in site II could still allow NtrC binding with sufficient affinity to exert some degree of repression.

The binding of NtrC to the *gdhA* promoter region bearing the mutations in site I or site II was tested using DNase I footprinting. As shown in Fig. 6, mutations in site I completely abolished the protected positions associated with this site, suggesting that these mutations
significantly affect NtrC binding. Interestingly, the binding of NtrC to site II in this mutant probe was also reduced since the protected sites in this box and the hypersensitive sites at position -23 were only evident at the highest NtrC concentrations. This indicates some degree of cooperativity in the NtrC binding to sites I and II. In addition, it was clear that the mutations in site I also reduced NtrC binding to the most upstream sites III and IV, since the positions in box III were less protected and the hypersensitive sites in box IV were less evident. Concomitantly, the periodic hypersensitive sites located between sites II and III, which were indicative of a loop of intervening DNA, were less evident with mutations in site I. This clearly suggests that the interactions between the NtrC dimers bound to the distant sites, and therefore the DNA looping, are diminished in this mutant.

The effect of the mutations in site II on the binding affinity of NtrC was difficult to establish because these mutations altered the protection/hypersensitivity pattern of site II. Nevertheless, it was evident that one new hypersensitive position, replacing that at -23, appeared at -24. Additionally, the surrounding positions with weak signals that appeared using this mutant probe were also protected by NtrC. Therefore, mutation of the NtrC site II did not completely abolish NtrC binding to this site. Again, reducing the binding of NtrC to site II by mutation of the site had a clear effect on the binding to site I and to the most upstream binding sites (Fig. 6).

**NtrC and NtrC<sup>D55E,S161F</sup> are able to bind and repress gdhA transcription.**

Since NtrC directly controls gdhA transcription in response to nitrogen availability, NtrC-mediated repression of gdhA should take place only under nitrogen-limiting conditions and should be released under conditions of nitrogen excess. One possibility to explain the nitrogen-mediated regulation of gdhA transcription is that NtrC can repress gdhA transcription only if it is phosphorylated. However, since transcription of ntrBC is eightfold
higher under nitrogen-limiting conditions than under nitrogen-excess conditions (Hervas, Canosa, and Santero 2008), another possibility is that both forms of NtrC can repress \textit{gdhA} transcription but repression only takes place under nitrogen-limited conditions because of the higher regulator concentration present under this condition.

The \textit{in vitro} experiments demonstrated that the mutant form of NtrC\textsuperscript{D55E,S161F} is able to bind the \textit{gdhA} promoter region and repress transcription (Figs. 3 and 4). This form of NtrC cannot be phosphorylated in response to limited nitrogen availability since the phosphorylated residue is substituted; however, it activates transcription under both nitrogen-limiting and nitrogen-sufficient conditions since it does not require phosphorylation to activate transcription. To establish whether NtrC repression is dependent on its phosphorylation state, wild-type NtrC was purified as previously described for the mutant NtrC\textsuperscript{D55E,S161F} (Hervas et al. 2009). Instead of using its partner NtrB for phosphorylation, NtrC was phosphorylated using acetyl phosphate, which has been successfully used as an artificial phosphate donor in phosphorylation reactions involving transcriptional activators belonging to the same family as NtrC and actually phosphorylates \textit{E. coli} NtrC \textit{in vivo} (Feng et al. 1995).

Phosphorylated NtrC (NtrC\textsuperscript{~P}) was tested for transcriptional activation of the \textit{glnK} promoter of \textit{P. putida} since it had been previously shown that open complex formation at this promoter was strictly dependent of NtrC (Hervas et al. 2009). As seen in Fig. 7, either NtrC or NtrC\textsuperscript{D55E,S161F} could activate transcription of \textit{glnK} \textit{in vitro}. As expected, the ability of the constitutive mutant NtrC\textsuperscript{D55E,S161F} to activate transcription was not affected by incubation with acetyl phosphate; if anything, acetyl phosphate had a slight negative effect. Also as expected, non-phosphorylated wild-type NtrC could not activate transcription from \textit{glnK} at all. However, incubation of wild-type NtrC with acetyl phosphate resulted in transcriptional activation that was even higher than that of NtrC\textsuperscript{D55E,S161F}. This result clearly indicates that \textit{P. putida} NtrC is efficiently phosphorylated by incubation with acetyl phosphate.
EMSA assays using the *gdhA* promoter region and the different forms of NtrC were performed in order to detect any effect of the phosphorylation of the constitutive double mutation on its binding affinity or on the mobility of the shifted complex. As shown in Fig. 8A, the presence of NtrC produced just a small change in the DNA mobility, just as it happens at the *glnK* promoter (not shown). Mobility of the shifted complex was slower at the highest NtrC concentrations, which indicated binding of additional NtrC dimers to the complex. Binding of either form of NtrC to the probe was evident as its concentration increased in the incubation mixture. Binding affinity of wild-type NtrC, assayed as the percentage of remaining unbound DNA, was slightly lower than that of NtrC<sup>D55E,S161F</sup> (Fig. 8B). Phosphorylation of NtrC had no apparent effect on either the binding affinity or on the mobility of the complex.

