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Expression, Purification, Crystallization and Preliminary X-Ray Crystallographic Analysis of CnrX from *Cupriavidus metallidurans* CH34

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The nickel and cobalt resistance of *Cupriavidus metallidurans* CH34 is mediated by the CnrCBA efflux pump encoded by the *cnrYHXCBAT* metal resistance determinant. The products of the three genes *cnrYXH* transcriptionally regulate expression of *cnr*. CnrY and CnrX are membrane-bound proteins, probably functioning as anti-sigma factors, whereas CnrH is a *cnr*-specific extracytoplasmic functions (ECF) sigma factor. The periplasmic domain of CnrX (residues 29-148) was cloned as a N-terminal His-tagged protein, expressed in *Escherichia coli*, and purified using affinity chromatography and gel filtration. The molecular mass was estimated to be about 13.6 kDa by size exclusion chromatography, corresponding to a monomer. The tetragonal bipyramid crystals were obtained by mixing an equal volume of protein in 50 mM Tris-HCl, pH 7.5, 1% glycerol, 100 mM NaCl, 1 mM DTT, and the reservoir solution of 15% w/v PEG 2000, 100 mM lithium chloride at 277 K in 2–4 days using hanging drop vapor diffusion. The protein concentration was 24 mg/ml. The crystal that diffracted to 2.42 Å resolution belongs to space group $P4_1$ or $P4_3$ with unit cell parameters of a=b=32.14 Å, c= 195.31 Å, α=β=γ=90°, with one molecule of CnrX in the asymmetric unit.

Keywords: *Cupriavidus metallidurans* CH34, nickel resistance, CnrX, periplasmic sensor, crystallization

Cupriavidus metallidurans CH34, previously known as *Ralstonia metallidurans*, *Ralstonia eutropha*, and *Alcaligenes eutrophus* CH34, is an aerobic Gram-negative nonspore bacillus that flourishes in millimolar concentrations of heavy metals such as cadmium, cobalt, zinc, thallium, copper, lead, chromium, and nickel [1, 14, 20]. It was first isolated in 1976 from the sludge of a zinc decantation tank that was heavily polluted with many heavy metals. Subsequent studies revealed a number of closely related strains that flourish in environments heavily polluted by heavy metals [6, 7, 13]. These strains have been successfully used to develop whole-cell- and protein-based metal-specific biosensors [2, 3], as well as treatment technologies for wastewater and polluted soils [4, 5, 20]. *C. metallidurans* CH34, thus far, is the best-studied representative of this particular group of microbes. Sequencing of the genome of *C. metallidurans* CH34 has been completed (http://genome.jgi-psf.org/draft_microbes/ralme/ralme.home.html) and its global analysis is ongoing [16, 22].

Heavy-metal-resistant bacteria do not have enzymes that are active under harsh condition, but have set resistant determinants that are themselves tools for the evaluation and remediation of heavy-metal-contaminated environments. The metal resistance determinants are located either on the bacterial chromosome or on one of the two indigenous plasmids; pMOL28 (180 kb) determines resistance to nickel, cobalt, mercury, thallium, and chromate, whereas pMOL30 (238 kb) determines resistance to cadmium, zinc, cobalt, mercury, lead, thallium, and copper [14]. Among the best characterized determinants are the *czc* operon (resistance to cobalt, cadmium, and zinc) on pMOL30 [19], the *cnr* operon (resistance to cobalt and nickel) located on pMOL28 [10, 13], and the closely related *ncc* (nickel-cobalt-cadmium resistance) of *Alcaligenes xylosoxidans* 31A [24].

The *cnr* genes are organized in two clusters, *cnrYXH* and *cnrCBAT* [8–10, 25]. The CnrA, CnrB, and CnrC proteins catalyze the energy-dependent efflux of nickel and cobalt, and likely form a membrane-bound protein complex, that functions as a proton/cation antiporter, whose topological orientation in the membrane resembles that of CzcABC [17]. The nickel transporter CnrT might complement for the missing cation diffusion facilitator (CDF) protein,

