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Overlapping repressor binding sites regulate expression of the *Methanococcus maripaludis glnK*$_1$ operon

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Abstract

The euryarchaeal transcriptional repressor NrpR regulates a variety of nitrogen assimilation genes by 2-oxoglutarate-reversible binding to conserved palindromic operators. The number and positioning of these operators varies among promoter regions of regulated genes, suggesting NrpR can bind in different patterns. Particularly intriguing is the contrast between the *nif* and *glnK*$_1$ promoter regions of *Methanococcus maripaludis*, where two operators are present but with different configurations. Here we study NrpR binding and regulation at the *glnK*$_1$ promoter, where the two operator sequences overlap and occur on opposite faces of the double helix. We find that both operators function in binding, with a dimer of NrpR binding simultaneously to each overlapping operator. We show in vivo that the first operator plays a primary role in regulation and the second operator plays an enhancing role. This is the first demonstration of overlapping operators functioning in Archaea.

Keywords

NrpR; *Methanococcus maripaludis*; *glnK*; transcription; regulation

Introduction

Most known transcriptional regulators in the Archaea belong to families that are better known in the Bacteria. This is the case even though the archaeal transcription machinery resembles a simplified version of the eukaryal RNA polymerase II system (Geiduschek & Ouhammouch, 2005). However, a few regulators that have been studied are primarily archaeal. NrpR is a prime example—it is widely distributed in the euryarchaeota but is known in only a few genera of Bacteria (Lie *et al.*, 2007). Discovered and characterized in *Methanococcus maripaludis*, all NrpR homologs appear to function similarly as transcriptional repressors of nitrogen assimilation genes. In *M. maripaludis*, NrpR represses transcription of genes for nitrogen fixation (*nif*, (Cohen-Kupiec *et al.*, 1997)), ammonia assimilation (glutamine synthetase, *glnA*, (Cohen-Kupiec *et al.*, 1999, Lie & Leigh, 2003)), and (shown here) a PII nitrogen sensing protein and an ammonium channel protein.

We are characterizing the mechanism of NrpR function in *M. maripaludis*. Work to date has focused on the regulation of the *nif* operon, whose promoter region contains two inverted repeat operators with the sequence GGAAN$_6$TTCC, spaced three helical turns apart (Fig. 1A). In vitro binding studies and in vivo mutagenesis showed that each operator binds a dimer of NrpR, and both operators together bind two NrpR dimers. Both operators are

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required for maximal repression, with the first operator playing the primary role (Cohen-Kupiec et al., 1997, Lie *et al.*, 2005, Lie & Leigh, 2002). The second operator, whose spacing from the first operator is critical, enhances binding affinity by adding a cooperative component, probably via tetramerization of two NrpR dimers. Binding is reversed by 2-oxoglutarate (2OG), which thus acts as the inducer ligand of NrpR. 2OG, the metabolic precursor of ammonia assimilation, is evidently a widespread indicator of nitrogen starvation in the Bacteria and the Euryarchaeota (Leigh & Dodsworth, 2007). In *M. maripaludis* 2OG varies in intracellular concentration in the range of 0.08 to 0.8 mM, under nitrogen excess and nitrogen deficient conditions respectively (Dodsworth *et al.*, 2005).

Besides the *nif* promoter, the sequence $GGAAN_6TTCC$ is found in the promoter regions of five additional nitrogen-regulated genes in *M. maripaludis: glnA*, genes for two molybdate transporters, a gene for an alanine transporter, and an operon containing the genes $glnK_1$ and $amtB_1$ (Xia *et al.*, 2009). All of these genes presumably function in nitrogen assimilation: besides the previously stated functions of Nif proteins and GlnA, molybdate transporters would help provide molybdenum as a component of the cofactor of nitrogenase, and alanine can serve as a nitrogen source. $glnK_1$ encodes a nitrogen sensor protein of the PII family (Leigh & Dodsworth, 2007), and $amtB_1$ encodes an ammonium channel protein. The $GGAAN_6TTCC$ sequence is completely conserved, suggesting a rigorous requirement for binding of NrpR. We refer to these sequences as nitrogen operators. Here we report on the roles of the nitrogen operators of the $glnK_1$-$amtB_1$ operon in NrpR binding and regulation. Like the *nif* operon, two operators are present, but with radically different configurations, suggesting two different patterns in which two NrpR dimers can bind to an operator pair.

Results

$glnK_1$ is expressed in an operon and regulated by the nitrogen source

The *M. maripaludis* genome sequence (Hendrickson *et al.*, 2004) revealed the presence of a gene cluster encoding three closely-related PII proteins and two ammonium channel proteins (Fig. 2). Two of the PII protein genes were designated $glnK_1$ and $glnK_2$ due to their presence immediately before the ammonium channel proteins $amtB_1$ and $amtB_2$, while the third PII protein gene was designated *glnB*. The sequences of the PII proteins are highly conserved and designation as *glnK* is usually made based on proximity to an *amtB* gene (Thomas *et al.*, 2000).

