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Regulation of *nif* Expression in *Methanococcus maripaludis*

ROLES OF THE EURYARCHAEAL REPRESSOR NrpR, 2-OXOGLUTARATE, AND TWO OPERATORS*

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The methanogenic archaean *Methanococcus maripaludis* can use ammonia, alanine, or dinitrogen as a nitrogen source for growth. The euryarchaeal nitrogen repressor NrpR controls the expression of the *nif* (nitrogen fixation) operon, resulting in full repression with ammonia, intermediate repression with alanine, and derepression with dinitrogen. NrpR binds to two tandem operators in the *nif* promoter region, nifOR_1 and nifOR_2. Here we have undertaken both *in vivo* and *in vitro* approaches to study the way in which NrpR, nifOR_1, nifOR_2, and the effector 2-oxoglutarate (2OG) combine to regulate *nif* expression, leading to a comprehensive understanding of this archaeal regulatory system. We show that NrpR binds as a dimer to nifOR_1 and cooperatively as two dimers to both operators. Cooperative binding occurs only with both operators present. nifOR_1 has stronger binding and by itself can mediate the repression of *nif* transcription during growth on ammonia, unlike the weakly binding nifOR_2. However, nifOR_2 in combination with nifOR_1 is critical for intermediate repression during growth on alanine. Accordingly, NrpR binds to both operators together with higher affinity than to nifOR_1 alone. NrpR responds directly to 2OG, which weakens its binding to the operators. Hence, 2OG is an intracellular indicator of nitrogen deficiency and acts as an inducer of *nif* transcription via NrpR. This model is upheld by the recent finding (J. A. Dodsworth and J. A. Leigh, submitted for publication) in our laboratory that 2OG levels in *M. maripaludis* vary with growth on different nitrogen sources.

Transcriptional regulation in the domain Archaea (1) is intriguing because, in many cases, homologs of bacterial regulators function in the context of the archaeal basal transcriptional apparatus, which resembles that of the Eukarya (2–5). Archaeal promoters consist of an AT-rich TATA box 24–26 bp upstream from the transcriptional initiation site and a purine-rich B recognition element immediately upstream of the TATA box. During transcription initiation, TATA-binding protein recognizes and binds to the TATA box. This interaction is stabilized by the binding of transcription factor B, and the DNA-TATA-binding protein-transcription factor B complex recruits the RNA polymerase. Transcription factor B also contacts the B recognition element, which determines the directionality of transcription (6). The complex containing these three factors is sufficient to initiate transcription in a cell-free system (7), although an additional factor, transcription factor E, increases transcription from some promoters (8, 9). Archaeal TATA-binding protein, transcription factors B and E, and the RNA polymerase are all homologs of the eukaryal transcription counterparts. However, homologs of bacterial transcriptional regulators are common in Archaea, and those that have been studied experimentally include the Lrp (10) homologs, which can function as either repressors or activators (11–14). Binding sites for these Lrp homologs can be upstream of the TATA box (15), overlapping it (12) or overlapping the transcriptional start site (16), indicating either activation or inhibition of different steps of the transcriptional initiation process. A few homologs of eukaryal transcriptional regulators as well as uniquely archaeal regulators are also known, and their regulatory actions have been investigated (9, 17–20). Histones and other non-sequence-specific DNA-binding proteins (21, 22) also have been found in some instances to play a role in the regulation of archaeal genes (23). Archaea thus possess a complex mix of transcriptional regulatory schemes.

Dinitrogen fixation occurs only in the domains Bacteria and Archaea (24). Nitrogen fixation has been demonstrated in various methanogens (24), and *nif* (nitrogen fixation) genes, which are organized into operons, have been detected (25–27). Nitrogen fixation is highly energy expensive and is regulated tightly both transcriptionally and post-translationally (28). The transcriptional regulation of *nif* genes in Bacteria involves diverse systems (28), whereas the *nif* regulation in those Archaea that have been studied is entirely different (see below)

Our laboratory has studied *nif* regulation as a model of transcriptional regulation in Archaea, using the species *Methanococcus maripaludis* (29, 30), in which a facile genetic system offers the unusual opportunity within Archaea to conduct *in vivo* as well as *in vitro* studies (31). *nif* expression in *M. maripaludis* is repressed by ammonia and derepressed when dinitrogen is the sole nitrogen source (32, 33). In addition, an intermediate level of expression occurs with alanine as a nitrogen source (34). Previously, we identified two tandem palindromes (nifOR_1 and nifOR_2) (see Fig. 1), both with the conserved sequence GGAA-N_6-TTCC (N_6 denotes six nucleotides), within the *nif* promoter region (33). Although both operators are highly similar to the consensus sequence, only nifOR_1 is needed for repression during growth on ammonia. However, nifOR_2 in combination with nifOR_1 is required for intermediate repression during growth on alanine (34). Furthermore, an unknown factor present in the cell extract binds more effectively to both operators together than to nifOR_1 alone. We demonstrated recently (18) that a novel regulator, NrpR, with homologs known only in the Euryarchaeota, is the repressor of *nif* expression.

