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NreB from *Achromobacter xylosoxidans* 31A Is a Nickel-Induced Transporter Conferring Nickel Resistance

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There are two distinct nickel resistance loci on plasmid pTOM9 from *Achromobacter xylosoxidans* 31A, *ncc* and *nre*. Expression of the *nreB* gene was specifically induced by nickel and conferred nickel resistance on both *A. xylosoxidans* 31A and *Escherichia coli*. *E. coli* cells expressing *nreB* showed reduced accumulation of Ni^{2+}, suggesting that NreB mediated nickel efflux. The histidine-rich C-terminal region of NreB was not essential but contributed to maximal Ni^{2+} resistance.

Nickel is the 24th most abundant element in the earth's crust and has been detected in different media in all parts of the biosphere. Nickel is classified as a borderline metal ion because it has both soft and hard metal properties and can bind to sulfur, nitrogen, and oxygen groups (3). In many bacteria, nickel is required for enzymes such as urease, CO dehydrogenase, and hydrogenase (5, 10). However, excess nickel is toxic. Nickel binds to proteins and nucleic acids and frequently inhibits enzymatic activity, DNA replication, transcription, and translation (1). Several nickel-resistant bacteria have been isolated from heavy-metal-contaminated sites. Well-studied examples include *Ralstonia metallidurans* CH34 and *Achromobacter xylosoxidans* 31A (8, 24). The determinant responsible for nickel resistance in *R. metallidurans* CH34, *cnr* (cobalt-nickel resistance), encodes three regulatory genes (*cnrY, cnrX*, and *cnrH*) and three structural genes encoding the subunits of the Co-Ni efflux pump (*cnrC, cnrB*, and *cnrA*) (8, 26). The *cnr* determinant is similar to the *ncc* determinant (nickel-cobalt-cadmium resistance) of *A. xylosoxidans* 31A. The proposed gene products for the efflux system CnrCBA and NccCBA are largely homologous to the gene products for the three subunits of the better-characterized CzcCBA cation-proton antiporter and probably have a similar function (16, 17, 27). In addition to the *ncc* locus, *A. xylosoxidans* 31A contains another distinct nickel resistance locus, *nre*, located on plasmid pTOM9. The *nre* locus confers low-level nickel resistance on both *Ralstonia* and *Escherichia coli* strains (24). The closest homologue of the deduced *nreB* gene product is NrsD from *Synechocystis* sp. strain PCC 6803 (6). Both NreB and NrsD belong to the major facilitator superfamily (MFS), and computer analysis indicates 12 putative transmembrane helices in each (11, 20). Additionally, both proteins possess histidine-rich C termini possibly implicated in metal binding (6).

In this study, we characterized the *nre* locus of *A. xylosoxidans* 31A and showed that only *nreB* is required for nickel resistance. In *A. xylosoxidans, nreB* was specifically induced by nickel but not by cobalt or zinc. The histidine-rich C terminus was not essential for NreB function but was necessary for maximum nickel resistance. *E. coli* cells harboring *nreB* showed reduced uptake of nickel compared to that of wild-type cells. The data support our hypothesis that NreB is a Ni^{2+} transporter responsible for Ni^{2+} efflux and resistance in *A. xylosoxidans* 31A and *E. coli*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used are listed in Table 1. *E. coli* strains were grown in Luria-Bertani broth or agar. Antibiotics were added, as appropriate, to the following final concentrations: ampicillin, 100 μg/ml; tetracycline, 12.5 μg/ml; kanamycin, 25 μg/ml for *E. coli* and 1 mg/ml for *A. xylosoxidans*. To induce gene expression under the *ptetA* promoter anhydrotetracycline (AHT; 0.2 μg/ml; Sigma-Genosys) was added. *A. xylosoxidans* 31A and *R. metallidurans* AE104 were grown in *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)- or Tris-buffered mineral salt medium containing a final concentration of 2 g of sodium gluconate per liter (13). $NiCl_2$ was added as indicated. For conjugative gene transfer, overnight cultures of donor strain *E. coli* S17-1 (25) and recipient strain *R. metallidurans* AE104 or *A. xylosoxidans* 31A were grown at 30°C in complex medium, mixed (1:1), and plated onto Luria-Bertani plates. After overnight growth, the bacteria were suspended in saline (8.5 g of NaCl/liter), diluted, and plated onto selective medium as previously described (15).

