Regulation of High Affinity Nickel Uptake in Bacteria

**Ni²⁺-DEPENDENT INTERACTION OF NikR WITH WILD-TYPE AND MUTANT OPERATOR SITES**

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The trace metal nickel is required by all organisms as an enzyme cofactor (1–3), and nickel-containing enzymes can account for several percent of the cellular protein in bacteria such as *Escherichia coli*. Free nickel ions can be toxic to cells by binding nonspecifically to biomolecules or by displacing other metals from their native binding sites. Clearly, molecular mechanisms must be present to preserve the balance between the level of nickel required for viability and the potentially lethal effects of excessive intracellular nickel. Nickel, present at nM to µM concentrations in the extracellular environment, is imported into *E. coli* via the ATP-dependent nickel permease encoded by the *nikABCDE* operon (4) (see Fig. 1). Transcription of this operon is highly regulated. It is activated by fumarate nitrate regulatory protein (Fnr), an oxygen-sensitive transcriptional factor, and repressed by NikR when intracellular nickel concentrations are high (5, 6). Hence, the *nik* operon is maximally expressed under anaerobic growth conditions when intracellular nickel is scarce.

Relatively little is currently known about NikR repressor or how it regulates gene expression. For example, the operator site to which NikR binds to control *nik* transcription has not been rigorously identified, and it is not clear whether NikR senses nickel directly or through mechanisms mediated by other proteins. We have recently shown that *E. coli* NikR is a member of the ribbon-helix-helix family of transcription factors (7). This family includes the Arc and Mnt repressors of bacteriophage P22 (8, 9), the MetJ repressor (10), the TraY protein (11), the CopG family of plasmid stability proteins (12), and the transcriptional activator AlgZ (13). The dimeric ribbon-helix-helix fold consists of an antiparallel β-sheet, formed by single β-strands from each subunit, and four α-helices. Crystallographic studies show that the β-sheets of MetJ, Arc, and CopG dimers bind in the major groove of operator DNA and make specific contacts with sequences of 4–6 base pairs (9, 10, 12). The operators of these repressors contain two or more dimer recognition sequences, usually related by dyad symmetry, and stable DNA binding requires stabilizing interactions between dimers bound to adjacent operator subsites (9, 12, 14).

All ribbon-helix-helix family members share homologous DNA-binding domains, but some also contain additional specialized domains or subdomains. For example, MetJ repressors contain a subdomain formed by the C-terminal 40 or so residues that helps form a binding site for the corepressor, S-adenosylmethionine (15). Mnt has a coiled coil domain of roughly 30 residues at its C terminus that mediates tetramerization (16, 17). Orthologs of NikR are present in some bacteria and many archaeal genomes (7). Each of these NikR proteins consists of an N-terminal ribbon-helix-helix domain of approximately 50 residues and a NikR-specific C-terminal domain of about 80 residues. The most striking feature of the C-terminal domain is a His-X₁₁-His-X₁₀-His-X₆-His-X₅-Cys sequence motif. Proteins rich in histidine and cysteine often bind divalent transition metals (18); in NikR, these residues may bind nickel ions (7).

Consistent with the role of NikR as a transcriptional repressor, we have previously demonstrated that the N-terminal domain dimer binds weakly to two regions within the *nikABCDE* promoter (Pₙⁱᵏ) (7). Here, we show that full-length NikR is a Ni²⁺-binding protein and characterize its binding to operator DNA. We demonstrate that high affinity binding of NikR to the operator in *vitro* is Ni²⁺-dependent, identify the operator bases most important for NikR binding, and then show that NikR binding to this operator site is required for efficient Ni²⁺-dependent repression of the *nikABCDE* operon in *vitro*. These experiments support a model for NikR function in which the N-terminal domain binds DNA subsites and the C-terminal domain binds Ni²⁺ and regulates operator binding via specific interactions between adjacentlly bound NikR dimers.

**EXPERIMENTAL PROCEDURES**

Molecular Biology—Synthetic oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) and were purified by electrophoresis on denaturing polyacrylamide gels (19). The *nik* gene was cloned by PCR² from genomic DNA of *E. coli* strain MC1061 largely

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² The abbreviations used are: PCR, polymerase chain reaction; NTA, nitrilotriacetic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)]-ethylglycine.

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1 Peter T. Chivers and Robert T. Sauer, unpublished observations.

