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RESEARCH LETTER

Distinct roles for NtrC and GlnK in nitrogen regulation of the *Pseudomonas* sp. strain ADP cyanuric acid utilization operon

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Abstract

The *Pseudomonas* sp. strain ADP *atzDEF* operon encodes the enzymes involved in cyanuric acid mineralization, the final stage of the *s*-triazine herbicide atrazine degradative pathway. We have previously shown that *atzDEF* is under nitrogen control in both its natural host and *Pseudomonas putida* KT2442. Expression of *atzDEF* requires the divergently encoded LysR-type transcriptional regulator AtzR. Here, we take advantage of the poor induction of *atzDEF* in *Escherichia coli* to identify *Pseudomonas* factors involved in nitrogen control of *atzDEF* expression. Simultaneous production of *P. putida* NtrC and GlnK, along with AtzR, restored the normal *atzDEF* regulatory pattern. Gene expression analysis in *E. coli* and *P. putida* indicated that NtrC activates *atzR* expression, while the role of GlnK is to promote AtzR activation of *atzDEF* under nitrogen limitation. Activation of *atzDEF* in a mutant background deficient in GlnK uridylylation suggests that post-translational modification is not strictly required for transduction of the nitrogen limitation signal to AtzR. The present data and our previous results are integrated in a regulatory circuit that describes all the known responses of the *atzDEF* operon.

Introduction

General nitrogen control is a global regulatory network that activates the expression of a variety of genes involved in assimilation of alternative nitrogen sources in response to decreased nitrogen availability. In many *Proteobacteria*, nitrogen control is mediated by the sensor kinase/phosphatase NtrB and the response regulator NtrC. A PII signal transduction protein transmits the nitrogen status to NtrB–NtrC by stimulating the phosphatase activity of NtrB under nitrogen excess, which results in decreased dephosphorylation of NtrC. NtrC-phosphate in turn activates transcription from a large number of promoters that are recognized by the alternative σ factor σ^N (Merrick & Edwards, 1995; Reitzer, 2003).

The widespread PII protein superfamily is comprised of small trimeric signal transduction proteins that integrate carbon, nitrogen and energy status signals. PII proteins regulate multiple cellular functions related to nitrogen metabolism, including ammonium transport and assimilation via glutamine synthetase, nitrogen fixation and nitrogen-responsive transcriptional regulation, by means of protein–protein interactions. PII protein activity is regulated by allosteric binding of 2-oxoglutarate, ADP and ATP. In addition, proteobacterial PII proteins are also regulated by GlnD-dependent reversible uridylylation in response to changes in the intracellular glutamine concentration. Details on PII protein structure and function have been reviewed recently (Ninfa & Atkinson, 2000; Arcondeguy *et al.*, 2001; Leigh & Dodsworth, 2007; Forchhammer, 2008). Some organisms harbor two PII proteins with partially overlapping functions. In the enterics, constitutively produced GlnB mediates most responses to reduced nitrogen

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availability, including modulation of NtrB phosphatase activity. GlnK, however, is produced during nitrogen limitation, can replace GlnB in some of its functions, and has been shown to have at least two specific targets: the high-affinity ammonium transporter AmtB and the nitrogen fixation-specific two-component system NifL–NifA (Leigh & Dodsworth, 2007; Forchhammer, 2008).

Little is known about nitrogen control in bacteria from the genus *Pseudomonas*. The sequenced genomes of *Pseudomonas* strains contain homologs to most enterobacterial nitrogen control genes, but only encode one PII protein, GlnK (Stover *et al.*, 2000; Nelson *et al.*, 2002; Buell *et al.*, 2003; Paulsen *et al.*, 2005). Our recent work underlines the central role of NtrC in the control of nitrogen assimilation in *Pseudomonas putida* (Hervás *et al.*, 2008, 2009).