The coding regions for $glnK_1$ and $amtB_1$ are closely contiguous, suggesting that these two genes may constitute an operon. We characterized the transcript by primer extension, Northern, and RT-PCR analyses. Primer extension analysis (Fig. S1 in supporting information) showed a distinct 5′ end of RNA 248 nucleotides upstream of the putative start codon of $glnK_1$. (This 5′ untranslated region is unusually long and its role in expression, if any, will be the subject of a future study.) A consensus TATA box (TTTATATA) is located 23 bp upstream of this transcription start site (Fig. 1B). For Northern analysis, high degrees of similarity between $glnK_1$, *glnB*, and $glnK_2$ and between $amtB_1$ and $amtB_2$ precluded specific probes in the coding regions. Consequently we used a probe for the 5′ untranslated region of $glnK_1$. Two separate Northern experiments were conducted (Fig. S2), and both showed a band of 2.1 to 2.3 kb. Smaller RNAs were also detected and could be degradation or termination products. A transcript extending from the transcription start site to the end of $amtB_1$ would be 1.8 kb and a transcript extending to the end of *glnB* would be 2.3 kb. We used RT-PCR to further determine the extent of the transcript (Fig. S3). RT-PCR products were detected that extended from the 5′ untranslated region into the coding regions of $glnK_1$ and $amtB_1$ but not *glnB*, indicating that the operon includes only $glnK_1$ and $amtB_1$.

nif and *glnA* expression varies with the nitrogen source (Lie et al., 2005, Lie & Leigh, 2002, Lie & Leigh, 2003, Cohen-Kupiec et al., 1997, Cohen-Kupiec et al., 1999). Both genes are repressed during growth with ammonia, expressed at intermediate levels during growth with alanine, and derepressed during growth with N_2. The Northern analysis of the *glnK*$_1$ transcript indicated a similar pattern of regulation (Fig. S2). One of the two Northern analyses also showed more mRNA fragmentation in preparations from N_2-grown cells than cells grown with the other two nitrogen sources. This effect may not be specific to the *glnK*$_1$ transcript, since we commonly observe fragmentation in mRNA preparations from N_2-grown cells.

The *glnK*$_1$ promoter region contains two overlapping nitrogen operators that bind NrpR

The promoter region of *glnK*$_1$ contains two potential NrpR binding sites, O_1 and O_2, each with complete agreement to the consensus nitrogen operator sequence, GGAAN$_6$TTCC (Fig. 1B). Unlike the *nif* promoter region, where two operators are centered 33 bp apart, in the *glnK*$_1$ promoter region the two putative operators are centered six bp apart, overlap, and are on opposite faces of the DNA helix. Because the juxtaposition of the two putative operators is so different, we wanted to determine if both of them could bind NrpR. Electrophoretic gel mobility shift assays (EMSA) were conducted to assess the binding of NrpR to DNA containing both operators, only one operator (the other operator altered), or neither operator. The mutant operators (Fig. 1B) were designed as inverted sequences of the original operators, eliminating the binding sites but maintaining the palindromic nature the sequences. Each operator alone bound NrpR to produce a single shifted band. Both operators together produced two shifted bands; a faster migrating band dominated at low NrpR concentrations and corresponded to the shift that occurred when only one operator was present, while a more slowly migrating band dominated at high NrpR concentrations (Fig. 3A). With both operators altered, no shift occurred. This gel-shift pattern was similar to the *nif* promoter where a single operator bound one NrpR dimer and two operators bound two NrpR dimers (Lie et al., 2005). These results suggest that despite their overlapping nature, both *glnK*$_1$ operators can simultaneously bind a dimer of NrpR. A binding curve for the wild type *glnK*$_1$ promoter DNA (Fig. 3B, C) indicated a binding dissociation constant (K_d) of 0.3 nM for both operators together, similar to *nif* (Lie et al., 2005). Furthermore, when both operators were present, each bound a dimer of NrpR with high affinity, since the band with the greater shift dominated at NrpR concentrations as low as 0.4 nM. Binding curves for DNA containing mutant operators indicated K_d values of 1.7 nM for O_1 and 3.6 nM for O_2. However, these values must be interpreted with caution since the alteration of each operator necessarily changed nucleotides flanking the eight conserved base pairs of the other operator.

DNAse I footprinting analysis was used to determine the region of DNA that is protected by binding of NrpR to the *glnK*$_1$ operators. Footprinting was performed with DNA containing only the first operator and DNA containing both operators. With DNA containing only O_1 (Fig. 4C and D), the footprint began 4 nucleotides 5′ of the inverted repeat on each strand and extended 11 and 7 nucleotides 3′ of the inverted repeat (coding and non-coding strand respectively). Hence, the footprint covered the inverted repeat and had a somewhat longer extension to the 3′ on each stand. With both operators present (Fig. 4A and B), the footprint conformed to that expected if two NrpR dimers bind, one to each inverted repeat. Hence, the upstream end of the footprint was similar: 4 nucleotides 5′ of the inverted repeat on the coding strand and 7 nucleotides 3′ on the non-coding strand. The downstream end was extended by the addition of the second inverted repeat: 8 nucleotides 3′ of the new inverted repeat on the coding strand and 3 nucleotides 5′ on the non-coding strand. With only O_1, hypersensitive sites were present a few nucleotides 3′ of the inverted repeat on each strand. Hypersensitive sites were not detected when both operators were present.

The difference in the juxtaposition of the two operators in the *glnK*$_1$ promoter region compared to the *nif* promoter region suggests that the configuration in which two NrpR molecules bind would be different. Additional evidence that this is the case comes from a comparative gel shift experiment. Using segments of DNA of similar length, gel shifts at varying NrpR concentrations were used to obtain bands where one or two NrpR dimers were bound, similar to the experiment in Fig. 3B above. With *nif* and *glnK*$_1$ operator regions, the migration was similar when one NrpR dimer was bound. However, with two NrpR dimers bound there was a greater shift with the *glnK*$_1$ operators than with the *nif* operators (Fig. 5). This suggests that the NrpR-DNA complex with two dimers bound is less compact for *glnK*$_1$ than for *nif*.

