

Regulation of the *cnr* Cobalt and Nickel Resistance Determinant of *Ralstonia eutropha* (*Alcaligenes eutrophus*) CH34

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The linked resistance to nickel and cobalt of *Ralstonia eutropha*-like strain CH34 (*Alcaligenes eutrophus* CH34) is encoded by the *cnr* operon, which is localized on the megaplasmid pMOL28. The regulatory genes *cnrYXH* have been cloned, overexpressed, and purified in *Escherichia coli*. CnrY fractionated as a 10.7-kDa protein in *in vitro* translation assays. CnrX, a periplasmic protein of 16.5 kDa, was overproduced and purified as a histidine-tagged fusion protein in *E. coli*. His-CnrX was found to possess a secondary structure content rich in alpha-helical and beta-sheet structures. CnrH, a sigma factor of the extracytoplasmic function family, was purified as an N-terminally histidine-tagged fusion. In gel shift mobility assays, His-CnrH, in the presence of *E. coli* core RNA polymerase enzyme, could retard at least two different promoter DNA targets, *cnrYp* and *cnrHp*, localized within the *cnrYXH* locus. These promoters and their transcription start sites were confirmed by primer extension. Purified His-CnrX did not inhibit the DNA-binding activity of His-CnrH and is therefore unlikely to be an anti-sigma factor, as previously hypothesized (EMBL M91650 description entry). To study the transcriptional response of the regulatory locus to metals and to probe promoter regions, transcriptional fusions were constructed between fragments of *cnrYXH* and the *luxCDABE*, luciferase reporter genes. Nickel and cobalt specifically induced the *cnrYXH-luxCDABE* fusion at optimal concentrations of 0.3 mM Ni²⁺ and 2.0 mM Co²⁺ in a noncomplexing medium for metals. The two promoter regions P_Y (upstream *cnrY*) and P_H (upstream *cnrH*) were probed and characterized using this vector and were found to control the nickel-inducible regulatory response of the *cnr* operon. The *cnrHp* promoter was responsible for full transcription of the *cnrCBA* structural resistance genes, while the *cnrYp* promoter was necessary to obtain metal-inducible transcription from the *cnrHp* promoter. The zinc resistance phenotype (ZinB) of a spontaneous *cnr* mutant strain, AE963, was investigated and could be attributed to an insertion of IS1087, a member of the IS2 family of insertion elements, within the *cnrY* gene.

The resistance of *Ralstonia eutropha*-like strain CH34 (*Alcaligenes eutrophus* CH34) against multiple heavy metals can be regarded as a phenomenon in its own right (15) and has found increasing applications within the field of environmental technology (29). The metal resistance determinants, which are localized predominantly on either of the two indigenous megaplasmids pMOL28 (15, 28) and pMOL30 (15), have been recently reviewed by Taghavi et al. (27). One important application has been the development of metal-specific biosensors based on *in vivo* and *in vitro* gene fusions of CH34 heavy metal resistance determinants. This was possible only through a detailed knowledge of the regulation of the metal resistances at the molecular level (2).

The *cnr* operon, located on pMOL28 (13, 28), encodes a phenotype of inducible resistance to 5 mM Co²⁺ and 3 mM Ni²⁺ in minimal medium. Resistance is mediated by an intricate coordination between the genes of the regulatory locus, *cnrYXH*, located upstream of the structural locus, *cnrCBA* (13). The latter encodes a three-component cation/proton antiporter (4), whose topological orientation in the membrane has been elucidated largely by comparison with the well-studied *czc* (cadmium-zinc-cobalt resistance) system (3, 17, 21), with which it shares close homology at the protein level (18, 20).

Despite the strong similarity between the structural resistance genes of the *czc* (17, 18), *czt* (9), *sil* (8), *cnr* (13), and *ncc* (25) operons, the regulation of both *cnr* and *ncc* (nickel resistance determinants) differs greatly from that of the other three-component-system-based heavy metal resistance operons. The *cnr* and *ncc* operons share an average of 66% identity in their regulatory loci and 79% in their structural loci. For *cnr*, regulation requires the activities of at least three genes, *cnrYXH*. Until now, the gene products of the regulatory locus have remained elusive. Only CnrH shares a close similarity (32%) with a known gene product, namely, σ^E of *Escherichia coli*, an alternative σ^{70} -like sigma factor of the extracytoplasmic function (ECF) family (14, 16, 22). CnrY and CnrX, however, show no homology to known proteins, and therefore their functional roles *in vivo* have remained open to debate. In this paper, we report the preliminary characterization of the Cnr regulatory proteins, through cloning and isolation from *E. coli* overexpression systems, and the molecular characterization of a spontaneous *cnr* mutant harboring zinc resistance. Furthermore, we identified promoter regions within the regulatory locus by use of transcriptional fusions with reporter bioluminescence genes and examined their heavy metal-dependent activity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *R. eutropha*-like strains were grown in either 869 medium (15) or Tris-buffered 284 medium with 0.2% (wt/vol)