2 The abbreviations used are: PCR, polymerase chain reaction; NTA, nitrilotriacetic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)]-ethylglycine.
Biochemical Corp.). Sequencing using Sequenase version 2.0 DNA sequencing kit (U. S. Biochemical Corp.) to construct a P\textsubscript{nik}-lacZ reporter fusion, a 500-base pair DNA fragment containing the nikABCDE promoter region was first subcloned from E. coli strain MC1061 by PCR using primers P\textsubscript{cl}118 (5'-GGC GGG CAA ACC TGC ATT TGC GCC GG-3') and P\textsubscript{cl}120 (5'-CAT TGT CGA CAG AAC CAA AAT GTA TAA AAA-3'). The non-coding bases corresponding to the ATG start codon of nik\textsubscript{a} are shown in bold. The purified fragment was digested with E\textsc{coi} and SalI and ligated into pACYC184 (20) digested with the same enzymes. The lac\textsubscript{Z} gene was amplified from E. coli strain CA8224.1 (E. coli Genetic Stock Center) by PCR with primers PC14a (5'-TTT GGA AGG TCG TCT GAG TCA ATC TTC CTT CGG CAA-3') and 100 mM NaCl. The resulting fragment was digested with SalI and BamHI and ligated into the pACYC184 backbone already containing the nik promoter. The resulting plasmid, designated pC163, was sequenced to confirm that the wild-type nik promoter sequence was correct.

Purification and characterization of the mutant strain BB101 (5' lac\textsuperscript{F} lac\textsuperscript{p} pro') DNA was amplified from E. coli strain CA8224.1 and cloned into the pACYC184 vector. The resulting plasmid was digested with SalI and BamHI and ligated into pC163 that had been digested with the same enzymes. The promoter and operator regions of the mutant plasmids were sequenced to confirm the expected structure.

Replicase Constructs—Operator mutations were generated as follows. A purified oligonucleotide containing the desired operator mutations was used as a primer together with PCR120 in a PCR reaction with plasmid pC163 as template. The resulting PCR product of approximately 100 base pairs was purified by electrophoresis on a non-denaturing 10% polyacrylamide gel and then used as a megaprimer in a subsequent PCR reaction that contained primer PCR118 and plasmid pC163. Each resulting 500-base pair fragment was purified by non-denaturing 6% polyacrylamide gel electrophoresis, digested with E\textsc{coi} and SalI, and cloned into pC163 that had been digested with the same enzymes. The promoter and operator regions of the mutant plasmids were sequenced to confirm the expected structure.

Purification of Full-length NikR—Bacterial lysate was centrifuged at 14,000 \texttimes g for 30 min, and the supernatant was retained. Nickel sulfate (10 mM stock solution in H\textsubscript{2}O) was added to the supernatant to a final concentration of 50 \textmu M followed by the addition of 1 \textmu M imidazole (pH 7.5) to a final concentration of 10 mM. The supernatant was then loaded onto a N\textsuperscript{32P}-NTA column (Qiagen, Inc., Chatsworth, CA) that had been pre-equilibrated with 10 volumes of 100 mM potassium phosphate (pH 8.0), 500 mM NaCl, and 10 mM imidazole. The N\textsuperscript{32P}-NTA resin was used per liter of initial cell culture. After loading, the column was washed with 50 volumes of the equilibration buffer, and bound protein was eluted with 5 volumes of 100 mM potassium phosphate, 10 mM Tris-HCl, and 250 mM imidazole (final pH 7.6). EDTA (0.5 mM stock solution in H\textsubscript{2}O) was added to the eluate to a final concentration of 10 mM to prevent aggregation, and the solution was dialyzed against 10 mM HEPES (pH 7.6), 100 mM NaCl, 1 mM EDTA (4 liters per 5 ml of eluate) and stored at 4°C. NikR samples were desalted on a PD-10 desalting column (Amersham Pharmacia Biotech) pre-equilibrated with 10 mM HEPES (pH 7.6), 10 mM glycerol, and 100 mM NaCl.

Protein concentration of full-length NikR was determined at pH 7 in 6 M guanidine hydrochloride using an extinction coefficient of \epsilon\textsubscript{495} = 4495 M\textsuperscript{-1} cm\textsuperscript{-1}. The protein concentration of the N-terminal domain was determined at pH 12.0 using an extinction coefficient of \epsilon\textsubscript{495} = 2600 M\textsuperscript{-1} cm\textsuperscript{-1}. Purified proteins were more than 95% pure as determined by SDS-PAGE.