NrpR binding to the operators in the *nif* promoter region is inhibited by 2OG (Lie et al., 2005). Binding curves for the wild type *glnK*$_1$ operator region (Fig. S4) enabled us to calculate K_d values with 0.1 mM and 0.4 mM 2OG of 1.4 nM and 6.7 nM respectively, compared to 0.3 nM in the absence of 2OG. Hence, in the case of both the *nif* and the *glnK*$_1$ promoter regions, 2OG indicates the nitrogen state of the cell and is the inducer ligand that controls NrpR binding to the operators.

The *glnK*$_1$ operators both mediate nitrogen repression in vivo

To test the roles of the *glnK*$_1$ operators in regulation of the *glnK*$_1$ operon, *lacZ* reporter fusions were constructed. DNA with promoter regions containing wild type and mutant operators identical to those used for the gel shift analyses were used to make reporter constructs, by fusing the *lacZ* coding region to the *glnK*$_1$ start codon. These constructs were incorporated into the *M. maripaludis* genome in single copy using a negative selection strategy (Moore & Leigh, 2005). In addition, an *nrpR* deletion mutation was introduced into the strain containing the *lacZ* fusion to the wild type promoter region. β-galactosidase assays were conducted with cultures grown on N_2, alanine, or ammonia (Fig. 6). With the wild type promoter region (both O_1 and O_2 present), expression was high during growth with N_2, partially repressed during growth with alanine, and markedly repressed during growth with ammonia, in agreement with the results of Northern blots (above). By comparison, no repression occurred when both operators were altered by mutation; in fact, β-galactosidase activities were higher with ammonia or alanine than with N_2. We do not know why growth with N_2 might decrease expression; however, nitrogen fixing cultures have higher energy demands and grow more slowly than non-nitrogen fixing cultures, complicating their direct comparison. Repression also did not occur with the wild type promoter region when *nrpR* was deleted, confirming the role of NrpR. In this case too, β-galactosidase activities were higher with ammonia or alanine than with N_2, though not as markedly. The results with the double operator mutant and the *nrpR* deletion mutant both support the role of NrpR binding to the operators. However, the quantitative difference between these two mutants was unexpected. A direct comparison is complicated by the observation that *nrpR* mutants grow more slowly than *nrpR*$^+$ strains for unknown reasons (Lie & Leigh, 2003).

The *lacZ* fusion strains were also useful in determining the role of each operator individually. O_1 alone mediated repression. This repression was not as marked as with both operators present, suggesting either that O_2 enhances repression or that the mutations altering O_2 lessened the effect of O_1 due to the nucleotide changes flanking O_1. O_2 alone had little discernible effect, since the expression pattern with this mutant resembled that with both operators mutant.

Discussion

Taken together, the results of NrpR binding experiments and the measurements of gene expression support a model in which NrpR binding to both operators results in maximal

repression. O_1 appears to play a primary role in mediating repression, and due to its proximity to the transcription start site may block some step in transcription initiation such as recruitment of RNA polymerase. Repression in vivo was more rigorous when both operators were unaltered than when O_2 was altered. This suggests that O_2 enhances repression, with the caveat that mutations flanking O_1 could have weakened it. NrpR binding studies bolster the idea that O_2 plays an enhancing role. Gel shift and footprinting analyses strongly suggest that NrpR can bind to either operator, and that two NrpR dimers bind to both operators simultaneously. Thus, a greater gel shift occurred when both operators were intact than when only one operator was present, and the footprint with both operators was larger by the expected amount than when only O_1 was present. Why should O_2, which has a perfect 8-bp consensus sequence as does O_1, play only an enhancing role in repression? One possibility is that the binding affinity of O_2 for NrpR is lower, possibly due to differences in flanking sequences. Another explanation is the greater distance of O_2 from the transcription start site, hampering its ability alone to block a step in transcription initiation. In either case, two mechanisms seem possible by which O_2 could enhance repression. First, O_2 could increase the affinity of NrpR binding to the operator region through cooperativity, either through a direct interaction of two NrpR dimers or by an effect on the conformation of the DNA, enhancing the binding of NrpR to O_1. The presence of DNase hypersensitive sites on NrpR-bound O_1 DNA suggests that effects on DNA conformation do occur. Second, NrpR binding to O_2 could exacerbate the effect of NrpR binding to O_1, by helping to block the same or a different step in transcription initiation. By any of these mechanisms, the enhancement of NrpR binding by O_2 would presumably increase the sensitivity of repression to the nitrogen starvation signal 2OG. In addition, if binding of NrpR is cooperative, the effect of 2OG might also be cooperative. Further experiments are needed to distinguish among these possibilities.

To our knowledge, this is the first example in Archaea where two transcriptional regulator molecules bind to overlapping binding sites, although several examples are known in the Bacteria (Augustus *et al.*, 2006, Lawson & Carey, 1993, Phillips & Stockley, 1994, Dahl *et al.*, 1994, White *et al.*, 1998). Structurally, NrpR appears to be unusually versatile in its ability to bind multiply to promoter regions in two different configurations, one where two operators overlap and one where two operators are separated. Models for binding to the *nif* and $glnK_1$ promoter regions are shown in Fig. 7. NrpR binding to the *nif* promoter region is known to be cooperative (Lie *et al.*, 2005), and tetramerization of two NrpR dimers involving faces distant from the DNA binding sites seems likely. In the case of the $glnK_1$ promoter region, if cooperativity occurs, either a different dimer-dimer interaction involving faces of NrpR close to the DNA binding sites, or an effect mediated by a DNA conformational change as mentioned above, may occur. Structural studies are underway to test these models.

