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Isolation and Characterization of the *nikR* Gene Encoding a Nickel-Responsive Regulator in *Escherichia coli*

KARINNE DE PINA,[1] VALERIE DESJARDIN,[1] MARIE-ANDREE MANDRAND-BERTHELOT,[1] GERARD GIORDANO,[2] AND LONG-FEI WU[2]*

Laboratoire de Génétique Moléculaire des Microorganismes et des Interactions Cellulaires, CNRS UMR5577, Institut National des Sciences Appliquées, 69621 Villeurbanne Cedex,[1] and Laboratoire de Chimie Bactérienne, CNRS UPR9043, Institut de Biologie Structurale et Microbiologie, 13402 Marseille Cedex 20,[2] France

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Expression of the nickel-specific transport system encoded by the *Escherichia coli nikABCDE* operon is repressed by a high concentration of nickel. By using random transposon Tn*10* insertion, we isolated mutants in which expression of the *nik* operon became constitutive with respect to nickel. We have identified the corresponding *nikR* gene which encodes a nickel-responsive regulator. Expression of *nikR* was partially controlled by Fnr through transcription from the *nikA* promoter region. In addition, a specific transcription start site for the constitutive expression of *nikR* was found 51 bp upstream of the *nikR* gene.

Nickel has been known for a long time as a heavy metal toxic to both eukaryotic and prokaryotic organisms (1, 16). Epidemiological studies have identified nickel as potentially carcinogenic and allergenic to humans (5, 9). Nickel is also an essential trace element for both eukaryotes and prokaryotes. The average daily requirement for humans is estimated to be 0.15 mg per day, and the total quantity in the body is about 10 mg (19). In microorganisms, nickel forms the active center of at least five classes of metalloenzymes: urease, NiFe-hydrogenase, methyl coenzyme M reductase, and CO dehydrogenase (7) and superoxide dismutase (26).

We demonstrated previously that nickel has an antagonistic effect on the fermentative growth of *Escherichia coli* (23). Nickel is essential for activities of three NiFe-hydrogenase isoenzymes and for bacterial fermentative growth. The successful production of these nickel-containing enzymes relies on the efficient uptake of nickel via the high-affinity, nickel-specific ABC transport system encoded by the *nikABCDE* operon (11, 21). However, when present at high concentrations (from 0.3 mM in rich medium), nickel inhibits growth and thus exhibits a toxic effect (11, 23). *E. coli* uses two strategies in response to the toxic concentration of nickel. First, it activates Tar- and NikA-dependent negative chemotaxis and swarms away from this repellent agent (4). Second, it blocks the entrance of nickel through the high-affinity nickel transport system. This is achieved by repression of the expression of the *nik* operon (21, 22). We report here the identification of the *nikR* gene which encodes a novel type of metallo-regulatory protein responding specifically to nickel.

Screening for Tn*10* insertions in nickel-responsive regulator element. To isolate mutants defective in the repression of *nik* at a high nickel concentration, we took advantage of the relatively simple phenotype plate screens for constitutive expression of β-galactosidase from the *nikA-lacZ* fusion. A random collection of 1,100 independent Tn*10* insertions in strain HYD723 [as MC4100, but *nikA*::MudI(*lacZ* Amp^r) (21)], which was performed by using phage lambda 1098 as described by Way et al. (20), was plated on MacConkey-lactose-tetracycline plates supplemented with 0.5 mM nickel at a density of about 200 cells per plate. Two colonies, KS01 and KS02, showing red color under anaerobic conditions were picked, purified, and characterized further. They exhibited constitutive expression of β-galactosidase activity in the presence of 0.5 mM nickel (Fig. 1). The constitutive expression of the *nik* operon in the mutants might be the result of insertion of Tn*10* into a regulatory element or a consequence of transposition of phage MudI from the *nik* operon to other places. In order to assess these possibilities, we analyzed the phenotype of these double mutants with respect to the restoration of hydrogenase activity by nickel. Two observations confirmed that they retained the correct *nikA*::MudI(*lacZ* Amp^r) genotype. First, these mutants showed a hydrogenase-negative phenotype, which was restored by 0.5 mM $NiCl_2$. Second, β-galactosidase activity and hydrogenase activity were detected only during anaerobic growth in these double mutants, which is the same phenotype as that of the parental *nik-lacZ* single mutant HYD723 (21).

