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# Control of expression of a periplasmic nickel efflux pump by periplasmic nickel concentrations

Gregor Grass[1], Beate Fricke[2] & Dietrich H. Nies[1,*]
[1]*Institut für Mikrobiologie, Martin-Luther-Universität Halle-Wittenberg, Kurt-Mothes-Str. 3, 06099, Halle, Germany, European Community;* [2]*Institut für Physiologische Chemie, Martin-Luther-Universität Halle-Wittenberg, Kurt-Mothes-Str. 3, 06099, Halle, Germany, European Community; *Author for correspondence (Fax +49-345-5527010; E-mail: d.nies@mikrobiologie.uni-halle.de)*

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**Abstract**

There is accumulating evidence that transenvelope efflux pumps of the resistance, nodulation, cell division protein family (RND) are excreting toxic substances from the periplasm across the outer membrane directly to the outside. This would mean that resistance of Gram-negative bacteria to organic toxins and heavy metals is in fact a two-step process: one set of resistance factors control the concentration of a toxic substance in the periplasm, another one that in the cytoplasm. Efficient periplasmic detoxification requires periplasmic toxin sensing and transduction of this signal into the cytoplasm to control expression of the periplasmic detoxification system. Such a signal transduction system was analyzed using the Cnr nickel resistance system from *Cupriavidus (Wautersia, Ralstonia, Alcaligenes) metallidurans* strain CH34. Resistance is based on nickel efflux 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 while CnrH is a *cnr*-specific extracytoplasmic functions (ECF) sigma factors. Experimental data provided here indicate a signal transduction chain leading from nickel in the periplasm to transcription initiation at the *cnr* promoters *cnrYp* and *cnrCp*, which control synthesis of the nickel efflux pump CnrCBA.

## Introduction and overview

### *RND-driven transenvelope efflux pumps*

The first RND (resistance, nodulation, cell division, (Saier *et al*. 1994; Tseng *et al*. 1999)) system cloned, sequenced, and characterized on the physiological, biochemical and systems theoretical level is the CzcCBA system, which mediates resistance to Co(II), Zn(II) and Cd(II) in the metal resistant $\beta$-proteobacterium strain CH34 (Nies *et al*. 1987, 1989; Nies & Silver 1989; Nies 1995; Rensing *et al*. 1997; Legatzki *et al*. 2003b). This bacterium keeps changing its name and moved from *Alcaligenes eutrophus* to *Ralstonia metallidurans, Wautersia metallidurans* and *Cupriavidus metallidurans* (Mergeay *et al*. 1985; Goris *et al*. 2001; Vandamme & Coenye 2004; Vaneechoutte *et al*. 2004), the last two name changes within the year 2004.

RND-driven systems are large tripartite membrane protein complexes that span the complete cell envelope of Gram-negative bacteria (Nies 2003). The trimeric RND protein transverses the cytoplasmic membrane with 12 transmembrane $\alpha$-helices per monomer and contains a periplasmic head portion in addition to the membrane-bound part. The three subunits form a common central

cavity at the inside of the periplasmic part. The cavity is connected to the periplasm by three tunnels between neighboring subunit pairs (Murakami *et al*. 2002). The second protein of the complex is an outer membrane channel-tunnel protein (OMF, (Paulsen *et al*. 1997)) such as TolC or OprM, trimeric tubes, which span the outer membrane with a $\beta$-barrel structure and extend into the periplasm towards the periplasmic head of the RND protein (Koronakis *et al*. 2000; Akama *et al*. 2004a). RND and OMF proteins are connected by a membrane fusion protein (MFP, (Dinh *et al*. 1994; Saier *et al*. 1994)) such as MexA, which probably forms a hexameric ring around the RND–OMF contact site (Akama *et al*. 2004b; Higgins *et al*. 2004).

RND proteins come in several protein families occurring in all three domains of life (Tseng *et al*. 1999). Most important in Gram-negative bacteria are the HME family of metal cation transporters and the HAE family of proteins involved in export of organic substances including antibiotics (Nikaido 1998; Tseng *et al*. 1999; Zgurskaya & Nikaido 2000; Nies 2003). The substrates for efflux are collected by the RND protein, maybe within its central cavity, then transported through the OMF protein and across the outer membrane directly to the outside (Murakami *et al*. 2002; Higgins *et al*. 2004). As shown with the HME–RND protein CzcA from *C. metallidurans*, this process is driven by the proton motive force (pmf). So, substrates are transported pmf-driven directly to the outside. However, where do the substrates of RND-driven efflux complexes come from, the cytoplasm, the periplasm, or both cellular compartments?

*Periplasmic efflux and RND systems*

In case of HAE–RND proteins the substrate binding sites are located in the periplasmic part of the RND protein as reviewed recently (Nies 2003; Yu *et al*. 2003). However, this is no argument for the actual source of the substrate. In case of the hydrophobic substrates of HAE–RND proteins, substrates may even originate from the interior of the cytoplasmic membrane. In the HME–RND protein CusA, which is part of the CusCBA efflux complex involved in copper resistance in *Escherichia coli*, essential amino acyl residues were identified, which are located in the periplasmic part of CusA and are part of potential copper-binding sites (Franke *et al*. 2003). Again, this does not indicate a periplasmic or cytoplasmic origin of the substrate copper.