*In vitro* transcriptional repression assays using the different forms of NtrC are shown in Fig. 9. In general, NtrC<sup>D55E,S161F</sup> efficiently repressed transcription, obtaining 50% repression with 600 nM of the protein, and repression was not affected by incubation with acetyl phosphate. Transcriptional repression by wild-type NtrC was less efficient since almost twice as much protein was required to achieve the same level of repression. Intriguingly, non-phosphorylated NtrC was able to repress *gdhA* transcription almost as efficiently as the phosphorylated form, since treatment with acetyl phosphate only increased its repression efficiency by 1.25-fold (Fig. 9B).

This *in vitro* result suggesting that the phosphorylation state of NtrC is not crucial for repressing *gdhA* is in strong contrast to the reported effect of phosphorylation on NtrC oligomerisation and acquisition of its ATPase activity required for activating transcription. In order to confirm that non-phosphorylated NtrC is also able to repress *Pseudomonas putida gdhA* transcription *in vivo*, the kinetics of *glnK* repression and *gdhA* induction after addition of ammonium to a culture grown under nitrogen limiting conditions was analysed. As shown
in Figure 10, glnK transcript decayed very rapidly, with a half-life shorter than 3 minutes, and reached its basal level of expression within the first 10 minutes after ammonium addition. This clearly indicated that glnK transcription activation was abolished almost immediately after ammonium addition, and therefore NtrC-P became de-phosphorylated by that time. On the other hand, gdhA induction had a lag of at least 10 minutes and maximal gdhA expression took 40 minutes. Since transcription of ntrC is auto-activated by NtrC-P, new production of NtrC is shut down shortly after ammonium addition and the NtrC concentration was progressively reduced by protein turnover and dilution by growth to sufficiently low levels along the 40 minutes. This result clearly indicated that by the time the activating function of NtrC, which is strictly dependent of phosphorylation, was completely lost the repressing function of NtrC still remained unaltered.

Discussion

Among the enzymes involved in nitrogen and amino acid metabolism in enterobacteria, the physiological importance of glutamate dehydrogenase in providing nitrogen is not particularly well understood. Its physiological role cannot be nitrogen scavenging when the availability of nitrogen is low (Reitzer, 2004) because the reaction usually goes in the opposite direction unless ammonium is present at a high concentration. Consistently, the expression of its encoding gene gdhA in E. coli (Riba et al. 1988) and Klebsiella aerogenes (Schwacha and Bender 1993) has been shown to be regulated by nitrogen availability, but in the opposite way—induced by nitrogen. Intriguingly, gdhA is not regulated by nitrogen availability in Salmonella enterica serovar Typhimurium (Brenchley, Baker, and Patil 1975), indicating that nitrogen-mediated regulation of gdhA may not be critical for all enteric bacteria. P. putida is a ubiquitous saprophytic soil bacterium with a very versatile metabolism. In spite of the differences with enterobacteria, gdhA in P. putida is also regulated by nitrogen and in the same way as E. coli gdhA; that is, it is induced by nitrogen
excess. This suggests that the function of its gene product is not related to nitrogen scavenging and ammonium assimilation.