Mapping, cloning, and sequencing of the *nikR* gene. In an attempt to quickly locate the mutations in mutants KS01 and KS02, the Tn*10* insertions were introduced into the wild-type strain MC4100 by transduction with P1*cml* (10). Transductants were selected for tetracycline resistance and then screened for ampicillin resistance. This experiment also allowed us to genetically purify the Tn*10* insertion mutants. In the two cases, a 98% linkage was found between ampicillin resistance and tetracycline resistance. This percentage of cotransduction indicated that the Tn*10* integration is approximately 0.02 min away from the *nikA-lacZ* fusion on the *E. coli* chromosome (25). Therefore, constitutive expression of the *nikA-lacZ* fusion in the mutants might be the consequence of Tn*10* insertion in the promoter-operator region of the *nik* operon or in a gene coding for a nickel-responsive regulator, which is located in the immediate vicinity.

To assess these possibilities, in *trans* complementation experiments were performed by using various plasmids carrying the *nik* operon and adjacent regions, which were described previously (22, 24). Plasmid pLW22 contains a 7-kb chromosome fragment which covers the entire *nik* operon as well as 0.9-kb upstream and 0.9-kb downstream adjacent regions (Fig.

* Corresponding author. Mailing address: Laboratoire de Chimie Bactérienne, UPR9043, Institut de Biologie Structurale et Microbiologie, CNRS, 31 chemin Joseph Aiguier, 13402 Marseille Cedex 20, France. Phone: (33) 4 91 16 44 31. Fax: (33) 4 91 71 89 14. E-mail: wu@ibsm.cnrs-mrs.fr.

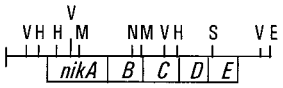
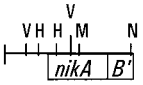
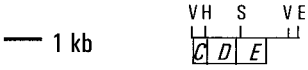
Plasmid	Insert	β-Galactosidase activity					
		HYD723		KS01		KS02	
		-$NiCl_2$	+$NiCl_2$	-$NiCl_2$	+$NiCl_2$	-$NiCl_2$	+$NiCl_2$
——	——	214±41	<1	234±23	210±05	225±23	218±25
pLW22		<1	<1	<1	<1	<1	<1
pLW25		209±19	<1	202±27	223±31	224±21	246±52
pLW26		214±40	<1	225±25	<1	218±21	<1

FIG. 1. Complementation analysis of Tn*10* insertion mutants with plasmids carrying the *nik* region. KS01 and KS02 are Tn*10* insertion derivatives of HYD723 (*nikA-lacZ*). Cells which carried the indicated plasmid were grown microaerobically at 37°C in LB medium supplemented with 2 μM ammonium molybdate, 2 μM sodium selenite, and kanamycin (25 μg/ml) when required (21). Plasmids pLW22, pLW25, and pLW26 were described previously (24). β-Galactosidase activity was measured for cells treated by addition of 0.0025% sodium dodecyl sulfate–5% chloroform, and the specific activity is expressed as nanomoles of *o*-nitrophenol produced per minute per milligram bacterial dry weight. Values quoted are the averages of three separate experiments. Symbols: E, *Eco*RI; H, *Hin*cII; M, *Mlu*I; N, *Nsi*I; S, *Ssp*I; V, *Eco*RV.

1). Introduction of plasmid pLW22 into mutants KS01 and KS02 completely abolished expression of the *nikA-lacZ* fusion (Fig. 1). Successful in *trans* complementation by plasmid pLW22 indicated that transposon Tn*10* had integrated into a repressor gene instead of the promoter-operator region of the *nik* operon in mutants KS01 and KS02. We designated the transposon-affected gene as *nikR* (for nickel-responsive regulator).