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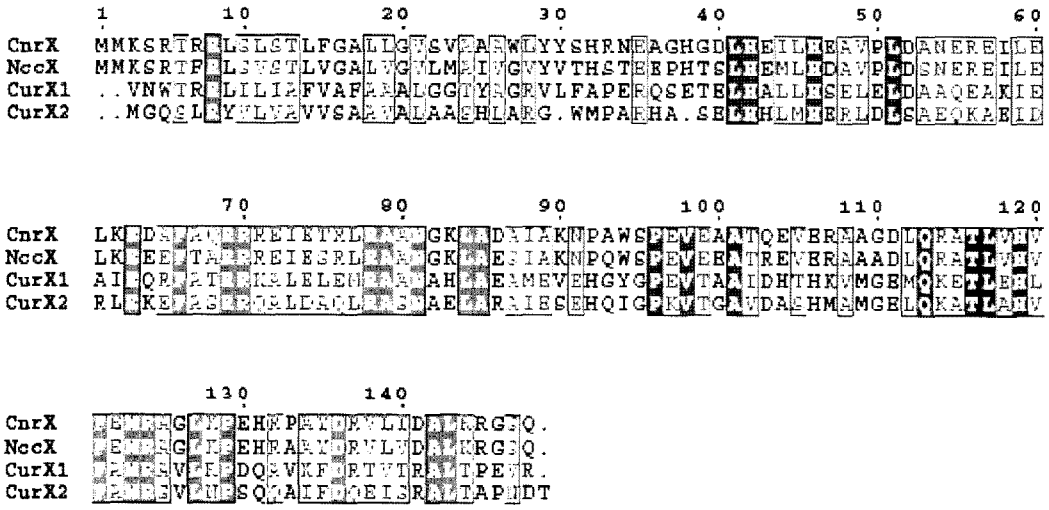

Fig. 1. Sequence alignment.
CnrX from *Ralstonia metallidurans CH34*, NccX (Swiss-Prot entry: Q44582) from *Alcaligenes xylosoxidans* 31A, CurX1 (Swiss-Prot entry: Q2G6E6) and CurX2 (Swiss-Prot entry: Q2G461) from *Novosphingobium aromaticivorans DSM12444* are aligned using Clustal W. The sequence identities to CnrX are 76.4%, 31.5%, and 33.1%, respectively.

similar to CzcD [18]. The genes *cnrYXH,* located upstream of *cnrCBA*, appear to be involved in regulation. The CnrH gene product is a sigma factor belonging to the sigma-70 factor family [11, 15, 21], and shares a close similarity with other sigma factors of the extracytoplasmic functions (ECF) family [8, 25]. CnrH alone is able to activate *cnr* expression; however, CnrY and CnrX are needed for nickel inducible expression of CnrH [8, 25], and both CnrY and CnrX are suggested to function as anti-sigma factors [9]. The CnrY and CnrX gene products, comprised of 95 and 148 amino acids, respectively, show homology to a very limited number of known proteins. CnrX shares sequence identity of 76.4% with NccX from *Alcaligenes xylosoxidans* 31A [24], and little over 30% identities with the putative CurX1 and CurX2 proteins from *Novosphingobium aromaticivorans DSM12444* (see Fig. 1). CurX1 and CurX2 are located upstream of ECF sigma factors, similar to CnrH, and seem to exert control over the expression of the two putative copper resistance operons present in *N. aromaticivorans DSM12444*. This strongly indicates that the CnrX represents a family of anti-sigma factors specialized in the control of heavy-metal resistances. As the first step towards the structure-function elucidation of this new group of proteins, we have overexpressed and crystallized the periplasmic domain of the CnrX protein from *C. metallidurans* CH34.

The *cnrX* gene was amplified by polymerase chain reaction (PCR) from the *pGEX4T-1-cnrX* plasmid using the primers 5'-GCGAATTCGTATTACTCGCATCGGAATG-AAGCCG-3' and 5'-AGACTCGAGTCACTGCGAGCC-GCGGC-3'. The PCR product was purified, digested with EcoRI and XhoI, and ligated into the pET21b expression vector (Novagen). This construction added a hexa-histidine tag with a thrombin cleavage site at the N-terminus of the recombinant CnrX amino acids 29–148. After confirmation of the DNA sequence, the resulting plasmid was transformed into *E. coli* BL21 (DE3). Cells were grown in Luria-Bertani medium at 37°C with ampicillin (100 μg/ml), and expression was induced by 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) at an optical density of about 0.6 at 600 nm. Cells were allowed to grow at 18°C for 16 h after the induction, and were harvested and resuspended in ice-cold lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM 2-mercaptoethanol, and 0.2 mM phenyl-methanesulfonyl fluoride]. The cells were disrupted by sonification and crude lysate was centrifuged at 16,000 ×*g* (Sorvall GSA rotor) for 30 min at 2°C and the cell debris was discarded.