^{63}Ni uptake. Uptake experiments were performed by filtration. Following growth in Luria-Bertani medium, cells were harvested and washed once with a buffer consisting of 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (pH 7.0), 100 mM sucrose, and 100 mM potassium phosphate. Transport assays were conducted by addition of $^{63}Ni^{2+}$ to a final concentration of 5 μM, with filtration at the indicated times. The nitrocellulose filters (0.45-μm pore size; Whatman) were washed with 10 ml of the same buffer. A blank value, obtained by filtering 0.1 ml of the assay mixture without cells and washing it with 10 ml of buffer, was subtracted from all measured values. $^{63}NiCl_2$ (1.25 μCi/ml) was from Amersham/Pharmacia.

Genetic techniques. Standard molecular genetic techniques were used (21). Transposon mutagenesis was conducted as described previously (24). Total RNA of *A. xylosoxidans* was isolated as described previously (8, 9). Northern blot analysis was performed as described by Große et al. (9). PCR was performed using *Pwo* (Roche) or *Taq* (Qiagen) DNA polymerase. DNA sequences were obtained by the dideoxy-mediated chain termination method of Sanger et al. using ^{35}S (22) or using an ABISEQ automatic sequencer.

Construction of the truncated *nreB* gene lacking the coding region for the histidine rich C terminus. To construct the truncated *nreB* gene, PCR amplification was performed using the primer pair 5′-GAGGAATTC$_{2460}$ATGCTTGATGTATTGAAGAACCGGA-3′ and 5′-CTCCTGCAG$_{3649}$GATGACATCTTC

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype	Reference or source
Strains		
A. xylosoxidans 31A	Nickel resistant (*nre*$^+$ *ncc*$^+$) pTOM8 pTOM9	24
A. xylosoxidans AX1	Φ(*nreB-lacZ*); transcriptional fusion on pTOM8	This work
R. metallidurans AE104	Ni^s	15
E. coli DH5α	F$^-$ *end-1 hsdR17 supE44 thi-1 recA1 deoR gyrA96 relA1* Δ(*argF-lacZYA*)*U169*	New England Biolabs
E. coli S17-1	*thi pro hsdR hsdM*$^+$ *recA tra*$^+$	25
E. coli W3110	Wild type	Laboratory stock
Plasmids		
pASK-IBA3	Overexpression vector (STREP TagII); Amp^r	IBA GmbH, Göttingen, Germany
pNREB	pASK-IBA3 with *nreB* under *ptet* control	This work
pNREB2	pASK-IBA3 with truncated *nreB* (Δ aa[a] 404–446) under *ptet* control	This work
pGEM-T-Easy	Cloning vector; Amp^r	Promega, Madison, Wis.
pLO18	Suicide vector; *sacB*	G. Grass and D. H. Nies, unpublished data
pNREB4	Φ(*nreB-lacZ*) transcriptional fusion in pLO18	This work
pVDZ′2	*incP* mob$^+$ Tc^r *lacZ*α	4
pVDZ′2::TEC9	*nre*$^+$; 4.2-kb *Eco*RI fragment, nt[b] 1–4189 (accession no. L31491)	23
pVDZ′2::TBK9	*ncc*$^+$; 11.9-kb *Bam*HI/*Kpn*I fragment	23
pVDZ′2::TBA9	*ncc*$^+$ *nre*$^+$; 14.5-kb *Bam*HI fragment	23
pVDZ′2::TBA9-*Eco*RI	*ncc*$^+$ *nre*$^+$; 14.5-kb *Bam*HI fragment with deleted *Eco*RI site in *ncc*; parallel orientation of *ncc* and *nre*	This work
pVDZ′2::TBA9-*Eco*RI-anti	*ncc*$^+$ *nre*$^+$; 13.5-kb *Bam*HI/*Eco*RI fragment with inverted internal *Eco*RI fragment of pVDZ′2::TBA9-*Eco*RI resulting in opposite orientation of *ncc* and *nre*	This work

[a] aa, amino acids.
[b] nt, nucleotides.