The Cus system is one of three copper homeostasis factors characterized in *E. coli*. The other two factors are the P-type ATPase CopA, which detoxifies the cytoplasm by copper efflux into the periplasm, and the periplasmic multicopper oxidase CueO, which oxidizes Cu(I) to Cu(II) and is involved in protection of periplasmic proteins against copper stress (Rensing *et al*. 1999, 2000; Grass & Rensing 2001a, b; Roberts *et al*. 2002; Grass *et al*. 2004). Cus seemed not to be involved in copper resistance of *E. coli*, neither in the presence nor the absence of CopA (Franke *et al*. 2001), but Cus becomes necessary for copper homeostasis when CueO is absent or not able to function (Grass & Rensing 2001b; Outten *et al*. 2001). Thus, a periplasmic enzyme that detoxifies periplasmic copper is able to fill in for the function of a transenvelope efflux system. Moreover, the Cus system contains in addition to CusCBA a periplasmic copper-binding protein, CusF, which may bind and transport copper to the CusCBA complex (Franke *et al*. 2003). This all indicated that an RND-driven complex is able to accept substrates from the periplasm (Grass & Rensing 2001b), but these data did not completely exclude an additional cytoplasmic substrate origin.

*C. metallidurans* contains a variety of RND-driven systems, P-type ATPases and proteins of the CDF (cation diffusion facilitator, (Nies & Silver 1995; Paulsen & Saier 1997)) protein family (von Rozycki *et al*. 2005), all located on the bacterial chromosome(s) and two indigenous megaplasmids, pMOL28 and pMOL30. Of the proteins with similarity to Zn/Cd/Pb-exporting P-type ATPases, two are located on the bacterial chromosome(s). One (ZntA) is predominantly a zinc efflux system, the other (CadA) a cadmium efflux system, but both are also able to export the respective other metal cation (Legatzki *et al*. 2003a). Deletion of both genes for these two transporters results in severe zinc and cadmium sensitivity, which can be complemented *in trans* by expression of the RND-driven CzcCBA system in case of zinc, but not completely in case of cadmium (Legatzki *et al*. 2003a). This indicated that CzcCBA, in *C. metallidurans* CH34 wild type encoded by plasmid pMOL30, is not able to detoxify cytoplasmic cadmium efficiently. Thus,

CzcCBA relies on a P-type ATPase for export of cadmium into the periplasm before a further export of the metal cation to the outside can happen. On the other side, CzcCBA was able to increase cadmium resistance in the Δ*cadA* Δ*zntA* double knockout strain 50-fold, which may indicate some provenience in cytoplasmic cadmium detoxification.

Cd(II) and Zn(II) could also be detoxified by CDF proteins. *C. metallidurans* contains three members of this family (von Rozycki *et al.* 2005). CzcD is the archetype of the CDF protein family, encoded by pMOL30 downstream of *czcCBA*, and able to export Co(II), Cd(II) and Zn(II) (Nies 1992; Anton *et al.* 1999, 2004). FieF probably exports Fe(II) like its namesake from *E. coli*. DmeF has a broad ion specificity centered around Co(II) (Munkelt *et al.* 2004; Grass *et al.* 2005). DmeF and FieF are both encoded by the bacterial chromosome(s). Deletion of *dmeF* in a plasmid-free strain that did not contain the RND-driven Co(II)-efflux systems CnrCBA and CnrCBA led to a severe decrease in cobalt resistance. In contrast to the comparable experiment with the P-type ATPases and cadmium, cobalt resistance of the Δ*dmeF* deletion strain cannot by increased by expression of CzcCBA *in trans*, although presence of the protein complex in the bacterial membrane can be demonstrated by Western blot (Munkelt *et al.* 2004). This indicated that detoxification of cobalt by an RND-driven efflux complex essentially requires a previous transport of Co(II) from the cytoplasm into the periplasm, giving gleaning evidence that CzcCBA is only able to export periplasmic but not cytoplasmic Co(II).

Since FieF and DmeF are able to export Zn(II) and to some extend Cd(II) (Munkelt *et al.* 2004), these chromosomally encoded CDF proteins may fill in for missing P-type ATPases, which might explain the full and partial complementation of zinc and cadmium resistance, respectively, of the Δ*cadA* Δ*zntA* deletion strain by CzcCBA. Deletion of *dmeF* decreased also cobalt resistance in the presence of the cobalt–nickel efflux system CnrCBA, which is encoded by the second megaplasmid of *C. metallidurans*, pMOL28 (Munkelt *et al.* 2004). Therefore, export of Co(II) is also a prerequisite for further export of cobalt by this HME–RND system. Although DmeF transports Ni(II), absence of DmeF does not lead to decreased nickel resistance (Munkelt *et al.* 2004). Again, additional cytoplasmic efflux proteins like the nickel transporter CnrT might complement for the missing CDF protein (Nies 2003; von Rozycki *et al.* 2005).

The data available at the moment strongly argue in favor of a purely periplasmic detoxification of heavy metal cations by HME–RND-driven transenvelope efflux systems. This would mean that periplasmic heavy metal cation concentrations are in a flow equilibrium resulting from (i) uptake from the outside by porins, (ii) efflux to the outside by RND systems, (iii) uptake into the cytoplasm, (iv) efflux from the cytoplasm and (v) binding to periplasmic components. Assuming that porin-mediated uptake is faster than uptake into the cytoplasm (Nikaido 2003), this would indicate a stepwise decrease of metal cation concentrations from the outside to the periplasm and further to the cytoplasm. From another point of view, heavy metal homeostasis could be a two-step process with concentrations adjusted separately in the cytoplasm and the periplasm. Jon Hobman and David Giedroc in this special issue in detail discuss structure and function of regulatory proteins involved in cytoplasmic metal homeostasis. Existence of periplasmic metal homeostasis, accomplished by RND-mediated periplasmic efflux, needs systems for periplasmic metal concentration sensing and usage of this information for expression control of the respective RND-driven efflux system.