Pseudomonas sp. strain ADP (Mandelbaum *et al.*, 1995) uses the *s*-triazine herbicide atrazine as the sole nitrogen source. This pathway is encoded by *atzA*, *atzB* and *atzC*, responsible for the conversion of atrazine into cyanuric acid, and the *atzDEF* operon, involved in cyanuric acid mineralization to carbon dioxide and ammonium (Martinez *et al.*, 2001; Wackett *et al.*, 2002). Expression of the *atzDEF* operon is induced in response to cyanuric acid, and nitrogen limitation (García-González *et al.*, 2005). The gene transcribed divergently from *atzDEF*, *atzR*, encodes a LysR-type transcriptional regulator. Transcription of *atzR* is activated by NtrC and autorepressed (García-González *et al.*, 2005; Porrúa *et al.*, 2009). AtzR activates *atzDEF* transcription in response to both signals, and is sufficient for the cyanuric acid response (Porrúa *et al.*, 2007; O. Porrúa, unpublished data). NtrC is required, along with AtzR, for nitrogen regulation of *atzDEF*, but its effect is likely indirect and the mechanisms involved are unclear. We have proposed a regulatory circuit in which AtzR-dependent activation of *atzDEF* transcription is modulated by cyanuric acid and a nitrogen limitation signal transduced by the general nitrogen control system (García-González *et al.*, 2005). However, the identity of the element that transmits this signal to AtzR remains unknown.

In the present work, we aim to identify the factors involved in nitrogen control of *atzDEF* and their specific roles in the regulation. With these findings, we expect to complete a regulatory circuit that takes into account all the known regulatory responses of the *atzDEF* operon.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains and their relevant genotypes are summarized in Table 1. Minimal medium (Mandelbaum *et al.*, 1993) contained $2\,g\,L^{-1}$ glucose (for *Escherichia coli*) or 25 mM sodium succinate (for *P. putida*) as the sole carbon source. Nitrogen sources were $1\,g\,L^{-1}$ ammonium chloride (nitrogen excess) or $1\,g\,L^{-1}$ L-arginine (nitrogen limitation) for *E. coli*, and $1\,g\,L^{-1}$ glutamine (nitrogen excess) or $1\,g\,L^{-1}$ serine plus $25\,mg\,L^{-1}$ glutamine (nitrogen limitation) for *P. putida*. Luria–Bertani medium was used as rich medium (Sambrook *et al.*, 2000). Antibiotics and other additions were used as described (García-González *et al.*, 2005; Porrúa *et al.*, 2007).

Plasmid and strain construction

Plasmids and oligonucleotides are summarized in Table 1. DNA manipulations were performed according to standard procedures (Sambrook *et al.*, 2000). Plasmid DNA was transferred to *E. coli* and *P. putida* strains by transformation (Inoue *et al.*, 1990), triparental mating (Espinosa-Urgel *et al.*, 2000) or electroporation (Koch *et al.*, 2001). *Escherichia coli* DH5α was used as a host in cloning procedures.

Plasmid pMPO307 was constructed by subcloning an EcoRI–PstI fragment containing the *P. putida glnAntrBC* operon into EcoRI- and PstI-digested pBR322. The *P. putida ntrC* coding sequence was excised from pMPO231 (Hervás *et al.*, 2009) with XbaI, and cloned in XbaI-cleaved pMPO301 to yield pMPO243. The *P. putida glnKamtB* operon and promoter region was cleaved from pMPO254 with EcoRI and ligated into EcoRI-digested pBluescriptII SK(+), yielding pMPO256. This insert was excised with EcoRV and PstI and cloned into PstI- and SspI-digested pBR322 to produce pMPO257. Plasmid pMPO258 was constructed by cleaving pMPO257 with PstI and SmaI and religating. Construction of pMPO270 was initiated by cloning PCR-amplified *P. putida glnK* into the P*tac*-based overproduction vector pVLT31 digested with EcoRI and HindIII. The resulting plasmid, pMPO267, was subsequently cleaved with EcoRI and MluI to remove P*tac*, which was replaced by a BamHI–MluI fragment from pMPO301 containing P*lac*UV5, yielding pMPO270. An EcoRV–SmaI fragment prom MPO256 containing *glnK* and its promoter region was cloned into Sma-linearized pKT230 to produce pMPO272.

The $glnK^{Y51F}$ mutant allele was generated by overlap extension PCR site-directed mutagenesis using mutagenic oligonucleotides Y51F-fwd and Y51F-rev and external universal oligonucleotides forward (−40) and reverse (−48) as primers, and pMPO262 as a template, essentially as described (Aiyar *et al.*, 1996). The PCR product was digested with SphI and HindIII and ligated into SphI- and HindIII-digested pBluescript II KS(+), resulting in pMPO286. Construction of pMPO288 was initiated by cloning a SacI–HindIII fragment of pMPO286 containing *P. putida* $glnK^{Y51F}$ into pVLT31 digested with SacI and HindIII. The resulting plasmid, pMPO287, was cleaved with XbaI and MluI to remove P*tac*, which was replaced by a BamHI–MluI fragment from pMPO301 containing P*lac*UV5 to yield pMPO288.