The *nikR* gene is thus located either on the 0.9-kb fragment upstream or on the 0.9-kb fragment downstream of the *nik* operon. Further complementation analysis showed that plasmid pLW26 containing the 0.9-kb downstream fragment was capable of complementing the Tn*10* insertion mutations in mutants KS01 and KS02, whereas plasmid pLW25 carrying the 0.9-kb upstream fragment was not able to do so (Fig. 1). Therefore, the *nikR* gene is located downstream of the *nik* operon.

It should be noted that plasmid pLW22, in contrast to plasmid pLW26, repressed *nikA* expression in the absence and presence of nickel. Since both plasmids carry the *nikR* gene, it seems unlikely that the constitutive repression generated by pLW22 results from multiple copies of *nikR*. Interestingly, plasmid pLW22 harbors in addition the entire *nik* operon encoding a functional nickel-specific transport system, which should increase the intracellular nickel availability required for repression. To minimize the effects resulting from multiple copies of the *nikABCDE* operon, we lowered plasmid copy number by introducing a *pcnB* allele into strain KS01/pLW22. Expression from the *nikA-lacZ* fusion was relieved to half the level of that of the KS01 strain without plasmid in the absence of nickel, demonstrating that overproduction of the high-affinity nickel uptake system was responsible for the constitutive repression.

The DNA sequence of the *nik* locus which covers the *nikAB CDE* genes and a 423-bp downstream region was previously reported (11). No open reading frame (ORF) was revealed by computer analysis of this 423-bp fragment. We rechecked the sequence of the 423-bp area and sequenced its 200-bp downstream region by using the T7 sequencing kit of Pharmacia with Deaza ^{35}Sequencing Mixes to overcome G-C compression. The new sequence corrected seven errors in the old one and was in full agreement with the *E. coli* genome sequence in this region (15). An ORF that predicted a polypeptide of 133 amino acids was revealed. This ORF, designated *nikR*, was transcribed in the same direction as the *nikE* gene and was separated from *nikE* by 5 bp. This ORF corresponded to the hypothetical 15.1-kDa YhhG protein described in the *E. coli* genome database.

Identification of the predicted polypeptide NikR. The sequence of the *nikR* gene predicts a polypeptide of 133 amino acids with a molecular weight of 15,093 and a calculated pI of 6.22. The hydropathy plot of the putative polypeptide revealed a highly hydrophilic protein. The product of the *nikR* gene was first identified by expression of *nikDER* genes from plasmid pHD4 by an in vivo T7 expression system (17). Autoradiography revealed two prominent bands with apparent molecular masses of 28 and 30 kDa and a much fainter band of 15 kDa (Fig. 2, lane 2). These bands were not observed in strain K38/pGP1-2 carrying vector pT7-6 alone without an insert (lane 1),

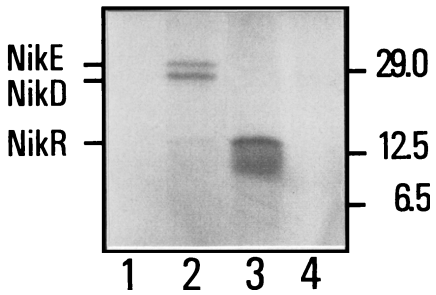


FIG. 2. Specific expression of the *nikR*, *nikD*, and *nikE* gene products, under control of the T7 ϕ10 promoter in *E. coli* K38/pGP1-2. Cells containing vector pT7-6 (lane 1), its derivative pHD4, harboring *nikDER* (lane 2), vector pKSM710 (lane 4), and its derivative p8611, harboring *nikR* (lane 3), were labeled with [^{35}S]methionine and [^{35}S]cysteine and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 17% denaturing polyacrylamide gel. Molecular mass standards (in kilodaltons) and NikE, NikD, and NikR proteins are indicated on the right and on the left, respectively.

and their apparent molecular masses correspond to those calculated for the *nikD* (26.5 kDa), *nikE* (29.6 kDa), and *nikR* (15.1 kDa) gene products, respectively. This result suggests that *nikR* may be expressed from the same transcriptional unit as the *nikD* and *nikE* genes from the T7 ϕ10 promoter under this condition, although its expression was much weaker than that of the other two genes.