### *Expression control of CnrCBA*

The CnrCBA system from *C. metallidurans* strain CH34 (Liesegang *et al.* 1993) is only expressed at high (upper $\mu$M) nickel concentrations. The genes *cnrYXH* located upstream of *cnrCBA* are required for regulation of Cnr (Liesegang *et al.* 1993; Grass *et al.* 2000; Tibazarwa *et al.* 2000). Transcription of the *cnr* determinant is initiated at two promoters, *cnrYp* and *cnrCp*, resulting in two tricistronic transcripts, the *cnrYXH*-mRNA and the *cnrCBA*-mRNA, and maybe additionally in a *cnrYXHCBA* hexacistronic mRNA (Grass *et al.* 2000). Tibazarwa *et al.* (2000) provided also data for the existence of a third Cnr-promoter, *cnrHp*, with a completely different −35 and −10 putative promoter consensus sequence. The DNA region containing *cnrHp* was physiologically inactive in our hands (Grass *et al.* 2000).

The sigma factor CnrH alone is a constitutive activator of *cnrCBA* expression. CnrY and CnrX

are both required for $Ni^{2+}$-dependent control of CnrH (Grass *et al*. 2000), which is a member of the ECF (extracytoplasmic function) sigma factor family (Lonetto *et al*. 1994; Missiakas & Raina 1998; Grass *et al*. 2000; Tibazarwa *et al*. 2000; Nies 2004). As indicated by *phoA* protein fusions, the carboxy-termini of the proteins CnrX and CnrY are located in the periplasm (Grass *et al*. 2000). CnrY is a small membrane-bound protein (Tibazarwa *et al*. 2000). As judged by a $\phi$(*cnrCBA–lacZ*) operon fusion, *cnrCBA* expression follows a saturation curve with half-maximum activation occurring at 49 $\mu$M $Ni^{2+}$ (Grass *et al*. 2000).

Thus, a signal transduction chain may exist that may reach from periplasmic nickel concentrations to transcription initiation at the *cnr* promoters, which control expression of the CnrCBA efflux pump. The following experimental part of this article adds genetic and biochemical evidence to show that this signal transduction is indeed able to function in such a manner.

## Materials and methods

### *Bacterial strains and growth conditions*

Tris-buffered mineral salts medium containing 2 g sodium gluconate $liter^{-1}$ (Mergeay *et al*. 1985) was used to cultivate *C. metallidurans* strain AE126(pMOL28) (Mergeay *et al*. 1985) and its derivative strains DN177(pMOL28-2, $\phi$ (*cnrCBA–lacZ*)) and DN190(pMOL28-3) (Grass *et al*. 2000), which is $\phi$(*cnrCBA–lacZ*), $\Delta$*cnrYXH*. Solid Tris-buffered media contained 20 g of agar $liter^{-1}$. $\beta$-galactosidase activity was determined in permeabilized cells as published previously (Nies 1992) with 1 U defined as the activity forming 1 nmol of *o*-nitrophenol per min (Pardee *et al*. 1959; Ullmann 1984) at 30 °C.

### *Genetic techniques*

Standard molecular genetic techniques were used (Nies *et al*. 1987; Sambrook *et al*. 1989). The *cnrYXH* region was amplified from plasmid pMOL28 DNA and cloned as a *Xba*I fragment into pGEM T Easy (Promega, Madison, WI, USA) to generate plasmid pECD566. Plasmid pDNA291 contains the *cnrYXH* region cloned from plasmid pECD566 into the broad host range plasmid pVDZ′2 (Deretic *et al*. 1987).

### *Yeast two hybrid*

The CytoTrap™ (Stratagene, La Jolla, CA, USA) system was used to study interaction between Cnr regulatory proteins. The *cnrY* gene was PCR-amplified from plasmid pMOL28, cloned (*Bam*HI/*Sal*I) into the "bait" vector pSOS (Stratagene), and a $A_{58}E/A_{59}R$ double amino acid substitutions were introduced to keep the CnrY′ protein soluble. This double mutation renders the single membrane-spanning segment of CnrY more hydrophilic. Thus, CnrY′ was no longer membrane-bound and could be expressed as a cytoplasmic protein. In addition, the *cnrYX* region was also cloned into pSOS and *cnrY* was mutated here as well. The *cnrH* gene, the *cnrX′* gene (*cnrX* without the N-terminal leader sequence) and the *cnrX′*H region were cloned into the "predator" vector pMyr (Stratagene) as *Eco*RI/*Sal*I-digested PCR fragments. All plasmids were verified by DNA sequence analysis and transformed into *Saccharomyces cerevisiae* strain cdc25H (Stratagene). Interaction studies were performed as described by the manufacturer's manual.

### *Mutagenesis*

The Quick-Change site-directed mutagenesis system was used (Stratagene, La Jolla, CA, USA). Target genes were cloned into pGEM T EASY (Promega, Madison, WI, USA) leading to plasmid pECD566 and propagated in a $Dam^+$ *E. coli* strain. Supercoiled vector plasmid was used as template in a PCR reaction carried out with an overlapping, antiparallel pair of primers, which contained the desired point mutations (16 PCR cycles plus one cycle per mutated base pair). The PCR product was treated with *Dpn*I, which exclusively degrades methylated template DNA. The resulting DNA was transformed into *E. coli* without prior ligation. Mutations were verified by DNA sequence analysis. Both genes, *cnrY* and *cnrX*, were mutated as part of the *cnrYXH* region. The resulting mutated *cnrYXH* regions were cloned into pVDZ′2 (Deretic *et al*. 1987) and transferred into *C. metallidurans*.