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Table 1. Strains and plasmids used in this work

Strain	Genotype/phenotype	Origin/references
P. putida		
KT2440	mt-2 *hsdR*1 ($r^- m^+$) Cm^r Rif^s	Franklin *et al*. (1981)
KT2442	mt-2 *hsdR*1 ($r^- m^+$) Cm^r Rif^r	Franklin *et al*. (1981)
MPO217	mt-2 *hsdR*1 ($r^- m^+$) Cm^r Rif^r Δ*glnK*::Km	This work
E. coli		
ET8000	*rbs lacZ*::IS*1 gyrA hutC*$_{Ka}$ Ntr^+	MacNeil *et al*. (1982)
ET8556	*rbs lacZ*::IS*1 gyrA hutC*$_{Ka}$ *ntrC1488*	MacNeil *et al*. (1982)
Plasmid	Relevant features	Origin/references
pMPO104	*atzR–lacZ* protein fusion in pMPO200. Ap^r	García-González *et al*. (2005)
pMPO200	Broad host-range translational fusion vector based in pBBR1MCS-4. Ap^r	García-González *et al*. (2005)
pMPO202	*atzD–lacZ* protein fusion in pMPO200. Ap^r	García-González *et al*. (2005)
pMPO204	*atzD–lacZ* protein fusion in pMPO200 containing *atzR*. Ap^r	García-González *et al*. (2005)
pMPO231	pT7-7-based *P. putida* NtrC overexpression vector	Hervás *et al*. (2009)
pMPO243	pACYC184 derivative harboring *P. putida ntrC* transcribed from P*lac*UV5 and *lacI*^{q}. Cm^r	This work
pMPO253	pLAFR3-derived cosmid harboring fragment of the *P. putida* KT2440 spanning ORFs PP5220 to PP5242*. Tc^r	This work
pMPO254	pLAFR3-derived cosmid harboring fragment of the *P. putida* KT2440 spanning ORFs PP5226 to PP5247*. Tc^r	This work
pMPO257	*P. putida glnKamtB* cloned in pBR322. Tc^r	This work
pMPO258	Deletion derivative of pMPO257 harboring only *glnK*. Constructed by PstI–SmaI digestion and religation. Tc^r	This work
pMPO260	Allelic replacement plasmid based on pEX18Tc, containing a kanamycin resistance gene flanked by *glnK* chromosomal flanking sequences. Tc^r Km^r	This work
pMPO262	*P. putida glnK* cloned in pBluescript SK(+). Ap^r	This work
pMPO268	pJB861 derivative expressing *atzR* from the P_m promoter. Gm^r Tra^- Mob^+ IncP	This work
pMPO270	pVLT31 derivative harboring *P. putida glnK* transcribed from P*lac*UV5, and *lacI*^{q}. Tc^r IncQ	This work
pMPO272	*P. putida glnK* cloned in pKT230. Str^r IncQ	This work
pMPO286	*P. putida glnK*^{Y51F} cloned in pBluescript KS(+). Ap^r	This work
pMPO287	pVLT31 derivative harboring *P. putida glnK*^{Y51T} transcribed from P*tac*, and *lacI*^{q}. Tc^r IncQ	This work
pMPO288	pVLT31 derivative harboring *P. putida glnK*^{Y51T} transcribed from P*lac*UV5, and *lacI*^{q}. Tc^r IncQ	This work
pMPO301	pACYC184 derivative harboring the P*lac*UV5 promoter and *lacI*^{q}. Cm^r	A.B. Hervás, unpublished data
pMPO307	*P. putida* KT2440 chromosomal fragment harboring *glnAntrBC* cloned in pBR322. Tc^r	This work
pUT-miniTn*5*-Km	MiniTn*5*-Km delivery plasmid. Ap^r Km^r	de Lorenzo *et al*. (1990)
pVLT31	Broad host-range expression vector harboring the P*tac* promoter and *lacI*^{q}. Tc^r Tra^- Mob^+ IncQ	de Lorenzo *et al*. (1993)
Oligonucleotide	Sequence (5′–3′)	
AmtB-fwd	CCGGATCCACCGATACCGACGCGATC	
Amt-rev	GCAAAGCTTGCGTTGAAACCGAACC	
Forward (−40)	GTTTTCCCAGTCACGAC	
PP5235-fwd	TCGAATTCATCAGCGGCTGACC	
PP5235-rev	CTGGATCCTGGCTGTGACTAGCTTC	
Reverse (−48)	AGCGGATAACAATTTCACACAGGA	
Y51F-fwd	GCGGTGCTGAATTCGTGGTCGATTTCC	
Y51F-rev	GGAAATCGACCACGAATTCAGCACCGC	

