Nur, a nickel-responsive regulator of the Fur family, regulates superoxide dismutases and nickel transport in *Streptomyces coelicolor*

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Summary

Nickel serves as a cofactor for various microbial enzymes including superoxide dismutase (SOD) found in *Streptomyces* spp. In *Streptomyces coelicolor*, nickel represses and induces production of Fe-containing and Ni-containing SODs, respectively, primarily at the transcriptional level. We identified the nickel-responsive regulator (Nur), a Fur (ferric-uptake regulator) homologue, which binds to the promoter region of the *sodF* gene encoding FeSOD in the presence of nickel. Disruption of the *nur* gene caused constitutive expression of FeSOD and no induction of NiSOD in the presence of nickel. The intracellular level of nickel was higher in a Δ*nur* mutant than in the wild type, suggesting that Nur also regulates nickel uptake in *S. coelicolor*. A putative nickel-transporter gene cluster (*nikABCDE*) was identified in the genome database. Its transcription was negatively regulated by Nur in the presence of nickel. Purified Nur protein bound to the *nikA* promoter region in a nickel-dependent way. These results support the action of Nur as a regulator of nickel homeostasis and antioxidative response in *S. coelicolor*, and add a novel nickel-responsive member to the list of versatile metal-specific regulators of the Fur family.

Introduction

Since the discovery of nickel-dependent growth of *Ralsto-*

nia (formerly *Hydrogenomonas*, Bartha and Ordal, 1965), studies on the role of nickel in living organisms have expanded. At physiological concentrations in the cell, nickel serves as an essential cofactor for microbial and plant enzymes (Mulrooney and Hausinger, 2003). When in excess, it is toxic to cells by generating reactive oxygen species (Stohs and Bagchi, 1995), and acts as a potent human carcinogen (Denkhaus and Salnikow, 2002). Therefore, it is critical for cells to keep homeostatic control of its level through sensing and transport systems, and to maintain protective measures against its potentially harmful effects.

Currently, nine enzymes that utilize nickel as a cofactor have been identified including urease, hydrogenase, carbon monoxide dehydrogenase, acetyl-CoA decarbonylase/synthase, methyl-CoM reductase, glyoxylase I, acireductone dioxygenase, methylenediurease and superoxide dismutase (SOD) (Mulrooney and Hausinger, 2003). Nickel-mediated induction of gene transcription has been reported in *Helicobacter pylori* for urease (van Vliet *et al.*, 2001), and in *Streptomyces coelicolor* for Ni-containing SOD (Kim *et al.*, 1998a), whereas repression has been reported for uptake systems in *Escherichia coli* (de Pina *et al.*, 1999) and for Fe-containing SODs in *S. coelicolor* and *Streptomyces griseus* (Kim *et al.*, 1998b; 2000).

In *Bradyrhizobium japonicum*, where nickel induces transcription of NiFe-hydrogenase, HypB acts as a nickel-sensor or nickel-delivery system for transcriptional regulation of hydrogenase (Olson *et al.*, 1997). The best characterized nickel sensor that also serves as a transcriptional regulator is NikR, which was first identified in *E. coli* (de Pina *et al.*, 1999). It is a member of the ribbon-helix-helix family of DNA-binding proteins that binds to a 28-bp palindromic operator site in the nickel-uptake operon *nikABCDE* in the presence of nickel (Chivers and Sauer, 2000). Recently, NmtR, a nickel-cobalt sensing ArsR/SmtB family protein that represses an ATPase efflux system for heavy metal resistance was identified in *Mycobacterium tuberculosis* (Cavet *et al.*, 2002). In *S. griseus*, a nickel sensor SrnQ forms a heterodimeric repressor complex with SrnR, a member of the ArsR family, and represses the transcription of the *sodF* gene encoding an Fe-containing SOD (Kim *et al.*, 2003).

The best studied and most widespread metal-responsive regulators that control metal homeostasis are

members of ferric-uptake-regulator (Fur) family. Fur was discovered as an iron-responsive repressor of the iron transport system in *E. coli* (Hantke, 1981; Bagg and Neilands, 1987). In a wide range of bacteria, Fur orthologues serve as global regulators for iron homeostasis, antioxidative response, and in some cases for virulence (Ratledge and Dover, 2000; Hantke, 2001). In addition to iron, other metals bind specifically to Fur-family proteins, and thus control their own homeostasis. For example, the zinc-uptake regulator (Zur) is a Fur homologue that regulates zinc transport in a zinc-responsive way (Patzer and Hantke, 1998). A manganese-uptake regulator, Mur, is another Fur homologue found in *Rhizobium leguminosarum* (Diaz-Mireles *et al.*, 2004). These metal-specific Fur members require their specific cognate metals to bind to their target promoters.

The *S. coelicolor* A3(2) genome encodes four Fur homologues (Bentley *et al.*, 2002). Two of them have been characterized as regulators of catalase genes; H_2O_2-sensitive CatR for the *catA* gene encoding the major catalase, and FurA for the *catC* gene encoding a catalase-peroxidase (Hahn *et al.*, 2000a,b). In this report we present a novel nickel-responsive regulator of Fur family. It was discovered through efforts to find the nickel-responsive regulator that binds to and represses transcription from the promoter of the *sodF* gene encoding Fe-containing SOD (Kim *et al.*, 1998b; Chung *et al.*, 1999). We present evidence that it represses Fe-SOD and nickel-uptake system, and at the same time induces Ni-SOD, in the presence of nickel.