The supernatant was loaded onto a nickel-chelated Hi-trap chelating column (Amersham Biosciences) and eluted with a linear gradient of 20–500 mM imidazole in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM 2-mercaptoethanol. Fractions containing the eluted N-terminal His-tagged CnrX were pooled based on SDS-PAGE anaylsis. The His-tagged CnrX was incubated with thrombin (1 mg/ml) at 4°C for 16 h in order to remove the His-tag, after which the CnrX domain was purified by gel filtration using a HiLoad 26/60 Superdex-75 prep-grade column (Amersham Biosciences), which was preequilibrated with buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% glycerol, 1 mM DTT). The AKTA FPLC system (Amersham-Pharmacia) was used throughout for protein purification. The molecular mass of the purified protein was estimated by gel-filtration to be about 13.6 kDa, which corresponds to the monomeric form of CnrX. The protein was concentrated using an Amicon Ultra-15 (Millipore, Billerica, MA,

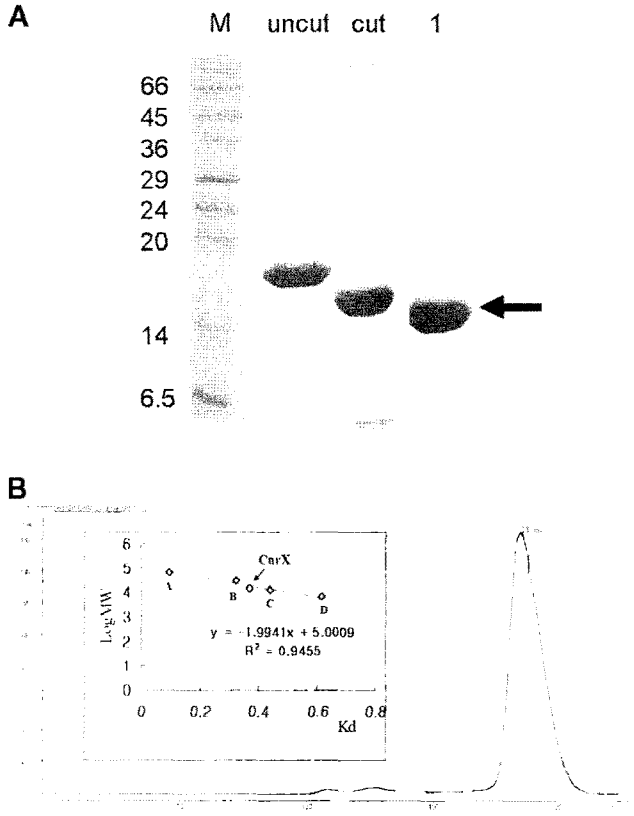

Fig. 2. SDS-PAGE and size-exclusion chromatographic analysis of the recombinant CnrX.

A. Lane M, molecular mass markers (kDa); before and after the removal of His-tag: lane 1, the purified CnrX. **B.** Chromatogram by size-exclusion chromatography with stand molecular markers. The standard markers: A, BSA (66 kDa); B, carbonic anhydrolase (29 kDa); C, cytochrome *c* (12.4 kDa); D, Aprotinin (6.5 kDa).

U.S.A.) and the concentration was determined using the Bio-Rad protein assay with BSA as a standard. The SDS-PAGE analysis and the size-exclusion chromatographic analysis of the recombinant CnrX are shown in Fig. 2.

Initial screening for the crystallization condition was carried out by the sitting-drop vapor diffusion method using 96-well Intelli plates (Hampton Research) and Hydra II plus One (MATRIX Technology) robotic system at 295 K. The protein concentration in 50 mM Tris-HCl, pH 7.5, 1% glycerol, 100 mM NaCl, and 1 mM DTT was 24 mg/ml. A sitting drop was prepared by mixing 200 nl of the protein solution and the reservoir solution, and equilibrated with 70 μl of the reservoir solution. The initial search for crystallization conditions was performed using commercially available kits from Hampton Research (Aliso Viejo, CA, U.S.A.), JENA Bioscience (Jena, Germany), and Emerald BioSystems (Bainbridge Island, WA, U.S.A.). Out of 1,200 conditions screened, twenty gave microcrystals after one to five days. These included 20% (w/v) polyethylene glycol 3350, 0.2 M trisodium citrate dihydrate; 20% (w/v) polyethylene glycol 3350, 0.2 M dipotassium hydrogen phosphate, pH 9.2; 20% (w/v) PEG-1000, 0.1 M cacodylate