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
<i>R. eutropha</i> strains		
CH34	Wild type, containing pMOL28 and pMOL30, <i>czc cnr chr mer cop pbr</i>	15
AE126	pMOL28, <i>cnr chr mer</i>	15
AE104	Plasmid free, metal sensitive	15
AE963	pMOL29, <i>cnrY963::IS1087 chr mer</i> , ZinB phenotype (due to derepression of <i>cnr</i>)	1
<i>E. coli</i> strains		
DH10B	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>dlacZ</i> ΔM15 Δ <i>lacX74 deoR recA1 endA1 araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU galK</i> λ ⁻ <i>rpsL nupG</i>	GIBCO BRL
CM1446	DH10B containing pMOL877	30
JM105	F ['] <i>traD36 lacI^qΔ(lacZ)</i> M15 <i>proA</i> ⁺ <i>B</i> ⁺ / <i>thi rpsL</i> (Str ^r) <i>endA sbcB15 sbcC hsdR4</i> (r _K ⁻ m _K ⁺) Δ(<i>lac-proAB</i>)	32
BL21(DE3)	F ⁻ <i>dcm ompT hsdS</i> (r _B ⁻ m _B ⁻) <i>galDE3</i>	Stratagene
TOP10 F [']	F ['] <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74 deoR recA1 araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (Str ^r) <i>endA1 nupG</i>	Invitrogen
DH5α	F ⁻ φ80d <i>lacZ</i> ΔM15 Δ(<i>lacZyA-argF</i>)U169 <i>deoR recA1 endA1 phoA hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44</i> λ ⁻ <i>thi-1 gyrAg6 relA1</i>	GIBCO BRL
pMOL877-based plasmids		
pMOL877	<i>luxCDABE</i> , Tet ^r	30
pMOL1550	<i>cnrYXH-luxCDABE</i> , Tet ^r	This study
pMOL1551	<i>cnrYXHC'-luxCDABE</i> , Tet ^r	This study
pMOL1583	<i>cnrYp-luxCDABE</i> , Tet ^r	This study
pMOL1561	<i>cnrYX^{aa} H-luxCDABE</i> , Tet ^r	This study
pMOL1586	<i>cnrYp-cnrYX-cnrHp-luxCDABE</i> , Tet ^r	This study
pMOL1587	<i>cnrHp-luxCDABE</i> , Tet ^r	This study
pMOL1588	<i>cnrHp-cnrH-luxCDABE</i> , Tet ^r	This study
pMOL1591	<i>cnrX-cnrHp-luxCDABE</i> , Tet ^r	This study
pMOL1592	<i>cnrYp-cnrY-luxCDABE</i> , Tet ^r	This study
pMOL1593	(<i>cnrY-cnrYp</i>) - <i>cnrX-cnrHp-luxCDABE</i> ^b , Tet ^r	This study
pMOL1596	<i>ivrHC-luxCDABE</i> , Tet ^r	This study
Other plasmids		
pTrc99A	Amp ^r	Pharmacia Biotech Ltd.
pTrcCnrX	Amp ^r , <i>cnrX</i> in pTrc99A	This study
pTrcCnrYa	Amp ^r , <i>cnrYa</i> in pTrc99A	This study
pTrcCnrYb	Amp ^r , <i>cnrYb</i> in pTrc99A	This study
pCnrXphoA1	Amp ^r Km ^r , pTrc- <i>cnrX'</i> in pGV4218	This study
pCnrXphoA2	Amp ^r Km ^r , <i>cnrX'</i> -pTrc in pGV4218	This study
pGV4218	Amp ^r Km ^r	5, 19
pVDZ'2-cnr	<i>cnr</i> as <i>EcoRI-PstI</i> fragment in pVDZ'2	13
pRSETA	Amp ^r	Invitrogen
pRSETCnrX	Amp ^r , <i>cnrX</i> in pRSETA	This study
pRSETCnrH	Amp ^r , <i>cnrH</i> in pRSETA	This study
pMOL29	pMOL28 containing <i>cnrY963::IS1087</i>	1

^a Point mutations in P_H region.