Results

Purification and identification of Nur

In order to identify the nickel-responsive protein binding to the *sodF* promoter region in the presence of nickel, we isolated the binding protein through chromatographies on DEAE-Sepharose, Q-Sepharose and heparin columns, and finally through DNA-affinity fractionation using streptavidin-coated magnetic beads linked to the *sodF* promoter DNA. Figure 1A demonstrates silver-stained protein patterns fractionated through DNA-affinity beads; unbound proteins (flow through; FT), and those eluted by 0.2–1.5 M NaCl (lanes 1–6). The *sodF* promoter-binding activity in each fraction was monitored by gel mobility shift assay. As demonstrated in Fig. 1B, the majority of *sodF*-binding activity was enriched in the fraction eluted by 1.0 M NaCl. In that fraction, a specific protein band of approximately 16 kDa was visible upon silver staining (Fig. 1A, lane 5). The protein band was excised, digested with trypsin in the gel, and subjected to MALDI-TOF mass analysis as described in *Experimental procedures*. The tryptic digest pattern matched with an uncharacterized gene product (SCO4180) encoding a putative Fur homo-
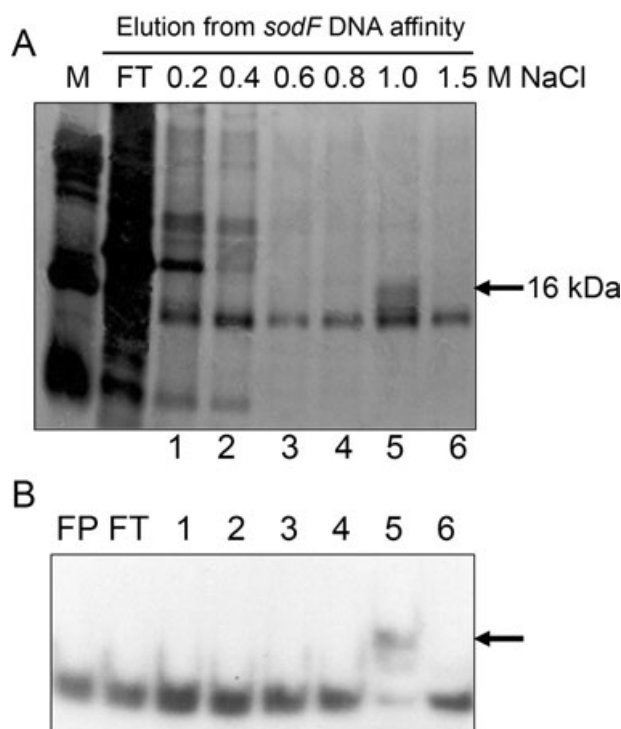

Fig. 1. Purification of Nur.
A. Proteins bound to DNA-affinity beads coated with *sodF* promoter DNA were washed with TE buffer containing 0.2, 0.4, 0.6, 0.8, 1.0 and 1.5 M NaCl (lanes 1–6). Proteins in flow-through (FT) and eluted fractions (1–6) were resolved on SDS-PAGE and stained with silver nitrate.
B. The proteins in the flow-through (FT) and eluted fractions (1–6) were examined for *sodF* promoter fragment-binding activity by gel mobility shift assay.

logue in the *S. coelicolor* A3(2) genome. We named this gene *nur* (for nickel-uptake regulator) based on its sequence similarity to the *fur* genes and its role in nickel uptake as described below.

Sequence comparison of Nur with other Fur homologues

The *nur* gene encodes a protein of 145 amino acids (16 229 Da), and is annotated as a putative iron-uptake regulatory protein in the genome database ScoDB (http://streptomyces.org.uk). The flanking genes in the *nur* locus were described in Fig. 2A. The four open reading frames (ORFs) (SCO4176, 4177, 4178 and 4179) preceding *nur* in the same orientation all encode conserved hypothetical proteins of unknown functions. The downstream ORFs encode homologues of a glycine cleavage T-protein (SCO4181), a D-tyrosyl-tRNA deacylase (SCO4182) and a transposase (SCO4183). The genes surrounding *nur* are also conserved in *Streptomyces avermitilis* (Ikeda *et al.*, 2003). However, they do not exhibit similarity with the flanking regions of *fur* or its paralogues in *E. coli* or *Bacillus subtilis*.
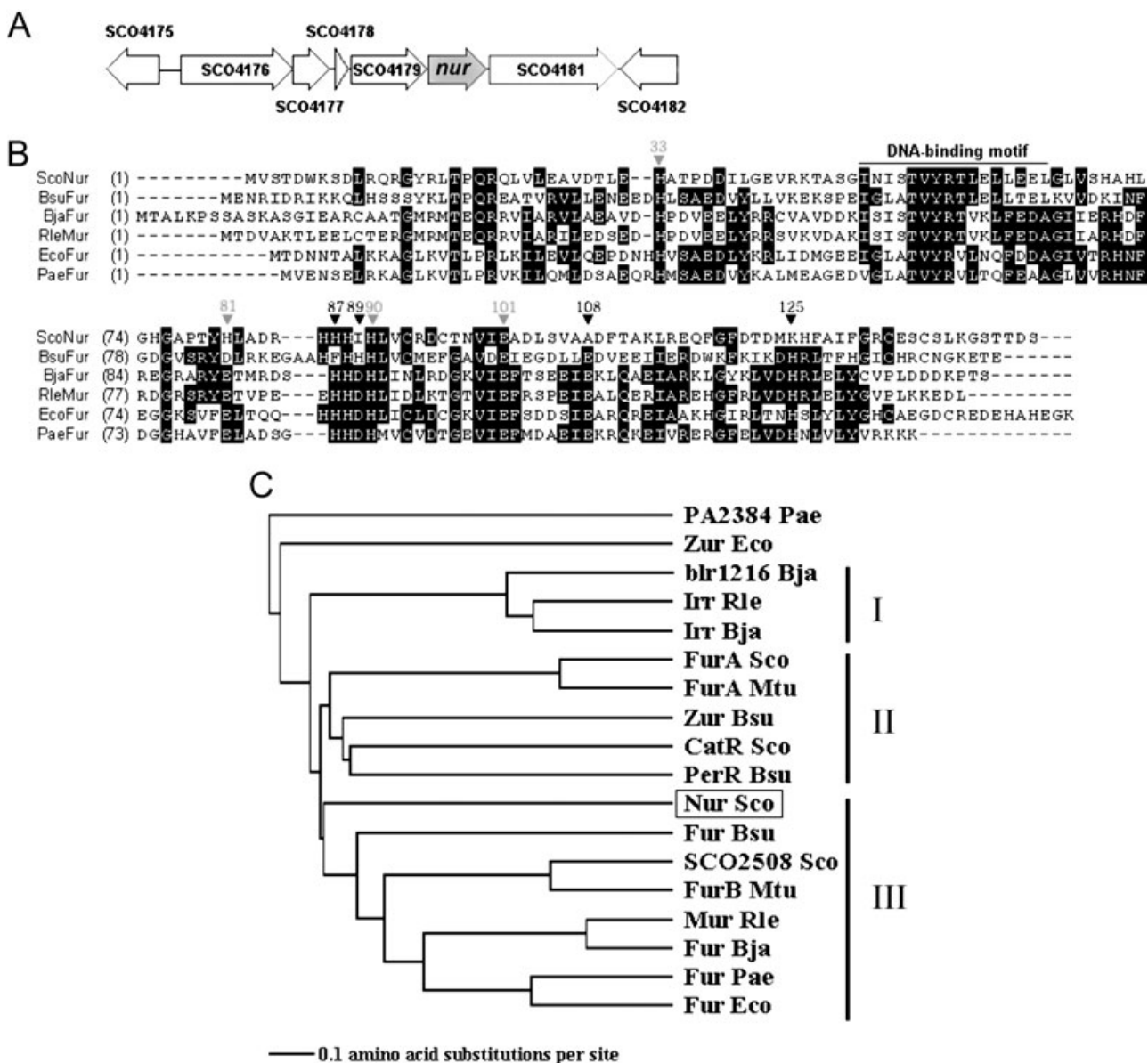
Fig. 2. Comparative analysis of bacterial Fur homologues with Nur.
A. Gene structure around the *nur* gene. SCO4175, SCO4176 and SCO4177; conserved hypothetical proteins. SCO4178: putative small membrane protein. SCO4179: a homologue of polynucleotide phosphorylase. SCO4181: a homologue of glycine cleavage T-protein. SCO4182: putative D-tyrosyl-tRNA deacylase-like protein.
B. Sequence comparison of Nur with other Fur homologues. Amino acid sequence of *S. coelicolor* Nur (ScoNur) was aligned with those of *B. subtilis* Fur (BsuFur), *P. aeruginosa* Fur (PaeFur), *E. coli* Fur (EcoFur), *R. leguminosarum* Mur (RleMur) and *Bradyrhizobium japonicum* Fur (BjaFur). The DNA binding motif, the predicted metal binding residues according to the *P. aeruginosa* Fur structure (Pohl *et al.*, 2003) for structural zinc (white arrowheads) and variable metal (black arrowheads) were indicated.
C. Phylogenic analysis of Nur and other Fur homologues. Amino acid sequences of 18 Fur homologues from seven selected bacteria were analysed as described in *Experimental procedures*. Reported names or locus_id from GenBank was used to designate each protein, followed by abbreviation of bacterial name. Eco, *E. coli*; Bsu, *B. subtilis*; Sco, *S. coelicolor*; Mtu, *M. tuberculosis*; Pae, *P. aeruginosa*; Bja, *B. japonicum*; Rle, *R. leguminosarum*. The length of horizontal bars represents numbers of amino acid substations per site as presented. Possible clusters were grouped as I, II and III.