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In this study, we extended our investigation of the roles of the nitrogen operators and NrpR and report that 2-oxoglutarate (2OG) [1] functions as an inducer. Using purified His-tagged NrpR for *in vitro* binding studies, we show the primary role played by $nifOR_1$, the importance of $nifOR_2$ in mediating cooperative binding of NrpR to DNA, and the direct role of 2OG in affecting the binding affinity of NrpR to the operators. The binding properties of purified NrpR to the *nif* operators at different concentrations of 2OG account for the regulatory effects observed *in vivo*.

EXPERIMENTAL PROCEDURES

Media and Growth Conditions—All *M. maripaludis* strains were maintained in complex McCas medium (35) or minimal nitrogen-free medium as described previously (36), with the addition of neomycin sulfate (1 mg/ml) or puromycin (2.5 μg/ml), as appropriate. NH_4Cl (10 mM), L-alanine (10 mM), or N_2 (15 p.s.i. of 80% N_2, 20% CO_2) served as a nitrogen source. Mm706 (+pWLG40RepHis) (described below) was grown in a 12-liter fermenter (model MMF14-171816, New Brunswick Microferm) as follows. Anaerobic McCas medium (10 liters) (containing both puromycin and neomycin) was inoculated with 250 ml of fresh culture grown in modified 1-liter bottles (37) and sparged with 800 ml/min H_2, 200 ml/min CO_2, and 20 ml/min 1% H_2S in N_2. Gases (except H_2S) were passed through a copper furnace set at 300 °C to scavenge trace amounts of O_2. The impeller agitation rate was 200 rpm, and the temperature was 37 °C.

Strain Construction—To construct Mm706 ($\Delta nrpR$, neo^r), plasmid pTJL11R4 (18), in which the EcoRV-EcoRI portion of *nrpR* had been replaced with a neomycin resistance cassette (38), was transformed into *M. maripaludis* S2 (wild type) by the polyethylene glycol method (39). Replacement of genomic *nrpR* by a homologous recombination was shown by Southern analysis, and the loss of NrpR DNA binding activity was confirmed by the gel shift assay. To construct C-terminal *his*-tagged *nrpR*, the *nrpR* gene was PCR amplified from pTJL11 (18) using forward primer RepHisBam (5′-GGGGATCCAGGAGGAGCATTATG-GACAGTAATATTG-3′), reverse primer RepHisXho (5′-GGCTC-GAGAATATCGTCGTAATGTGTTAGTTC-3′), and *Pfu* (Stratagene) as follows: 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 40 °C for 30 s, 68 °C for 5 min, and 68 °C for 10 min. The product was digested with BamHI and XhoI and cloned into pET-24(+) (Novagen) to yield pRepHis14. This plasmid contained *nrpR-his*, encoding NrpR with the amino acids Leu, Glu, and His_6 residues fused to the C terminus. *nrpR-his* was then amplified from pRepHis14 (forward primer RepHisPst 5′-CCCCTGCA-GATGGACAGTAATATTGATGTTG-3′ and reverse primer RepHisXba 5′-CCTCTAGACCTCCTTTCAGCAAAAAACCC-3′) as above, digested with PstI and XbaI, and cloned into NsiI and XbaI-digested pWLG40+lacZ (18, 40) to yield plasmid pWLG40RepHis. This plasmid, which is replicative in *M. maripaludis*, contains *nrpR-his* fused to an archaeal histone promoter (41), allowing for constitutive expression in *M. maripaludis*. This plasmid was sequenced to determine that no errors were introduced during PCR amplification. pWLG40RepHis was then transformed into Mm706. A control strain was generated similarly by transforming the vector pWLG40+lacZ into Mm706.