^b *cnrY-cnrYp* region cloned in the opposite orientation as *cnrX-cnrHp-luxCDABE*.

gluconate (15) as a carbon source. Bioluminescence assays were carried out in either 284-gluconate liquid medium or RM medium (284 medium supplemented with 0.1% [wt/vol] gluconate, MOPS [morpholinepropanesulfonic acid], and beta-glycerol phosphate replacing Tris and inorganic phosphate, respectively) (2). For *E. coli*, 869 medium supplemented with antibiotics was used for propagation, while Luria broth (LB) supplemented with antibiotics and/or 2% (wt/vol) glucose was used in protein overexpression cultures. Antibiotic concentrations were 20 μg of tetracycline (TET) per ml and 100 μg of ampicillin (AMP) per ml. NiCl₂ and CoCl₂ were used for *lux* induction experiments. *R. eutropha* and *E. coli* strains were grown at 30 or 37°C, respectively, unless stated otherwise.

Genetic techniques. Standard molecular genetic techniques were used (23). The pMOL28 plasmid (15) was used as the source for the *cnr* genes and was extracted from AE126 by the procedure of Taghavi et al. (26). Electroporation (26) was used to introduce plasmid DNA into *R. eutropha* and *E. coli*.

Construction of Cnr protein overexpression clones. The *cnrY* gene was cloned in its two possible open reading frames (ORFs) (13). The *cnrYa* ORF (positions 970 to 1266, according to the numbering of Liesegang et al. [13]) was PCR amplified as an *NcoI-BamHI* fragment from pMOL28, using 5'-GATCTCCATGGAGGTTTGCCACGGC-3' as the upstream primer and 5'-GCGGCAAGGATCTCTGTCAGC-3' as the downstream primer. The resulting fragment spanned the region from position 962 to 1747 of the *cnr* sequence. The *cnrYb* ORF (positions 982 to 1270) was amplified as an *NcoI-HindIII* fragment with the

upstream and downstream primers 5'-GTTTGCCATGGCAGACGTGGAAG A-3' and 5'-ATGGCAAGCTTGCCGACGCC-3', respectively, spanning positions 981 to 1516. The inserts were cloned into the expression vector pTrc99A, linearized with either *NcoI-BamHI* or *NcoI-HindIII*. The *cnrX* gene (positions 1272 to 1727) was cloned as an *RcaI-BamHI* fragment using the primers 5'-CGGTAAATCATGAAATCTCGT-3' (upstream) and 5'-GCGGCAAGGATCTCTGTCAGC-3' (downstream) into pTrc99A, linearized with *NcoI* and *BamHI*. Following ligation, plasmid constructs were transformed into *E. coli* JM105 and selected on LB-AMP. Positive transformants were sequenced to ensure the in-frame insertion of the cloned fragments within the expression vector.

Expression of *cnr* regulatory genes in a cell-free system. The protein expression of pTrcCnrX, pTrcCnrYa, and pTrcCnrYb in *E. coli* JM105 was tested in a cell-free system. Plasmid DNAs (4 μg) of the clones were used as templates (in both linearized and circularized forms) in an in vitro coupled transcription-translation protocol, using the Promega (Madison, Wis.) *E. coli* S30 Extract System For Linear Templates kit, as specified by the manufacturer. L-[4,5-³H]-Leucine (Amersham Pharmacia Biotech, Rainham, United Kingdom) was used to label nascent polypeptides. The translation products were separated on a 16.5% T, 6% C Tricine sodium dodecyl sulfate (SDS)-polyacrylamide gel (24) and visualized by autoradiography. ¹⁴C-labeled protein markers (Amersham Pharmacia Biotech) were included as standards.