Some of the cloning procedures are simplified. Detailed descriptions are available upon request.*ORF designations according to the *Pseudomonas putida* KT2442 sequencing project nomenclature (Nelson *et al*., 2002).

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For construction of the Δ*glnK*::Km mutant MPO217, the allelic replacement plasmid pMPO260 was generated. Initially, ~700 bp from the chromosomal regions flanking *glnK* were PCR-amplified with oligonucleotide pairs PP5235-fwd and PP5235-rev (upstream region), or AmtB-fwd and Amt-rev (downstream region). The PCR products were cleaved with EcoRI and BamHI or BamHI and HindIII (for upstream or downstream flanking sequences, respectively) and three-way ligated into EcoRI- and HindIII-digested pEX18Tc. The BamHI-excised kanamycin resistance gene from pUTminiTn*5*-Km was then cloned into the BamHI site generated between the upstream and the downstream flanking sequences yielding pMPO260. This plasmid was transferred to *P. putida* KT2442 by electroporation, and selection of integration and allelic replacement was performed essentially as described (Hoang *et al.*, 1998; Llamas *et al.*, 2000).

β-Galactosidase assays

Steady-state β-galactosidase assays were used to examine the expression of *lacZ* fusions in *E. coli* and *P. putida*. Fusion-bearing strains were grown as described (García-González *et al.*, 2005), and β-galactosidase activity was determined from sodium dodecyl sulfate- and chloroform-permeabilized cells (Miller, 1992).

Results and discussion

Pseudomonas putida NtrC stimulates *atzR* and *atzDEF* expression in *E. coli*

We attempted to replicate nitrogen control of the *atzDEF* operon in the Ntr$^+$ *E. coli* strain ET8000 (MacNeil *et al.*, 1982) using *lacZ* translational fusions to *atzR* (pMPO104), or *atzD* (pMPO204, also harboring the complete *atzR*) (García-González *et al.*, 2005). Expression was tested in medium containing ammonium (nitrogen excess) or arginine (nitrogen limitation) as the sole nitrogen source. The inducer cyanuric acid was not used in these assays, as nitrogen regulation and cyanuric acid induction occur independently. Expression of both fusions was very low and only marginally responsive to nitrogen limitation in *E. coli* (Table 2). Plasmid pMPO307, harboring the *P. putida glnAntrBC* operon, stimulated *atzR–lacZ* expression in response to nitrogen limitation, but failed to increase *atzD–lacZ* expression under nitrogen limitation. These results are consistent with our observation that NtrC directly activates transcription from the P*atzR* promoter (Porrúa *et al.*, 2009), and suggest that NtrC is not directly responsible for P*atzDEF* activation. It may be argued that the concentration of AtzR, which is required for *atzDEF* expression, may be limiting under these conditions. However, this is not the case, as the *atzD–lacZ* fusion was induced 100-fold

Table 2. Effect of *Pseudomonas putida* GlnK on expression of *atzD–lacZ* and *atzR–lacZ* fusions in *Escherichia coli*