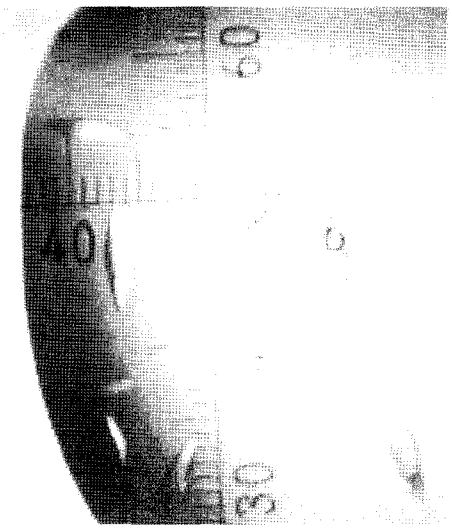

Fig. 3. A crystal of CnrX from *Cupriavidus metallidurans* CH34. The 10 unit is 0.3 mm in length.

pH 6.5, 0.2 M $MgCl_2$; 30% (v/v) PEG-400, 0.1 M CHES pH 9.5; 15 % (w/v) PEG 400, 100 mM MES sodium salt pH6.5; 15% (w/v) PEG 2000, 100 mM LiCl.

Subsequently, these conditions were optimized using hanging drop vapor diffusion experiments. Each hanging drop was prepared by mixing 1 μl of the protein solution and 1 μl of the reservoir solution and equilibrated over 0.5 ml of reservoir solution. Optimization gave a limited number of reasonable size crystals, but they were generally not suitable for data collection. Diffraction quality crystals were obtained with a reservoir solution of 17% w/v PEG 2000, 100 mM lithium chloride at 277 K, an optimization of JB Screen Membrane1 No. 15. The tetragonal-bipyramid crystals grew in 2–4 days (see Fig. 3).

For X-ray data collection, a crystal was transferred to a solution consisting of 17% w/v PEG 2000, 100 mM lithium chloride, and 25% (w/v) glycerol, before being flash-frozen. X-ray diffraction data were collected at 100 K with an ADSC Quantum CCD detector (Madison, WI, U.S.A.) at the 6C beamline of Pohang Light Source (PLS), Korea. The crystal to detector distance was set to 180 mm. A total of 200 images were collected with 1.0° oscillation and 10 s exposure per image. The wavelength used was 1.12714 Å.

Diffraction data were collected to 2.42 Å resolution, and were processed and scaled with the programs DENZO and SCALEPACK from the HKL suite [23]. The crystals belong to the tetragonal space group, $P4_1$ or $P4_3$, with a=b=32.140 Å, c=195.314 Å, $\alpha=\beta=\gamma=90^p$. A total of 126,273 reflections were measured to yield 7,534 unique reflections with an R_{merge} (on intensity) of 5.9%. The merged data set is 94.0% complete to 2.42 Å resolution. The presence of one molecule of CnrX in the asymmetric unit gives a crystal volume per protein mass (V_M) of 3.0 $Å^3$ Da^{-1}, with the corresponding solvent content of 57.9% [12]. The

Table 1. Data collection statistics of CnrX crystals from *Cupriavidus metallidurans* CH34. Values in parentheses are for the highest resolution shell.

	CnrX
Wavelength (Å)	1.12714 Pohang Light Source, beamline 6C
Space group	$P4_1$ or $P4_3$
Unit cell parameters	a=b=32.14 Å, c=195.31 Å $\alpha=\beta=\gamma=90^\circ$
Temperature (K)	100.
Resolution range (Å)	50.0–2.42 (2.51–2.42)
Total reflections	126,273
Unique reflections	7,534
Completeness (%)	94.0 (70.9)
Mean I/σ (I)	29.8 (8.9)
R_{merge} (%)[a]	5.9 (9.1)

[a] $R_{merge}=\sum_h\sum_i|I(h,i)-\langle I(h)\rangle|/\sum_h\sum_i I(h,i)$, where I(h, i) is the intensity of the i^{th} measurement of reflection h and $\langle I(h)\rangle$ is the mean value of I(h, i) for all i measurements.

statistics for data collection are summarized in Table 1. There are two methionine residues in CnrX, and currently, efforts toward structure determination using MAD with selenomethione-substitute CnrX crystals are in progress.

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