GTCGCGTGACG (the underlined sequences are restriction sites), resulting in a fragment lacking 3′ positions 3650 to 3786. The PCR fragment was then cloned into overexpression vector pASK-IBA3 using the *Eco*RI and *Pst*I restriction sites introduced by PCR. The complete *nreB* gene was amplified using the primer pair 5′ GAG$\underline{\text{GAATTC}}_{2460}$ATGCTTGATGTATTGAAGAACCGGA-3′ and 5′-AT $\underline{\text{TCTGCAG}}_{3780}$ATGCGCGTCGGGCCATCG-3′ and also cloned into vector pASK-IBA3.

Reporter gene fusion. To construct a Φ(*nreB-lacZ*) transcriptional fusion in strain *A. xylosoxidans* 31A, a 1,273-bp fragment, *nreB*′ (amplified with the primer pair 5′-CCG$\underline{\text{GTCGAC}}_{2498}$GTTCACGGCACAGGTGATCCC-3′ and 5′-$_{3783}$AT TCTAATGCGCGTCGGGCCA-3′), containing the 3′ end of *nreB* was amplified as a *Sal*I/*Pst*I fragment from *A. xylosoxidans* 31A and cloned into pGEM T-Easy (Promega, Madison, Wis.). A promoterless *lacZ* gene was cloned into the single *Pst*I site directly downstream of *nreB*′. The *lacZ* gene was amplified from chromosomal DNA of *E. coli* W3110, introducing *Pst*I sites at each end. The correct orientation of *lacZ* was confirmed by restriction analysis, and the Φ(*nreB-lacZ*) fragment was subcloned as a *Sal*I-fragment into suicide vector pLO18 taking advantage of an additional *Sal*I site in pGEM-T-Easy. The resulting plasmid was used to insert the *lacZ* gene by recombination immediately downstream of *nreB* in *A. xylosoxidans* as described by Groβe et al. (9), creating strain AX1 Φ(*nreB-lacZ*). The β-galactosidase activity in permeabilized cells was determined as described previously (14).

RESULTS

The *nre* locus is a second distinct nickel resistance determinant of *A. xylosoxidans* 31A. Previously, the *ncc* (nickel, cobalt, cadmium) determinant on plasmid pTOM9 of *A. xylosoxidans* 31A was identified as a nickel resistance determinant (23, 24). However, a 14.5-kb *Bam*HI fragment containing not only the *ncc* determinant but also a 4.2-kb *Eco*RI fragment downstream of *ncc* rendered cells of AE104 more nickel resistant than a fragment containing only *ncc* (Table 2). This suggested the presence of an additional nickel resistance determinant downstream of *ncc*. Expression of this determinant is independent of *ncc*, because inversion of the 4.2-kb *Eco*RI fragment did not alter the resistance pattern (Table 2). The 4.2-kb *Eco*RI fragment alone was able to confer nickel resistance on both *R. metallidurans* AE104 and *E. coli* (Table 2). Sequence analysis of the 4.2-kb *Eco*RI fragment suggested the presence of four open reading frames (accession no. L31491). Subsequent sub-

TABLE 2. MICs for different constructs in *R. metallidurans* AE104 and *E. coli* S17-1

Plasmid[a]	MIC[b] of Ni^{2+} (mM) for:	
	R. metallidurans AE104	*E. coli* S17-1
pVDZ′2	0.5	0.3
pVDZ′2::TEC9a	3	5
pVDZ′2::TEC9b	3	5
pVDZ′2::TBK9a	50	0.3
pVDZ′2::TBK9b	45	0.3
pVDZ′2::TBA9a	70	5
pVDZ′2::TBA9b	70	5
pVDZ′2::TBA9a-*Eco*RI	70	5
pVDZ′2::TBA9b-*Eco*RI	70	5
pVDZ′2::TBA9b-antiTEC9a	70	5

[a] Fragments were cloned in both orientations as follows: a; under the control of *plac*; b, opposite orientation with respect to *plac*.
[b] The MIC was defined as the concentration at which no growth was observed. Cells were streaked on TES agar with $NiCl_2$ at 0, 0.3, 0.5, 1.5, 3, 5, 7, 9, 11, 15, 20, 30, 40, 45, 50 60, or 70 mM, and growth was monitored after 4 days at 30°C (*R. metallidurans*) or 37°C (*E. coli*).