RESULTS

In a previous study (7), we showed that the purified ribbon-helix-helix domain of NikR protected two regions of DNA within the nik promoter. These protected regions overlapped an extensive inverted repeat sequence in which 28 of 38 base pairs were related by an axis of 2-fold symmetry located near the -10 position of P\textsubscript{nik} (Fig. 1). Such dyad-symmetric DNA sequences are typical of many operator sites for bacterial repressors. As described below, we first studied nickel binding by the intact NikR repressor, then assayed NikR binding to a DNA fragment containing the entire nik promoter region by electrophoretic mobility shift experiments, and finally used footprinting and mutational studies to define the operator site and its functional role in greater detail.

Protein Purification and Ni\textsuperscript{2+} Binding—Intact NikR repressor was cloned, overexpressed, and purified by chromatography on a N\textsuperscript{32P}-NTA column. In the absence of excess Ni\textsuperscript{2+} during column loading, NikR stripped nickel from the column and failed to bind efficiently (<10% bound). Because the N\textsuperscript{32P}-NTA equilibrium dissociation constant is roughly 3 psi (24), this result suggested that NikR contains at least one high affinity binding site for nickel ions. Addition of 50 \textmu M Ni\textsuperscript{2+} to the loading buffer, however, resulted in >95% binding of NikR to the resin and allowed efficient single step purification. Following desalting into nickel-free buffer, full-length purified NikR had a UV-visible absorbance spectrum with maxima at 245, 262, 302, 362, 460, and 570 nm, suggestive of ligand-to-metal transitions at the shorter wavelengths (>302 nm) and nickel d-d transitions at the longer wavelengths (Fig. 2a). Circular dichroism spectroscopy also showed peaks at wavelengths corresponding to peaks in the UV-visible spectrum (Fig. 2b). In both the

Footprinting and DNA Binding—DNase I footprinting was performed as described previously (7) but in a binding buffer containing 10 mm HEPES (pH 7.6), 100 mM KCl, 3 mM MgCl\textsubscript{2}, 1.5 mM CaCl\textsubscript{2}, and 50 \mu M NiSO\textsubscript{4}. A DNA fragment extending from position -131 to +57 relative to the starting point of P\textsubscript{nik} transcription was end-labeled with \textsuperscript{32P} and incubated with NikR at 20°C for 30 min. DNase I (Worthington) was then added to a final concentration of 200 ng/ml. EDTA was not included in any of the buffers for DNA binding assays. DNA methylation protection experiments were performed as described previously (22).

Electrophoretic mobility shift assays were performed using 7% acrylamide gels and electrophoresis buffer containing 75 mM Tris, 300 mM boric acid (final pH 7.5). The binding buffer was 20 mM Tris (pH 7.6), 100 mM KCl, 0.1% Nonidet P-40, and 5% glycerol. Ni\textsuperscript{2+} was added to the binding buffer, the electrophoresis buffers, and the gel solution before polymerization to final concentrations as described under "Results." An end-labeled DNA fragment extending from position -43 to +57 relative to the starting point of P\textsubscript{nik} transcription was incubated with NikR at 20°C for 15 min in a volume of 50 \mu l, and 20 \mu l was then loaded directly onto a running gel (300 V). No dyes were added to the binding reactions. After loading all samples, the electrophoresis voltage was reduced to 150 V.

Reporter Assays—\beta-galactosidase assays were performed in E. coli strain MC1061 containing an F\textsuperscript{kan} lac\textsuperscript{p} episome (obtained from A. Grossman, Massachusetts Institute of Technology), plasmid pNIK103 (which expresses NikR under control of a T7 promoter and lac operator), and either the wild-type or mutant variants of the P\textsubscript{nik}-lacZ reporter plasmid. This strain lacks a gene for T7 RNA polymerase, but sufficient NikR was still synthesized from the chromosomal and/or pNIK103 copies of the nik\textsubscript{r} gene to repress P\textsubscript{nik}-lacZ expression in the presence of nickel. The presence of the episomal lac\textsuperscript{I} allele, which encodes Lac repressor, was important for determining the dynamic range of the reporter assay. Without the lac\textsuperscript{I} gene, P\textsubscript{nik}-lacZ expression was low even in the absence of added nickel, suggesting that too much NikR was being synthesized.