*Protein purification*

CnrX, CnrY′ and CnrH were purified using the IMPACT$^{TM}$ protein purification system (NEW ENGLAND BioLabs). Each gene was fused to an intein with a chitin binding domain coding-region. Protein expression in *E. coli* strain ER2566 (NEW ENGLAND BioLabs) was started by addition of 0.25 mM isopropyl-thio-galactopyranoside and shaking for 16 h in LB at 18 °C (Sambrook *et al.* 1989). Cultures were harvested by centrifugation, suspended in 0.1 Vol. of HEPES-buffer (20 mM HEPES–HCl (pH 8.0), 500 mM NaCl, 2 ml $l^{-1}$ Tween 20, 5 ml $l^{-1}$ Triton X-100) and lysed by sonication (10 times 15 s at 60 W, UW60, Uniequip, Martinsried, Germany) on ice. The debris was removed by centrifugation (30 min 7650 *g*, 4 °C) and the clear supernatant applied onto a chitin bead affinity column (1 ml bed volume, equilibrated with 10 ml of HEPES-buffer). The column was washed with 17 bed volumes of HEPES-buffer followed by 3 bed volumes of HEPES cleavage buffer (20 mM HEPES (pH 8.0), 50 mM NaCl, 1 ml $l^{-1}$ Triton X-100, 40 mM dithiothreitol (DTT)). Cleavage buffer remained in the column for 16 h at 4 °C to allow autocatalytic cleavage of the fusion protein. Finally, the target protein was eluted with 3 bed volumes of HEPES cleavage buffer and fractions of 1 ml were collected.

The *cnrX* gene was amplified by PCR from plasmid pMOL28 DNA (primers: supplementary material), cloned into pTYB2 (NEW ENGLAND BioLabs), verified by DNA sequencing, and transformed into *E. coli* strain ER2566 (NEW ENGLAND BioLabs) for overexpression of the CnrX/intein/chitin binding domain fusion protein. The protein contained an additional glycine residue at its carboxy terminus to facilitate the splicing process. PCR-cloning, expression and purification of CnrH was performed alike, including the glycine residue, which was attached to the carboxy terminus of CnrH. In addition, the start codon of the *cnrH* gene was mutated from "GTG" into "ATG". For purification of CnrY, the *cnrY* gene was also PCR cloned. Since *E. coli* ER2566 (NEW ENGLAND BioLabs) cells died rapidly after expression of the membrane-bound CnrY protein and other expression systems were also not successful, *cnrY* was mutated to introduce an $A_{58}R/A_{59}R$ double substitution in CnrY to enhance solubility. The mutant protein CnrY′ was purified similar to CnrH or CnrX. The N-terminal sequence of the protein could be confirmed.

*Immunoblotting*

*C. metallidurans* cells were cultivated under various conditions and the cell density was determined from the optical density with an equilibration curve (Nies & Silver 1989). A sample representing a dry weight of 25 $\mu$g was mixed with threefold sample buffer (final concentrations 50 mM Tris–HCl, pH 6.8, 10 g $l^{-1}$ SDS; 10 ml $l^{-1}$ $\beta$-mercaptoethanol, 2 mM EDTA, 0.1 g $l^{-1}$ bromphenole blue, 100 g $l^{-1}$ sucrose), incubated for 20 min at 95 °C and loaded onto a SDS polyacrylamid gel (Laemmli 1970). Purified CnrX protein was used as a control. The gel was blotted with a semi dry blot (Biometra, Göttingen, Germany) for 30 min, 100 V, 250 mA, 250 V onto a PVDF-membrane (Roche, Mannheim, Germany). The membrane was dried by air and then blocked in PBS (4 mM $KH_2PO_4$, 16 mM $Na_2HPO_4$, 115 mM NaCl), 5 ml Tween20 and 50 g skimmed milk per liter with shaking at 4 °C for 16 h. The PVDF-membrane was washed three times 5 min with PBS–Tween (PBS, 1 ml Tween20 $l^{-1}$) and additional 5 min with PBS at 23 °C. The membrane was incubated for 1 h at 23 °C with shaking after the first antibody (polyclonal CzcX-antibody, diluted in PBS–Tween 1:50,000) was added. The membrane was washed three times for 15 min with PBS–Tween and 5 min with PBS and incubated with the second antibody (a monoclonal anti-rabbit-IgG conjugated with horseradish peroxidase (Sigma-Aldrich, Deisenhofen, Germany) and diluted 1:50,000 in PBS–Tween) for 1 h at 23 °C with shaking. Unbound antibody was washed off the membrane for three times for 15 min with PBS–Tween and once 5 min with PBS. For signal detection, the PVDF-membrane was incubated 1:1 with solution 1 (0.1 M Tris–HCl (pH 8.5), 0.4 mM *p*-coumaric acid, 2.5 mM luminol) and solution 2 (0.1 M Tris–HCl (pH 8.5), 5.4 M $H_2O_2$) for 1 min. Excess liquid was wiped off and the membrane was exposed for 5 sec to 5 min to ECL-hyperfilm (Amersham Pharmacia Biotech, Uppsala, Sweden). The hyperfilm was scanned (Apple Computer, Ismaning, Germany) and the intensities of the signals were determined with NIH Image (National Institutes of Health, USA).

*Run off transcription assays*

The *cnrYp*- and the *cnrCp*-promoter regions were amplified by PCR from plasmid pECD566, which is *cnrYXH* in pGEM T Easy (Promega, Madison, WI, USA). Both fragments contained a region of 300 bp (*cnrYp*) and 200 bp (*cnrCp*) downstream of the promoter sequences. Since plasmid pECD566 contained the phage T7 promoter on the vector, two additional fragments (T7-marker 1 and T7-marker 2) were PCR amplified from this plasmid. Both fragments started upstream of the T7 promoter and continued for 180 bp into *cnrY* (marker 1) or for 340 bp into *cnrX* (marker 2). Both marker fragments were used to construct RNA size markers. The two PCR fragments were used as templates for T7 RNA polymerase with the DIG RNA labeling kit (Roche, Mannheim, Germany), however, α-[$^{32}$P]-UTP was used instead of DIG-UTP.