In order to increase the specific expression of the *nikR* gene, we then introduced a *Nco*I site at the position of the ATG initiation codon of *nikR* and cloned *nikR* into plasmid pKSM710 (8). In the resulting plasmid, p8611, the *nikR* gene can be expressed optimally from the T7 ϕ10 promoter. Introduction of the *Nco*I site substituted the second codon, GAA (Glu), for CAA (Gln). Insertion of plasmid p8611 into strain K38/pGP1-2 led to the specific synthesis of a polypeptide with an apparent molecular mass of 15 kDa (Fig. 2; compare lanes 3 and 4), similar to that predicted from the nucleotide sequence of *nikR*. We concluded that the *nikR* gene indeed encodes a polypeptide chain. The authenticity of this polypeptide as the product of the *nikR* gene was proven by determination of the N-terminal sequence. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel (15%) electrophoresis and then electroblotted onto a polyvinylidene difluoride membrane. After being stained with PONCEAU S (Sigma), the NikR band was excised. The N-terminal amino acid sequence of NikR was determined by automated Edman degradation of NikR using an Applied Biosystems gas-phase sequencer. Over a stretch of seven amino acids (Met-Glu-Arg-Val-Thr-Ile-Thr), the sequence was identical to the first seven amino acids predicted from the *nikR* nucleotide sequence, except for the second amino acid, which was changed from Gln to Glu after introduction of the *Nco*I site.

Regulation of *nikR* gene expression. The dependence on nickel for the NikR-mediated repression of expression of the *nik* operon suggests that nickel functions as either a corepressor of NikR or an inducer for the expression of *nikR*. In order to study *nikR* expression, a *nikR-uidA* operon fusion was constructed. The promoterless *uidA*-Kan^r cassette encoding β-glucuronidase was obtained by *Sma*I digestion of plasmid pUIDK3 (2) and was inserted into the unique *Apa*LI site in the *nikR* gene of plasmid pKS1014, which is a derivative of pACYC184 containing the 7-kb *Bam*HI-*Hin*dIII fragment of pLW21 (24). The resulting plasmid, pKS1015, was checked by restriction endonuclease digestion and was found to contain the correct fusion of the *uidA*-Kan^r cassette within the *nikR* gene. Insertion of the *uidA*-Kan^r cassette into *nikR* was also confirmed by the defect in *nikR* function. Compared with pKS1014, pKS1015 was no longer able to repress the expression of the *nikA-lacZ* fusion in the double mutant KS01 [*nikA*::MudI(*lacZ* Amp^r) *nikR*::Tn*10*] (data not shown).

Expression of the *nikR* gene was first analyzed by monitoring activity of the β-glucuronidase produced as a result of plasmid pKS1015. β-Glucuronidase was not detectable in the wild-type strain, NM522 [F′ *lacI*^{q} Δ(*lacZ*)M15 *proA*$^+$*B*$^+$/*supE thi* Δ(*lac-proAB*) Δ(*hsdMS-mcrB*)*5*], harboring the parental plasmid pKS1014, suggesting that the chromosomal copy of the *uidA* gene was not expressed under these growth conditions in the absence of inducer. Introduction of plasmid pKS1015 (*nikR-uidA*) conferred on strain NM522 a specific β-glucuronidase activity of about 400 units, which was increased more than sixfold under anaerobic conditions (Table 1). Neither aerobic nor anaerobic expression of *nikR* was affected by the addition of 0.5 mM nickel in the growth medium. Therefore, expression of the *nikR* gene is independent of nickel, which may function as a corepressor of NikR in the regulation of expression of the *nik* operon.