Cells freshly transformed with the P\textsubscript{nik}-lacZ reporter plasmids were grown anaerobically without shaking at 37°C in screw-capped 1.5-ml microfuge tubes containing LB broth plus 100 \mu g/ml ampicillin and 25 \mu g/ml chloramphenicol. NiSO\textsubscript{4} was added to a final concentration of 250 \mu M when required. After overnight growth for 12–14 h (A\textsubscript{600} of 0.5 to 0.7), the culture tubes were opened, and cells were lysed immediately with chloroforom and SDS. \beta-galactosidase assays were then performed as described (23).

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UV-visible and CD spectra, the peaks attributed to nickel binding required the presence of both the metal ion and NikR and were absent following incubation with 50 mM EDTA for 12 h at room temperature. Both the spectral properties and activity of apoNikR were restored upon addition of excess nickel, showing that nickel binding is reversible.

The stoichiometry of high affinity nickel binding to NikR was determined by titration of a sample of 17.5 mM apoNikR with Ni\(^{2+}\) (Fig. 2c) using purified protein that had been incubated with EDTA and desalted into buffer without chelating agents. The increase in absorbance at 302 nm was measured after the addition of each Ni\(^{2+}\) aliquot. The absorbance increase was initially linear, indicating a binding site of submicromolar affinity, and then saturated at a Ni\(^{2+}\) concentration of roughly 1 nickel ion for each NikR subunit, or 2 ions bound for each NikR dimer (7). Assuming that nickel ions bind to the His-X\(_9\)-His-X\(_{10}\)-His-X\(_9\)-Cys sequences, this suggests the presence of a single high affinity Ni\(^{2+}\)-binding site in each C-terminal domain of the dimer. After saturation of the high affinity site, no significant absorbance changes were observed up to a concentration of 50 mM Ni\(^{2+}\). Addition of higher concentrations of Ni\(^{2+}\) resulted in protein aggregation at the NikR concentrations used in this experiment. The small positive slope of the plateau region in Fig. 2c may result from a small amount of protein aggregation.

**Operator Binding**—In electrophoretic mobility shift experiments performed in the presence of 50 mM Ni\(^{2+}\), purified NikR half-maximally bound to a 100-base pair DNA fragment containing the **nik** promoter-operator region at a protein concentration of roughly 15 pM (Fig. 3, a and c). Nickel had to be present in the electrophoresis buffer and in the gel at a concentration of at least 20 mM for a mobility shift to be observed in these experiments. No mobility shift complexes were observed in the presence of 5 mM Ni\(^{2+}\) (data not shown) or in the absence of Ni\(^{2+}\) (Fig. 3b). Moreover, NikR that had been quickly desalted and hence had nickel bound just to the high affinity site also did not produce a mobility shift complex. These observations suggest that the formation of a stable
NikR-operator complex is dependent upon a rapidly exchanging and thus weakly bound nickel ion and show that nickel bound only to the high affinity site is not sufficient to produce a stable protein-DNA complex. In experiments using the purified N-terminal domain of NikR, no mobility shift complex was observed at protein concentrations as high as 10 μM, either in the presence or absence of nickel (data not shown), indicating that the C-terminal domain is required for the formation of a stable mobility shift complex.

In DNase I footprinting experiments, the intact NikR protein protected approximately 40 contiguous base pairs centered near the −10 position of the Pnik promoter (Fig. 4a). The outer flanks of the NikR footprint were similar to those of the isolated N-terminal domain footprint (7). However, unlike the N-terminal domain alone, full-length NikR also protected the central region of the operator site from DNase I digestion.

To identify potential sites of close contact between NikR and the operator site, we performed dimethylsulfate protection experiments. The N7 positions of four guanines, two on each DNA strand, were protected from methylation by bound NikR (Fig. 4b). Each protected G was located in a dyad-symmetric 5'-GTATG-3' sequence located on opposite strands and on opposite sides of the −10 promoter hexamer. Moreover, these sequences were roughly centered within the two regions protected by the ribbon-helix-helix domain of NikR. No significant protection of guanines in other regions of the operator was observed. These results and the fact that other ribbon-helix-helix dimers recognize DNA subsites of 4–6 base pairs suggested that operator bases within or near these 5'-GTATG-3' sequences were likely to comprise the principal base-specific determinants of NikR recognition.