The amino acid sequence of Nur exhibits significant similarity to other members of Fur family (Fig. 2B). Comparison with *Pseudomonas aeruginosa* Fur (PaFur), whose crystal structure has been resolved (Pohl *et al.*, 2003), revealed partial conservation of metal-binding residues. Three of the four residues, predicted to constitute the structural zinc-binding site, His-33, His-90 and Glu-101, are conserved as in other Fur homologues. In Nur, His-81 replaces either aspartate or glutamate found in other Fur proteins. The residues predicted to constitute

variable metal site (His-87, Ile-89, Ala-108 and Lys-125) according to the *P. aeruginosa* Fur template, are poorly conserved, showing only one match out of four. The predicted structure of Nur *in silico* by SWISS-MODEL (Schwede *et al.*, 2003) using the structural data of PaFur in PDB as a template, show close similarity in overall secondary and tertiary structural shapes, consistent with sequence similarity of the two proteins. However, the residues predicted for variable metal binding in Nur inferred from PaFur structure are neither ideal nor well-positioned for metal coordination. This implies that the variable metal-binding site may be different from what is proposed for PaFur.

Comparative analysis of 18 Fur homologues from seven selected bacteria of different taxa was performed as described in *Experimental procedures*. As demonstrated in Fig. 2C, three clusters can be proposed, where Nur is grouped in a cluster (III) containing *E. coli* and *B. subtilis* Fur, but is most distantly related within the group. Nur shares 27% identical and 48% similar residues with *E. coli* Fur, whereas it shares 29% identical and 38% similar residues with more distantly related *E. coli* Zur. Close relatives of Nur were found mainly in actinomycetes such as *Frankia* (Franean1DRAFT_3571; 59% identity and 69% similarity), *Nocardioides* (NocaDRAFT_0210; 55% identity and 68% similarity), *Dehalococcoides (*cbdb_A24; 42% identity and 58% similarity) and *Kineococcus* (KradDRAFT_0398).

Disruption of the nur *gene*

To create a *Δnur* mutant, the entire coding region of the *nur* gene was replaced with an apramycin-resistance cassette by polymerase chain reaction (PCR)-targeted mutagenesis (Gust *et al.*, 2002; see *Experimental procedures*). Cell extracts prepared from the *Δnur* mutant were examined for the presence of *sodF* promoter-binding activity by gel mobility shift assay. Figure 3 demonstrates
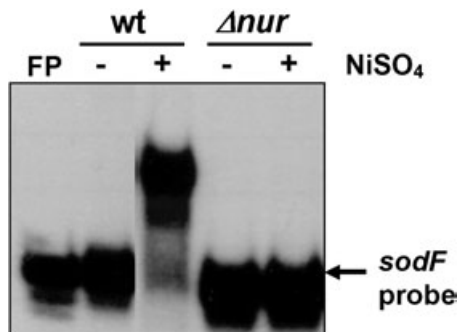

Fig. 3. Effect of *Δnur* mutation on *sodF* DNA binding. Cell extracts of wild type (wt) and *Δnur* mutant grown in YEME liquid media with or without 50 μM NiSO$_4$ were assayed for binding with the *sodF* promoter fragment.

clearly that the nickel-responsive DNA binding protein is absent in *Δnur* cell extracts. The growth rate or morphological phenotypes of the *Δnur* mutant were indistinguishable from the wild type when grown on NA, R2YE, or YEME media. No significant difference in the sensitivity toward nickel up to 5 mM was observed between the wild type and mutant (data not shown).

