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Nickel-Resistant Determinant from *Leptospirillum ferriphilum*▽

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Leptospirillum ferriphilum **strain UBK03 isolated from a mine in Jiangxi, China, is resistant to Ni^{2+} (30 to 40 mM). A four-gene nickel resistance cluster was identified and, when transformed into *Escherichia coli*, enabled growth in 6 mM nickel. Mutation experiments revealed that the genes *ncrA*, *ncrB*, and *ncrC* could confer nickel resistance in *Escherichia coli*, whereas the gene *ncrY* could have a negative effect on nickel resistance.**

Bioleaching for the recovery of metals from ores recently received increased attention. This technology has the potential to overcome many problems faced by metal industries (14, 20, 36). The success of a bioleaching operation depends largely on the consortium of microorganisms present in the mining environment. As these mining bacteria can grow and thrive under ore conditions having high concentrations of heavy metals, they are naturally resistant to heavy metal toxicity (2, 9, 24). Although Rawlings et al. (6, 28, 35) have demonstrated that *Leptospirillum ferriphilum* is the most important microorganism in bioleaching to date, only one gene cluster, related to arsenic resistance (35), has been isolated from this organism so far. However, many metal resistance genes have been cloned from other acidophilic bacteria, such as the zinc and nickel resistance genes from *Acidiphilium multivorum* GS19h (13), the copper resistance genes from *Acidithiobacillus ferrooxidans* (8), and the cadmium resistance genes from *Acidiphilium symbioticum* (20).

Bacteria resistant to nickel have been isolated from ecosystems polluted by heavy metals, such as wastewater, mine refuse, industrial composts, and cooling waters of the metal processing industry (26). These bacteria are mainly *Ralstonia eutropha* CH34 (4, 5, 15, 19, 21), *Alcaligenes denitrificans* 4a-2 (33), *Alcaligenes xylosoxydans* 31A (32), *Ralstonia eutropha* KTO2 (32), *Klebsiella oxytoca* CCUG 15788 (34), *Hafnia alvei* 5-5 (25, 26), and *Escherichia coli* (29). The mechanism of nickel resistance in bacteria is due to the action of an operon-encoded, energy-dependent specific efflux system that pumps the cation from the cell, thereby lowering the intracellular concentration of the toxic metal (25).

The present study is aimed at characterizing the metal resistance of a new strain, *L. ferriphilum* UBK03, cloning its nickel resistance determinant, and studying the functions of these genes.

Characterization of *L. ferriphilum* UBK03. *L. ferriphilum* UBK03 is a gram-negative, vibrio- or spiral-shaped bacterium that was isolated from a mine in Jiangxi, China (Table 1). It is an obligate chemolithotroph and grows optimally at 37°C in 9K inorganic medium within the pH range 1.3 to 2.0 (6). To identify this bacterium at the genetic level, a partial 16S rRNA gene of UBK03 was obtained by PCR using the primer pair 16sF/16sR (Table 1). The 16S rRNA gene sequence of UBK03 (GenBank accession no. DQ534052) was compared with other sequences in the NCBI nucleotide database and found to be identical to those of the *L. ferriphilum* strains Fairview (GenBank accession no. AF356830.1) and ATCC 49881 (GenBank accession no. AF356829.1).

Metal resistance of *L. ferriphilum* UBK03. Because *L. ferriphilum* has been proven by Rawlings et al. (6, 28, 35) to play the most important role in bioleaching, it might be resistant to metals. Thus, its resistance to some metals was examined by monitoring ferrous iron oxidation using a method to determine ferrous iron in the presence of ferric iron (17). The metal tolerance of strain UBK03 in 9K medium followed this order: Ni^{2+} (30 mM to 40 mM) > Zn^{2+} (20 mM to 30 mM) > Co^{2+} (5 mM to 10 mM) > Cu^{2+} (<5 mM) ≈ Cd^{2+} (<5 mM).

Cloning of nickel resistance genes from *L. ferriphilum* UBK03. The genomic DNA of *L. ferriphilum* UBK03 was isolated as described previously (6). Partially digested 3- to 10-kb HindIII DNA fragments were used to construct a gene bank in the HindIII site of the vector pUC19 (Table 1). The recombinant vectors were introduced into *E. coli* JM109 with electroporation using Gene Pulser (Bio-Rad). When transformants were selected on LB plates (22, 31) containing 100 μg/ml of ampicillin and 3 mM $NiCl_2$, three colonies appeared on the plates in 24 h, indicating that they expressed the genes for nickel resistance. The plasmids of these three positive colonies were isolated and analyzed with restriction enzymes. The results demonstrated that the recombinant plasmids, designated pNR21, pNR22, and pNR23, had 4.0-, 7.3-, and 5.6-kb DNA insertions in the vector pUC19, respectively. To determine whether the nickel resistance determinant was indeed located within these plasmids, the recombinant plasmids were extracted and transformed into the *E. coli* strain Top10. All three plasmids endowed *E. coli* Top10 with nickel resistance. Although the plasmids included DNA fragments of different lengths, all strains exhibited the same MIC of nickel, 6 mM.