**Operator Base Pairs Required for High Affinity NikR Binding and Repression**—To define the operator determinants required for NikR binding in greater detail, base substitution mutations were constructed at each base in the 5'-GTATG-3' sequences and at four flanking base pairs. Individual mutants contained two symmetric changes, one in each half of the operator, and were named according to the distance of the mutations from the dyad-symmetry axis. Binding of the mutant operators to NikR in the gel mobility shift assay revealed severe reductions in affinity (>1000-fold) for five of the mutants (−14/14, −13/13, −11/11, −10/10, and −9/9) and a modest reduction (5-fold) for another mutant (−12/12) (Fig. 5). At the three remaining positions (−15/15, −8/8, and −7/7), the mutations caused no change in apparent affinity. These experiments indicate that the 5'-GTATGA-3' subsite sequences play critical roles in determining the affinity of NikR binding.

We also constructed an operator mutant (C11→G14) containing transversion mutations at each of the 11 base pairs from operator positions −6 to +5 (Fig. 5). This mutant bound NikR with wild-type affinity. This result makes it highly unlikely that NikR makes base-specific interactions with this central region of the operator. A mutant operator in which the entire right subsite was substituted (rhs7/15) showed no binding to NikR in the mobility shift assay. Moreover, no intermediate species indicative of a dimer-DNA complex was observed for this or any other operator variant, suggesting that complexes between single NikR dimers and the operator are too unstable to be detected in this assay. As a result, any NikR-operator complex that is observed in mobility shift experiments is likely to be stabilized by dimer-dimer interactions of some type. Because both Ni2+ ions and the C-terminal domain are also required for the observation of stable complexes, it seems likely that Ni2+ dependent interactions between the C-terminal domains of NikR mediate dimer-dimer contacts.

Does binding of NikR to the operator site identified here regulate nikABCDE expression in vivo? To answer this question, we constructed Pnik-lacZ reporter fusions with wild-type operators and six of the mutant operators. In the context of the wild-type promoter and operator, very little β-galactosidase expression (<20 units) was detected during aerobic growth (data not shown), and expression under anaerobic growth was repressed approximately 5-fold in the presence of Ni2+ (Fig. 6). Four of the operator mutants that severely reduced NikR binding in vitro were found to reduce Ni2+-dependent repression of β-galactosidase expression in vivo (Fig. 6). By contrast, two operator mutants that showed NikR binding levels within 5-fold of wild type still mediated Ni2+-dependent repression of the nikABCDE operon. Five of six operator mutants also resulted in increased levels of β-galactosidase expression in the absence of added Ni2+, suggesting that these mutations may increase intrinsic promoter strength and/or abolish repression by NikR that occurs in the absence of Ni2+ or in the presence of very low levels of Ni2+.

**DISCUSSION**

The experiments reported here establish that NikR is a direct sensor of nickel ions and acts as a negative regulator of
nikABCDE expression by binding to an operator consisting of two 5'-GTATGA-3' half-sites related by dyad symmetry and separated by 16 base pairs. A low affinity interaction between NikR and 20–50 μM Ni²⁺ results in tight binding of NikR to the operator. Under these conditions, NikR half-maximally bound to the nIK operator at a concentration of roughly 15 pM. Binding was dramatically reduced if nickel was not present throughout the binding assay or if the NikR C-terminal domain was removed. For example, although the isolated N-terminal domain of NikR protects the operator in footprinting experiments (7), this binding is observed at concentrations 10,000-fold higher than those required for binding of intact NikR. In principle, the C-terminal domain might help mediate direct Ni²⁺-dependent contacts with operator DNA and/or mediate cooperative inter-

![Diagram](http://www.jbc.org/)

**FIG. 4.** DNase I and dimethylsulfate footprinting of the nIK operator. a, DNase I footprint on the top strand of the nIK operator. Lane 1 contained operator DNA alone. Lane 2 contained operator DNA, 1 mM NikR, and 50 μM Ni²⁺. The bracketed region shows the bases protected by NikR. The gray bars indicate bases previously shown to be protected by the NikR N-terminal domain (7). b, dimethylsulfate protection of the nIK operator. Densitometry traces of the top and bottom strands of the promoter region are shown above and below the promoter sequence. Peaks correspond to DNA cleavage at N7-methylated guanine residues. Arrows and filled diamonds indicate guanine residues protected by NikR from methylation.