Expression and production of *cnrX* gene products in *E. coli*. Overnight cultures of *E. coli* (pTrcCnrX) were diluted 100-fold in 5 ml of LB-AMP and grown to an optical density at 600 nm (OD_{600}) of 1.0. IPTG (isopropyl- β -D-thiogalactopyranoside) (1 mM) was added to induce protein overexpression for 4 to 5 h. The cells were harvested and lysed by sonication in lysis solution (25 mM Tris [pH 7.5], 0.1 M NaCl, 1 mM EDTA, 15% [vol/vol] glycerol) with 0.1 mg of phenylmethylsulfonyl fluoride added as a protease inhibitor. The pellet fraction was collected following centrifugation at 18,000 rpm for 10 min (Sorvall centrifuge, SS34 rotor) and solubilized in 0.25% (wt/vol) Thesit (Roche Diagnostics, Brussels, Belgium) plus 10 mM EDTA, followed by incubation at 4°C for 30 min. The samples were centrifuged at 40,000 rpm for 2 h at 4°C, using a Beckman ultracentrifuge. The supernatant was collected, mixed with Laemmli loading buffer, fractionated on a 16.5% Tricine SDS-polyacrylamide gel (24), and visualized with Coomassie blue R-250.

Construction of PhoA (alkaline phosphatase) fusions with CnrX. A gene fusion between the nucleotide sequence of *cnrX'* encoding the first 27 amino acids of CnrX (MMKSRT...AAWL) and that of the ORF encoding the mature form of alkaline phosphatase (*phoA*) was constructed in vitro. In this construct, transcription of *cnrX* and that of *phoA* are coupled and directed from the pTrc promoter, resulting in a hybrid CnrX-PhoA fusion protein. The pTrc promoter together with *cnrX* were obtained by PCR amplification of pTrcCnrX using the downstream primer 5'-CGCGAATGGATCCGCTCATGTTTGAC-3' (*Bam*HI site) and the upstream primer 5'-TGCAGTAGATCTGCCATGCCGC-3' (*Bgl*II site). The *cnrX'* fragment was cloned as a *Bam*HI-*Bgl*II insert in the *Bgl*II-linearized and dephosphorylated plasmid pGV4218 (5, 19). The hybrid plasmid was used to transform *E. coli* DH5 α . Transconjugants were selected directly on LB plates with 25 μ g of kanamycin per ml, 100 μ g of AMP per ml, 1 mM IPTG, and 0.5 mg of XP (5-bromo-4-chloro-3-indolylphosphate, 4-toluidine salt) (Boehringer Mannheim) per ml. This resulted in two plasmids, pCnrXphoA1 and pCnrXphoA2, having the pTrc-*cnrX'* fragment cloned with *phoA* in the same and opposite orientations, respectively. The orientations of the cloned inserts were verified by PCR (5), while the structural integrity of the constructs was confirmed by sequence analysis. Constructs with plasmid pCnrXphoA1 formed small, dark blue colonies (PhoA⁺), while PhoA⁻ transconjugants with the insert in the opposite orientation formed larger, light green colonies with a white halo or simply uniform white colonies.

Construction of histidine-tagged fusion proteins. Both *cnrX* and *cnrH* were cloned as tagged fusions incorporating an N-terminal His tag. For the construction of pRSETCnrX, the *cnrX* ORF was amplified from pMOL28 as a *Bam*HI-*Pst*I fragment with downstream and upstream primers 5'-TTTGGATCCATGATGAAATCTCGTACCCGACGG-3' and 5'-TTTCTGCAGCGTCTTCCGGA TTCCTGCGAGCCGG-3', which includes the *cnrX* region from position 1260 to 1738. The *cnrH* ORF was amplified as a *Bam*HI-*Pst*I fragment with primers 5'-TTTGGATCCAAATCCGGAAGACGCTGACAGAATCC-3' and 5'-TTTGACTGCAGACTTATTTTCCGAGTCAGCATCCAGC-3', which includes the *cnrH* region from position 1711 to 2296. The pRSETA vector was isolated from *E. coli* TOP10 F' using a Plasmid Midi-prep kit for high-copy-number plasmids and then linearized with *Bam*HI and *Pst*I. Following ligation of the vector and insert, plasmid constructions were transformed into electrocompetent *E. coli* BL21(DE3), and transconjugants were selected on LB-AMP plates.