In *E. coli* and *K. aerogenes*, the nitrogen-mediated regulation of *gdhA* is dependent on NtrC. However, NtrC is only indirectly involved in this regulation. The transcription of enterobacterial *gdhA* is negatively regulated by the dual regulator Nac under nitrogen-limiting conditions. *P. putida* lacks Nac, and all of the genes induced under nitrogen-limiting conditions appear to be directly activated by NtrC (Hervas et al. 2009). The regulatory role of enterobacterial Nac in genes that are negatively controlled is apparently also a function of NtrC in *Pseudomonas*, and NtrC is the regulator that directly represses *gdhA* transcription without the need of an intermediate repressor. Therefore, the global Ntr regulatory network in *Pseudomonas* is simpler compared to that in enterobacteria, since NtrC directly performs both the positive and the negative regulation of nitrogen-regulated genes, thereby avoiding the second stage of regulation carried out by Nac.

The binding of NtrC to the *gdhA* promoter and *in vitro* transcription assays show that NtrC directly represses *gdhA* transcription. NtrC is an activator of σ^N^-dependent promoters that function to promote the isomerisation of closed complexes between σ^N^-RNA polymerase and its cognate promoter into open complexes in a reaction dependent on ATP hydrolysis. Some transcriptional activators of σ^N^-dependent promoters such as NtrC itself (Dixon 1984; MacFarlane and Merrick 1985; Reitzer and Magasanik 1985; Schwab et al. 2007) or SfnR (Kouzuma et al. 2008) can repress their own transcription in different bacteria. However, this autoregulatory negative control does not respond to the signals these regulators normally respond. Nitrogen-mediated regulation of *gdhA* in *P. putida* is therefore the first example of regulation of an operon in response to a particular signal, which involves direct negative control by a transcriptional activator of σ^N^-dependent promoters.
Consistent with its negative control of \textit{gdhA} transcription, NtrC binds to four boxes, two of which are located very close to the promoter, and the other two are located further upstream (Fig. 3). NtrC footprinting also showed a pattern of hypersensitive positions in the intervening region between sites II and III, indicating that this region is distorted upon NtrC binding. This suggests an interaction between the NtrC dimers bound to sites I and II and those bound to sites III and IV. This view is further supported by the NtrC footprints on probes containing mutations in sites I or II, which showed that these mutations also have an effect on binding to sites III and IV and on the hypersensitive positions between sites II and III, which are less evident in the mutant probes (Fig. 6). The mutational analysis also showed cooperative binding to sites I and II. According to these data, we propose a model of \textit{gdhA} repression in which NtrC dimers cooperatively bind to the four sites and form a repressor loop, similar to what is found at \textit{lac} (Oehler et al. 1990) or \textit{gal} (Adhya et al. 1998) promoters (Fig. 11). Nac also binds to two separate sites in the enterobacterial \textit{gdhA} promoter region (Goss, Janes, and Bender 2002), and tetramer formation is essential for Nac repression (Rosario and Bender 2005), which suggests that a repressor loop is formed between the distantly bound Nac. This arrangement seems particularly appropriate since cooperative binding to several operators in the promoter region and formation of higher order structures by protein-protein interactions increase the repression of regulators in such a way that repression efficiency does not have to rely on the individual affinity of the repressor for its binding sites (Rojo 2001). Simultaneous deletion of sites III and IV resulted in reduced repression efficiency by NtrC but did not abolish repression (Fig. 5), suggesting that NtrC still could repress, to some extent, by using only sites I and II. Therefore, sites III and IV can be considered secondary operators that contribute to repression by allowing formation of a higher order complex containing a repressor loop that increases repression, similar to the \textit{lac} operators O$_2$ and O$_3$. The mutational analysis indicated that site I, located in the transcribed
region, was a critical operator for NtrC-mediated repression since its mutation almost completely abolished repression (Fig. 5) even though NtrC was still partially bound to site II (Fig. 6). It is difficult to determine the specific contribution of site II to repression since its mutation, which caused partial derepression, showed partial binding to site II but also reduced binding to site I. Therefore, the partial derepression in this mutant promoter could also be explained by the reduced NtrC occupancy of the essential site I.

Regarding the mechanism of repression, the \textit{in vitro} repression assays clearly demonstrated that NtrC could not repress transcription if formation of the open complexes was previously allowed (Fig. 4). Therefore, NtrC cannot prevent promoter clearance or transcription elongation from the \textit{gdhA} promoter. Rather, it inhibits the early steps of transcription initiation such as RNA polymerase binding or the isomerisation of the closed promoter complex into an open complex (Rojo 2001). The arrangement of NtrC binding sites, above all the location of site II, suggests that NtrC might prevent RNA polymerase binding. However, our results are fully compatible with either mechanism of repression.