			Nitrogen source	
			Ammonium	Arginine
E. coli strain	Fusion plasmid	Other plasmids	β-Galactosidase activity	
ET8000 (Ntr$^+$)	pMPO104 (*atzR–lacZ*)	None	3 ± 1	12 ± 3
		pMPO307 (NtrB$^+$C$^+$)	3 ± 1	289 ± 20
	pMPO204 (*atzR–atzD–lacZ*)	None	1 ± 0	5 ± 1
		pMPO307 (NtrB$^+$C$^+$)	1 ± 0	4 ± 1
ET8556 (*ntrC*$^-$)	pMPO204 (*atzR–atzD–lacZ*)	pMPO243 (P*lac*UV5-*ntrC*)	1 ± 0	4 ± 1
		pMPO243 (P*lac*UV5-*ntrC*)+ pMPO253 (GlnK$^+$ AmtB$^+$ cosmid)	3 ± 0	45 ± 1
		pMPO243 (P*lac*UV5-*ntrC*)+ pMPO254 (GlnK$^+$ AmtB$^+$ cosmid)	3 ± 0	63 ± 14
		pMPO243 (P*lac*UV5-*ntrC*)+ pMPO257 (GlnK$^+$ AmtB$^+$ plasmid)	2 ± 0	433 ± 71
		pMPO243 (P*lac*UV5-*ntrC*)+ pMPO258 (GlnK$^+$ AmtB$^-$ plasmid)	2 ± 0	556 ± 158
	pMPO202 (*atzD–lacZ*)	pMPO243 (P*lac*UV5-*ntrC*)	2 ± 0	4 ± 0
		pMPO243 (P*lac*UV5-*ntrC*)+ pMPO258 (GlnK$^+$ AmtB$^-$ plasmid)	2 ± 0	9 ± 1
	pMPO104 (*atzR–lacZ*)	pMPO243 (P*lac*UV5-*ntrC*)	3 ± 1	158 ± 45
		pMPO243 (P*lac*UV5-*ntrC*)+ pMPO258 (GlnK$^+$ AmtB$^-$ plasmid)	3 ± 0	11 ± 2

The β-galactosidase activity is expressed in Miller units. Values are the average and SD of at least three independent measurements. IPTG induction of the P*lac*UV5 promoter in pMPO243 was not required.
IPTG, isopropyl-β-D-thiogalactopyranoside.

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in the presence of 0.1 mM cyanuric acid (data not shown), a process known to be mediated by AtzR (Porrúa *et al.*, 2009).

It is intriguing that *E. coli* NtrC, encoded in the ET8000 genome, did not stimulate *atzR* expression under our assay conditions. However, *E. coli* NtrC readily activated *atzR* when expressed from the P*lac*UV5 promoter in a plasmid (data not shown). Because NtrC does not have a binding site at the *atzR–atzDEF* promoter region (Porrúa *et al.*, 2009), activation may require a higher NtrC concentration that may be naturally achieved in *P. putida*, but not in *E. coli*. In addition, the stronger binding of *P. putida* E-σ^N to its cognate promoters (Bernardo *et al.*, 2009) may also contribute to UAS-independent activation (Porrúa *et al.*, 2009).

Pseudomonas putida GlnK stimulates *atzDEF* expression in response to nitrogen limitation in *E. coli*

To screen for *Pseudomonas* factors required for *atzDEF* activation in response to nitrogen limitation, a *P. putida* KT2440 gene bank in pLAFR3 (Staskawicz *et al.*, 1987) was used to complement the P*atzDEF* activation defect under nitrogen limitation in an *E. coli* indicator strain. For this purpose, we chose ET8556 (an *ntrC*$^-$ derivative of ET8000), bearing pMPO243, which expresses *P. putida ntrC* from the P*lac*UV5 promoter, and the *atzD–lacZ* fusion plasmid pMPO204. The presence of pMPO243 restored growth of ET8556 on arginine as the sole nitrogen source (a widely used indicator of the Ntr system function), and stimulated *atzR–lacZ* expression similar to pMPO307, but failed to stimulate *atzD–lacZ* expression (Table 2). Upon transfer of the gene bank to the indicator strain, two clones were selected that formed blue colonies on nitrogen-limited minimal plates containing X-gal and displayed increased *atzD–lacZ* expression (11–15-fold) in nitrogen-limited medium (Table 2). The cosmids in these clones, designated pMPO253 and pMPO254, bore overlapping inserts that included the *P. putida glnK-amtB* operon, encoding the only *P. putida* PII protein and a high-affinity ammonium transporter. Plasmids pMPO257 and pMPO258, harboring the complete *P. putida glnKamtB* operon or only *glnK*, respectively, were constructed and their effect on *atzD–lacZ* expression was tested. Both plasmids mimicked the behavior of the cosmids, but expression was stimulated an additional ~10-fold (Table 2). These results indicate that *glnK* is the factor required for *atzD–lacZ* upregulation under nitrogen limitation. In contrast, pMPO258 induced a 13-fold decrease in *atzR–lacZ* expression under nitrogen limitation. Despite this reduction, AtzR was still required for *atzDEF* activation, as pMPO258 had no effect on expression of an *atzD–lacZ* fusion lacking *atzR* (see pMPO202 in Table 2). Taken together, our results indicate that NtrC and GlnK have separate functions in nitrogen control of *atzDEF* expression, the role of NtrC being activation of *atzR*, while GlnK is, along with AtzR, responsible for activation of *atzDEF*. As shown above for NtrC, neither of the two *E. coli* PII proteins can functionally replace their *P. putida* counterpart under our assay conditions. We have not yet sought an explanation for this defect.