Production and purification of His-CnrX and His-CnrH in *E. coli*. Overnight cultures of *E. coli* BL21(DE3) bearing pRSETCnrH were diluted 100-fold into 3.3 liters of LB plus 2% (wt/vol) glucose and AMP and grown to an OD_{600} of 0.5. The cells were collected by centrifugation and washed once with fresh LB-AMP medium. The cells were suspended in the same volume of fresh LB-AMP. For induction of protein overexpression, 1 mM IPTG was added and the cells were incubated for 16 h at 28°C with shaking. Subsequently, the cells were harvested by centrifugation and suspended in 50 ml of lysis buffer (500 mM NaCl, 50 mM Tris-Cl, pH 8) plus the protease inhibitors 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) (0.1 mg/ml) and leupeptin (1 μ g/ml). Cells were lysed by a double passage through a French pressure disruption cell. The cell pellet was recovered following centrifugation at 12,000 rpm (Sorvall centrifuge, SS34 rotor) at 4°C for 30 min. The recovered pellet was suspended in 30 ml of immobilized metal chromatography (IMAC) buffer G (6 M GdCl₂, 50 mM Tris [pH 7.9], 0.5 M NaCl) and left on a rotary mixer for 1 h at 4°C. The denatured proteins were recovered in the supernatant fraction following centrifugation at 18,000 rpm at 4°C for 30 min using a Beckman centrifuge. The guanidine HCl-solubilized supernatant fraction was mixed with 6 ml of Ni-nitrilotriacetic acid (Ni-NTA) resin (QIAGEN, Westburg B. V., The Netherlands) in a Falcon tube and left to mix on a rotary mixer for 1 h at 4°C. The Ni-NTA resin-His-CnrH mix was packed in batch fashion into a 5-ml column (Amersham Pharmacia Biotech). The purification was carried out using the Pharmacia fast protein liquid chromatography system at 4°C. The column was washed with IMAC buffer G for 10 min at 1 ml/min until the OD_{280} attained the baseline value. The adsorbed, denatured protein was allowed to slowly renature by the application of a 6 to 0 M GuCl₂ gradient, which was achieved by applying a continuous gradient beginning with IMAC buffer G and ending with IMAC buffer B (50 mM Tris-Cl [pH 8.0], 1 M NaCl, 20% [vol/vol] glycerol) over 4 h at 1 ml/min. His-CnrH was eluted with 10 column volumes (40 ml) of imidazole-containing IMAC buffer I (0.5 M imidazole, 1 M NaCl, 50 mM Tris-Cl, pH 7.6) at 1 ml/min. Two-milliliter fractions were collected. Fractions containing His-CnrH were pooled and further purified by gel

filtration chromatography, using a Superdex 75 (16/60) column (Amersham Pharmacia) equilibrated with gel filtration buffer (50 mM Tris-Cl [pH 8.0], 200 mM KCl, 10 mM MgCl₂, 1 mM EDTA, 5 mM 2-mercaptoethanol, 10% [vol/vol] glycerol). The pure protein was stored in aliquots in 50% (vol/vol) glycerol at -80°C. A similar purification protocol was followed for the pRSETCnrX overexpression constructs. The following buffers replaced the equivalents used for His-CnrH: IMAC buffer Ix (0.5 M imidazole, 1 M NaCl, 50 mM Tris-Cl [pH 7.6], 0.1% [wt/vol] Thesit) and gel filtration buffer X (50 mM Tris-Cl [pH 8.0], 200 mM KCl, 10 mM MgCl₂, 1 mM EDTA, 5 mM 2-mercaptoethanol, 10% [vol/vol] glycerol, 0.1% [wt/vol] Thesit).

Gel shift mobility assays. DNA targets were obtained by PCR amplification from pMOL28. The amplified products corresponded to the following targets: P_Y fragment (positions 820 to 1028 according to Liesegang et al. [13]), P_H fragment (positions 1600 to 1800), and ivrHC fragment, containing the intervening region between *cnrH* and *cnrC* (positions 2270 to 2482). The DNA was end labeled with [γ -³²P]dATP using polynucleotide kinase (GIBCO BRL, Life Technologies N. V., Merelbeke, Belgium), according to the manufacturer's specifications. The labeled DNA was recovered using a MicroSpin column (Amersham Pharmacia Biotech) as specified by the manufacturer. The protocol of Landini et al. (12) was used as the basis for the gel shift mobility assays. Purified His-CnrH (see above) was concentrated to approximately 0.2 mg/ml (\approx 9 μ M) using a Centricon instrument (Millipore, Bedford, Mass.). The protein concentration was determined spectrophotometrically at 280 nm. Reconstitution of His-CnrH with the *E. coli* RNA polymerase core enzyme (Biozym, Landgraaf, The Netherlands) was allowed to occur by incubation of various concentrations of His-CnrH with the core enzyme (50 nM) in vitro at 37°C for 20 min in the presence of binding buffer (50 mM Tris-Cl [pH 8], 1 mM EDTA, 1 mM dithiothreitol). The reconstituted mixture was allowed to interact with 1.5 pmol of labeled DNA (10 ng) according to the protocol of Landini et al. (12) (37°C for 30 min). Subsequently, the reaction mixture was separated on a 5% nondenaturing acrylamide gel, using 0.5 \times Tris-borate-EDTA as the running buffer. DNA bands were visualized using autoradiography.