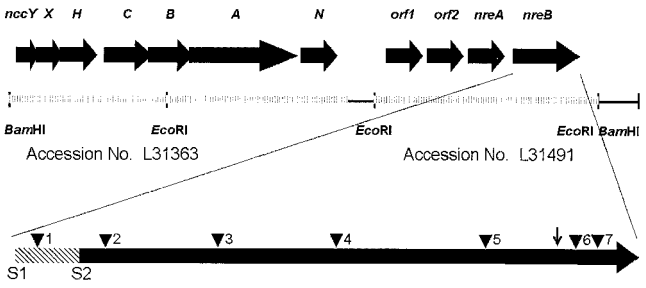

FIG. 1. Genetic organization of the nickel resistance determinants *ncc* and *nre* from *A. xylosoxidans* 31A (accession no. L31491 and L31363) on a 14.5-kb *Bam*HI fragment. Arrows indicate the transcriptional orientation of the respective genes. Triangles represent loci of Tn*5* insertions (nucleotide numbering according to the sequence with accession number L31491) as follows: 1, 2397; 2, 2535; 3, 2876; 4, 3239; 5, 3573; 6, 3703; 7, 3718. The two possible start codons are indicated by S1 (nucleotide 2376) and S2 (nucleotide 2460). The vertical arrow marks the 3′ end of the truncated *nreB* gene (nucleotides 2460 to 3649). Accession number L31363 is a 4.2-kb *Eco*RI fragment (shaded), and accession number L31491 is an 8.1-kb fragment (shaded). Distances are not drawn to scale.

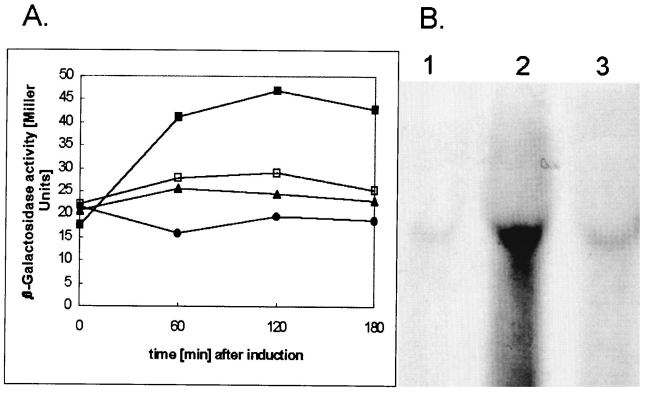

FIG. 2. Induction of *nreB*. Induction of β-galactosidase activity in an *nreB-lacZ* mutant strain. (A) Cells of *A. xylosoxidans* AX1 containing a ϕ(*nreB-lacZ*) operon fusion on plasmid pTOM9 were diluted 15-fold to an optical density at 600 nm of 0.15 into fresh medium containing no added metal (□) or were induced after 5 h of growth with 0.3 mM Ni^{2+} (■), Co^{2+} (▲), or Zn^{2+} (●). Incubation was continued with shaking at 30°C, and the β-galactosidase activity was determined as Miller units (14). (B) Northern blot analysis of *nreB* transcription. Total RNA was separated by electrophoresis in 1.5% agarose, transferred to a nylon membrane, and hybridized with an *nreB*-specific probe (all lanes). Total RNA was isolated from *nreB*-containing strain *A. xylosoxidans* 31A that was cultivated without toxic concentrations of heavy-metal cations (lane 1) or induced for 30 min with 300 μM Ni^{2+} (lane 2) or Co^{2+} (lane 3). Blebs and compressions are due to the 16S and 23S rRNAs. The original photograph was scanned with Ofoto 2.0 (Light Source Computer Images, Inc.) and processed with Adobe Photoshop 3.0 (Adobe Systems, Inc.).

cloning (12) of this 4.2-kb *Eco*RI fragment showed that only the 3′ 2-kb fragment is necessary for nickel resistance in *A. xylosoxidans* 31A. This distinct nickel resistance determinant possessing two putative genes, *nreA* and *nreB*, was termed *nre* (Fig. 1).