To avoid multiple-copy effect, the *nikR-uidA* fusion was recombined back into the chromosome of the *recD* strain, D355 [F$^-$ *lac-3350 galK2 galT22* λ$^-$ *recD1014 rpsL179* IN(*rrnD-rrnE*)*1*] (13), after linearization of plasmid pKS1015 with *Eco*RI and *Kpn*I. The resulting *nikR* mutation was then moved into strain MC4100 (*araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 flbB5301 ptsF25 deoC1 rbsR*) via P1*cml* transduction (10), selecting for kanamycin resistance and giving rise to strain KS04. To ascertain a successful recombination, biosynthesis of NikA was analyzed by immunoblotting, as described previously (4). As expected, the NikA protein was not detected by anti-NikA antiserum in a crude extract of the wild-type parental strain, MC4100, grown in the presence of 0.5 mM nickel. In contrast, KS04 synthesized NikA constitutively in the absence or presence of nickel in the growth medium, indicating the defect of the chromosomal *nikR* gene (data not shown). Compared with expression from plasmid pKS1015, the β-glucuronidase activity was reduced by 10-fold and 6-fold in strain KS04 grown under aerobic and anaerobic conditions, respectively (Table 1). However, *nikR* expression remained inducible by anaerobic conditions and independent of nickel concentration.

The *fnr* gene product is required for the anaerobic expression of several respiratory enzymes (6). Expression of the *nik* operon has been reported to be under the positive control of Fnr (21). To test whether or not the anaerobic induction of the *nikR-uidA* expression depends on Fnr, *nikR* expression was examined in an *fnr* background. The *fnr* derivative of KS04 was constructed by transduction with a P1*cml* lysate grown on strain MC4100nir (as MC4100, but *fnr-22 zcj-261*::Tn*10*) (21), selecting for tetracycline resistance and then scoring for the absence of nitrate reductase. Since the *fnr* mutation led to a 3.5-fold reduction in anaerobic *nikR-uidA* expression in the resulting double mutant KS04Fnr compared with expression in the parental single mutant KS04, *nikR* expression seems to be regulated partially by Fnr (Table 1).

To assess whether *nikR* is autoregulated, the wild-type *nikR* allele was provided in *trans* to mutant KS04 by plasmid pKS1014 carrying the entire *nik* operon in addition to *nikR*. The anaerobic β-glucuronidase activity was reduced by about twofold in the resulting strain, KS04/pKS1014, compared with that in KS04 carrying vector pACYC184 without an insert. Therefore, *nikR* appears to be partially autoregulated.

The partial autoregulation and the Fnr-mediated activation of *nikR* expression could be the consequence of transcriptional regulation of *nikR* at the level of the promoter of the *nik* operon. This hypothesis is supported by the finding, in the

TABLE 1. Expression of the *nikR-uidA* fusion under different growth and genetic conditions

Strain/plasmid[a]	Relevant genotype	β-Glucuronidase activity[b]			
		Aerobic growth		Anaerobic growth	
		− $NiCl_2$	+ $NiCl_2$	− $NiCl_2$	+ $NiCl_2$
NM522/pKS1014	Wild type/(*nikA-R*)$^+$	< 1	< 1	< 1	< 1
NM522/pKS1015	Wild type/(*nikA-E*)$^+$ *nikR-uidA*	440 ± 37	400 ± 35	2,490 ± 83	2,534 ± 61
MC4100	Wild type	< 1	< 1	< 1	< 1
KS04	*nikR-uidA*	38 ± 01	33 ± 02	369 ± 32	379 ± 05
KS04Fnr	*nikR-uidA fnr*	34 ± 02	ND	105 ± 06	ND
KS04/pACYC184	*nikR-uidA*/vector	37 ± 02	34 ± 02	349 ± 20	ND
KS04/pKS1014	*nikR-uidA*/(*nikA-R*)$^+$	35 ± 02	33 ± 01	188 ± 08	191 ± 02
HYD723	*nikA*::MudI	< 1	< 1	< 1	< 1
KS06	*nikA*::MudI *nikR-uidA*	34 ± 04	39 ± 05	127 ± 07	111 ± 04
KS06Fnr	*nikA*::MudI *nikR-uidA fnr*	32 ± 02	ND	96 ± 04	ND
KS06/pACYC184	*nikA*::MudI *nikR-uidA*/vector	38 ± 05	32 ± 02	92 ± 02	100 ± 06
KS06/pKS1014	*nikA*::MudI *nikR-uidA*/(*nikA-R*)$^+$	37 ± 01	37 ± 03	92 ± 02	100 ± 06