GlnK mediates nitrogen regulation of *atzDEF* in *P. putida*

To test the effect of GlnK on *atzR* and *atzDEF* expression in a *Pseudomonas* strain, a Δ*glnK* mutant, designated MPO217, was constructed in the *P. putida* KT2442 background. We have used this organism widely for *atz* gene expression studies, because *Pseudomonas* sp. ADP is extremely resistant to genetic manipulation (García-González *et al.*, 2005; Porrúa *et al.*, 2007; Porrúa *et al.*, 2009). Expression from the *atzR–lacZ* and *atzD–lacZ* fusion plasmids pMPO104 and pMPO204 was monitored in KT2442 and MPO217. As a control, pMPO224, bearing a *lacZ* fusion to the NtrC-dependent *nifLA* operon of *Klebsiella pneumoniae*, was also tested (Table 3).

As expected, all three fusions were induced (25–45-fold) in KT2442 under nitrogen limitation. Expression of the NtrC-activated *nifLA–lacZ* and *atzR–lacZ* fusions was near-constitutive in the Δ*glnK* strain (induction ratio was ~2-fold). Constitutive expression of NtrC-activated genes has been shown in *E. coli glnB glnK* mutants, lacking both PII proteins (Atkinson & Ninfa, 1998), and is attributed to decreased NtrB-dependent dephosphorylation of NtrC under nitrogen excess. Nitrogen regulation of both fusions was restored when the assays were performed in the Δ*glnK* strain harboring pMPO270, which expresses *P. putida glnK* from the P*lac*UV5 promoter. The extent of nitrogen regulation of the *atzR–lacZ* fusion in the complemented strain was comparable to that in KT2442 (24- vs. 45-fold), although the absolute expression levels were somewhat reduced (four- to sevenfold). These results suggest that *P. putida* GlnK assumes the role of the major PII protein in *E. coli*, GlnB, in controlling NtrB-dependent dephosphorylation of NtrC (Reitzer, 2003). In addition, the nitrogen responsiveness of constitutively produced GlnK suggests that post-translational modification of GlnK (likely via GlnD-dependent uridylylation) prevents its interaction with NtrB.

Expression of *atzD–lacZ* in MPO217 was low and unaffected by nitrogen limitation (Table 3), consistent with the hypothesis that GlnK helps AtzR to activate *atzDEF* in response to nitrogen limitation. Constitutive production of GlnK from P*lac*UV5 in pMPO270 resulted in constitutive *atzD–lacZ* expression, while plasmid pMPO272, which produces GlnK from its native nitrogen-regulated promoter (Hervás *et al.*, 2008), restored nitrogen regulation comparable to that in KT2442 (35-fold), albeit the absolute

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Table 3. Effect of GlnK on nitrogen regulation of *atzR* and *atzDEF* in *Pseudomonas putida*

			Growth conditions		
			High N*	Low N*	
P. putida strain	Fusion plasmid	Other plasmids	β-Galactosidase activity		Fold induction†
KT2442 (wild type)	pMPO224 (*nifLA–lacZ*)	None	302 ± 26	12 000 ± 350	40
	pMPO104 (*atzR–lacZ*)	None	18 ± 3	814 ± 85	45
	pMPO204 (*atzR–atzD–lacZ*)	None	370 ± 27	9430 ± 494	25
MPO217 (Δ*glnK*)	pMPO224 (*nifLA–lacZ*)	None	9420 ± 1720	22 600 ± 350	2
		pMPO270 (P*lac*UV5-*glnK*)	200 ± 60	8000 ± 160	40
	pMPO104 (*atzR–lacZ*)	None	1080 ± 196	2280 ± 390	2
		pMPO270 (P*lac*UV5-*glnK*)	5 ± 3	122 ± 38	24
	pMPO204 (*atzR–atzD–lacZ*)	None	671 ± 98	542 ± 42	< 1
		pMPO270 (P*lac*UV5-*glnK*)	23 100 ± 6450	15 100 ± 1890	< 1
		pMPO272 (P*glnK-glnK*)	1350 ± 397	47 500 ± 9640	35

The β-galactosidase activity is expressed in Miller units. Values are the average ± SD of at least three independent measurements. IPTG induction of the P*lac*UV5 promoter in pMPO243 was not required.