The *nreB* gene is induced by nickel but not by zinc and cobalt in *A. xylosoxidans* 31A. The *nre* determinant has metal ion specificity different from that of the *ncc* determinant. Whereas the *ncc* determinant confers Ni^{2+}, Co^{2+}, and Cd^{2+} resistance, the *nre* determinant confers only Ni^{2+} resistance. Regulation of *nre* transcription was examined by Northern blot analysis and by a Φ(*nreB-lacZ*) operon fusion. For Northern blot analysis, cells of *A. xylosoxidans* 31A were grown in Tris minimal medium (13) to mid-log phase, at which point either $NiCl_2$ or $CoCl_2$ was added. As a control, a culture was grown with no added metal. An increase in an *nreB*-specific transcript was detected only after addition of Ni^{2+} (Fig. 2B). Addition of Co^{2+} did not result in an increase in transcription compared to that of the control with no metal added (Fig. 2B). The actual size of the transcript could not be determined because of compressions due to rRNA and unstable transcripts. Transcription of a Φ(*nreB-lacZ*) operon fusion was specifically induced by nickel. Neither zinc nor cobalt induced transcription (Fig. 2A). These results show specific induction of *nreB* expression by nickel and not by cobalt or zinc, consistent with the specificity of metal resistance. Induction of *nreB* expression was also dependent on the nickel concentration in the medium. After 1 h, the β-galactosidase activity (in Miller units) of a Φ(*nreB-lacZ*) operon fusion was 28 without added metal, 55.85 with 0.5 mM Ni^{2+} added, and 78.2 with 1 mM Ni^{2+} added. Maximal induction was obtained after 2 h with 3 and 0.5 mM Ni^{2+} and after 1 h with 1 mM Ni^{2+}. After 3 h, a slight decrease in *nreB* transcription could be observed for all of the concentrations tested (data not shown).

NreB confers nickel resistance on *E. coli*. There are two possible ATG start codons for NreB. However, transposon mutagenesis (Fig. 1; Table 3) and codon usage (2) suggest that the second start codon is used. Therefore, *nreB* starting with the second start codon was cloned into the expression vector pASK-IBA3 (Sigma-Genosys, The Woodlands, Tex.) to create pNREB. Expression of *nreB* in *E. coli* W3110(pNREB) rendered cells more nickel resistant than *E. coli* W3110(pASK-IBA3) lacking the *nreB* gene (Fig. 3). Although maximal nickel resistance was obtained after addition of the inducer AHT, a substantial increase in nickel resistance was also observed in the absence of induction, indicating that the *ptet* promoter was leaky and that only minute amounts of NreB were necessary for an increase in nickel resistance (Fig. 3). Expression of the

TABLE 3. Effects of different transposon insertions in *nreB* on nickel resistance of *E. coli* strain S17-1

Plasmid, Tn*5* insertion[a] (nucleotide no.)	MIC[b] of Ni^{2+} (mM)
pVDZ′2	0.3
pVDZ′2::TEC9	5
pVDZ′2::TEC9, Tn*5-1* (2397)	3
pVDZ′2::TEC9, Tn*5-2* (2535)	0.3
pVDZ′2::TEC9, Tn*5-3* (2876)	0.3
pVDZ′2::TEC9, Tn*5-4* (3239)	0.3
pVDZ′2::TEC9, Tn*5-5* (3573)	0.3
pVDZ′2::TEC9, Tn*5-6* (3703)	3
pVDZ′2::TEC9, Tn*5-7* (3718)	3

[a] Tn*5* insertion loci were determined by DNA sequencing. The nucleotide numbers shown correspond to the sequence with accession no. L31491.

[b] The MIC was defined as the concentration at which no growth was observed. Cells were grown overnight, resuspended in saline (0.85%, wt/vol), and streaked on TES agar with $NiCl_2$ at 0, 0.3, 0.5, 1.5, 3, 5, or 7 mM, and growth was monitored at 37°C.