Purification of His-tagged NrpR—Mm706 (+pWLGRepHis) was grown to an OD_{660} of approximately 1 in a fermenter, as described above. Cells were harvested by spinning 400-ml aliquots at 10,000 × *g* for 30 min at 4 °C. Cell pellets were resuspended in 10 ml of 50 mM Tris, pH 7.5, 50% glycerol, 376 mM NaCl and stored at −80 °C. For purification of His-tagged NrpR, frozen cell suspensions were thawed on ice and spun at 10,000 × *g* for 30 min at 4 °C. Cells were resuspended in 50 mM Tris, pH 7.5, 1 mM phenylmethylsulfonyl fluoride and lysed by sonication at 4 °C. Cellular debris was removed (10,000 × *g* for 15 min at 4 °C), and the supernatant was subjected to ammonium sulfate fractionation. The fraction that precipitated between 40 and 80% ammonium sulfate was resuspended in binding buffer (25 mM imidazole, 1 M NaCl, 5% glycerol, 50 mM NaH_2PO_4, pH 6.82) and allowed to incubate with nickel resin (catalog no. 70666-4, Novagen) with shaking at 4 °C for at least 1 h. The slurry was then added to 10-ml gravity columns, and the void was discarded. The resin was washed three times with wash buffer (35 mM imidazole, 1 M NaCl, 5% glycerol, 50 mM NaH_2PO_4, pH 6.82) and eluted with elution buffer (1 M imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 5.26). The eluate (1.5-ml portions) was dialyzed overnight at 4 °C against 1 liter of 50 mM Tris, pH 7.5, loaded on a Hi-Trap™ heparin column (Amersham Biosciences) at room temperature, and eluted with a gradient of 200–700 mM NaCl in 50 mM Tris, pH 7.5. Fractions of 1.5 ml each were collected, for a total of 12 ml. Fractions that were determined by SDS-PAGE analysis to contain His-tagged NrpR (total of 3 ml) were pooled, dialyzed overnight at 4 °C against 1 liter of 50% glycerol, 50 mM Tris, pH 7.5, and stored at −20 °C until needed. The purity of His-tagged NrpR was determined by SDS-PAGE (12% acrylamide) of serial dilutions and visualized by silver staining.

Gel Mobility Shift Assays—Gel shift conditions were those described previously (34), with modifications. The binding buffer was 10 mM Tris·HCl, pH 7.5, 10 mM dithiothreitol, 300 μg/ml bovine serum albumin (BSA), 50 μg/ml poly(dIdC·dIdC)·(dIdC·dIdC), and 80 mM KCl in a final volume of 80 μl. Radiolabeled probes were added to a final concentration of 0.02 nM. 2OG, pH 7.5, and His-tagged NrpR were added to the final concentrations indicated. Binding mixtures were incubated at 37 °C for 30 min, then 50% glycerol was added to a final concentration of 10%, and 20-μl portions were loaded into wells. Gels were run at 150 V at room temperature. Band intensities were measured with a PhosphorImager (Amersham Biosciences). DNA probes (see Fig. 1) used for gel shift assays were WT *nif* (91 bp), ct1ag1 (91 bp), ct2ag2 (91 bp), ct4a (85 bp), and a double mutant (91 bp). The synthesis and use of probes were carried out as described previously (34). ct4a was amplified from plasmid pnifmutCT4A (33). K_d values were His-tagged NrpR concentrations at which 50% of the DNA probe was bound.

Expression Assays—Northern analysis and β-galactosidase assays were performed as described previously (34).

Molecular Mass Determinations—Native molecular masses were determined by the Ferguson method (42, 43). Binding conditions were the same as for the gel mobility shift assays with the omission of BSA and poly(dIdC·dIdC)·(dIdC·dIdC). BSA migrated similarly to NrpR, making visualization of the NrpR difficult. The omission of BSA and poly(dIdC·dIdC)·(dIdC·dIdC) did not affect the migration of NrpR or NrpR-*nif* DNA complexes. His-tagged NrpR was 2 μM (monomer concentration), and probes were 104 nM. These concentrations of NrpR-His and DNA yielded complexes with the same migrations as those in the standard gel shift assay described above (data not shown). After incubation, binding reactions (20 μl) were mixed with equal volumes of loading buffer (125 mM Tris·HCl, pH 7.5, 20% glycerol, 0.001% bromphenol blue) and loaded onto gels of varying acrylamide concentrations (6,7, 8, and 9%). Protein standards (α-lactalbumin (14,200 Da) 0.144 μg/μl, carbonic anhydrase (29,000 Da) 0.1 μg/μl, chicken egg albumin (45,000 Da) 0.1 μg/μl, BSA (66,000-Da monomer, 132,000-Da dimer) 0.1 μg/μl, and urease (272,000-Da trimer, 545,000-Da hexamer) 0.144 μg/μl) (catalog no. MW-ND-500, Sigma) were prepared according to the manufacturer's instructions and incubated under binding conditions as described above. Gels were run until the tracking dye reached the end of the gel and were silver-stained (PLUS silver stain, Bio-Rad). R_F values were determined, and $100\cdot[\log_{10}(R_F\cdot 100)]$ was plotted against the acrylamide concentration for each protein standard. Linear fits of the curves were generated in Microsoft Excel, slopes were determined, and a Ferguson plot (negative slope *versus* molecular mass) was made. The slope values of the experimental samples were applied to the plot to determine the respective molecular masses. Unbound DNAs in experimental samples were also detected by silver staining, and molecular masses were determined as above.