Purified CnrH protein was dialyzed against HEPES buffer (20 mM HEPES (pH 8.0), 50 mM NaCl, 1 ml $l^{-1}$ Triton X-100) for 16 h. A volume of 16 μl of CnrH (2 μg protein) was added to one unit of *E. coli* RNA polymerase core enzyme in 4 μl of fivefold transcription buffer (200 mM Tris–HCl (pH 8.0), 50 mM $MgCl_2$, 0.5 mM EDTA, 0.75 M KCl, 2 mM $K_3PO_4$, 0.5 mM DTT, 0.05 g $l^{-1}$ BSA). Diethylpyrocarbonate-treated water (DEPC-$H_2O$) instead of CnrH-containing solution served as a negative control. At 4 °C, 4 μl of fivefold transcription buffer, 2 μl of NTPs (final concentration 1 mM each), 1 μCi α-[$^{32}$P]-UTP (> 800 Ci/mmol, NEN, Köln, Germany), 1 μl RNAse inhibitor (50 units, MBI-Fermentas, St. Leon-Rot, Germany), DNA template (final concentration 20 nM) and DEPC-$H_2O$ were added to a final volume of 40 μl. The mixture was incubated for 20 min or 1 h at 37 °C. The RNA was precipitated by addition of 4 μl 3 M sodium acetate (pH 5.2). After centrifugation, the RNA pellet was dried and suspended in 6 μl of DEPC-$H_2O$ and 10 μl of buffer (ad 10 ml formamide: 10 mg xylene cyanol FF, 10 mg bromphenole blue, 200 μl 0.5 M EDTA (pH 8.0)), heated for 2 min at 85 °C, and applied onto a 60 g $l^{-1}$ polyacrylamide gel (6%)containing 380 g $l^{-1}$ urea.

*Inductively coupled plasma optical emission spectroscopy (ICP-OES)*

Since CnrX was not soluble due to its hydrophobic N-terminal membrane anchor, a CnrX′ protein that did not contain the first 23 amino acids of the amino-terminal leader sequence was purified as a strep tag protein as published (Grass & Rensing 2001a). The same procedure was employed to purify the three CnrX′ derivatives CnrX′$H_{31}$R/$H_{37}$R, CnrX′$H_{41}$R/$H_{45}$R, and CnrX′$H_{118}$R/$H_{130}$R. CnrX′ and its variants were incubated with 0.1 mM $NiCl_2$ on ice for 30 min and subsequently washed several times with 0.1 M Tris–HCl buffer (pH 8.0) using Viva Spin columns (Sartorius, Göttingen, Germany). Trace element concentrations were determined by ICP-OES using an IRIS Advantage Duo ER/S (Thermo Jarrell Ash, Franklin, MA).

## Results

*CnrX is a membrane-bound protein with a periplasmic carboxy terminus*

The three proteins CnrX, CnrY and CnrH are essential but sufficient for regulation of the *cnr* determinant by nickel (Grass *et al.* 2000). Of these three proteins, only CnrX has a periplasmic part and is the only one containing His residues that may form metal binding sites. CnrX is mostly hydrophilic (Liesegang *et al.* 1993) except for its amino-terminal hydrophobic region that probably serves as a signal sequence for export of CnrX into the periplasm. So, if periplasmic nickel is the inducer of *cnr*, CnrX, which could be a soluble or a membrane-attached protein, should be the nickel sensor.

CnrX was purified after heterologous expression in *E. coli*. The purified protein had a size of approximately 16 kDa as predicted for the full-length protein (16.5 kDa). Only a weak band corresponding to a possible processed form (14.1 kDa) was visible in CnrX preparations (Figure 1 lane 1). After raising antibodies in rabbits, the size of CnrX was also determined in the native host *C. metallidurans*. Again, full-length protein of 16.5 kDa was found in *C. metallidurans* cells while no protein could be detected in the cells of a negative control, a Δ*cnrYXH* deletion strain (Figure 1). As expected for a constituent of the nickel inducible Cnr system, the amount of CnrX in *C. metallidurans* increased about sevenfold after the cells were incubated with 500 μM $Ni^{2+}$ (Figure 1, lanes 2 and 3). Thus, CnrX is probably

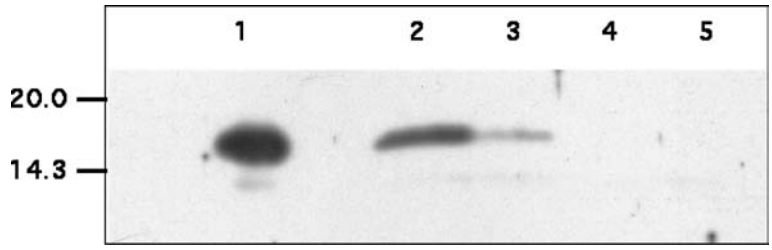


*Figure 1.* Western blot of CnrX in *C. metallidurans* cells. Cells of *C. metallidurans* strain DN177(pMOL28-2, $\phi$(*cnrCBA–lacZ*)) (lanes 2 and 3) and DN190(pMOL28-3, $\phi$(*cnrCBA–lacZ*), $\Delta$*cnrYXH*) (lanes 4 and 5) were grown in the presence of 500 $\mu$M $Ni^{2+}$ (lanes 2 and 4) or without $Ni^{2+}$ (lanes 3 and 5). Crude extracts were prepared and separated on a SDS-PAGE-gel, blotted, and analyzed with CnrX-specific antibodies. Lane 1 contains 20 ng of CnrX protein expressed in *E. coli* and purified thereafter.

composed of a 23 amino acyl (aa) amino-terminal hydrophobic membrane anchor that may serve also as signal sequence for the export into the periplasm, and a 124 aa carboxy-terminal part, which is attached at the periplasmic face of the cytoplasmic membrane (Grass *et al.* 2000; Tibazarwa *et al.* 2000).