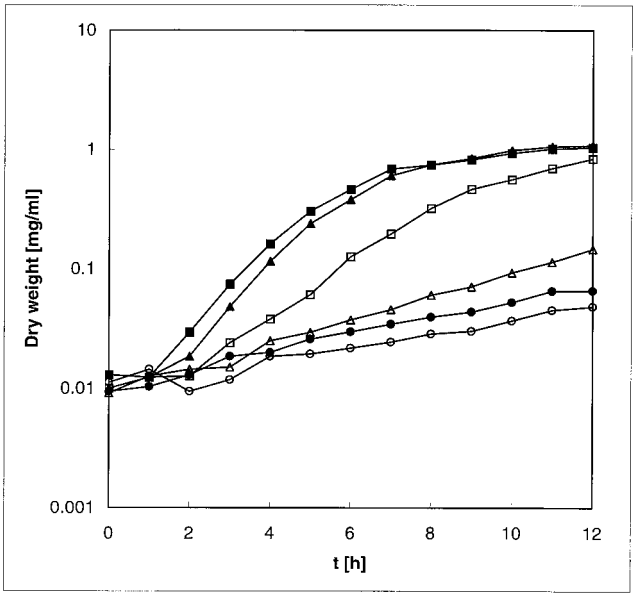

FIG. 3. Effect of 3 mM Ni^{2+} on the growth of *E. coli* W3110 containing specific constructs. Cells were grown overnight in Luria-Bertani medium, diluted 200-fold into fresh Luria-Bertani medium containing 3 mM $NiCl_2$, and grown at 37°C with shaking. The optical density at 600 nm was monitored hourly for 12 h and converted to dry weight (milligrams per milliliter) and is presented as a semilogarithmic plot. Symbols: ● and ○, pASK-IBA3; ■ and □, pNREB; ▲ and △, pNREB2. The inducer AHT was either not added (open symbols) or was added to a final concentration of 0.2 μg/ml (filled symbols).

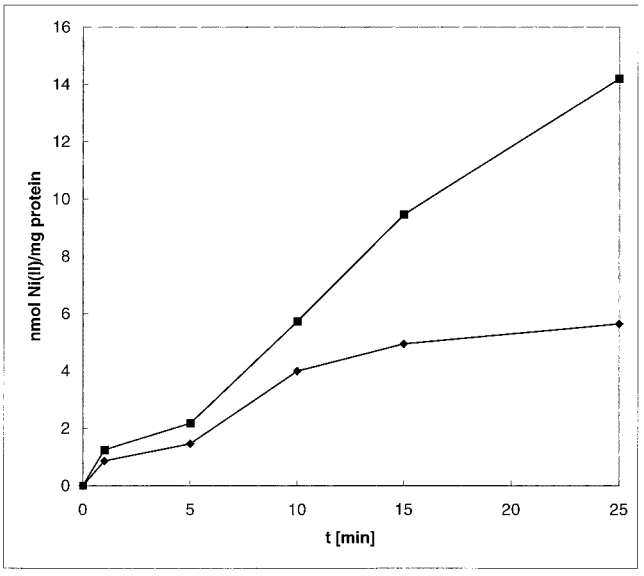

FIG. 4. $^{63}Ni^{2+}$ uptake by *E. coli* W3110 expressing *nreB*. Cells were grown overnight in Luria-Bertani medium and diluted 100-fold into fresh Luria-Bertani medium. The cells were grown to an optical density at 600 nm of 0.8 and induced with AHT at 0.2 μg/ml. After growth for 2.5 h, the cells were washed with buffer and concentrated fourfold in buffer. $^{63}Ni^{2+}$ was added to a final concentration of 5 μM. The cells were incubated at 37°C, and 0.1-ml aliquots were filtered through a nitrocellulose membrane (0.45-μm pore size) at various times and immediately washed with 10 ml of buffer. The membranes were dried, and radioactivity was determined using a liquid scintillation counter. The protein concentration was determined using the bicinchoninic acid kit (Sigma), and the Ni^{2+} concentration per milligram of protein was calculated. *E. coli* W3110(pASK-IBA3), ■; *E. coli* W3110(pNREB), ◆.

NreB protein could not be visualized by silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

The C-terminal histidine-rich region of NreB is not essential but contributes to maximal nickel resistance. The C-terminal histidine-rich region in the homologous *nrsD* protein from *Synechocystis* sp. strain PCC 6803 had previously been shown to bind metals (6). To assess if the C-terminal region is essential for NreB, the C-terminal region was deleted (amino acids 404 to 446) and the truncated NreB protein was expressed in *E. coli* W3110(pNREB2). The truncated NreB protein was still able to confer a significant increase in nickel resistance compared to *E. coli* W3110(pASK-IBA3) (Fig. 3). Indeed, the truncated NreB protein was able to confer nearly the same resistance as the full-length protein when induced with AHT (Fig. 3). However, a significant loss of nickel resistance was observed when no inducer (AHT) was added (Fig. 3). These results are also in agreement with insertional mutants generated by transposon mutagenesis. Transposon insertions into the *nreB* gene at sites encoding the histidine-rich C-terminal domain resulted in only a slight decrease in nickel resistance, whereas insertions into other parts of the gene completely abolished nickel resistance (Table 3).