*Nitrogen sources were 1 g L^{-1} glutamine (high N) or 1 g L^{-1} serine plus 25 mg L^{-1} glutamine (low N).

†Fold induction was calculated as the ratio of the expression levels in low-N and high-N growth medium.

IPTG, isopropyl-β-D-thiogalactopyranoside.

expression levels were somewhat increased (four- to five-fold) relative to the wild-type strain. These results indicate that nitrogen control of GlnK synthesis is critical for correct *atzDEF* regulation. In addition, physiological modulation of GlnK activity may not be essential for stimulating *atzDEF* expression, provided sufficient GlnK is present. Finally, the basis for the indirect effect of NtrC on *atzDEF* expression is fully explained, because NtrC stimulates the synthesis of both AtzR (García-González *et al.*, 2005) and GlnK (Hervás *et al.*, 2008, 2009).

GlnK uridylylation is not strictly required for *atzDEF* activation

To test whether post-translational modification of GlnK is necessary for *atzDEF* regulation, a mutant *glnK* gene encoding the Y51F GlnK variant was constructed. Tyrosine-51 is the conserved uridylylation site, and substitutions at this position have been shown to prevent PII protein uridylylation (Ninfa & Atkinson, 2000; Arcondeguy *et al.*, 2001; Leigh & Dodsworth, 2007). Plasmid pMPO288, producing $GlnK^{Y51F}$ from the P*lac*UV5 promoter, was constructed and its ability to stimulate P*atzDEF* expression under nitrogen excess was determined in the Δ*glnK* mutant *P. putida* strain MPO217. The empty vector, pVLT31, and plasmid pMPO270, producing the wild-type GlnK protein, were used as controls. Because GlnK indirectly influences AtzR expression by controlling NtrC activity, we used an *atzD–lacZ* fusion (pMPO202) that does not harbor *atzR*, and produced AtzR in an NtrC-independent fashion from the 3-methyl benzoate-induced P_m promoter of the toluene/xylene catabolic pathway (Winther-Larsen *et al.*, 2000) in plasmid pMPO268.

Expression of *atzD–lacZ* under nitrogen excess was low (465 ± 29 Miller units) in the Δ*glnK* mutant carrying pVLT31, but was increased over 40-fold (19 800 ± 4420 Miller units) in the presence of wild-type GlnK, to levels comparable to those attained with AtzR produced from its own promoter (see Table 3). Under these conditions, the $GlnK^{Y51F}$ mutant also elicited a 16-fold increase in *atzD–lacZ* expression (7330 ± 1800 Miller units), confirming that GlnK uridylylation is not *conditio sine qua non* for *atzDEF* activation. Taken together, our results support a model in which GlnK modulates the activities of both NtrC (likely via NtrB) and AtzR. However, while the uridylylation/deuridylylation switch appears to be necessary for correct control of the Ntr system, AtzR stimulation may still occur in the absence of GlnK modification.

The *atzR–atzDEF* regulatory cascade

The results presented allow a complete picture of the regulatory cascade that controls the expression of the *atzDEF* operon to be drawn (Fig. 1). AtzR synthesis is induced under nitrogen limitation by the general nitrogen control system: the P*atzR* promoter is transcribed by E-σ^N, activated by NtrC and repressed by its own gene product (García-González *et al.*, 2005; Porrúa *et al.*, 2007, 2009). We have shown that GlnK transmits the nitrogen limitation signal to NtrC, most likely by stimulating NtrB-dependent NtrC dephosphorylation. GlnK, the sole PII protein in *P. putida*, assumes this role, played by the major PII protein GlnB in enterobacteria (Ninfa & Atkinson, 2000; Arcondeguy *et al.*, 2001; Leigh & Dodsworth, 2007; Forchhammer, 2008). Interestingly, NtrC and GlnK are reciprocally regulated, as NtrC also stimulates *glnK* transcription (Hervás