Experimental procedures

Cultivation of *M. maripaludis*

Cells were grown under strict anaerobic condition at 37° C as described (Balch *et al.*, 1979). Nitrogen-free medium (Blank *et al.*, 1995, Lie & Leigh, 2002) was supplemented with L-alanine (10 mM), NH_4Cl (10 mM), or N_2 (40% H_2:20% CO_2:40% N_2 replaced 80% H_2:20% CO_2). Growth of *M. maripaludis* in a chemostat was done using a minimal defined medium as described (Xia et al., 2009, Haydock *et al.*, 2004).

Plasmid construction, site directed mutagenesis, and strain construction

Plasmids are listed in Table S1. *M. maripaludis* DNA was extracted using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies) according to the

manufacturer's protocol. The wild type promoter region of *glnK*$_1$ was PCR amplified from *M. maripaludis* S2 DNA with Herculase® polymerase (Stratagene) and primers PglnK1fw and PglnKrv. Digestion of the PCR product with SpeI and NsiI was followed by ligation into vector pWLG40 resulting in pWLG40K$_1$. Similar plasmids with mutant operators were made as follows: PCR products were generated from *M. maripaludis* S2 DNA with Taq-polymerase, forward primer PglnK1fw, and either of three reverse primers, deltaO1rv, deltaO2rv or deltaO1/O2rv to produce mutations in O_1, O_2, and both operators respectively. The products served as “megaprimers” (Sarkar & Sommer, 1990) together with primer Pglnk1rv in a second set of PCR amplifications from *M. maripaludis* S2 DNA to generate products, which were cloned into pCR2.1®-TOPO, excised with SpeI and NsiI, and ligated into pWLG40+lacZ resulting in pWLG40glnK$_1$O$_1$, pWLG40glnK$_1$O$_2$, and pWLG40glnK$_1$O$_1$+O$_2$. pCR2.1glnK$_1$pro was constructed by amplifying the *glnK$_1$* promoter region from S2 genomic DNA with primers FpglnKFNotI and FpglnKRNotI and cloning into pCR2.1 TOPO® (Invitrogen).

A plasmid containing the wild type *glnK$_1$* promoter fused to *lacZ* was constructed as follows: pWLG40K$_1$ DNA was used as template with primers PglnKAscIfw and LacZAscIrv to generate a product that was treated with AscI followed by ligation into pBLprt resulting in pBLPrtK$_1$. Similar plasmids with mutant operators were constructed from pWLG40glnK$_1$O$_1$, pWLG40glnK$_1$O$_2$ and pWLG40glnK$_1$O$_1$+O$_2$ DNA by PCR with primers PglnK1fw and PglnKrv, digestion with AscI, and insertion into pBLPrt to yield pBLPrtglnK$_1$O$_1$, pBLPrtglnK$_1$O$_2$ and pBLPrtglnK$_1$O$_1$+O$_2$. Each plasmid contained the *glnK$_1$* promoter-*lacZ* construct in reverse orientation to *upt*. Constructs were confirmed by sequencing.

Strains are listed in Table S1. Strains Mm1127, Mm1213, Mm1128 and Mm1164 were made by transforming Mm900 with plasmids pBLPrtK$_1$, pBLPrtglnK$_1$O$_1$, pBLPrtglnK$_1$O$_2$ and pBLPrtglnK$_1$O$_1$+O$_2$. Transformation (Tumbula *et al.*, 1994) and selection were carried out as described (Moore & Leigh, 2005), resulting in replacement of the *upt* site with each reporter construct. Constructs were confirmed by sequencing. Strain Mm1198 containing an in-frame deletion of *nrpR* was made by transforming Mm1127 with the plasmid pCRPrtNeoΔnrp and selecting for integration and vector removal as described (Moore & Leigh, 2005). The construct was confirmed by PCR analysis using the primers LfRepKO1 and RtRepKO1.

Electrophoretic mobility shift assays

Probes for EMSA were obtained by PCR from plasmid templates, gel purified, and end labeled with T4 polynucleotide kinase and ^{32}P- γ-ATP. For the experiment in Fig. 3, probes containing the wild type operators or mutations in the first, second or both operators region were amplified from pWLG40K$_1$, pWLG40glnK$_1$O$_1$, pWLG40glnK$_1$O$_2$, or pWLG40glnK$_1$O$_1$+O$_2$ respectively with primers P7 and P8, P9 and P13, P10 and P12, or P11 and P14. For the experiment in Fig. 5, probes containing the *nif* or *glnK$_1$* operators were amplified from pMmp1.1 or pCR2.1glnK$_1$pro respectively with primers Mmniffor1 and Mmnifrev, or FPglnKF and FPglnKrev2, resulting in products 161 and 154 bp in length. Conditions for gel-shift and image analysis were as described previously (Lie et al., 2005). 2OG and His-tagged NrpR (Lie et al., 2005) were added to the final concentrations indicated.