[a] All strains, except NM522, are derivatives of MC4100. Plasmid pKS1014 contains a 7-kb chromosome fragment which covers the entire *nik* operon. Plasmid pKS1015, a derivative of pKS1014, was constructed as described in the text. Cells were grown either aerobically or microaerobically at 37°C in LB medium supplemented with 2 μM ammonium molybdate, 2 μM sodium selenite and, when required, 20 μg of chloramphenicol per ml. When noted, 0.5 mM $NiCl_2$ was added in the growth medium.

[b] β-Glucuronidase activity was measured and expressed as described in Fig. 1. Values quoted (with standard deviations) are the averages of three separate experiments.

promoter-operator region of the *nik* operon (11), of an inverted repeated sequence composed of a 14-base dyad, AA TCAGTATGACGA-N10-TCGTCATACTTATT, which may serve as a NikR binding site and of a partially conserved FNR box located just upstream. Indeed, introduction, by P1*cml*-mediated transduction, of the *nikA*::MudI insertion upstream of the *nikR* gene in mutant KS06 had a strong polar effect on *nikR-uidA* expression (Table 1), which was reduced to a level similar to that of KS04Fnr. In addition, neither the *fnr* mutation nor the presence of multiple copies of *nikR*$^+$ in *trans* had further effect on the *nikR-uidA* expression in mutant KS06 (Table 1). These results suggest that the invariable aerobic β-glucuronidase activity and the remaining anaerobic β-glucuronidase activity in the double mutant KS06 could be more a consequence of *nikR-uidA* expression from another promoter than that of the *nikABCDE* operon.

Mapping of the transcriptional start site for *nikR*. To identify the promoter responsible for constitutive *nikR* transcription, total RNA isolated from wild-type strain NM522 harboring plasmid pLW22 was subjected to primer extension analysis by using the synthetic oligonucleotide NikR (5′-GCTCAGGC GATCCAGCG-3′). This oligonucleotide is complementary to the DNA sequence from bp 59 to bp 44 downstream of the translation start codon of *nikR*. The same extension product of 111 bp was detected from cultures grown anaerobically without or with 0.5 mM $NiCl_2$ (Fig. 3), identifying the A residue located 51 bp upstream of the ATG of *nikR* as the transcription start site. A putative −10 box (TACAAA) was found, but a sequence homologous to the −35 box with the proper spacing was not identified. This result confirms that expression of *nikR* is independent of nickel concentration and indicates that this gene is constitutively expressed from its own promoter.

Functional similarity between NikR and Fur proteins. Bacteria require numerous metal ions, such as iron, nickel, and cobalt, for growth and have consequently evolved several distinct, high-affinity uptake systems (14). However, at high concentrations, metal ions are also potentially toxic elements because they can catalyze the formation of dangerously reactive hydroxyl radicals, which can damage virtually all cellular constituents. Therefore, expression of these systems is generally tightly regulated. A common feature of many of these uptake systems is that their expression is induced by metal ion starvation and repressed by high concentrations of metal. The best known example is the iron-responsive regulation of the expression of the high-affinity iron uptake pathway and bacterial virulence factors (12). It is mediated by the ferric uptake regulation (Fur) repressor protein or diphtheria toxin repressor (DtxR) (14, 18).