Nickel specificity of purified Nur

The Nur protein of *S. coelicolor* was overproduced and purified from *E. coli* as described in *Experimental procedures*. Gel mobility shift assay was performed to verify whether recombinant Nur forms specific complexes with *sodF* probes and to determine metal specificity. As demonstrated in Fig. 4A, purified Nur formed a single complex band at low concentration (at 5 nM) and an additional one at higher concentrations. These bands comigrated with those formed by cell extracts. The complex bands formed with recombinant Nur protein were verified to be specific for *sodF* probes based on competition analysis (data not shown). To examine its metal specificity, various divalent metals were added to the binding buffer for gel mobility shift assay. As demonstrated in Fig. 4B, addition of 0.1 mM NiSO$_4$ allowed binding of Nur protein whereas 1 mM FeSO$_4$, ZnCl$_2$, CoCl$_2$, CuSO$_4$, MnSO$_4$ and CdCl$_2$ did not confer DNA binding activity to Nur. The finding that Nur specifically required nickel for binding to the *sodF* promoter region is consistent with the previous observation that a nickel-responsive protein, most likely a repressor, binds to the *sodF* promoter in the presence of nickel (Chung *et al.*, 1999). The specific action of nickel on Nur binding was further examined by lowering the concentration of nickel salt in the binding buffer. We found that 1 μM NiSO$_4$ is sufficient to confer binding activity even in the presence of 1 mM EDTA in the binding buffer (Fig. 4C), confirming the pronounced nickel specific action on Nur. The Nur protein purified from *E. coli* exhibited somewhat restricted binding behaviour compared with that in *S. coelicolor* cell extracts. Whereas cobalt at 1 mM was partially effective to increase activity in cell extracts (Chung *et al.*, 1999), purified recombinant Nur was not activated by cobalt at 1 mM. This may reflect some difference in the efficiency in incorporating metals, in some detailed structure, or contribution from other cellular factors, between the purified recombinant Nur and Nur in *S. coelicolor* cell extracts.

Changes in the expression of Fe- and Ni-SODs in Δnur mutant

Kim *et al.* (1998a,b) previously reported that nickel induces *sodN*, and represses *sodF* expression in *S. coelicolor* Müller strain. We examined expression of the
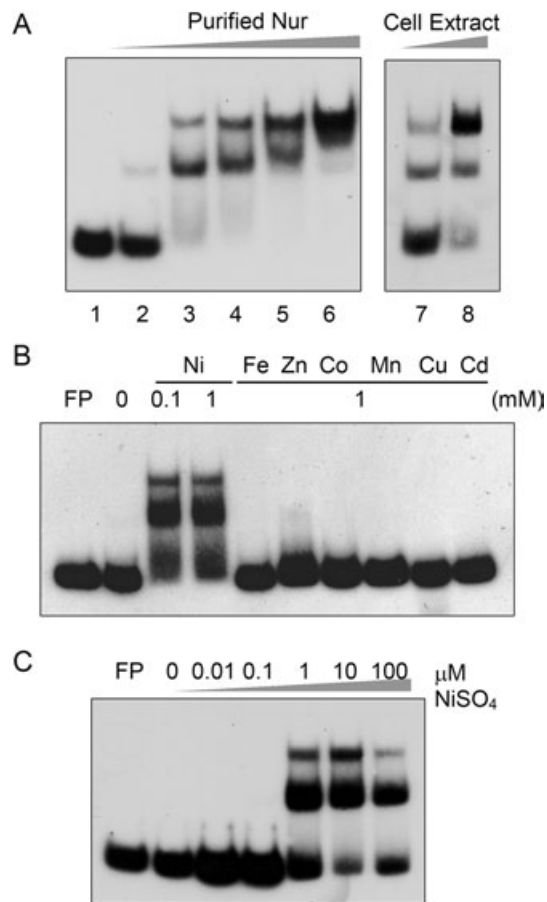
A

Purified Nur   Cell Extract

1   2   3   4   5   6   7   8

B

Ni   Fe Zn Co Mn Cu Cd

FP   0   0.1 1   1   (mM)

C

FP 0 0.01 0.1 1 10 100   μM
                              NiSO$_4$

Fig. 4. Nickel-specific binding of purified Nur to *sodF* DNA.
A. Formation of multiple complex bands between purified recombinant Nur and *sodF* DNA. Increasing amount of purified Nur protein (0, 5, 25, 50, 100 and 200 nM for lanes 1–6) and cell extracts (20 and 50 μg for lanes 7 and 8) were incubated with the *sodF* promoter fragment.
B. Effect of different metals on Nur-binding activity. Purified recombinant Nur proteins were incubated with *sodF* promoter in the presence of various metals. Concentrations of added metals in the binding reaction were indicated in mM for NiSO$_4$, FeSO$_4$, ZnCl$_2$, CoCl$_2$, CuSO$_4$, MnSO$_4$ and CdCl$_2$.
C. Minimum concentration of nickel required for Nur binding activity *in vitro*. Various amounts of NiSO$_4$ (from 0.01 to 100 μM) were added in the binding reaction between purified Nur and *sodF* DNA.

genes encoding Fe-containing (*sodF* and *sodF2*) and Ni-containing SODs (*sodN*) in the wild type (M145) and *Δnur* mutant. Cell extracts were electrophoresed on non-denaturing polyacrylamide gel, and the SOD enzyme activity was detected in the gel. Results in Fig. 5A confirm nickel-responsive expression of Ni-SOD and Fe-SOD in M145 strain as previously observed in *S. coelicolor* Müller strain. In the *Δnur* mutant, Fe-SOD was constitutively produced independent of nickel regulation, consistent with the proposal that Nur is the nickel-responsive repressor of the *sodF* gene. On the other hand, Ni-SOD was not expressed at all even in the presence of nickel in *Δnur* mutant, suggesting its role as an activator. In order to test

whether these SOD activity patterns result from changes in transcript level, we monitored *sodF, sodF2 and sodN* transcripts in the same samples by S1 mapping. Results in Fig. 5B demonstrate that the constitutive production of Fe-SOD and no production of Ni-SOD in *Δnur* mutant are due primarily to changes in the transcript level. To confirm that the changed SOD expression pattern in *Δnur* mutant is indeed due to the absence of Nur, and not caused by any unexpected polar effect caused by the *nur* mutation, we introduced a wild-type *nur* gene to the chromosome at a different location (*att* site). The complemented strain showed a wild-type pattern of SOD expression, confirming the action of Nur as a direct repressor of *sodF* and as a positive regulator for *sodN (*data not shown).