NtrC activates transcription only under nitrogen-limiting conditions. This is because the kinase NtrB phosphorylates NtrC under these conditions. Phosphorylation does not greatly affect NtrC binding to its sites, but it stimulates cooperative binding and oligomerisation through the central domain determinants and is required for acquisition of the ATPase activity essential for activation of transcription (Porter et al. 1993; Harrod et al. 2004). NtrC~P is also the form that represses \textit{gdhA} transcription under physiological conditions. However, in spite of its dramatic effect on the activation of \textit{glnK} transcription (Fig. 7), phosphorylation of NtrC did not substantially affect its binding to the \textit{gdhA} promoter region (Fig. 8) and had only minor effects on its ability to repress transcription \textit{in vitro} (Fig. 9). This intriguing result was confirmed \textit{in vivo} by showing that during transition to a nitrogen excess condition, \textit{gdhA} was still fully repressed by the time NtrC was already unable to activate
$glnK$ transcription (Fig 10). Therefore, both results suggest that repression can take place regardless of the NtrC phosphorylation state. As previously discussed, the cooperative binding and oligomerisation of NtrC dimers when bound to distant sites may be important for efficient repression. However, the protein-protein interactions important for efficient repression do not appear to depend on phosphorylation. Phosphorylation-independent oligomerisation of NtrC dimers through their C-terminal domains leads to cooperative binding, as has been previously described for enterobacterial NtrC (Yang et al. 2004). Therefore, we propose that these dimer-dimer interactions are important for the efficient repression of $gdhA$ in *P. putida*.

Therefore, negative regulation of $gdhA$ by NtrC under nitrogen-limiting conditions does not simply rely on the phosphorylated form of NtrC but mainly on the increased production of NtrC under these conditions. *ntrC* transcripts are eightfold more abundant under nitrogen-limiting conditions than under nitrogen-excess conditions (Hervas et al. 2009). *In vitro*, an eightfold difference in the NtrC concentration is enough to allow full regulation of $gdhA$ (Figs. 4, 5 and 9). This difference in NtrC concentration can also have a dramatic effect on the *in vivo* $gdhA$ transcription levels since formation of higher order structures and cooperative binding stimulates fast and strong shutdown of a promoter when the repressor concentration rises above a certain level (Rojo 2001).
Experimental procedures

Bacterial strains and growth conditions

The bacterial strains used in this work and their genotypes are summarised in Table 1. Cells were grown in minimal medium (Mandelbaum, Wackett, and Allan 1993) containing 25 mM sodium succinate. The nitrogen sources were ammonium chloride or L-serine (1 g l⁻¹). When required, Luria-Bertani (LB) was used as a rich medium (Sambrook 2000). Cultures were grown in culture tubes or flasks with shaking (180 r.p.m.) at 30°C. Antibiotics and other additives were used at the following concentrations when required: carbenicillin, 500 mg l⁻¹; rifampicin, 20 mg l⁻¹; tetracycline, 5 mg l⁻¹ or 20 mg l⁻¹; and 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal), 25 mg l⁻¹. All reagents were purchased from Sigma-Aldrich.

Plasmid construction

The plasmids used in this work are summarised in Table 1. All DNA manipulations were made using standard protocols (Sambrook 2000). Plasmid DNA preparation and purification kits were purchased from Macherey-Nagel and GE Healthcare, respectively, and used according to the manufacturers’ instructions. Plasmid DNA was transferred to E. coli strains using transformation (Inoue, Nojima, and Okayama 1990) or to P. putida strains using triparental mating (Espinosa-Urgel, Salido, and Ramos 2000). E. coli DH5α was used as a host strain in the cloning procedures.

lacZ under the control of the gdhA promoter (pMPO323) was constructed by PCR amplification of the promoter region, using genomic DNA from P. putida KT2442 as a template and the oligonucleotides PgdhAfwdlargo and PgdhArev as primers. This PCR product was cloned in the transcriptional fusion vector pMPO234. pMPO325 was constructed by cloning the same fragment into the in vitro transcription vector pTE103. pMPO349, which carries a deleted version of the gdhA promoter, was constructed by cloning a fragment obtained by PCR using the oligonucleotides PgdhAdeI1 and PgdhArev into pTE103. Finally,
pMO350 and pMPO351, which contain the gdhA promoter with mutations in the NtrC binding site II and I, respectively, were constructed by overlapping PCR, as previously described (Camacho and Casadesus 2005). The following oligonucleotides were used: pMPO350, gdhAsitio2rev and gdhAsitio2fwd were used as mutagenic oligonucleotides and PgdhAfwdlargo and PgdhArev were used as external oligonucleotides; pMPO351, gdhAsitio1rev and gdhAsitio1fwd were used as mutagenic oligonucleotides and PgdhAfwdlargo and PgdhArev were used as external oligonucleotides. The overlapping PCR products were cloned into pTE103. The sequences of primers used in this work are shown in Table 1. All cloned PCR products were subsequently sequenced.