Expression of NreB is responsible for reduced uptake of $^{63}Ni^{2+}$ in *E. coli* W3110. Since NreB is predicted to be a cytoplasmic membrane protein and responsible for Ni^{2+} resistance, we examined whether cells expressing *nreB* exhibit decreased Ni^{2+} uptake in *E. coli* W3110, reflecting increased efflux. Alternately, resistance could be due to binding of the metal by the C-terminal histidine-rich domain of NreB. Binding should increase the amount of metal ions in resistant cells compared to that in sensitive ones, while efflux should decrease the concentration of cytosolic metal ions. When levels of cell-bound metal ions in cells with and without expression of *nreB* were compared, resistant cells contained only 40% of the $^{63}Ni^{2+}$ that the respective control cells contained after 25 min (Fig. 4). Thus, expression of *nreB* resulted in reduced accumulation of Ni^{2+}.

DISCUSSION

The results of this study suggest that NreB from *A. xylosoxidans* is responsible for nickel resistance by efflux. NreB is most closely related to proteins of the DHA3 family of the MFS (TC no. 2.A.1.21.5). MFS transporters have been shown to transport small solutes in response to a chemiosmotic gradient (20). Prior to this study, proteins of the MFS had not been shown to transport metals. The closest homolog of NreB is the NrsD protein from *Synechocystis* sp. strain PCC 6803 (6). Computer analysis of both NreB and NrsD suggests the presence of 12 transmembrane helices and histidine-rich C-termini in both proteins. Metal binding of the histidine-rich domain from NrsD was evaluated using metal affinity chromatography and showed that this domain has a low specificity for metal binding (6). In this report, we show that the histidine-rich domain is not essential for NreB function. Transposon insertions into the

nreB gene at sequences encoding the histidine-rich domain slightly reduced nickel resistance but did not abolish it. However, insertions into other regions of the *nreB* gene resulted in complete loss of nickel resistance. Furthermore, truncated NreB still conferred nickel resistance on *E. coli* when the gene encoding it was induced with AHT. However, in cells where *nreB* was not induced, there was a significant loss of nickel resistance compared to cells producing the complete protein. These results suggest that this domain binds nickel, thereby making nickel transport more efficient.

The mechanism of NreB resistance is most likely the result of nickel efflux coupled to a chemiosmotic gradient. Consistent with this hypothesis, cells of *E. coli* W3110 expressing *nreB* exhibited reduced uptake of nickel compared to a control without *nreB*. This is the first example of an MFS protein catalyzing metal ion transport. However, at this point, the actual substrate of NreB is not known, as it might be the free cation or a metal conjugate. Other nickel resistance determinants, such as those encoded by *cnr* and *ncc*, are close homologues of the better-studied *czc* determinant from *R. metallidurans* CH34. The *czcCBA* gene products form a membrane-bound protein complex catalyzing Co^{2+}, Zn^{2+}, and Cd^{2+} efflux by a proton-cation antiporter in *R. metallidurans* CH34 (7, 16, 19). The actual substrate transported is also not known for CzcCBA, NccCBA, or CnrCBA. NreB is more specific for nickel, while NccCBA and CnrCBA have a broad range of metal ion substrates. In addition to nickel, they also transport cobalt and cadmium (*ncc*) and cobalt and zinc (*cnr*) (28). It is not known how *nreB* is regulated; however, *nreB* is induced by nickel and not by cobalt or zinc, consistent with the specificity of metal resistance. Transcriptional analysis was performed with *A. xylosoxidans* 31A harboring both the *ncc* and *nre* determinants. Therefore, over time, the decrease in transcription of a ϕ(*nreB-lacZ*) operon fusion can be attributed to nickel efflux by NreB and NccCBA. This is similar to the self-repression of the *ars* operon that occurs when the ArsAB pump extrudes the inducer arsenite and transcript levels return to uninduced levels (18).

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