Footprinting

Forward (FpglnKF) and reverse (FpglnKrev2) primers for amplifying wildtype (with pCR2.1glnK$_1$pro as a template) or O$_1$ only (with pBLPrtglnK$_1$O$_2$) *glnK$_1$* promoter regions were labeled at the 5′ end using [γ–^{32}P] ATP and T4 Polynucleotide kinase (NEB), ethanol

precipitated, and washed before being used for PCR amplification. PCR products were purified (Qiagen) and resolved on a non-denaturing polyacrylamide gel (5%). Product (154 bp) was identified by UV shadowing, cut out, and incubated at 37°C in DNA elution buffer (10 mM TRIS pH 8, 1 mM EDTA, 1M LiCl, 0.1% SDS) overnight without agitation. The eluate was filtered through 4 mm PVDF syringe filters (National Scientific) with 0.2 μm pore size, ethanol precipitated, phenol-chloroform extracted, and ethanol precipitated again. The purity and concentration of product were determined spectrophotometrically. Products were then diluted to a final concentration of 200 nM in TE and stored at -80°C until needed. A G+A ladder of the PCR product was generated as described (Mukherjee & Sousa, 2003). Binding reactions contained DNA (2 nM), TRIS pH 7.5 (10 mM), glycerol (10%), KCl (150 mM), DTT (10 mM) and NrpR in a final volume of 50 μl, and were incubated at 37°C for 30 minutes. DNAse I co-factor solution (50 μl of 10 mM $MgCl_2$ and 5 mM $CaCl_2$) was then added followed by DNAse I (Fermentas), and incubation was continued for 2 min before addition of stop buffer (100 μl of 200 mM KCl, 1% SDS, 20 mM EDTA). Samples were then extracted with phenol-chloroform, ethanol precipitated, resuspended in TE, and frozen at -80°C until used. Samples were run on 8% non-denaturing polyacrylamide sequencing gels. Gels were dried, exposed to phosphor screens, and imaged with Storm® imaging software.

Reporter gene assays

Reporter strains were grown in triplicate with N_2, alanine, or ammonia and β-glactosidase assays were carried out with 0.2 ml of each culture as described (Lie & Leigh, 2002). Samples were taken from cultures grown to OD_{660} between 0.3 and 0.7.

Primer extension analysis

RNA was extracted with the RNAeasy kit (Qiagen) with modifications as described previously (Lie & Leigh, 2002). The reaction contained 2 pmol of ^{32}P-labeled primer Exglnk1a, 30 μg RNA from a nitrogen-fixing culture of *M. maripaludis* strain Mm900, and 0.5 μl of each deoxynucleoside triphosphate in 12 μl H_2O. The reaction was heated to 70°C and slowly cooled to 42°C. The reverse transcription was carried out in 20 μl reaction buffer (Invitrogen Life Technologies) containing 10 mM dithiotreithol and 200 U of Superscript II Reverse Transcriptase, according to the supplier's instructions. After 30 min at 42°C nucleic acids were precipitated with isopropanol, resuspended in 6 μl H_2O and 4 μl Sequenase stop solution (Sequenase Version 2 DNA Sequencing Kit, USB) and separated on a polyacrylamide sequencing gel next to a sequencing reaction performed with the same primer. The template for sequencing was generated by PCR amplification from a DNA bank of *M. maripaludis* using primers PglnK1fw and PglnKrv and ligating the product into pCR2.1®-TOPO. The sequencing reaction was carried out with the Sequenase Version 2 DNA Sequencing Kit, USB, according to the manufacturer's protocol.

Northern blots

RNA extractions and Northern analyses were done as described previously (Lie & Leigh, 2002). The probe (see Fig. 2) was amplified from plasmid pWLG40K_1 with primers utrglnK1fw and utrglnK1rv. 10 (Fig. S2A) or 5 (Fig. S2B) μg of total RNA was loaded into each lane.

Reverse transcription analysis of the *glnK$_1$* transcript

PCR and RT-PCR were performed using the OneStep RT-PCR Kit (Qiagen) following the manufacturer's protocol. Conditions were: 50°C 30 min; 95°C 15 min; 30 cycles of 95°C 45 sec, 54°C 45 sec, and 72°C 3 min; and 72°C 10 min. For non-RT reactions, enzyme supplied in the kit was substituted with Platinum Taq DNA polymerase (Invitrogen). Primers were

(forward) Glnkutr and (reverse) Glnk1rtr (Fig. S3 lanes 4, 5, and 8), Amtbrtr (lanes 3, 6, and 9), or Glnbrtr (lanes 2, 8, and 10). RNA was extracted from a nitrogen-fixing chemostat culture of Mm900 using the RNAeasy kit (Qiagen) with modifications as described previously ((Xia *et al.*, 2006)). Genomic DNA was purified from *M. maripaludis* strain S2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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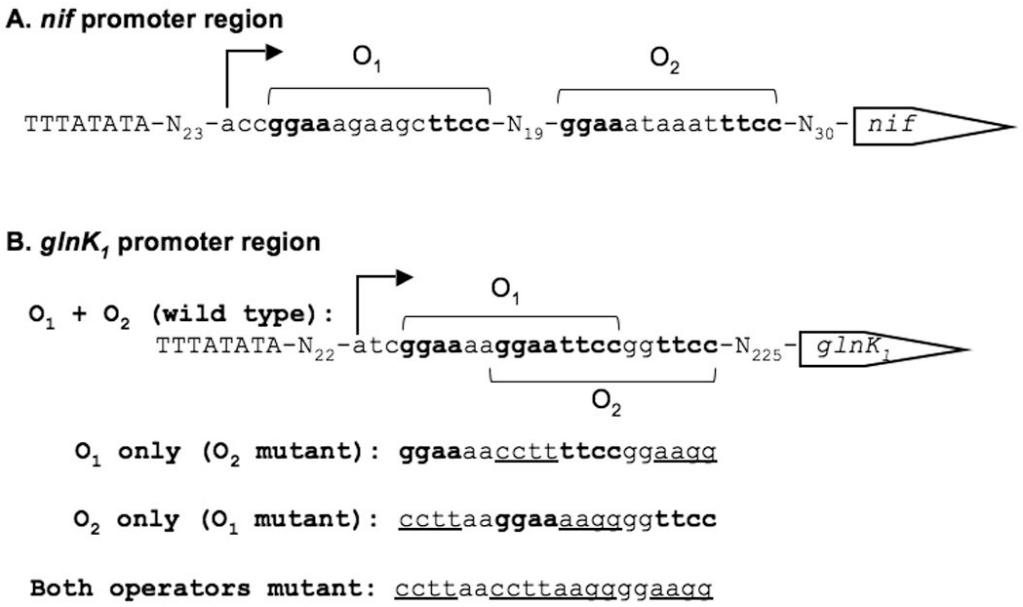