*Binding of nickel to CnrX*

To investigate if CnrX is able to bind nickel, the 124 aa carboxy-terminal part of CnrX was purified. This CnrX′ polypeptide was incubated with 0.1 mM $Ni^{2+}$ and washed several times with 0.1 M Tris–HCl (pH 8.0). Nickel content of the washed protein was analyzed by ICP-OES (data not shown). CnrX′ contained 0.18 mol $Ni^{2+}$ per mol of protein. This indicated rather loose binding of nickel by CnrX.

CnrX contains six histidine residues (Liesegang *et al.* 1993), which could be arranged in three pairs (supplementary material). All six His residues were individually changed into Arg residues. Second, double mutations comprising H $\rightarrow$ R changes in both His residues of each His pair were produced by a second round of site-directed mutagenesis. Additionally, the $E_{34}$ and $E_{42}$ residues close to some His residues were mutated to $D_{34}$, $D_{42}$, $Q_{34}$ or $Q_{42}$, respectively. The influence of the resulting CnrX mutant proteins on Cnr regulation was tested in strain DN190(pMOL28-3), $\phi$(*cnrCBA–lacZ*), $\Delta$*cnrYXH* (Grass *et al.* 2000). This strain was complemented *in trans* with plasmid pVDZ′2 (Deretic *et al.* 1987), which harbored the, respectively, mutated or wild-type *cnrYXH* gene regions.

Strains carrying a $H_{31}R$ single or a $H_{31}R/H_{37}R$ double mutation in CnrX were nickel resistant (Table 1). Expression of a $\phi$(*cnrCBA–lacZ*) reporter gene fusion was elevated without nickel, and increase in $\beta$-galactosidase activity after nickel induction was about twofold higher than in the positive control strain (Table 1). A strain carrying a single $H_{37}R$ amino acid substitution in CnrX exhibited an intermediate level with respect to both, resistance and expression of *cnr*. The carboxy-terminal part of the CnrX′$H_{31}R/H_{37}R$ mutant protein was purified and bound 80% as much nickel as wild type CnrX. Thus, the mutations in the $H_{31}/H_{37}$ pair led to an elevated expression of Cnr in the absence of nickel but nickel was still able to induce Cnr via these CnrX protein variants. Mutation of $E_{34}$ and $E_{42}$ located close to these and the following His residues had no effect on nickel resistance (Table 1, data not shown for $E_{34}$).

The single mutations $H_{41}R$ or $H_{45}R$, or the $H_{41}R/H_{45}R$ double mutation in CnrX led to diminished expression of *cnr* after nickel induction (between 10% and 20% of the positive control value) and to metal sensitivity (Table 1). The purified carboxy-terminal part of the CnrX′$H_{41}R/H_{45}R$ mutant protein bound only 25% as much nickel as the wild type polypeptide. Thus, these two H $\rightarrow$ R mutations diminished nickel binding of CnrX and almost completely abolished inducibility and metal resistance marking $H_{41}$ and $H_{45}$ as of central importance for induction of Cnr by $Ni^{2+}$.

Mutant strains carrying $H_{118}R$ or $H_{130}R$ variants of CnrX were slightly more resistant to nickel than strains with $H_{41}R$ or $H_{45}R$ mutations (Table 1). Increase in $\beta$-galactosidase activity was about 25% of that of the positive control strain. In contrast to $H_{41}R/H_{45}R$, the strain with the $H_{118}R/H_{130}R$ double mutation in CnrX was more resistant to nickel than each strain with the respective single amino acid substitution. Moreover, the influence of $Ni^{2+}$ on the increase in $\beta$-galactosidase activity was very small; expression of $\phi$(*cnrCBA–lacZ*) in the

*Table 1.* Induction of a $\phi$(*cnrCBA–lacZ*) fusion by $Ni^{2+}$ in various mutant strains.

| Phenotype of the mutation | MIC (mM $Ni^{2+}$) | Induction of a $\phi$ (*cnrCBA–lacZ*) fusion | | |
|---|---|---|---|---|
| | | *a*, (U $mg^{-1}$) $t=0$ | *b*, (U $mg^{-1}$ $h^{-1}$) | |
| | | | 0 mM $Ni^{2+}$ | 0.5 mM $Ni^{2+}$ |
| Wild type | 4.0 | 36.8 ± 0.4 | 0 | 37 |
| no *cnrYXH* | 0.5 | 32.0 ± 0.7 | 4 | 3 |
| *Mutations in cnrX* | | | | |
| $H_{31}R$ | 4.5 | 515 ± 23 | 13 | 70 |
| $H_{37}R$ | 2.0 | 134 ± 4 | 0.6 | 26 |
| $H_{31}R$, $H_{37}R$ | 4.0 | 314 ± 7 | 26 | 75 |
| $H_{41}R$ | 0.5 | 43.5 ± 0.5 | 0.6 | 4.2 |
| $H_{45}R$ | 0.5 | 44 ± 1 | 1.8 | 8.4 |
| $H_{41}R$, $H_{45}R$ | 0.5 | 35 ± 2 | 3.0 | 7.8 |
| $H_{118}R$ | 0.6 | 56.5 ± 1.5 | 1.8 | 11 |
| $H_{130}R$ | 0.6 | 54 ± 2 | 3.0 | 11 |
| $H_{118}R$, $H_{130}R$ | 1.0 | 146 ± 4 | 7.8 | 9.6 |
| $E_{42}D$ | 2.0 | 36 ± 18 | 0 | 25 |
| $E_{42}Q$ | 2.5 | 33 ± 10 | 0 | 22 |