**β-galactosidase assays**

To examine the expression of the gdhA-lacZ fusion in *P. putida* KT2442 and MPO201, preinocula of *P. putida* strains were grown to saturation in minimal medium under nitrogen-excess conditions (ammonium chloride). The cells were then diluted in minimal medium under nitrogen-excess or under nitrogen-limiting (L-serine) conditions, and the diluted cultures were grown for 16–24 h to the mid-exponential phase. Samples of the cultures were then taken, and β-galactosidase activity was determined as previously described (Miller 1992). The β-galactosidase activity is reported as the average of at least three independent cultures.

**Protein purification**

Wild-type and constitutively active *P. putida* NtrC (NtrC<sup>D55E, S161F</sup>) were purified using selective precipitation with ammonium sulphate in the range of 30% to 40% saturation, as previously described (Hervas et al, 2009). Proteins were dialysed against 2 liters of storage buffer (50 mM Tris HCl pH 8, 20% glycerol, 0.1 mM EDTA, 1 mM DTT and 10 mM NaCl) and the purity was estimated visually to be ≥ 90% using SDS-PAGE. The concentration was
determined using a Bradford protein assay and is expressed as µM of a dimer. Protein samples were stored at -80°C.

**RNA preparation and primer extension**

Total RNA from *P. putida* KT2442 grown to mid-exponential phase under nitrogen-excess or nitrogen-limiting conditions was prepared as previously described (Garcia-Gonzalez et al. 2005). Primer extension reactions were performed using 20 µg of RNA in each condition as the template, 32P end-labelled primer PEXgdhA, and Superscript II reverse transcriptase (Invitrogen, Carlsbad, California), as previously described (Govantes, Albrecht, and Gunsalus 2000). Sequencing reactions were performed using the Thermo Sequenase Cycle Sequencing Kit (USB, Cleveland, Ohio), according to the manufacturer's instructions. The samples were run on 6% polyacrylamide-urea sequencing gels in Tris-borate-EDTA buffer. The gels were then dried, exposed to radiosensitive screens, and finally scanned in a Typhoon 9410 scanner (GE Healthcare).

**DNase I footprinting**

NtrC footprint assays were performed as described previously (Porrua et al. 2007) except for the footprinting buffer (10 mM Tris-acetate pH 8, 100 mM potassium acetate, 8 mM magnesium acetate, 27 mM ammonium acetate, 5% glycerol, 0.67 mM CaCl2, 1 mM DTT, 0.33 mg ml⁻¹ salmon sperm DNA, and 5 µg ml⁻¹ BSA). The probes for DNase I footprinting were generated by PCR amplification using the oligonucleotides footgdhA1-B and PgdhArevfoot for the top strand, footgdhA2 and PgdhAfwdfoot for the bottom strand, and footgdhA2-mut and PgdhAfwdlargo for the mutant probes experiments. The amplified probes were digested, and the strands were specifically labelled with [α32P]-dCTP by filling in the 5’ overhanging ends using Klenow fragment. A sequencing reaction performed with the Sequenase 2.0 kit (USB) using an oligonucleotide specific for the labelled strand in each case (secgdhA1-B for the top strand, secgdhA2 for the bottom strand, and secgdhA2-mut for
the mutant probe experiments) was run with the partially digested DNA used as a size marker. The gels were processed and analysed as described for the primer extension analysis.