Primer extension for determination of mRNA transcription start sites. Total RNA extraction from the *R. eutropha* CH34 derivatives AE104, AE963, and AE126 was carried out using the RNeasy purification kit (QIAGEN), following the manufacturer's guide, with minor modifications for cell lysis. The CH34 derivative strains were grown in 284-gluconate medium to stationary phase and then diluted 100-fold in fresh medium and grown to an OD_{600} of 0.5. The cells were induced with NiCl₂ to a final concentration of 0.4 mM for 20 min and immediately chilled on ice. The pellet was recovered by centrifugation and then resuspended in Insta-Pure solution (EUROGENTEC S. A., Seraing, Belgium) as specified by the manufacturer. Chloroform was added to 1/10 volume, and the mixture was centrifuged at 3000 rpm for 15 min (Sorvall centrifuge, Sg3 rotor). Subsequent purification steps were as specified in the QIAGEN RNeasy purification kit manual.

For the determination of transcription start sites, 40 pmol of each of the oligonucleotides, *cnrYp* (5'-GGGCCGCTCTGCAGTGTG-3'), *cnrHp* (5'-GCCACAAGTTGGCCGAATG-3'), and *cnrCp* (5'-GCTGCAACAGGTTTCG G-3'), was labeled using 5 μ l of [γ -³²P]dATP (3,000 mCi/mmol) (Amersham) and polynucleotide kinase (New England Biolabs) by standard protocols. The final oligonucleotide concentration was adjusted to 0.8 pmol/ μ l. For primer extension experiments, a modification of the Promega protocol was used. Hybridization was carried out using 2 pmol of the labeled primer and 10 to 20 μ g of total RNA extract at 45°C for 3 h in 30 μ l of hybridization buffer (40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid) [pH 6.4], 400 mM NaCl, 80% formamide). The extension reaction was carried out in a 50- μ l volume of extension mix, containing 10 mM dithiothreitol, 1 \times First Strand Synthesis Buffer (GIBCO BRL), and 1 mM deoxynucleoside triphosphates. Superscript reverse transcriptase (GIBCO BRL, Life Technologies) (200 U) was added for extension, which was carried out for 1 h at 42°C. Following heat denaturation of the enzyme and RNase treatment, the extension products were recovered by ethanol precipitation, resuspended in formamide loading buffer, and separated on a 5% acrylamide sequencing gel next to a sequencing ladder. Sequencing reactions were carried out using the Pharmacia Amersham Biotech Thermosequenase kit according to the manufacturer's protocol for dTTP cycle sequencing: the sequencing ladder of pUC18 DNA generated using the forward universal primer (5'-GTTTTCAGTTCACGACGTTGTA-3') was used to as a reference to mark the positions of the primer extension products, in base pairs, from the site of primer hybridization.

Construction of transcriptional fusions. Different regions of the *cnr* regulatory locus were amplified by PCR. *Eco*RI sites were introduced into the primer sequences to clone the inserts into the unique *Eco*RI site of pMOL877. The inserts were obtained by PCR amplification using pMOL28 DNA as a template (see Table 2). For the construction of point mutations within *cnrYXH*, a strand overlap extension protocol was used. Two primary PCRs were carried out on *cnr* DNA using the primer pairs CNRLUX1-PXLUX1 and CNRLUX2-PXLUX2; the resulting amplicons corresponded to the 5' and 3' ends of *cnrYXH*, respectively. The point mutations incorporated in PXLUX1 and PXLUX2 affected the positions 1657 (G to A), 1659 (A to G), and 1662 (T to C). This changed the presumed *cnrHp* promoter sequence (based on the AAC consensus for RpoE-like ECF factors) from 5'-CCGGAACATCG-3' to 5'-CCGAAAGCACCG-3'. The second PCR step used the products of the first PCR step as the DNA