Cells of *C. metallidurans* strain DN190(pMOL28-3,$\phi$ (*cnrCBA–lacZ*),Δ*cnrYXH*) were complemented *in trans* with plasmid pDNA291 which contained various mutant derivatives of the *cnrYXH*-region. Minimal inhibitory concentrations (MIC) were determined on solid Tris-buffered mineral salts medium plates containing 2 g sodium gluconate per liter as carbon source after 3 days at 30 °C. Experiments were performed twice with identical results. To cells growing in the early exponential phase in liquid culture in the same medium, no nickel or 0.5 mM $Ni^{2+}$ was added at $t = 0$. The increase in $\beta$-galactosidase activity, which was the result of the action of the various *cnrYXH* derivatives on the *cnrCBA–lacZ* reporter, was determined as U $mg^{-1}$ dry weight for at least 3 h and plotted against time. The *a*-value gives the $\beta$-galactosidase activity at $t = 0$ before addition of nickel in U $mg^{-1}$ as mean values ($n \geq 3$) with standard deviations. The *b*-values are the slope of the linear increase in U $mg^{-1}$ $h^{-1}$ in the cultures with or without added nickel. Deviations here were below 20% of the values and are not shown for sake of clarity. n. d., not determined.

double mutant was nearly constitutive. The purified carboxy-terminal part of the CnrX′$H_{118}R/H_{130}R$ mutant protein bound half as much nickel as the wild type polypeptide. Thus, the histidine residues $H_{118}$ and $H_{130}$ were also involved in nickel-dependent induction of *cnr*.

Therefore, CnrX is a membrane-bound protein at the periplasmic face of the cytoplasmic membrane. CnrX is able to bind nickel loosely to His residues and alteration of some of these His residues abolished nickel-dependent induction of Cnr. This marks CnrX as a periplasmic nickel sensor involved in control of expression of the CnrCBA nickel efflux pump.

### *Interaction of the CnrX and CnrY proteins*

Control of ECF sigma factors such as CnrH is usually managed by reversible sequestration of the sigma factor to the cytoplasmic face of the cytoplasmic membrane by an anti-sigma factor complex (Nies 2004). However, the only part of CnrX that might stick out off the cytoplasmic face of the cytoplasmic membrane is MKSRTRR, which is probably too small for efficient sequestration of CnrH. Therefore, CnrY is needed for sequestration of the sigma factor CnrH in the absence of nickel. CnrY is membrane-bound, essential for Cnr expression (Grass *et al.* 2000), and has a predicted cytoplasmic portion much longer than that of CnrX (supplementary material).

To investigate CnrX–CnrY interaction with a yeast two hybrid system, the gene for a soluble $A_{58}R/A_{59}R$ mutant derivative of CnrY was PCR amplified and cloned into the CytoTrap™ bait vector. In addition and to yield an independent redundant result, the *cnrY′–cnrX′* region was cloned into the bait vector. CnrX′ and the *cnrX′–cnrH′* region were cloned into the predator vector. The resulting constructs plus controls provided by the manufacturer were transformed into the temperature-sensitive *Saccharomyces cerevisiae* strain cdc25H, and growth on galactose-containing agar plates at 37 °C was monitored. Yeast strains

containing *cnrY′* in the bait vector and *cnrX′* in the predator vector (Figure 2) were able to grow at elevated temperature, which indicated protein–protein interaction of CnrY and CnrX. Interestingly, combination *cnrY′*/cnrH did not allow growth of the temperature-sensitive *S. cerevisiae* strain (Figure 2). Therefore, CnrY alone was not able to bind to CnrH, and CnrX and CnrY might have to form a protein complex for sequestration of CnrH.

*CnrH is a sigma-factor required for initiation of transcription at the promoters cnrYp* and *cnrCp*

Binding of CnrH to *cnr* promoters has been shown (Tibazarwa *et al.* 2000), but not functional transcription initiation activity of this protein. Thus, CnrH was purified (data not shown) and used in run off transcription assays with RNA polymerase core enzyme from *E. coli* and the promoter regions of *cnrCp* and *cnrYp*. No transcription occurred from these promoters without CnrH. Only in the presence of CnrH, transcription initiation from *cnrCp* and *cnrYp* was observed (Figure 3). Therefore, it could be shown for the first time that CnrH not only was able to bind to promoter regions of *cnr*, but also to initiate transcription, and all this in the absence of Ni(II).

*Conclusions*

Expression of the CnrCBA efflux complex needs CnrH, the CnrYX complex and sufficient Ni(II) concentrations. Sigma factor CnrH was indeed able to initiate transcription initiation from *cnrYp* and *cnrCp*. This process did not need Ni(II) *in vitro* (run-off assays) and *in vivo* (constitutive expression of *cnr* by CnrH in the absence of CnrYX). Therefore, CnrYX are required to repress action of CnrH in the absence of Ni(II), probably by sequestration of CnrH to CnrYX. CnrY and CnrX interacted and were both required for repression of CnrH action. CnrX was able to bind Ni(II). His residues in the periplasmic part of CnrX were required for nickel binding. Therefore, periplasmic Ni(II) but not cytoplasmic Ni(II) cations are