**Electrophoretic mobility shift assay**

Electrophoretic mobility shift assays (EMSAs) of the NtrC-DNA complexes were performed as previously described (Porrua et al. 2007). Probes containing the wild-type and mutated versions of the NtrC binding sites were obtained by restriction digest of pMPO325, pMPO349, pMPO350 or pMPO351. The probes were labelled with [α³²P]-dCTP by filling in the 5’ overhangs using Klenow fragment. The reactions were performed in a volume of 15 µl in binding buffer (10 mM Tris-acetate (pH 8), 100 mM potassium acetate, 8 mM magnesium acetate, 27 mM ammonium acetate, 5% glycerol, 1 mM DTT, 20 ng µl⁻¹ poly-(dI-dC) DNA, 5 µg BSA) with 1.3 nM probe and increasing concentrations of NtrCwt or NtrC^{D55E,S161F}. After a 20-min incubation at room temperature, the reactions were stopped with 3 µl of loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 10 mM Tris-HCl (pH 8), 1 mM EDTA, 30% glycerol), and the samples were separated on an 8% native polyacrylamide gel in Tris-borate-EDTA buffer at 4°C. The gels were dried, exposed to radiosensitive screens, scanned in a Typhoon 9410 scanner (GE healthcare), and analysed using ImageQuant software (GE Healthcare).

**In vitro transcription (IVT)**

Multiround in vitro transcription reactions were performed (Porrua et al. 2009) in a final volume of 20 µl containing 35 mM Tris-acetate (pH 7.9), 70 mM potassium acetate, 5 mM magnesium acetate, 20 mM ammonium acetate, 5% glycerol, 1 mM DTT, 5 µg BSA, and 12.6 µM of a supercoiled plasmid template that contained PgdhA (pMPO325 for the wild-type promoter, pMPO349 for deletion of sites III and IV, pMPO350 for mutation of site II, and pMPO351 for mutation of site I) or PglnK (pMPO316), as previously described. For the
glnK promoter activation experiments, E. coli core RNA polymerase (Epicentre) (100 nM) and P. putida σ\(^{54}\) factor (200 nM) were added and subsequently incubated for 10 min at 30°C. Different concentrations of NtrC were then added, and the reactions were incubated for an additional 10 min at 30°C. For the gdhA promoter repression experiments, different concentrations of NtrC were added and, after 10 min of incubation at 30°C, E. coli σ\(^{70}\) holoenzyme (Epicentre) was added to 55 nM. The reactions were then incubated for an additional 10 min at 30°C. When indicated, the order of addition of the E. coli σ\(^{70}\) holoenzyme and NtrC was reversed.

After 20 min of incubation, a mixture of ATP, GTP, CTP (final concentration 0.4 mM each), UTP (0.07 mM), and \([\alpha^{32}P]\)-UTP (0.033 μM, Perkin Elmer) was added to initiate multiround in vitro transcription. After a 5-min incubation at 30°C, re-initiation was prevented by the addition of heparin (final concentration 0.1 mg ml\(^{-1}\)). The samples were incubated for an additional 5 min at 30 °C, and the reactions were terminated by the addition of 5 μl of stop buffer (150 mM EDTA, 1.05 M NaCl, 14 M urea, 3% glycerol, 0.075% xylene cyanol, and 0.075% bromophenol blue). The samples were run in 6% polyacrylamide-urea gels in Tris-borate-EDTA buffer at room temperature. The gels were dried, exposed to radiosensitive screens, scanned in a Typhoon 9410 scanner (GE healthcare), and analysed using ImageQuant software (GE Healthcare).

**Phosphorylation of NtrC by acetyl-phosphate**

The in vitro phosphorylation of NtrC is based on the experiments described by Feng et al (Feng et al. 1995). The reactions were performed in a total volume of 5 μl of binding buffer if the subsequent experiment was an EMSA or 5 μl of reaction buffer if the subsequent experiment was an IVT. Each reaction contained 2.5 μg of BSA, 0–2 μM of NtrC\(^{wt}\) or NtrC\(^{D55E,S161F}\), and 30 mM (for EMSA experiments) or 40 mM (for IVT experiments) acetyl-
phosphate (a concentration of 10 mM in the final reaction). The mixture was incubated for 10 min at room temperature before use in the EMSAs or IVT reactions.

Kinetics of *glnK* and *gdhA* transcription after ammonium addition

To examine de mRNA levels of *glnK* and *gdhA* genes after ammonium addition, preinocula of *P. putida* KT2442 were grown to saturation in minimal medium under nitrogen-limiting conditions (L-serine). Cells were then diluted in minimal medium under nitrogen-limiting conditions, and the diluted cultures were grown to mid-exponential phase (OD<sub>600</sub> = 0.3-0.4). Then, 1 g l<sup>−1</sup> of ammonium chloride was added to the culture, samples were taken at different times after the addition of ammonium. Total RNA from the samples was prepared as previously described (Garcia-Gonzalez et al. 2005). Finally, total RNA was retrotranscribed and quantitative PCR was performed as previously described (Yuste et al. 2006). The results shown are the average of two independent quantitative PCR of each of two independent cultures.