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TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant characteristics or sequence[a]	Source or reference(s)
Strains		
L. ferrophilum		
UBK03	Wild type	This study
E. coli		
JM109	Host for plasmid amplification	Promega
Top10	Host for plasmid amplification	Invitrogen
BL21	F^- *dcm ompT hsdS* (r_B^2 m_B^2) *galDE3*	Novagen
CC118	Δ(*ara-leu*)*7697* Δ*lacX74* Δ*phoA20 galE galK thi rpsE rpoB argE*(*am*) *recA1*	7, 10, 27
Plasmids		
pUC19	*lacZ′*; ColE1 replicon, cloning vector, Amp^r	Promega
pNR21	4.0-kb HindIII fragment from genomic DNA of strain UBK03, Amp^r	This study
pHA-4	Contains PhoA reporter gene	7, 10, 27
pWaldo-TEV-GFP	Contains GFP reporter gene	7, 10, 27
pNDA	pNR21 derivative deleting *ncrA*, Amp^r	This study
pNDB	pNR21 derivative deleting *ncrB*, Amp^r	This study
pNDC	pNR21 derivative deleting *ncrC*, Amp^r	This study
pNDY	pNR21 derivative deleting *ncrY*, Amp^r	This study
pLAFR1	Contains tetracycline resistance cassette, Amp^r, Tc^r	37
pNTA	pNR21 containing insertion in *ncrA*, Amp^r, Tc^r	This study
pNTB	pNR21 containing insertion in *ncrB*, Amp^r, Tc^r	This study
pNTC	pNR21 containing insertion in *ncrC*, Amp^r, Tc^r	This study
pNTY	pNR21 containing insertion in *ncrY*, Amp^r, Tc^r	This study
Primers		
16sF	5′-CATGGCCCATCAGCTAGTTG-3′	This study
16sR	5′-GCGATTCCGACTTCATGAGG-3′	This study
ncrAF	5′-**CTCGAG**GATGCTCAACATTCTTTCTAA-3′	This study
ncrAR	5′-**GGATCC**CTTCTCTTTCTTCGGCCAGG-3′	This study
ncrCF	5′-**CTCGAG**ATGACTGATTTTTCCACTCTTTT-3′	This study
ncrCR	5′-**GGATCC**GCTCGTTACGCCGATCCAGC-3′	This study
BU_F	5′-**AAGCTT**AGGAGAGGATAAGCCGATAA-3′	This study
YD_R	5′-**GAGCTC**TTAACGGTGAAATTAAGGTTCT-3′	This study
AU_F	5′-**AAGCTT**TCATTTTTTTCAGGTCCCTCT-3′	This study
AU_R	5′-**GGATCC**ATCCTCTCCTGATACGTTGT-3′	This study
CD_F	5′-**GGATCC**TATCTTTAAGGCAAGCGAAACA-3′	This study
BU_R	5′-**GGATCC**AGAGTGGAAAAATCAGTCATG-3′	This study
YD_F	5′-**GGATCC**GTCGGCATTTACATGGGCTATC-3′	This study
CU_R	5′-**GGATCC**TCAGGCGTGAGATTCATACAG-3′	This study

[a] Amp, ampicillin; Km, kanamycin; Tc, tetracycline. The restriction enzyme sites incorporated into the primers are shown in boldface in the primer sequences.

Together, these results suggested that the genes conferring nickel resistance were coded by the DNA fragment in each plasmid.

Sequence analysis of nickel resistance genes. Sequencing of the three plasmids revealed a common 3,991-bp segment containing four open reading frames (NcrA, NcrB, NcrC, and NcrY), which localized to the 3,991-bp HindIII-HindIII fragment of pNR21 (GenBank accession no. DQ517331), as shown in Fig. 1. BLAST (1) and Pfam (3) searches were performed to identify putative gene functions. The similarities of these proteins with related sequences are shown in Table 2. As analyzed by TMHMM (18) and PSORT (23), the proteins NcrA, NcrC, and NcrY were predicted to be membrane proteins, whereas NcrB was predicted to be a globular protein expressed in the cytoplasm.