The results described above indicate that NikR resembles a Fur or DtxR counterpart and functions as a nickel-responsive

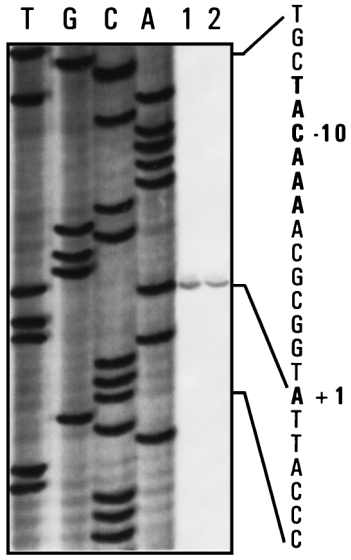


FIG. 3. Determination of the constitutive *nikR* transcription start site. Total RNAs (50 μg) isolated from *E. coli* NM522/pLW22 grown anaerobically in Luria broth in the absence (lane 1) or presence (lane 2) of 0.5 mM $NiCl_2$ were analyzed by primer extension with primer NikR (see text). The DNA sequence ladder (lanes TGCA) was obtained with the same primer and plasmid pLW22 as a template. Part of the sequence and the *nikR* transcription start site are indicated on the right.

regulator in nickel metabolism. Interestingly, when NikR was used as a query sequence to scan the nr database (all nonredundant GenBank CDS translations+PDB+SwissProt+PIR+PRF by using the Blastp program (http://www.expasy.ch/cgi-bin/blast-ncbi.p1), the 12 best-scoring sequences were as follows, in decreasing order: six conserved hypothetical proteins from *Methanococcus jannaschii* (Y549_METJA), *Archaeoglobus fulgidus* (AE001054), *Helicobacter pylori* (AE000635), *Methanobacterium thermoautotrophicum* (AE000835 and AE000842), and *Methanococcus jannaschii* (Y767_METJA); Fur protein from *Vibrio cholerae* (FUR_VIBCH); Rho1 GDP-GTP exchange protein 2 from *Saccharomyces cerevisiae* (ROM2_YEAST); SCP-1 from *Homo sapiens* (D67035); and three Fur proteins, from *Vibrio anguillarum* (FUR_VIBAN), *Vibrio vulnificus* (FUR_VIBVU), and *Vibrio parahaemolyticus* (AB003752). Therefore, the only apparent functionally related proteins are the four ferric uptake regulator proteins. The Smallest Sum Probability P(N) score obtained by sequence comparison between NikR and these Fur proteins ranges from 0.60 to 0.97, which is too low to establish sequence relatedness between them. However, NikR contains two motifs which are perfectly conserved in the four Fur proteins described above. The first motif consists of STQHHHXXL, corresponding to residues 73 to 81 of NikR. This motif is located in a region that shows the highest solvent accessibility score in NikR and that is composed of a histidine-rich stretch of 5 amino acids. The sequence of the stretch is His-His-His-His-Asp (HHHHD) in NikR and (His)-His-His-Asp-His [(H)HHDH] in 16 Fur-like proteins from gram-negative bacteria. The corresponding histidine-rich region of Fur-like proteins from gram-positive bacteria and *Synechococcus* does not contain aspartate. Some of these residues may provide a ligand(s) for metal coordination, and the position of the Asp might be of importance for metal specificity. The second motif, DXGXVXXFXDDXIXXR, corresponds to residues 104 to 119 of NikR and it is not as well conserved as the first one in the 16 Fur-like proteins.

To assess a possible functional substitution of NikR with Fur, we tested the effect of a *fur* deletion on *nikA-lacZ* expression by introducing *fur*::Tn*903* from strain QC1732 into HYD723 or by complementation of the *nikR* deficiency in KS01 by the *fur* gene. In neither case was the regulation pattern of the *nik* operon expression altered (data not shown). The nonexchangeability may rely on the low level of similarity between the NikR and FurR proteins and on the difference in the structures of the repressor-binding sites. The potential NikR-binding site contains two half sites of 14 bp separated by 10 bp (see above), whereas the perfect dyad of the Fur box is separated by one nucleotide (3). In vivo and in vitro experiments will help to determine the precise DNA motif involved in the contact with NikR.

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