Acknowledgments

IHF from *E. coli* and the alternative sigma factor σ<sup>54</sup> from *P. putida* were kind gifts from Ray Dixon (Norwich, UK) and Victoria Shingler (Umea, Sweden), respectively. We are grateful to all members of the laboratory for their insights and helpful suggestions, and Guadalupe Martín Cabello and Nuria Pérez Claros for technical help. Work in the authors’ laboratory is funded by the Spanish Ministry of Science and Innovation, grants BIO2007-63754, BIO2008-01805 and CSD2007-00005, and by the Andalusian government, grant P07-CVI-2518.
# Table 1. Bacterial strains, plasmids and oligonucleotides used in this work

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<td>(Franklin et al., 1981)</td>
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1. Coordinates are related to the transcriptional start of the gene
Figure legends

Figure 1. In vivo expression from the *P. putida gdhA* promoter. Expression was measured as β-galactosidase activity of a *gdhA-lacZ* transcriptional fusion under nitrogen-excess (ammonium + serine) or nitrogen-limiting (serine) conditions in the KT2442 wild-type strain and the *ntrC* mutant strain. Gray bars, empty vector pMPO234; white bars, *gdhA-lacZ* fusion pMPO323. The values are presented as the average of at least three independent assays. The error bars indicate the standard deviation of the means.

Figure 2. Primer extension analysis of the *gdhA* transcript. (A) Primer extension reaction with total RNA prepared from cultures of *P. putida* KT2442 grown under nitrogen-sufficient (NS, ammonium plus serine) or nitrogen-limiting (S, serine) conditions. The GATC lanes show the sequencing ladder (non-coding strand). (B) Sequence of the *gdhA* promoter region showing the putative NtrC binding sites (open boxes), the transcription initiation sites (two consecutive T in bold with an arrow), and the translation initiation site (underlined ATG).

Figure 3. DNase I footprint of the *gdhA* promoter region. (A) Predicted NtrC binding sites (open boxes), non-predicted NtrC binding site (dotted box), protected regions (black bars), and hypersensitive positions (dots) are marked. The NtrC concentrations were 0, 0.15, 0.3, 0.75 and 1.5 µM. The coordinates are relative to the *gdhA* transcripational start site. (B) Schematic representation of the protected regions on the sequence of the *gdhA* promoter, with the same indications as for the footprint pattern.

Figure 4. In vitro transcriptional repression of *gdhA*. *In vitro* transcription from the *PgdhA* promoter region in the presence of 0, 0.15, 0.3, 0.75, or 1.5 µM NtrC<sup>D55E,S161F</sup>. (A) *In vitro*
transcription following the addition of NtrC to templates pre-incubated with E-σ70 (upper panel) or the addition of E-σ70 to templates pre-incubated with NtrC (lower panel). (B) The average value of the transcript levels from two independent experiments plotted against the concentration of NtrC. The values obtained in the absence of NtrC were set as 100%, and the transcript levels are expressed relative to this value. The error bars represent the standard deviation. (■) in vitro transcription adding the RNA polymerase first; (□) in vitro transcription adding NtrC first.

**Figure 5.** The role of the NtrC binding sites on gdhA transcriptional repression. A comparison of the NtrC to repress transcription from gdhA promoters carrying mutations in the NtrC-binding sites. (A) *In vitro* transcription assays were performed with σ70-RNAP and NtrC\(^{D55E,S161F}\) and transcription templates carrying the mutations in sites I, II, and III+IV (see experimental procedures). The NtrC concentrations were 0, 0.3, 0.5, 0.7, and 1 µM. (B) The average value of the transcript levels from two independent experiments plotted against the concentration of NtrC. The values obtained with the wild type promoter in the absence of NtrC were set as 100%, and the transcript levels are expressed relative to this value. The error bars represent the standard deviation. (♦) wild type promoter, (●) site I mutant, (▲) site II mutant and (■) sites III and IV deletion mutant.