RESULTS

$nifOR_2$ Is Critical for Intermediate Repression during Growth on Alanine—Previously (33), a series of *nif* promoter-*lacZ* fusions were used to study the roles of $nifOR_1$ and $nifOR_2$ (Fig. 1) in *nif* repression. In addition to the wild type promoter region, mutant promoter regions were made in which the sequence of each operator was altered while preserving its palindromic nature. Only $nifOR_1$ is required for repression by ammonia, but $nifOR_1$ and $nifOR_2$ are both required for repression by alanine (34). Here we also tested the effect of altering the distance between $nifOR_1$ and $nifOR_2$. Fusions of wild type and mutant *nif* promoter regions with *lacZ* were introduced into *M. maripaludis*, and β-galactosidase assays were performed with cultures grown on the three nitrogen sources. As expected, the wild type *nif* promoter region resulted in low, intermediate, and high levels of expression with ammonia, alanine, and dinitrogen, respectively (Fig. 1). Mutagenesis of

[1] The abbreviations used are: 2OG, 2-oxoglutarate; BSA, bovine serum albumin; WT, wild type; nifOR, operator in *nif* promoter region.

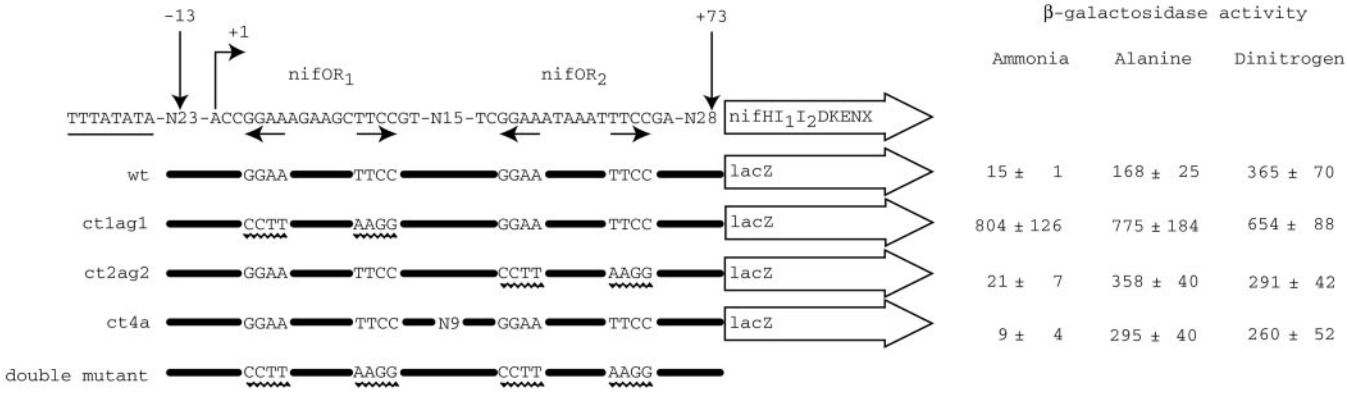

FIG. 1. **Wild type and mutant *nif* operator regions and expression levels.** *Top row*, the sequence of the *M. maripaludis nif* promoter region is shown. *Underlining*, TATA box; *bent arrow*, transcription start site; *inverted arrows*, *nif* operators $nifOR_1$ and $nifOR_2$; *large open arrows*, *nif* operon or *lacZ*. *Bottom four rows*, wild type and mutant *nif-lacZ* constructs are shown. *Squiggly underlines* indicate altered *nif* operator sequences. *nif-lacZ* expression levels *in vivo* (β-galactosidase activities) are shown. *−13* and *+73* indicate the region included in DNA probes for binding studies.

$nifOR_1$ (ct1ag1) abolished all repression, confirming the role of $nifOR_1$ as the "primary" operator. Mutagenesis of $nifOR_2$ (ct2ag2) removed repression by alanine only.

We tested an additional mutant (33) in which the spacing between $nifOR_1$ and $nifOR_2$ was decreased by 6 bp (ct4a) (Fig. 1). Repression occurred with ammonia but not with alanine (Fig. 1). Hence, both the sequence of $nifOR_2$ and its distance from $nifOR_1$ are important for the intermediate level of repression that normally occurs with alanine. Changing the distance between the two operators could destroy the ability of the repressor to bind to both operators at the same time. In the wild type *nif* promoter region, $nifOR_1$ and $nifOR_2$ were centered 3.1 helical turns apart and were thus on the same face of the DNA (in phase), whereas in the mutant, the two operators were centered 2.6 helical turns apart.