Southern hybridization analysis to confirm the source of the 3,991-bp fragment. To determine whether the 3,991-bp fragment in pNR21 was derived from *L. ferriphilum* UBK03, Southern hybridization was performed. *L. ferriphilum* UBK03 total DNA was digested by HindIII, electrophoresed on a 1%

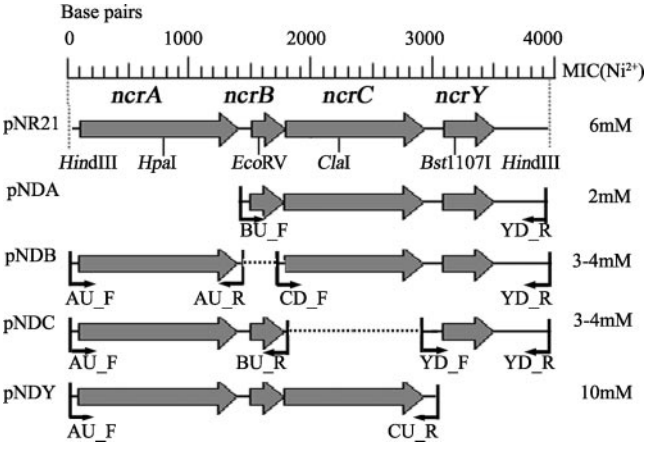

FIG. 1. Physical map of the sequences of pNR21, pNDA, pNDB, pNDC, and pNDY. Gray arrows indicate putative open reading frames. Black little arrows indicate the primers (Table 1) used in constructing the plasmid. The dashed line represents the deleted DNA fragment. The values in the right column are the MICs of Ni^{2+} for *Escherichia coli* JM109 bearing the corresponding plasmids.

TABLE 2. Comparison of proteins encoded by *L. ferriphilum* UBK03 with similar proteins

Protein name	Sequence length (aa[a])	Homologous protein (sequence length [aa], sequence identity [%], accession no.)
NcrA	432	NirA (356, 77.3, AAR82963.1) NreB-like protein (382, 83, AAK38164) NreB (440, 68, NP_436142.1)
NcrB	89	NirB (89, 98, AAR82964.1) Protein of unknown function (88, 62, ZP_00427384.1) Protein of unknown function (92, 58, ZP_00509556.1)
NcrC	376	NirC (307, 79, AAR82965.1) Putative transmembrane protein (377, 71, NP_745112.1) Putative transmembrane protein (373, 67, ZP_00509558.1)
NcrY	136	NirD (164, 75, AAR82966.1) NcrY (63, 44, AF322866_4) Possible exported protein (130, 35, NP_806781.1)

[a] aa, amino acids.

TABLE 3. Protein activities of PhoA and GFP fusion proteins[a]

Fusion	PhoA activity	Raw GFP activity
NcrA	0.6 ± 0.6	565.8 ± 17.6
NcrC	33.5 ± 1.5	66.2 ± 4.1
CK	1.9 ± 0.6	64.3 ± 3.3

[a] The data shown represent means ± standard deviations. The CKs were *E. coli* CC118 containing pHA-4 and *E. coli* BL21 containing pWaldo-TEV-GFPe.

agarose gel, transferred to a nylon membrane, and hybridized using sequences upstream of *ncrA* (1 to 747 bp) as the probe. The resulting Southern blot revealed a band around 4 kb, the same size as the cloned fragment (data not shown). This result indicated that the 3,991-bp nickel resistance gene cluster originated from *L. ferriphilum* UBK03 and is present as a single copy.

Topology analysis of proteins NcrA and NcrC. Using C-terminal tagging with alkaline phosphatase (PhoA) and green fluorescent protein (GFP) (7, 10, 27), the locations of the C termini of NcrA and NcrC, which were exposed to the cytoplasm or the periplasm, were detected. Fragments of *ncrA* and *ncrC* were amplified by PCR using the ncrAF/ncrAR and ncrCF/ncrCR primer pairs (Table 1), and the resulting products were verified by sequencing. These two DNA fragments were inserted into the reporter vectors pHA-4 and pWaldo-TEV-GFP using the XhoI and BamHI restriction sites, respectively. Constructs of PhoA or GFP fusions were transformed into *E. coli* CC118 or *E. coli* BL21(DE3)/pLysS, respectively. The plasmids pHA-4 in *E. coli* CC118 and pWaldo-TEV-GFPe in *E. coli* BL21 were used as controls. The protein activities of PhoA and GFP were assayed as described previously (7, 10), and the results are shown in Table 3. GFP activity was detected only for the NcrA fusion, and PhoA activity was detected only for the NcrC fusion, indicating that the C terminus of NcrA was cytoplasmic and the C terminus of NcrC was periplasmic. Using this information, the topology models of these two proteins were derived (Fig. 2).