Fig. 1. *nif* and *glnK*$_1$ promoter regions

A. *nif* promoter region.

B. *glnK*$_1$ promoter region. TATA boxes (caps), transcription start sites (bent arrows), and operators (bold) are shown. Mutant *glnK*$_1$ operators are underlined.

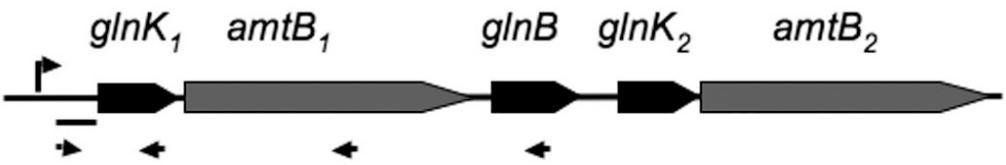

Fig. 2. The *glnK* gene cluster

The location of the transcription start site is indicated by a bent arrow. The probe used for Northern blots is indicated by a short horizontal line. Primers used for RT-PCR are shown as arrows.

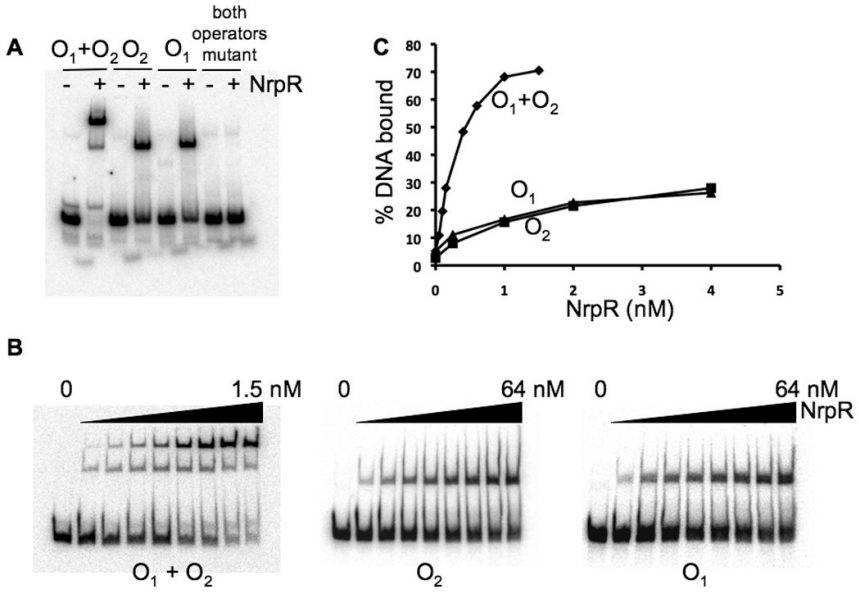

Fig. 3. Electrophoretic mobility shift analysis (EMSA) of NrpR binding to operator DNA

A. DNA probes containing operators shown in Fig. 1: wild type (O_1+O_2), O_2 only (O_1 mutant), O_1 only (O_2 mutant), and both operators mutant. No (-) or 120 nM (+) NrpR was mixed with the DNA.

B. EMSA at different NrpR concentrations: O_1+O_2, 0, 0.05, 0.1, 0.15, 0.2, 0.4, 0.6, 1.0, and 1.5 nM; O_2 and O_1, 0, 0.25, 1, 2, 4, 8, 16, 32, and 64 nM.