Nur regulates nickel uptake in S. coelicolor

The possible involvement of Nur in nickel homeostasis was examined by measuring the amount of total nickel accumulated in the wild type and *Δnur* mutant. Inductively coupled plasma – mass spectrometry (ICP-MS) analysis revealed that *Δnur* accumulated significantly higher levels of nickel in the mutant than the wild type in the range of added nickel concentrations tested (0.5–10 μM) (Fig. 6).
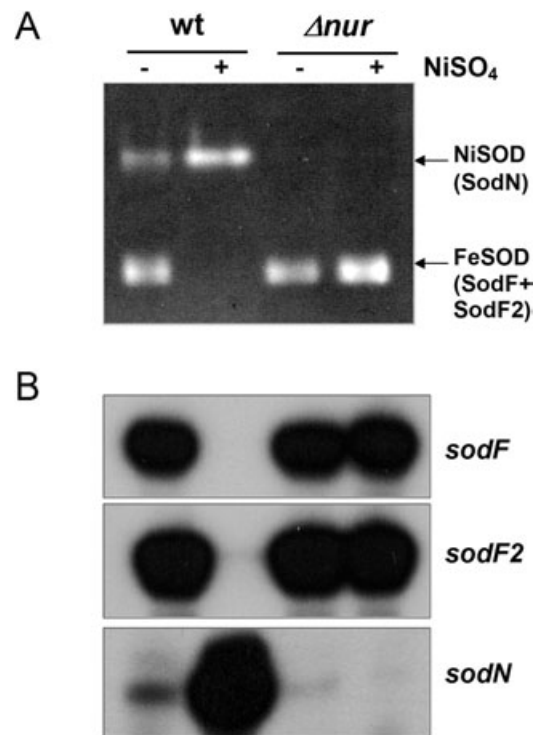
A

wt   *Δnur*

-    +   -   +   NiSO$_4$

← NiSOD (SodN)

← FeSOD (SodF+ SodF2)

B

sodF

sodF2

sodN

Fig. 5. Expression of *sod* genes in wild type (wt) and *Δnur* mutant.
A. SOD enzyme activity staining. Crude extracts of wt and *Δnur* mutant grown in YEME media with or without 50 μM NiSO$_4$ were electrophoresed on a 5% native PAGE and stained for enzyme activity.
B. RNA analysis by S1 mapping. RNAs from the same samples as in A were analysed for *sodF*, *sodF2* and *sodN* transcripts by S1 mapping.
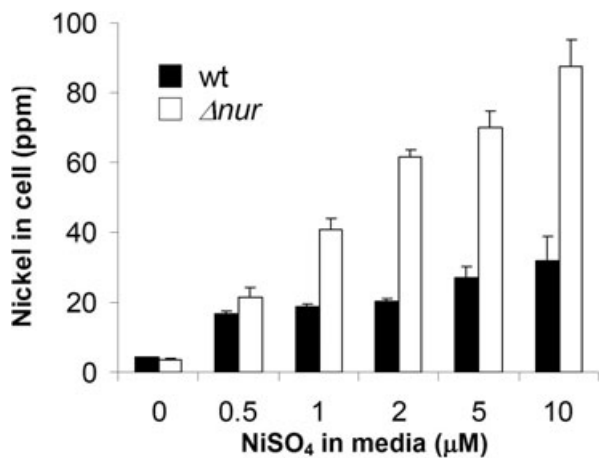
Fig. 6. Accumulation of nickel in wild type (wt) and *Δnur* mutant. Wild type (M145) and *nur* mutant cells were grown in NB media containing various concentrations of NiSO₄ (0, 0.5, 1, 2, 5, 10 µM). The content of nickel in each sample was analysed by ICP-MS. The values were presented as p.p.m. (µg of nickel g⁻¹ dry weight of cells). One p.p.m. corresponds to approximately 0.8 µmole per litre of wet packed mycelium.

This parallels the effect of Fur and Zur in negatively regulating iron and zinc uptake, respectively, in *E. coli* (Hantke, 1981; Patzer and Hantke, 1998).

Homology searches for a nickel transport system (ScoDB Blast; http://streptomyces.org.uk/blast/blast_form.html) revealed a gene cluster (SCO6451–6455) that shared significant sequence similarity with the *nikABCDE* genes encoding an ABC-type nickel transporter of *E. coli* (Fig. 7A). We named this locus in the same way as in *E. coli*. For NikA which serves as the nickel-binding component in the transporter (de Pina *et al.*, 1995), the two share 26% identical and 44% similar amino acids. Similar levels of homology exist between the rest of the genes; 26 and 38% for NikB, 32 and 48% for NikC, 28 and 39% for NikD, 33 and 53% for NikE, for identical and similar residues.

In order to test nickel-responsive gene expression of the *nikA* gene, we analysed *nikA* transcripts by S1 mapping in the wild type and *Δnur* mutant grown in the presence and the absence of added nickel. Figure 7B demonstrates that the *nikA* gene in the wild type is negatively regulated by nickel, and in *Δnur* mutant its expression is constitutive. This implies that the *nikA* operon is negatively regulated by Nur. Based on these observations, binding of Nur to *nikA* promoter region was examined. Purified recombinant Nur protein bound to the *nikA* promoter DNA in the presence of nickel (Fig. 7C). To provide a negative control with a known non-binding probe, parallel experiment with *sodN* probe was carried out. These results support the proposal that Nur is indeed a nickel-uptake regulator that represses *nikA* operon, a putative nickel uptake system in *S. coelicolor*, when sufficient amount of nickel is present in the growth medium.
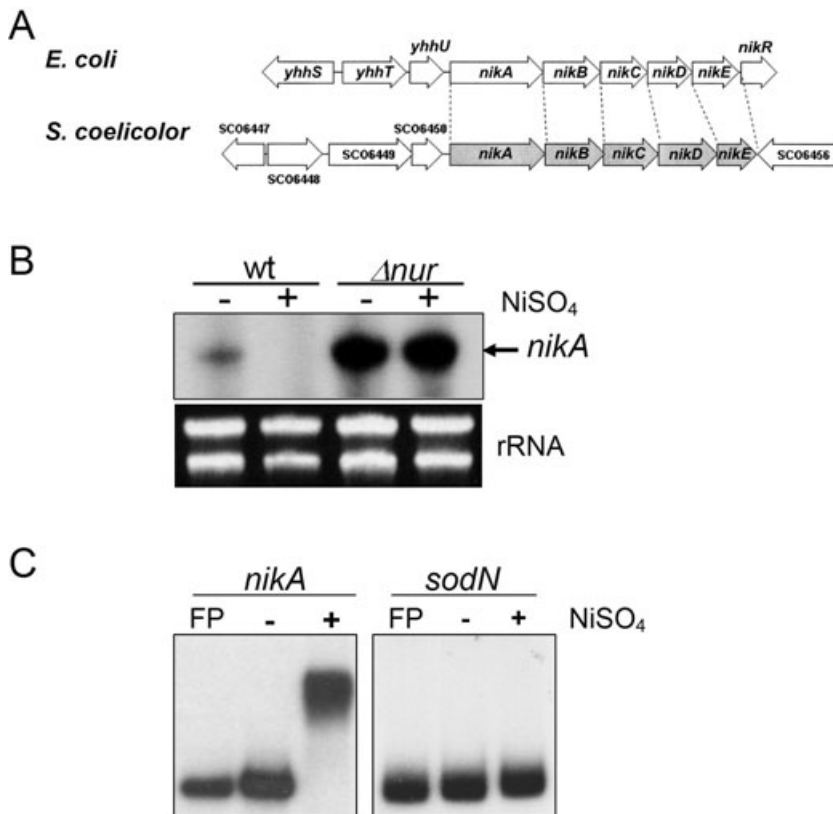