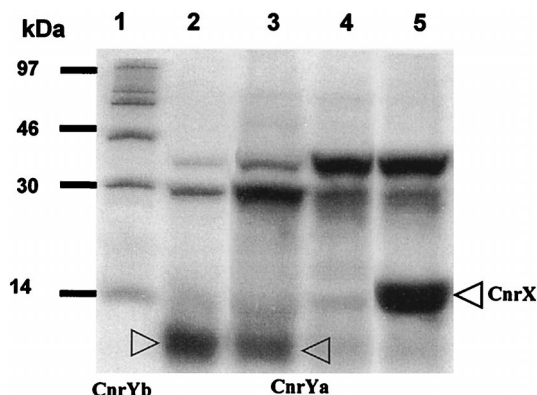


FIG. 1. Coupled in vitro transcription-translation of *cnrY* and *cnrX*. Plasmids pTrcCnrX, pTrcCnrYa, and pTrcCnrYb were used as DNA templates in an in vitro transcription-translation reaction. The templates were linearized or circularized to maximize efficient transcription from the pTrc promoter; a linearized template with a predominant 30-kDa band suggests inefficient transcription-translation coupling. The protein products are indicated. Lane 1, size marker; lane 2, pTrcCnrYb, linearized template; lane 3, pTrcCnrYa, linearized template; lane 4, pTrc99A; circularized template; lane 5, pTrcCnrX, circularized template.

template and the primer pair CNRLUX1-CNRLUX2. Plasmid pMOL877 was isolated from CM1446 using the GIBCO BRL Plasmid Midi-prep kit for low-copy-number plasmids. Constructs were transformed into *E. coli* DH10B by electroporation, and transconjugants were selected on 869 medium supplemented with 20 μ g of TET per ml. Plasmid DNA of transconjugants was isolated and subsequently electroporated into the *R. eutropha* strains AE104 and CH34. Transconjugants were selected on 869 medium supplemented with 20 μ g of TET per ml. Ninety-six clones were selected and inoculated onto different Ni induction plates (0.3 mM Ni), which were examined using autoradiography. Those constructs showing Ni-responsive bioluminescence (black spots on X-ray film) were further characterized.

Luminometry assays. The bioluminescence of *cnr-lux* constructs was measured with a LUCY1 luminometer (Anthos Labtech B.V., Heerhugowaard, The Netherlands) at 23°C. Cells were grown overnight in 869 medium plus 20 μ g of TET per ml to an OD₆₆₀ of 1.0. The cells were harvested by centrifugation, washed once with RM-gluconate (0.1%, wt/vol) plus MOPS and beta-glycerol phosphate, and suspended in fresh medium at an OD₆₆₀ of 0.3. Subsequently, 20 μ l of the metal salt solutions was added to the microtiter plates at 10 \times the working concentrations immediately before 180 μ l of the test cultures was added to the well. Water was used as a negative control. The bioluminescence emitted in absolute light units (ALU) was measured over 16 h at 30-min intervals, using the MIKROWIN software of the Anthos LUCY1 luminometer. Data processing was carried out using EXCEL 7.0 (Microsoft). Constructs harboring pMOL877 in CH34 gave a noise level of approximately 100 ALU.

Nucleotide sequence accession number. The sequence of IS1087 has been entered in the EMBL database under accession no. AJ243722.

RESULTS

In vitro transcription-translation of *cnrY*. Based on sequence data, two possible ORFs have been hypothesized for *cnrY* (13). In *nccY*, which is closely related to *cnr*, only the ORFb reading frame is present. Translational fusions of the ORFa and ORFb reading frames were constructed in the *E. coli* expression vector pTrc99A, resulting in pTrcCnrYa and pTrcCnrYb, respectively, and subsequently tested in an in vitro coupled transcription-translation system. As shown in Fig. 1, both could be expressed in vitro. However, the protein product corresponding to the ORFb coding frame appeared more abundant (also by comparing the intensity of the 28-kDa internal control). Since in the homologous *nccYXH* system ORFa does not exist, in all likelihood the CnrY protein is translated from ORFb in vivo. It was not possible to overproduce the CnrY protein in *E. coli* cultures. Sequence analysis confirmed that both pTrcCnrYa and pTrcCnrYb had the correct structural integrity, and both plasmids were stably maintained in *E. coli*. Therefore, protein instability due to toxicity may have been the basis of this result.