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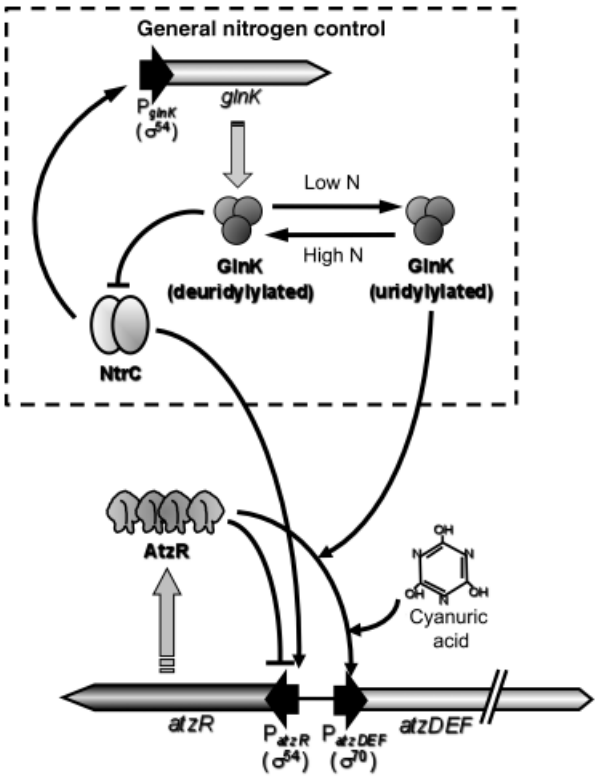

Fig. 1. Regulatory circuit of the *atzDEF* operon. Cartoon depicting the regulatory cascade involved in *atzDEF* regulation. The dashed box indicates the general nitrogen control system. Factors with assumed roles for which we have no direct experimental evidence (NtrB and GlnD) have been omitted for simplicity.

et al., 2008, 2009). The *atzDEF* operon is activated by AtzR in response to nitrogen limitation and cyanuric acid in an additive fashion (García-González *et al.*, 2005), and AtzR alone is accountable for the cyanuric acid response (Porrúa *et al.*, 2007; O. Porrúa & F. Govantes, unpublished data). In addition, AtzR perceives a nitrogen limitation signal transduced by GlnK. Although we cannot rule out an indirect effect of GlnK on AtzR, the fact that *P. putida* GlnK (but not *E. coli* GlnB or GlnK) stimulates *atzDEF* expression in an *E. coli* strain harboring all components of the general nitrogen control cascade suggests that both proteins specifically interact *in vivo*. This interaction appears to require sufficiently high intracellular levels of both AtzR and GlnK, and is not strictly dependent on GlnK uridylylation. However, because synthesis of both GlnK and AtzR is induced under nitrogen limitation (García-González *et al.*, 2005; Hervás *et al.*, 2008, 2009), it is likely that physiological stimulation will only occur under these conditions, in which both proteins are present at higher concentrations and GlnK is uridylylated. The possibility that GlnK may contact some of its targets (such as AtzR) both in its modified and in its unmodified form underscores the importance of nitrogen-regulated GlnK synthesis as a safety mechanism to prevent unwanted interactions during nitrogen-sufficient growth.

The regulatory cascade controlling the *atzDEF* operon regulatory circuit is reminiscent of that in the *K. pneumoniae nif* genes (Martínez-Argudo *et al.*, 2004; Leigh & Dodsworth, 2007). In both systems, nitrogen control operates at two levels. Firstly, transcription of the regulatory genes (*atzR* and *nifLA*) is enhanced by NtrC. Secondly, activity of the regulators is modulated by two dissimilar signals, one sensed directly (redox status by NifL, cyanuric acid by AtzR) and one (nitrogen availability) transduced by GlnK. Furthermore, modulation of NifL (He *et al.*, 1998) and AtzR appears to be independent of the post-translational modification of GlnK. A recent paper has shown the involvement of GlnK in the regulation of ammonium assimilation and nitrogen fixation in *Pseudomonas stutzeri* (He *et al.*, 2008). Dual signaling to a LysR-type regulator via a soluble inducer and a signal transduction protein has not been described previously.

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