His-tagged NrpR Regulates nif Expression Like Wild Type NrpR in Vivo—In preparation for the purification of the nitrogen repressor NrpR, we constructed a gene encoding a C-terminal His-tagged NrpR (NrpR-His) and tested its activity *in vivo*. We transformed the replicative plasmid pWLG40RepHis containing constitutively expressed *nrpR-his* into *M. maripaludis* strain Mm706, in which the internal portion of *nrpR* had been replaced with a neomycin resistance cassette. We made cell extracts and tested for the presence of active NrpR by its ability to bind to *nif* operator DNA in gel mobility shift assays as described previously (34). As expected, Mm706 contained no binding activity nor did a control strain contain the vector pWLG40+lacZ in an Mm706 background (data not shown). However, strong binding activity was restored in Mm706 (+pWLG40RepHis). Northern analysis confirmed that *nif* expression was fully repressed when Mm706 (+pWLG40RepHis) was grown with ammonia compared with dinitrogen (data not shown). Thus, NrpR-His is highly active *in vivo*.

NrpR-His Binds to WT nif and Single Operator Mutant Probes with Different Affinities in Vitro—We purified NrpR-His to >95% purity. We used gel mobility shift analysis to determine the ability of NrpR-His to bind to wild type and mutant forms of the DNA corresponding to the portion of the *nif* promoter region that contains the *nif* operators (Fig. 1, nucleotides −13 to +73). NrpR-His (0.4 nM) bound to DNA containing both operators (Fig. 2*A*, *WT nif*) or $nifOR_1$ alone (Fig. 2*A*, *ct2ag2*) but not to DNA containing $nifOR_2$ alone (Fig. 2*A*, *ct1ag1*) or to DNA lacking both operators (Fig. 2*A*, *dm*). NrpR-His also bound to DNA in which both operators were present, but the distance between the operators was decreased by 6 bp (Fig. 2*A*, *ct4a*). Increasing concentrations of NrpR-His resulted in increased binding, and except for the weakly binding construct ct1ag1, binding increased up to the point at which unbound DNA was nearly depleted (Fig. 2, *B* and *C*). Equilibrium dissociation constants (K_d) estimated from the binding curves (Fig. 2*C*) were WT *nif*, 0.3 nM, ct2ag2 and ct4a, both 0.2 nM, and ct1ag1, >7 nM. These results showed that $nifOR_1$ is essential for significant binding, in agreement with the requirement for $nifOR_1$, for any repression to occur *in vivo*. $nifOR_2$, despite its similarity in sequence to nifOR1, has much weaker binding that must result from the nucleotides flanking the conserved portions of the operator.

NrpR-His Binds to Single Operators as a Dimer and Cooperatively to Two Operators as a Dimer Pair—NrpR-His binding to $nifOR_1$ (ct2ag2) or $nifOR_2$ (ct1ag1) alone or to the deletion construct ct4a produced a single shifted band, always of the same mobility (Fig. 2, *A* and *B*, *band A*). NrpR-His binding to both operators together (WT *nif*) produced a band of the same mobility at low concentrations of NrpR-His (Fig. 2, *A* and *B*, *band A*) but also produced a slower migrating band (Fig. 2, *A* and *B*, *band B*) at higher concentrations of NrpR-His. We hypothesized that band A was a NrpR-His dimer bound to a single operator, whereas band B was two NrpR dimers, one bound to each operator. To test this hypothesis, we determined the binding stoichiometries of NrpR-His to wild type and mutant *nif* operator DNAs by generating Ferguson plots (43), a method in which the R_F values of unknown protein complexes at different acrylamide concentrations are compared against those of native molecular mass standards to derive their native molecular masses. Unbound NrpR-His was 160,000 Da, NrpR-His bound to WT *nif* operator DNA was 329,000 Da, NrpR-His-ct2ag2 was 185,000 Da, and NrpR-His-ct4a was 178,000 Da (Fig. 3). Subtracting the molecular masses of unbound DNA determined from the same acrylamide gel concentrations (WT *nif* was 47,000 Da, ct2ag2 was 49,000 Da, and ct4a was 38,000 Da) and dividing by the molecular mass of the NrpR-His monomer (60,811 Da) indicated that 2.6 monomers were present in free NrpR-His, 4.6 monomers were bound to WT *nif*, 2.2 monomers were bound to ct2ag2, and 2.3 monomers were bound to ct4a. We concluded that bands A and B (Fig. 2, *A* and *B*) contained a dimer and a dimer pair of NrpR-His, respectively. Because NrpR-His barely bound $nifOR_2$ alone even at high concentrations, we concluded that interactions between two NrpR-His dimers formed a pair that bound cooperatively to the two operators. Cooperative binding of dimer pairs to ct4a may not be possible because the spacing between the two operators is unfavorable or because the two operators are not on the same face of the helix, as seen in analogous bacterial and λ repression systems (44, 45).