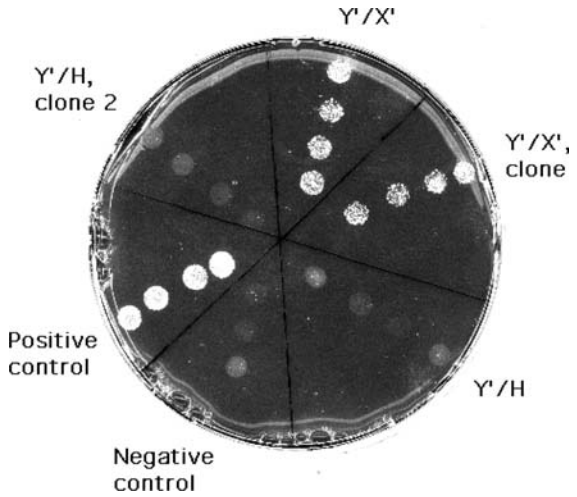


*Figure 2.* Interaction between CnrX and CnrY. A representative CytoTrap$^{TM}$ yeast two hybrid experiment is shown. Protein–protein interactions in the cytoplasm of specific *Saccharomyces cerevisiae* strains were detected by recruitment of the human Sos gene product (hSos) to the membrane of the cell where it activates the Ras pathway. The CytoTrap$^{TM}$ system uses the unique yeast strain cdc25H, which contains a temperature-sensitive mutation in the *cdc25* gene, the yeast homologue for the gene encoding hSos. This protein, a guanyl nucleotide exchange factor, is essential for activation of the Ras pathway and ultimately for the survival and growth of the cell at elevated temperatures. The mutation in the gene encoding the Cdc25 protein renders yeast cells temperature sensitive; cells can grow at 25 °C but not at 37 °C. This *cdc25* mutation can be complemented by the hSos gene product, allowing growth at 37 °C, provided that the hSos protein is localized to the membrane via a protein–protein interaction, in this case between two Cnr regulatory proteins. Positive control refers to the MAFB/MAFB interaction, negative control to the interaction between the lamin C protein and collagenase as described in the protocol provided by the manufacturer. Y′, X′ and H are the genes for the proteins *cnrY, cnrX* and *cnrH* cloned in the CytoTrap vectors as bait or predator. Experiments were performed using the soluble derivatives CnrX′ and CnrY′. In case of ''Y′/H, clone 2'', cnrY′X was cloned into the Bait vector. In case of ''Y′/X′ clone 2'', cnrX′H was cloned into the predator vector. In both ''clone 2'' cases, these constructs served as reproducibility tests since yeast will not use dicistronic mRNAs.

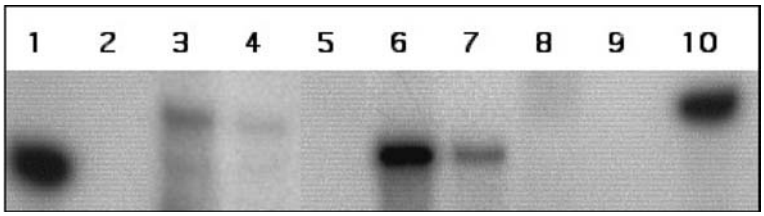


*Figure 3*. Run off transcription with sigma factor CnrH on Cnr promoters. CnrH protein was purified and added to RNA polymerase core enzyme from *E. coli*. The resulting holoenzyme was added to 1 mM of NTP, $\alpha$-[$^{32}$P]-UTP, and PCR fragments containing the *cnrYp* (lanes 3 and 4) or the *cnrCp* (lanes 6 and 7) promoters. The reaction was terminated after 20 min (lanes 4 and 7) or 60 min (lanes 3 and 6), the resulting RNA applied to polyacrylamide gel electrophoresis. Negative controls are *cnrYp* (lane 5) or *cnrCp* (lane 8) incubated for 60 min with core enzyme only. Lanes 2 and 9 are empty, lanes 1 (180 nt) and 10 (340 nt) contained size marker RNAs.

probably able to control expression of the CnrCBA efflux pump. This may indicate the existence of a control circuit that keeps the periplasmic Ni(II) concentration in homeostasis (Figure 4).

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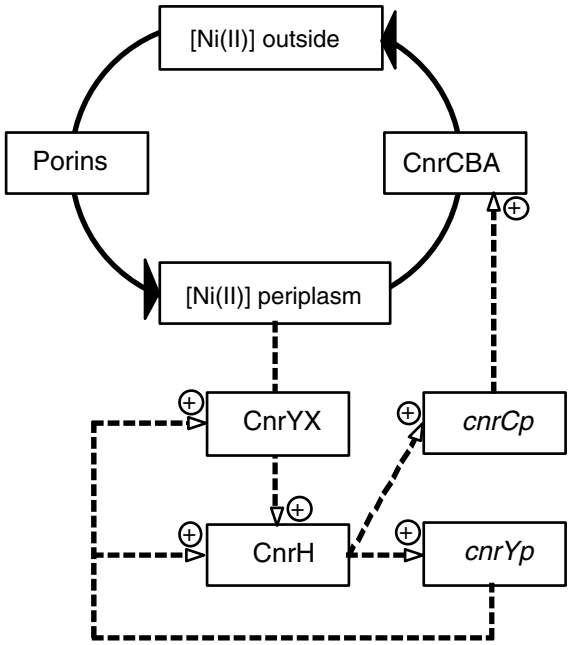


*Figure 4*. Control circuit for periplasmic nickel homeostasis on *C. metallidurans*. In this model, Ni(II) is transported into the periplasm by porin-mediated passive diffusion and back out by the RND-driven transenvelope efflux system CnrCBA (thick arrows). The resulting flow equilibrium depends on the number of CnrCBA protein complexes that in turn depends on transcription initiation rate from the structural gene promoter *cnrCp* upstream of the *cnrCBA* operon (dashed arrows indicating signal flows). The ECF sigma factor CnrH controls this promoter and that of the *cnrYXH* operon upstream of *cnrCBA*. Availability of CnrH is regulated by binding to the membrane bound anti sigma factor complex CnrYX, which releases CnrH upon nickel binding to His residues of CnrX.

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