C. Plot of data from B.

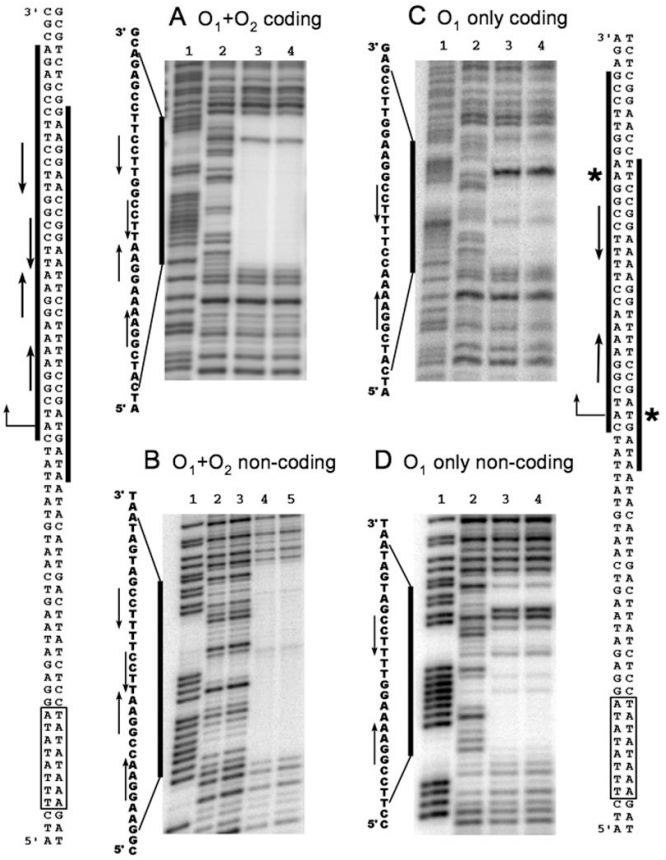

Fig. 4. DNAse I footprinting analysis of NrpR binding to operator DNA

Footprints are delineated by bold lines and indicated with respect to the sequence. Double stranded DNA sequences on left and right represent O_1+O_2 and O_1 only, respectively. Boxed sequences, TATA box; bent arrow, transcription start site; inverted arrow pairs, operator(s); asterisks, hypersensitive sites.

A, C and D. Lane 1, G+A ladder; lane 2, no NrpR; lane 3, 200 nM NrpR; lane 4, 400 nM NrpR. Units of DNAse I, 0.005

B. Lane 1, G+A ladder; lanes 2 and 3, no NrpR, lanes 4 and 5, 400 nM NrpR.; Units of DNAse I: lanes 2 and 4, 0.001; lanes 3 and 5, 0.002.

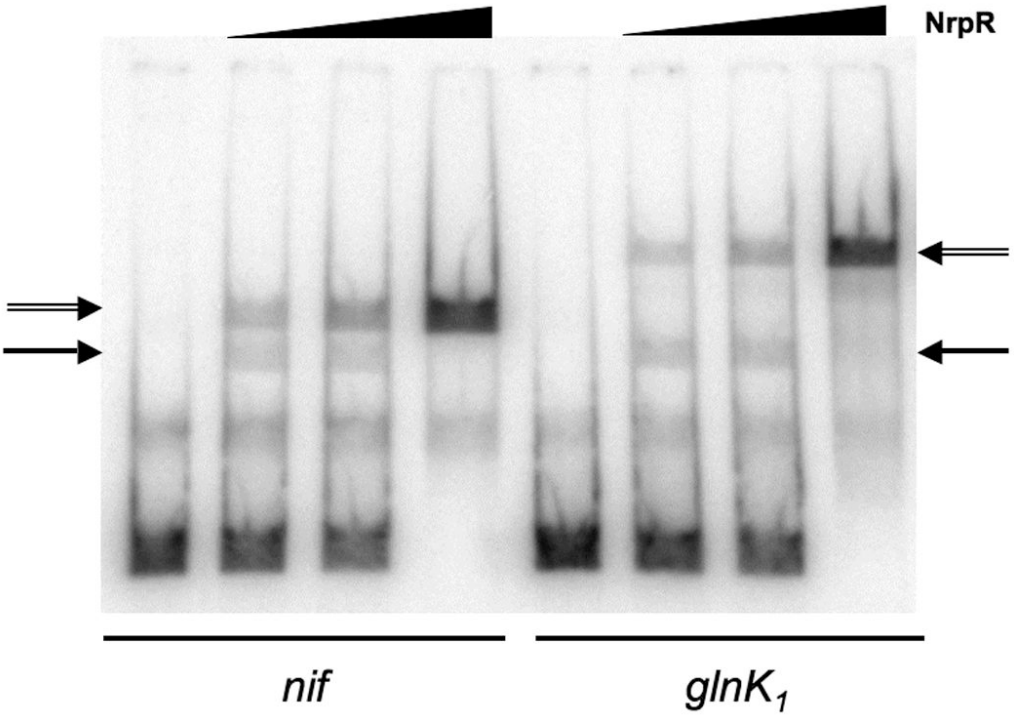

Fig. 5. EMSA of NrpR binding to *nif* and *glnK*$_1$ operators

DNA probes of equal length contained *nif* $O_1 + O_2$ or *glnK*$_1$ $O_1 + O_2$. Single and double arrows indicate shifts corresponding to NrpR binding to one or two operators respectively. NrpR concentrations were 0, 0.05, 0.1, and 2.0 nM.