**FIG. 5.** Dissection of the E. coli NikR operator site. The boxed region indicates the region protected from DNase I cleavage. Filled diamonds indicate guanine residues protected from methylation by NikR. The center of approximate dyad symmetry of the region is indicated by a black vertical bar. The sequence changes (coding strand) and relative half-maximal binding compared with wild type of nine symmetric double mutants, one mutant with 11 substitutions in the central region of the operator, and one mutant with 9 changes in the right subsite are shown.
actions between NikR molecules bound to each operator subsite. We note, however, that our results suggest that there are no sequence-specific interactions between NikR and the central or spacer region of the operator. Moreover, removal of one of the operator subsites also reduced NikR binding dramatically, suggesting that some specific interactions between adjacent binding sites are necessary for high affinity operator binding.

Our results indicate that NikR contains two distinct types of nickel-binding sites. Purified NikR binds two nickel ions per dimer with high affinity in a manner that gives rise to distinctive changes in both the UV absorbance and circular dichroism spectra, although the function of nickel binding to this site is unknown. The ability of NikR to strip nickel from Ni²⁺-NTA resin suggests that the Kd ratio is considerable. However, no evidence for low affinity nickel binding was obtained from UV-visible spectroscopic measurements, suggesting that Ni²⁺ bound at this site has a small extinction coefficient or that occupancy also requires the presence of operator DNA.

The NikR half-sites are similar in size to subsites recognized by other ribbon-helix-helix proteins, but the spacing between these sites is much greater than for other family members. For example, the center-to-center spacing of half-sites for NikR is 22 base pairs, roughly two full turns of the DNA helix, whereas the next largest subsite spacing is 11 base pairs for the Arc, Mnt, and CopG repressors. For Arc, Mnt, MetJ, and CopG, the subsite spacing is close enough to allow cooperative contacts between adjacent half sites. Thus, the spacing of subsites could be responsible for the function of NikR or an additional transcription factor?

Homology searches identified putative operator sequences for NikR in Salmonella typhimurium, Klebsiella pneumoniae, Helicobacter pylori, and Bradyrhizobium japonicum (Fig. 7). The Klebsiella site is in the promoter for NikA, whereas the Salmonella site is in the promoter for an Fnr-regulated operon encoding an ABC-type nickel/peptide transporter related to NikA (25). The Helicobacter site is in the NikR promoter, suggesting that NikR may negatively regulate its own synthesis. Finally, the Bradyrhizobium site is in the promoter region of hupN, a gene encoding a transmembrane protein thought to be involved in high affinity nickel uptake in this organism (26). In the E. coli operator, residues important for NikR binding account for fewer than half of the base pairs that are related by dyad symmetry. Is the extended inverted repeat sequence important for the function of NikR or an additional transcription factor?

Ribbon-helix-helix proteins use surface residues in the β-sheet to make specific operator DNA contacts (7, 27–31). Surprisingly, NikR and CopG have the same Arg-X-Thr-X-Thr sequence in the β-sheet but recognize completely different subsite sequences (5'-GTATGA-3' versus 5'-TTGA3'). Clearly, factors in addition to the surface β-sheet residues must determine how these ribbon-helix-helix proteins interact with operator half-sites. This suggests that it may not always be straightforward to predict the dimer recognition subsites for other NikR family members.

The experiments presented here and elsewhere (6, 7) support the general model shown in Fig. 1. By binding to an operator within P_nik, NikR represses nikABCDE transcription in the presence of Ni²⁺. When nickel concentrations in the cell fall below some critical level, the affinity of NikR for the operator decreases, and the operon is derepressed, allowing synthesis of NikABCDE, which can catalyze increased levels of nickel uptake. Based on the experiments presented here, it seems likely that the critical nickel threshold is in the 20 μM range. It remains to be determined what role, if any, is played by nickel binding to the high affinity site. Occupancy of this site may be required but not sufficient for strong operator binding and/or

![Fig. 6. Effect of operator mutations on P_nik-mediated expression of β-galactosidase in vivo. Shaded boxes indicate positions that have significantly reduced half-maximal binding in vitro. Repression ratio is P_nik expression in the absence of nickel divided by expression in the presence of nickel. nd, not determined.](http://www.jbc.org/content/283/26/19740.full)

![Fig. 7. Homologous NikR operator sites in other bacteria. The left column lists the organism and the gene immediately downstream of the putative NikR operator site. Highly conserved positions are shaded. Bases in each operator that constitute an inverted repeat in each operator are underlined.](http://www.jbc.org/content/283/26/19740.full)
repression. Alternatively, high affinity nickel binding could play a role in other aspects of nickel homeostasis. The experiments presented here provide a foundation for future studies designed to test these possibilities.

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