**Figure 6.** Effect of the mutations in NtrC binding sites I and II on binding to the gdhA promoter. DNase I footprint of the gdhA promoter region carrying mutations in site I or site II, with the NtrC binding sites (open boxes), protected regions (black bars), and hypersensitive positions (dots) marked. The mutations are marked in the corresponding site with an asterisk The bottom strand was labelled. The concentrations of NtrC\(^{D55E,S161F}\) used were 0, 0.15, 0.3, 0.75, and 1.5 µM.
Figure 7. Effect of NtrC phosphorylation on its transcriptional activation. *In vitro* transcription activation from the *glnK* promoter using σ^N^-RNA polymerase, IHF, and NtrC. Both wild-type NtrC and NtrC^D55E,S161F were incubated in the presence (+P) or absence (-P) of acetyl phosphate. The concentrations of NtrC used were 0, 0.2, 0.35, 0.5, and 0.7 µM.

Figure 8. Effect of phosphorylation on the binding affinity of NtrC and NtrC^D55E,S161F to the *gdhA* promoter region. (A) EMSA of a linear fragment containing the whole *gdhA* promoter region in the presence (+P) or absence (-P) of acetyl phosphate. The NtrC and NtrC^D55E,S161F concentrations were 0, 0.5, 0.7, 1, and 1.5 µM. (B) The average values of non-retarded band quantified from two independent experiments plotted against the concentration of NtrC. The values obtained in the absence of NtrC were set as 100%, and non-bound DNA was expressed relative to this value. The error bars represent the standard deviation. (◆) wild type NtrC without phosphate, (■) wild type NtrC with phosphate, (▲) NtrC^D55E,S161F without phosphate and (●) NtrC^D55E,S161F with phosphate.

Figure 9. Effect of phosphorylation of NtrC and NtrC^D55E,S161F on the repression of *PgdhA* transcription. (A) *In vitro* transcription from *gdhA* using different concentrations of NtrC and NtrC^D55E,S161F incubated in the presence (+P) or absence (-P) of acetyl phosphate. The NtrC concentrations used are indicated on top of each lane. (B) The average values of transcript levels from two independent experiments plotted against the concentration of NtrC. The values obtained in the absence of NtrC were set as 100%, and the transcript levels are expressed relative to this value. The error bars represent the standard deviation. (◆) wild type NtrC without phosphate, (■) wild type NtrC with phosphate, (▲) NtrC^D55E,S161F without phosphate and (●) NtrC^D55E,S161F with phosphate.
Figure 10. Kinetics of *glnK* and *gdhA* transcriptional response to ammonium addition.

Time course of *glnK* and *gdhA* mRNA levels estimated by quantitative RT-PCR after addition of 1 g l$^{-1}$ ammonium to cultures of *P. putida* KT2442 grown in nitrogen limitation (serine as the nitrogen source) to mid-exponential phase (OD$_{600}$ = 0.3-0.4). (●) *glnK*, (■) *gdhA*.

Figure 11. Loop formation during *gdhA* transcriptional repression mediated by NtrC.

Schematic representation of NtrC bound to its four sites in the *gdhA* promoter region, the interactions between the NtrC dimers bound to the neighbouring sites, and the interactions between the dimers bound to the distant sites, which result in DNA looping. The arrow indicates the transcriptional start site.
REFERENCES


Figure 1

\[ \text{β-galactosidase activity (Miller units)} \]

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Figure 3

A

B
Figure 4

A

polymerase → NtrC

NtrC → polymerase

B

% Transcripts vs [NtrC] (µM)
Figure 5

A

B

% transcripts

[NtrC] (µM)
Figure 7

![Diagram showing the comparison of NtrC<sub>wt</sub> and NtrC<sub>D55E,S161F</sub> with and without phosphate (P).]
Figure 8

A

\begin{align*}
\text{NtrC}^{\text{wt}} & \quad \text{NtrC}^{D55E,S161F} \\
- \text{P} & \quad - \text{P} \\
+ \text{P} & \quad + \text{P}
\end{align*}

B

\begin{align*}
\text{% non retarded} & \quad \text{[NtrC] (µM)} \\
\hline
0 & 0.25 & 0.5 & 0.75 & 1 & 1.25 & 1.5 \\
120 & 100 & 80 & 60 & 40 & 20 & 0
\end{align*}
Figure 9

A

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RNA1
gdhA

B

% transcripts

[NtrC] (µM)
Figure 10