Fig. 7. Nur-dependent expression of *nikABCDE* operon.
A. Comparison of gene structure at the *nikABCDE* locus between *S. coelicolor* and *E. coli*. SCO6447; putative NAD(P)H oxidoreductase, SCO6448; AraC-like transcriptional regulator, SCO6449; putative dehydrogenase, SCO6450; hypothetical protein, SCO6456; putative hydrolytic protein.
B. Repression of *nikA* transcription by Nur. RNAs from the wild type (wt) and *Δnur* mutant grown in YEME with or without 50 µM NiSO₄ were analysed for *nikA* transcripts by S1 mapping. As RNA loading control, the amount of rRNAs in each sample was monitored through a separate agarose gel electrophoresis.
C. Gel mobility shift assay with *nikA* promoter DNA and Nur protein in the presence or absence of 100 µM NiSO₄ in the binding buffer. The absence of Nur binding to the *sodN* promoter fragment (from −280 to +90 nt relative to the transcription start site) was presented as a comparison. FP, free probe.

Discussion

In *S. coelicolor*, expression of FeSOD and NiSOD respond to nickel in opposite ways. This phenomenon is common in other species of *Streptomyces* such as *S. griseus* and *S. lividans*. It has been reported that in *S. griseus*, SrnQ and SrnR, which are encoded by genes immediately downstream of *sodF*, form a heterodimeric repressor of FeSOD expression in a nickel-dependent way (Kim *et al.*, 2003). Because the *sodF* promoter region of *S. coelicolor* shares some sequence similarity with that of *S. griseus* (Kim *et al.*, 2000), the regulator of *S. coelicolor sodF* was initially hypothesized to be a homologue of SrnRQ system. However, no homologue of SrnQR was found in *S. coelicolor* genome sequence (Bentley *et al.*, 2002). While both *S. griseus* and *S. coelicolor* display nickel-responsive repression of the *sodF* gene, Nur and SrnQR share no similarity in amino acid sequence and regulatory mechanisms. In *S. griseus*, SrnQR does not seem to affect NiSOD activity (Kim *et al.*, 2003). This exemplifies an evolution of dissimilar regulatory mechanism for the same function among species of the same genus.

In contrast to the direct binding of Nur to the *sodF* promoter region, no significant binding of Nur was detected with the *sodN* promoter region spanning 280 nt upstream from the transcription start site (Fig. 7C). If Nur does not act as a direct activator of *sodN*, a possible mechanism could involve a double negative system, where Nur represses the expression of the repressor for *sodN* expression, as proposed for the mechanism of positive action of Fur in *E. coli* and *P. aeruginosa*. Fur directly binds to the promoter and represses the expression of the *sodA* gene encoding MnSOD in *E. coli* (Niederhoffer *et al.*, 1990). On the other hand, it activates the expression of *sodB* encoding FeSOD by repressing the synthesis of RyhB, a small regulatory RNA that destabilizes *sodB* and several other transcripts positively regulated by Fur (Masse and Gottesman, 2002; Geissman and Touati, 2004). A similar mechanism for the positive action of Fur has been demonstrated in *P. aeruginosa* (Wilderman *et al.*, 2004). However, Kim *et al.* (1998a) previously reported that Ni does not significantly change the half-life of the *sodN* transcript, and sequence searches reveal no putative Hfq homologue in *S. coelicolor*. Therefore, the regulatory mechanism of Nur-mediated *sodN* induction might be different from Hfq- and RyhB-mediated regulation of *sodB* in *E. coli*. A systematic approach with combined bioinformatic and genetic analyses is expected to reveal the existence of any direct regulator(s) of *sodN* in *S. coelicolor*.

The increased nickel-uptake in Δ*nur* mutant indicates that Nur indeed controls nickel homeostasis in *S. coelicolor*. Part of this action should involve controlling *nikABCDE* operon encoding a putative nickel-transporter system. Even though this operon sequence matches closely that of its orthologue in *E. coli*, regulatory factors involved in their transcription are entirely different from each other. In *E. coli*, NikR, a nickel-responsive repressor, and FNR, an anaerobiosis-triggered global transcription activator, control the *nikABCDE* operon (Wu *et al.*, 1989). NikR is a metallo-regulatory protein whose function is homologous to Nur as a nickel-responsive repressor of the nickel uptake system (de Pina *et al.*, 1999), but its primary, secondary and tertiary structures are different from that of Fur-family proteins. FNR turns on *nikABCDE* transcription only under anoxic conditions. This regulation is understood to be necessary to minimize the toxic effect of nickel that can generate reactive oxygen species in the presence of oxygen (Stohs and Bagchi, 1995). In contrast, *S. coelicolor* utilizes nickel under aerobic conditions. *S. coelicolor* might have developed a robust regulatory system for nickel utilization, involving NiSOD and Nur, to avoid possible nickel-driven toxicity under aerobic conditions. It can also be hypothesized that in the presence of nickel, Nur could act to limit the utilization of Fe which is another potent mediator of oxygen toxicity.