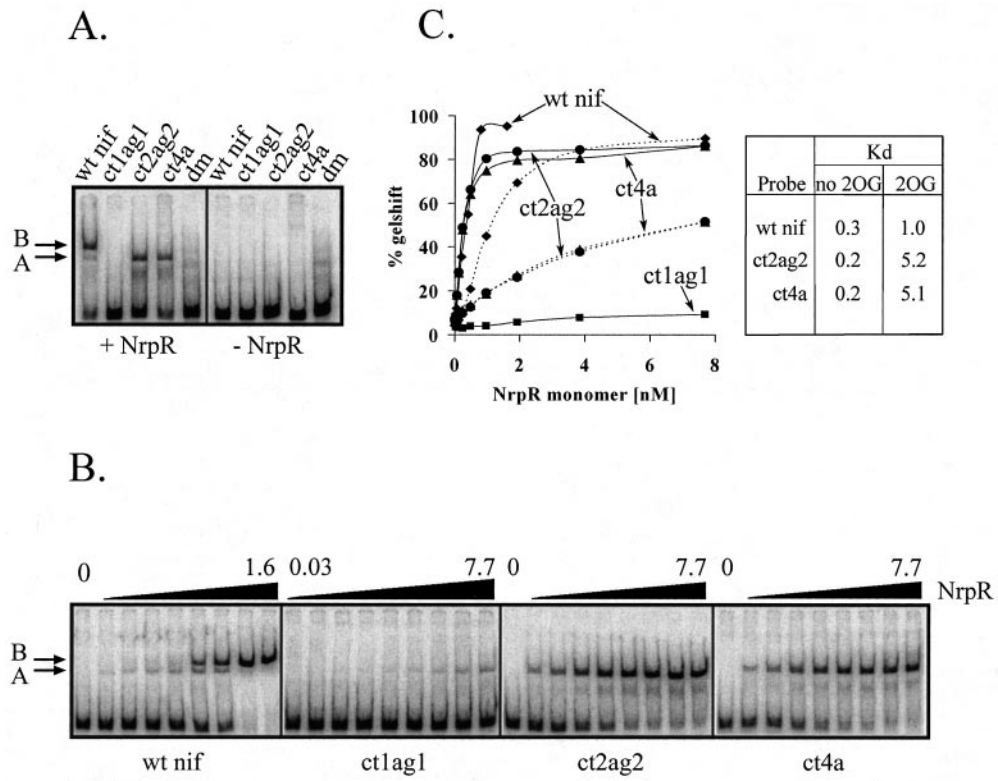

FIG. 2. **Binding of NrpR-His to *nif* operator DNA.** *A*, gel mobility shift assay is shown of NrpR (0.4 nM) binding to the *nif* DNA probes (0.02 nM), shown in Fig. 1. *dm*, DNA with both operators mutated. *B*, binding responses to varying NrpR concentrations are shown. *C*, *solid lines*, graphical representation of data shown in *B*; *dotted lines*, similar analysis in the presence of 2OG (0.1 mM). ♦, WT *nif* DNA; ▲, ct4a DNA; ●, ct2ag2 DNA; ■, ct1ag1 DNA. K_d values derived from the graph are tabulated. NrpR concentrations for WT *nif* (without 2OG) are 0, 0.003, 0.01, 0.03, 0.05, 0.2, 0.4, 0.8, and 1.6 nM; for WT *nif* (with 2OG) values are 0, 0.06, 0.1, 0.2, 0.5, 1, 1.9, 3.8, and 7.7 nM; for ct1ag1 (without and with 2OG) values are 0.03, 0.06, 0.1, 0.2, 0.5, 1, 1.9, 3.8, and 7.7 nM; and for ct2ag2 and ct4a (without and with 2OG, respectively), values are the same as for WT *nif* (with 2OG). *A* and *B* indicate bands described under "Results."

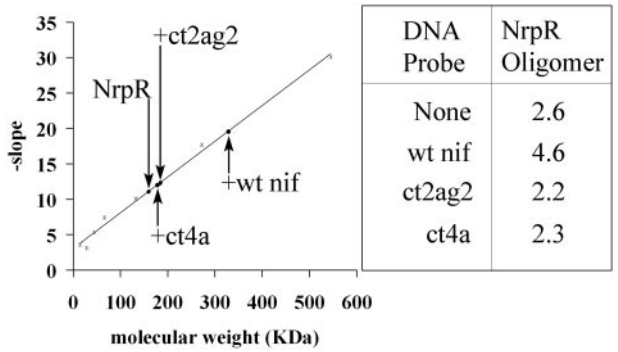

FIG. 3. **Molecular masses of NrpR and NrpR-DNA complexes.** A Ferguson plot shows the molecular mass standard (*X*) and the NrpR complex (●). Multiplicities of NrpR monomers deduced from the plot are tabulated.