Identification of the nickel resistance of each cloned gene. To evaluate the effect of each gene on nickel resistance, a series of independent deletion and TC-box insertion mutants were constructed using standard molecular genetic techniques (31). All constructs are listed in Table 1 and were obtained as described below.

We constructed the deletion plasmids pNDA, pNDB, pNDC, and pNDY (Table 1; Fig. 1) carrying *ncrBCY*, *ncrACY*, *ncrABY*, and *ncrABC*, respectively, by deleting *ncrA* (bp 1 to 1398), *ncrB* (bp 1409 to 1740), *ncrC* (bp 1782 to 2844), and *ncrY* (bp 2921 to 3337). Plasmids pNDA and pNDY (Fig. 1) were constructed by inserting the PCR products containing *ncrBCY* and *ncrABC* into pUC19 using HindIII-BamHI. Plasmids pNDB and pNDC (Fig. 1) were constructed by inserting DNA fragments *ncrA* and *ncrAB* into pUC19 using HindIII-BamHI and then ligating the PCR products containing *ncrCY* and *ncrY* into the generated plasmid using BamHI-SacI. The MICs of strains harboring the corresponding plasmids were measured, as shown in Fig. 1.

Insertion mutations were constructed by ligating the 1,188-bp tetracycline resistance cassette of plasmid pLAFR1 (TC box) (37) into unique restriction sites of plasmid pNR21. These sites, HpaI, EcoRV, ClaI, and Bst1107I, are located within *ncrA*, *ncrB*, *ncrC*, and *ncrY*, and were used to construct the insertion plasmids pNTA, pNTB, pNTC, and pNTY, respectively. The MICs of these strains containing plasmids pNTA, pNTB, pNTC, and pNTY were 2 mM, 4 to 5 mM, 4 to 5 mM, and 9 mM, respectively.

The MICs of strains harboring different mutant plasmids were analyzed by one-way analysis of variance, followed by Duncan's multiple-range test with an α value of 0.05. As a result, there were significant differences among mutations in the *ncrA*, *ncrB*, and *ncrY* strains. However, there were not significant differences between mutations in the *ncrB* and *ncrC* strains, as well as mutations in the *ncrA* strain and the control (pUC19).

Among the proteins encoded by pNR21, NcrA was the base of the nickel resistance system. When *ncrA* was mutated by either deletion (pNDA) or insertion (pNTA), the strains had the same MICs of nickel (2 mM) as the control (pUC19). NcrA was found to contain 10 transmembrane helices (Fig. 2), belongs to the major facilitator superfamily (Pfam accession no. PF07690) (30), and possesses a histidine-rich region in the C terminus, which might have high affinity to nickel (Fig. 2). This protein may form a transporter in the membrane, be the foundation of the nickel resistance complex (12), and require the presence of accessory proteins for maximal function.

NcrB is a cytoplasmic, histidine-rich, 89-amino-acid protein with unknown function (Pfam accession no. PF02583). When this gene was mutated by deletion (pNDB) or insertion (pNTB), the MIC of nickel was reduced from 6 mM (pNR21) to 3 to 4 mM. These results suggest that NcrB may assist in nickel efflux.

NcrC, similar to NcrA, is a membrane protein belonging to the high-affinity nickel transport protein family (Pfam accession no. PF03824). When this gene was mutated by deletion (pNDC) or insertion (pNTC), the MIC of nickel was reduced from 6 mM (pNR21) to 3 to 5 mM. These results suggest that NcrC also significantly contributes to nickel resistance. Topology analysis of NcrC (Fig. 2) revealed that at least half of the

FIG. 2. Predicted topology of proteins NcrA (A) and NcrC (B). The membrane-associated amino acid residues are boxed, the periplasmic residues are in bold, and the remaining residues are cytoplasmic. The regions are named using the letters M, P, and C for membrane, periplasm, and cytoplasm, respectively.

residues in the mid-region of the protein are cytoplasmic, with high frequencies of histidine (9%) and charged residues (41%), which are aspartic acid, glutamic acid, histidine, lysine, and arginine. These residues have high affinity for nickel and might act to chelate the nickel cation in the cytoplasm (11, 16, 29).

In contrast to NcrA, NcrB, and NcrC, which endow *E. coli* JM109 with nickel resistance, NcrY acts in the opposite manner. Mutations in the *ncrY* strain (pNDY and pNTY) increased the MIC of nickel from 6 mM (pNR21) to 9 to 10 mM. According to Pfam, NcrY is a predicted protein with unknown function (Pfam accession no. PF04076). In our system, NcrY could have a negative effect on nickel resistance through an unknown mechanism which will be studied in the future.

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