Metal utilization in aerobes would inevitably accompany oxidative stress. Changes in redox potential of some metals under oxygenic condition produce reactive oxygen species. In addition, reaction of some metals with peroxide produces more harmful reactive oxygen species such as hydroxyl radical. This connection between metal ions and oxidative stress might have allowed evolution of Fur proteins to regulate not only metal homeostasis but also response to oxidative stress. Observations that Fur-family proteins are involved in regulating response genes against oxidative stresses are accumulating. In *E. coli*, Fur regulates two SODs and oxidative stress defence proteins (Niederhoffer *et al.*, 1990; Touati, 2000), and Fur itself is induced by OxyR and SoxR in response to oxidative stress (Zheng *et al.*, 1999). Other Fur homologues that regulate oxidative defence genes primarily in response to oxidative stress include PerR from *B. subtilis* (Bsat *et al.*, 1998), CatR from *S. coelicolor* (Hahn *et al.*, 2000a), and Mycobacterial FurA (Zahrt *et al.*, 2001) that sense peroxides and regulate expression of proteins to cope with such stresses. Recent results from transcriptome analysis in various bacterial systems also show that Fur homolgues participate in regulation of SODs, catalases, or other defence systems for oxidative damage (Thompson *et al.*, 2002; Grifantini *et al.*, 2004; Palyada *et al.*, 2004).

It is reasonable to propose that a nickel-responsive regulator is involved in nickel utilization by analogy with other Fur homologues that regulate the metabolism of their cognate metals. In addition to Zur and Mur, the discovery of Nur, another novel member of Fur family specified for nickel, expands the spectrum of metal specificity for Fur homologues.

Experimental procedures

Bacterial strains and culture conditions

Streptomyces coelicolor A3(2) M145 strain and its derivatives were grown and maintained according to standard procedures (Kieser *et al.*, 2000). For liquid culture, YEME and Nutrient Broth (NB) media were used. Pre-germinated spores were inoculated into seed media and cultivated for 24–48 h. One percent (v/v) of the seed culture was innoculated to the main media and grown for 18 h in NB, or 24 h in YEME. For plate culture, 10^7 pregerminated spores or patches of mycelia were streaked on R2YE, NA, or SFM media.

Gel mobility shift assay for DNA-binding proteins

DNA probes were generated by PCR. For *sodF* promoter fragment, a forward primer whose 5′ end corresponds to 114 nucleotides upstream from the start codon (5′-CCG TGC GGG GAA GCT TCG TGT GCG-3′; HindIII site underlined) and a reverse primer whose 5′ end corresponds to the start codon (5′-CAT GGC GGA TCC CTC CGG-3′; BamHI site underlined) were used. The amplified DNA fragments were cloned in pUC18 and recovered by digestion with BamHI and HindIII. This generates a 90 bp *sodF* promoter probe that spans from −60 to +30 nt relative to the transcription start site. For *sodN* promoter fragment, a forward primer whose 5′ end corresponds to 290 nucleotides upstream from the transcription start site (5′-GGA AGG GGT ACC GCA GCA CGA CCA CGT C-3′; KpnI site underlined) and a reverse primer whose 5′ end corresponds to 106 nucleotide upstream from the transcription start site (5′-CAG GGT CGT GCA TGC CGC AGG GCA GGT CG-3′; SphI site underlined) were used. The amplified DNA fragments were cloned in pUC18 and recovered by digestion with KpnI and SphI. This generates a 370 bp *sodF* promoter probe that spans from −280 to +90 nt relative to the transcription start site. For *nikA* promoter fragment, a forward primer whose 5′ end corresponds to 271 nucleotides upstream from the start codon (5′-CGC CCG GAT CCC CGT CGA GAC GGT GGC-3′) and a reverse primer whose 5′ end corresponds to 60 nucleotides downstream from the start codon (5′-ACT CCG TAT CGG AAG CTT CCG GTC GGT CC-3′) were used for PCR. The PCR products were labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Binding reactions were performed with approximately 10 fmole probe and 10–20 μg cell extracts or 0.5–1 μg purified Nur in 20 μl of the binding buffer; 4 mM Tris-HCl, pH 7.8, 1 mM EDTA, 4 mM DTT, 5 mM $MgCl_2$, 20 mM KCl, 0.3 mg ml^{-1} BSA, 10% (v/v) glycerol, and 2 μg poly(dI-dC), with or without 100 μM $NiSO_4$. Reaction mixture was loaded on a 5% non-denaturing polyacrylamide gel, and run in 0.5× TBE buffer at 4°C at less than 20 mA. After electrophoresis, gels were vacuum-dried and exposed to the X-ray films.

Purification of Nur from *S. coelicolor*

Streptomyces coelicolor M145 cells were grown for 24 h in YEME media with 100 μM $NiSO_4$ and harvested by centrifugation, followed by washing twice with TE buffer (20 mM Tris, pH 7.8, 1 mM EDTA, 5 mM $MgCl_2$) containing 50 mM NaCl.

Fifty grams of the harvested mycelia were disrupted by sonication (XL-2020, Misonix) and the suspension was clarified by centrifugation at 15 000 *g* for 30 min. The supernatant was loaded on a DEAE-sepharose (Pharmacia) column equilibrated with TE buffer and eluted with 0.1–1 M NaCl gradient. The *sodF* promoter binding activity in the eluted fractions was monitored by gel mobility shift assay. Fractions containing the binding activity were pooled, and further purified through Q-sepharose (Pharmacia) and heparin (Pharmacia) columns. The eluants from the heparin column were then applied to DNA-affinity resin containing the *sodF* promoter fragments linked to magnetic beads as described previously (Folcher *et al.*, 2001). The *sodF* promoter region (−60~+30 nt relative to transcription start site), were amplified by PCR using 5′-biotinylated reverse primer. The PCR products were purified by PCR purification kit (Qiagen) and 25 μg of biotinylated DNA was incubated with 10 mg of streptavidin-coated magnetic beads (Roche). The DNA-coated beads were incubated with the partially purified protein mixture eluted from the heparin column for 30 min at room temperature, separated by using a magnet, and washed with TE buffer containing 50 mM NaCl. Non-specific DNA-binding proteins were removed by washing beads three times with TE buffer containing 50 mM NaCl and 100 μg poly(dI-dC). The *sodF*-binding activity was eluted through stepwise washing with TE buffer containing increasing concentrations of NaCl (0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1, 1.5 M).

MALDI-TOF mass spectrometry

The protein fractions from the *sodF* DNA-affinity beads were precipitated by adding 5 vols of acetone and separated on SDS-PAGE. The gel was stained with Brilliant blue G (Aldrich) and the protein bands were sliced and destained by washing with 50% acetonitrile (Merck), followed by rinsing in tertiary distilled water (Rosenfeld *et al.*, 1992). The gel slices were crushed and treated with 2 units of trypsin (Promega) for 10 h at 37°C. The digested peptides were analysed by MALDI-TOF Mass Spectrometer (Voyager-DE™ STR Biospectrometry Workstation, Applied Biosystems) at the National Center for Inter-University Research Facilities (NCIRF) at Seoul National University. The peptide fingerprints were analysed through Mascot program (http://www.matrixscience.com/) linked to the primary sequence database of *S. coelicolor*.