2OG Inhibits NrpR-His Binding to WT nif—We found previously (18) that NrpR binding activity was similar in cell extracts of ammonia-, alanine-, and dinitrogen-grown cultures. This observation suggested that some additional factor must modulate NrpR binding to operator DNA *in vivo*. Gel mobility assays showed that 2OG (5 mM) markedly inhibited the binding of NrpR-His to WT *nif* DNA (Fig. 4*A*). Alanine, glutamine, or glutamate had no effect. Increasing the concentrations of 2OG had increasing effects (Fig. 4*B*), and estimates of K_d (Fig. 4*C*) showed that increasing 2OG decreased the binding affinity of NrpR-His over a range of less than 0.1 mM to 1.6 mM 2OG (Fig. 4*D*). 2OG may act as an inducer in concert with NrpR to regulate *nif* expression.

NrpR-His Has Higher Binding Affinity to WT nif than Mutant Operator DNAs in the Presence of 2OG—Because NrpR-His binds cooperatively to $nifOR_1$ and $nifOR_2$ together (as described above), one would expect higher affinities for WT *nif* than for ct2ag2 or ct4a. Although this was not observed in the absence of 2OG (Fig. 2*C*), it was the case in the presence of a moderate concentration of 2OG. Thus, the presence of as little as 0.1 mM 2OG was sufficient to render the affinities for the double and single operator configurations different by 5-fold (Fig. 2*C*). The situation in the absence of 2OG may correspond to ammonia-grown cells, in which 2OG levels may be very low and marked repression occurs similarly with both operators, $nifOR_1$ alone, or $nifOR_1$ with $nifOR_2$ at an altered distance. The situation with concentrations of 0.1 mM 2OG (or higher) may correspond to alanine-grown cells, in which both operators appropriately spaced are required for repression. In dinitrogen-grown cells, 2OG levels are likely so high that NrpR cannot bind even to both operators together.

DISCUSSION

The results presented here lead to a model for an inducer-controlled binding of the repressor NrpR to *nif* operator DNA that accounts for the regulatory effects observed *in vivo* (Fig. 5). The wild type configuration of the *nif* promoter region, containing both operators $nifOR_1$ and $nifOR_2$ appropriately spaced, cooperatively binds a NrpR dimer pair with high affinity. This binding results in repression during growth on ammonia, in which cellular 2OG levels are low, and repression during growth on alanine, in which cellular 2OG levels are intermediate. Only during growth on dinitrogen are 2OG levels sufficiently high to disrupt cooperative binding, resulting in derepression. Alteration of the primary operator $nifOR_1$ radically decreases the binding affinity, which explains the complete loss of repression. Altering the auxiliary operator $nifOR_2$ or moving

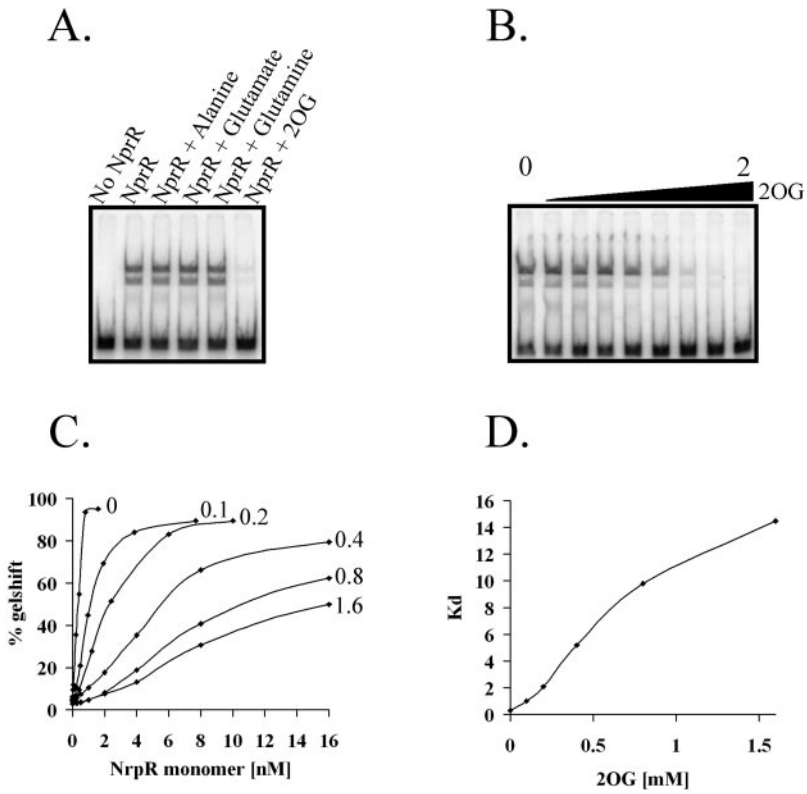

FIG. 4. **2OG inhibits binding of NrpR to operator DNA.** *A*, gel shift of NrpR-His (0.4 nM) binding to WT *nif* (0.02 nM) in the absence (*No NrpR*) or presence of potential effectors (5 mM). *B*, effect of increasing 2OG concentration (0, 0.001, 0.003, 0.009, 0.03, 0.08, 0.2, 0.7, and 2 mM). *C*, binding *versus* NrpR-His concentration at various 2OG concentrations (mM). *D*, K_d *versus* 2OG concentration using data from *C*.