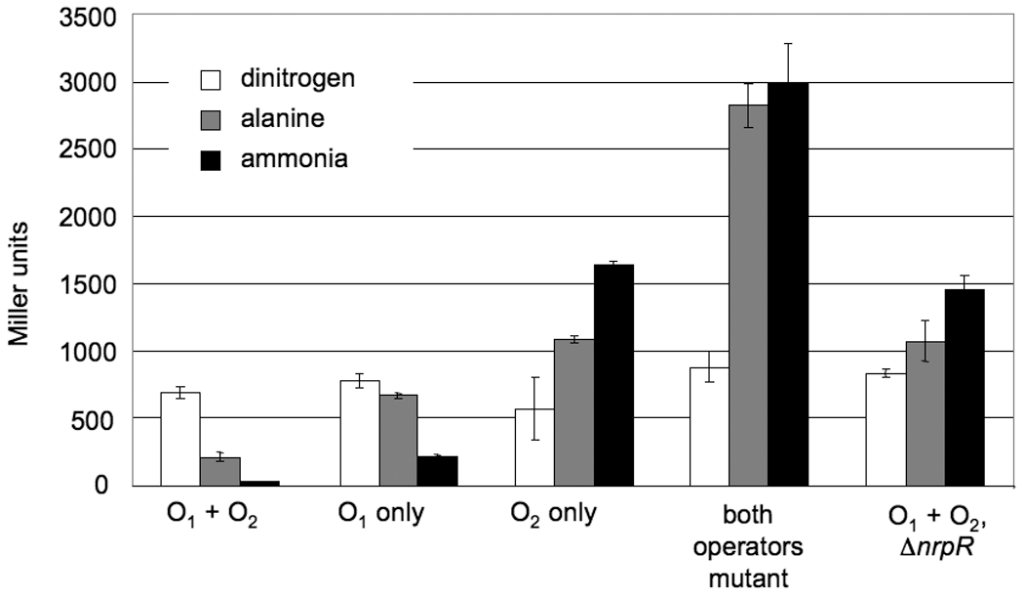

Fig. 6. In vivo regulation of wild type and mutant *glnK*$_1$ promoter reporter fusions

Strains containing *lacZ* fusions to promoters with wild type (O_1 + O_2) and mutant (O_1 only, O_2 only, or both operators mutant) operators were grown under three nitrogen conditions as indicated, and β-galactosidase assays performed as described in Experimental Procedures.

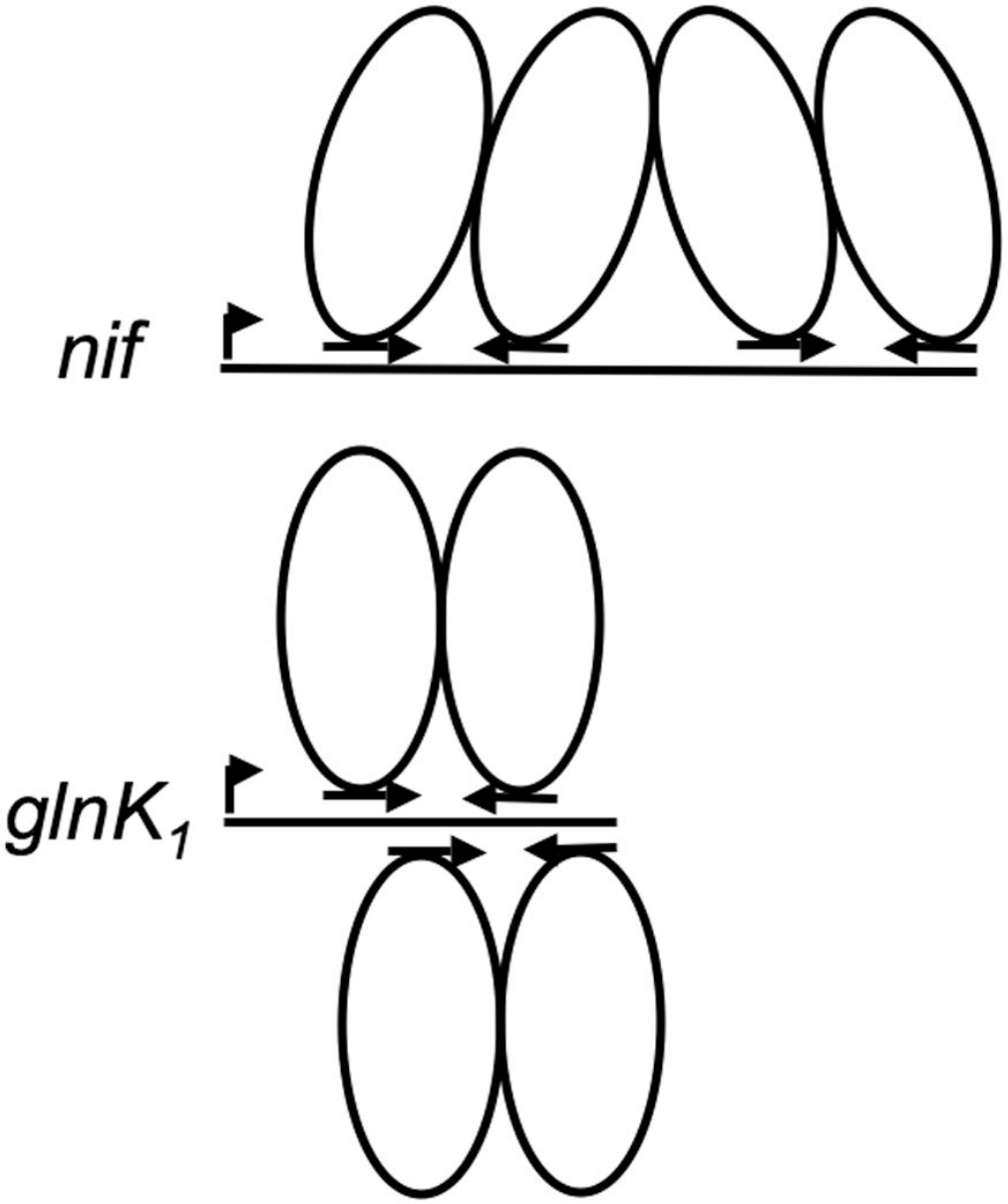

Fig. 7. Models for the binding of NrpR to the *nif* and *glnK*$_1$ promoter regions

A dimer of dimers (tetramer) binds to the *nif* promoter region, which contains two operators centered three helical turns apart on the same face of the DNA helix. Two separate dimers bind on opposite sides of the helix in the *glnK*$_1$ promoter region, which contains two overlapping operators spaced only 6 bp apart.