Bioinformatic analysis of Nur sequence

Eighteen Fur homologues from *E. coli* (2), *B. subtilis* (3), *S. coelicolor* (4), *M. tuberculosis* (2), *P. aeruginosa* (2), *B. japonicum* (3) and *R. leguminosarum* (2) were selected by BLAST search (McGinnis and Madden, 2004). Multiple alignment was carried out by MUSCLE (Edgar, 2004), and a rooted tree (Fig. 2C) was generated by DRAWGRAM in PHYLIP (Felsenstein, 2004). The structure of Nur was predicted by SWISS-MODEL (Schwede *et al.*, 2003) with Fur from *P. aeruginosa* as a template (PDB code: 1mzb). Visualization of modelled structure and comparative analysis with *P. aeruginosa* Fur was carried out using Vector NTI™ (Invitrogen).

Overproduction and purification of S. coelicolor *Nur from* E. coli

The entire coding region of the *nur* gene was amplified by PCR using a mutagenic forward primer (5′-GAC TCG TCA TAT GGT GAG CAC CGA-3′; NdeI site underlined) and a reverse primer (5′-CAT AGC CGG ATC CTA CGA CTC GCT-3′; BamHI site underlined). The PCR product was digested with NdeI and BamHI and cloned into pET3a (Novagen). *E. coli* BL21(DE3)pLysS cells harbouring the recombinant plasmid were grown to A_{600} of 0.5, and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h before harvest. The harvested cells were disrupted by sonication, cleared by centrifugation at 20 000 *g* for 30 min. The Nur protein was purified through sequential chromatographies on DEAE, Q-sepharose and heparin columns as described above.

PCR-targeted disruption of the nur gene

The PCR-targeted mutagenesis of the *nur* gene was carried out as described previously (Gust *et al.*, 2002). PCR primers were designed to contain 39 nucleotides matching the *S. coelicolor* sequence flanking the start or stop codon of the target gene to be inactivated at 5′ sides, and 19 or 20 nucleotides matching the left or right end of the disruption cassette containing apramycin-resistance gene *aac(3)IV*. To disrupt the *nur* gene, a forward primer (5′-GGG ATC GCT TTC TTC AAG TAG TCC TAG ACT CGT GGT GTG ATT CCG GGG ATC GTC GAC C-3′; the start codon underlined) and a reverse primer (5′-GCA GGG GGC TCT TCA TAC GCC CAG CCT ACG ACT CGC TTA TGT AGG CTG GAG CTG CTT C-3′; the complementary sequence of the stop codon underlined) were used for PCR to amplify a 1384-bp cassette from pIJ773. The purified PCR product (100 ng) was introduced by electroporation to *E. coli* BW25113 strain that harbours the λ RED recombination plasmid pIJ790 with chloramphenicol resistance marker and *S. coelicolor* cosmid D66 that contains the *nur* gene and resistance markers for ampicilin and kanamycin. Transformants resistant to ampicilin, kanamycin and chloramphenicol were selected and the target-replaced cosmid was verified by SacI restriction analysis. The mutagenized cosmid was introduced to *E. coli* ET12567/pUZ8002 and then to *S. coelicolor* M145 by conjugation. Apramycin-resistant exconjugants were screened for kanamycin sensitivity, which results from a double-crossover allelic exchange in *S. coelicolor*. The expected gene structure in Δ*nur* mutant was confirmed by genomic PCR and Southern hybridization analyses.

Complementation of the Δnur *mutant with the wild-type* nur *gene*

The 1198 bp NaeI fragment containing the entire coding region of the *nur* gene with flanking sequences was cloned into the modified pSET152 vector (Bierman *et al.*, 1992) that contains a hygromycin cassette from pIJ963 (Kieser *et al.*, 2000). The recombinant plasmid was introduced to the Δ*nur* mutant and allowed to integrate into the chromosome via the *attP* site.

Enzyme activity staining of SOD in gel

Preparation of cell extracts, gel electrophoresis and the in-gel staining of SOD enzyme activity were performed as described previously (Kim *et al.*, 1996).

RNA isolation and S1 mapping analysis

RNAs were isolated from wild type (M145) and Δ*nur* mutant cells grown in YEME to A_{600} of 0.6–0.7. Probes for *sodF*, *sodF2*, *sodN* and *nikA* transcripts were generated by PCR using M145 chromosomal DNA as a template. The primer pairs used were 5′-TCC GAA CAA CAC ACC TAA GG-3′ (5′ end at 165 nt upstream from the start codon) and 5′-GAC TGA TCA CGG GAG CCA GC-3′ (5′ end at 64 nt downstream from the start codon) for *sodF*, 5′-GGT TGA ATG CTG TGA TCC GAC C-3′ (5′ end at 131 nt upstream from the start codon) and 5′-GTC GTT CGC ACC CTT CAC GTA C-3′ (5′ end at 126 nt downstream from the start codon) for *sodF2*, 5′-CTC GGT CTC CTG CGA CAG TTG CTC-3′ (5′ end at 175 nt upstream from the start codon) and 5′-CAT CTT CTC CTG GAC GGC CTT CAC-3′ (5′ end at 126 nt downstream from the start codon) for *sodN*, 5′-CGC CCG GAT CCC CGT CGA GAC GGT GGC (5′ end at 235 nt upstream from the start codon) and 5′-CCG ATG ACG ACG GAT TCG GCC-3′ (5′ end at 164 nt downstream from the start codon) for *nikA*. PCR-generated probes were labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Hybridization and S1 nuclease digestion were carried out according to standard procedures, and the protected fragments were analysed on a 5% polyacrylamide gel containing 7 M urea.

Measurement of nickel content using inductively coupled plasma – mass spectrometry (ICP-MS)

The amount of nickel accumulated in the wild type and Δ*nur* mutant was determined for cells grown for 18 h in NB liquid media supplemented with various concentrations of NiSO$_4$ (0, 0.5, 1, 2, 5 and 10 μM). The harvested mycelia were washed three times in tertiary distilled water and dried in an 50°C oven for 1 day. The nickel content was analysed using ELAN 6100 ICP-MS (Perkin-Elmer SCIEX) at the National Center for Inter-University Research Facilities (NCIRF) at Seoul National University.

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