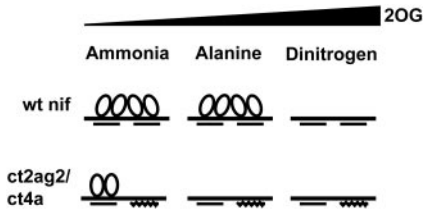

FIG. 5. **Model for repressor-operator binding resulting in the regulation of *nif* transcription in *M. maripaludis*.** The *wedge* represents intracellular 2OG concentration, which varies with growth on three different nitrogen sources. *Ovals* represent NrpR monomers. *Underlines* represent wild type (*straight*) and altered or repositioned (*squiggly*) operator sequences.

it with respect to nifOR$_1$ removes the ability of NrpR dimer pairs to bind cooperatively, so only a single dimer binds to nifOR$_1$ at a lower affinity compared with cooperative binding to both operators together. As a result, repression occurs only during growth on ammonia in which 2OG levels are low, whereas during growth on alanine, 2OG levels are sufficient to disrupt the relatively weak binding of the NrpR dimer to the nifOR$_1$. Our laboratory[2] has shown recently that cellular 2OG levels indeed vary in *M. maripaludis* with nitrogen conditions. Although absolute intracellular metabolite levels are difficult to measure accurately, the relative levels differed by 10-fold, with approximate levels of 0.08 mM in ammonia-grown cells and 0.8 mM in dinitrogen-grown cells. These values correspond roughly to the 10-fold range of 2OG concentrations (0.1–1.6 mM) over which the K_d for NrpR binding to the *nif* operators varies (Fig. 4*D*).

2OG is a common indicator of cellular nitrogen status in a variety of Bacteria because it serves as the main precursor for ammonia assimilation. 2OG serves as a carbon skeleton for the incorporation of amino groups in the glutamate synthase (GOGAT)-glutamine synthetase pathway. In *Escherichia coli*, 2OG and glutamine are both signals of nitrogen status and together signal nitrogen sufficiency (high glutamine, low 2OG) or nitrogen deficiency (low glutamine, high 2OG). In *E. coli*, 2OG interacts directly with P_{II} nitrogen sensor proteins, whereas glutamine controls their covalent modification (28). P_{II} in turn regulates nitrogen assimilation functions at several levels, including transcription by interaction with the NtrB-NtrC two component regulators (47). In other instances, 2OG alone seems to signal nitrogen status. In *Azotobacter vinelandii*, 2OG binds to NifA, releasing it from its inactive NifA-NifL complex, and in cyanobacteria, 2OG binds directly to the transcriptional regulator NtcA, in both cases activating *nif* transcription (48–50). Our laboratory[2] has shown recently in cell extracts of *M. maripaludis* that 2OG reverses the negative effect that the P_{II} homologs NifI$_1$ and NifI$_2$ (30, 51) have on nitrogenase activity. NrpR provides another instance in which 2OG affects a nitrogen regulator.

This work has led to an unusual level of understanding of an archaeal regulatory system (3) because the roles of a repressor, an inducer, and two operators are now known *in vitro* and *in vivo*. The system invites comparison with well studied bacterial repression systems such as LacI (52, 53) and λ CI (46). Like NrpR with *nif* regulation, each of these systems uses auxiliary operators, but they differ as well. In the LacI system, two auxiliary operators are 92 and 401 bp from the primary operator. LacI is a tetramer, and DNA looping evidently allows it to bind simultaneously to the primary operator and one of the auxiliary operators. In contrast, CI is a dimer, two of which interact to bind cooperatively to two operators, which are centered only 24 bp apart (46). The protein-DNA binding system in NrpR appears to be more similar to CI, in which the repressor is a dimer and the operators are centered 31 bp apart. On the other hand, the mechanism that modulates binding is more like LacI, in which an inducing ligand decreases binding affinity, rather than CI, in which the cellular concentration of the intact repressor itself determines the amount bound. (It is interesting to note, however, that an auxiliary operator is not always

[2] J. A. Dodsworth and J. A. Leigh, submitted for publication.

necessary for full repression by NrpR because a single operator, which may have high affinity for NrpR, appears sufficient in the case of *glnA* (34)). In any case, even though NrpR is a regulator known only in the Euryarchaeota, its basic mechanism appears analogous to bacterial repression, and NrpR presumably occludes the binding or action of some components of the archaeal transcription initiation machinery.

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