Purification and characterization of CnrX. The *cnrX* gene was cloned in the pTrc99A expression vector, and after induction with IPTG, CnrX was over expressed in *E. coli* as a 16.5-kDa protein which was localized in the membrane fractions (Fig. 2A, compare membrane fractions in lanes 2 and 3 with soluble fractions in lanes 6 and 7, respectively). In whole-cell preparations of IPTG-induced cultures, a lower-molecular-mass band of approximately 14 kDa was also observed to co-migrate in SDS-polyacrylamide gels (Fig. 2A, lane 9), which suggested that CnrX may exist as processed and nonprocessed forms, with the latter being located in the membrane fractions. The existence of a membrane-translocating leader sequence at the N terminus of CnrX, as suggested on the basis of the amino acid sequence (MMKSRTRRLSSTLFLGALLGVSVAAA | WLY [the bar represents the cleavage site, and hydrophobic residues are in boldface]), was supported by studies with pTrc-*cnrX'*-*phoA* fusions. A fusion between the N-terminal 27 amino acids of CnrX and a *phoA* reporter system where PhoA lacks its own signal sequence (19) resulted in strong PhoA activity; such transformants formed small, dark blue colonies when plated on selective medium (see Materials and Methods). Transformants having the pTrcCnrX' fused to *phoA* in the opposite orientation (pCnrXphoA2) did not show PhoA

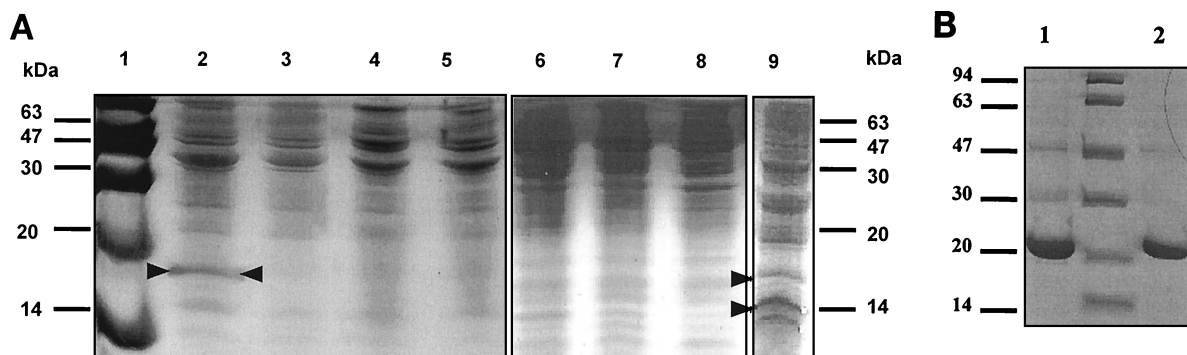


FIG. 2. Overproduction of CnrX and purification of His-CnrX. (A) Constructs bearing pTrcCnrX expressed CnrX as a 16.5-kDa protein fractionating in membrane components of cell lysates. Lane 1, size marker; lanes 2 and 3, protein extracts from induced and noninduced cultures of pTrcCnrX, respectively; lanes 4 and 5, protein extracts from induced and noninduced cultures, respectively, of the pTrc99A control with no insert; lanes 6 to 8, soluble fractions of induced pTrcCnrX, noninduced pTrcCnrX, and control pTrc99A cultures, respectively; lane 9, whole-cell preparation showing two forms of CnrX. (B) His-CnrX was overproduced as a 21-kDa protein and purified by Ni-NTA IMAC and gel filtration chromatography. Lane 1, pooled Ni-NTA IMAC fractions; lane 2, pooled gel filtration fractions.

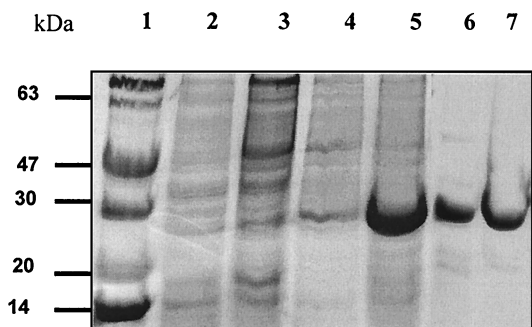


FIG. 3. Overproduction and purification of His-CnrH. Constructs bearing pRSETCnrH overproduced His-CnrH as a 28-kDa protein in inclusion bodies. Lane 1, molecular mass marker; lanes 2 and 3, supernatant and pellet protein fractions of noninduced cultures, respectively; lanes 4 and 5, supernatant and pellet protein fractions of induced cultures, respectively. His-CnrH inclusion bodies were purified by Ni-NTA IMAC followed by gel filtration chromatography. Lane 6, pooled IMAC fractions; lane 7, gel filtration fraction.

activity. These data are consistent with those in the accompanying study by Grass et al. (6).

The levels of overproduction of CnrX in *E. coli* (Fig. 2A) were considered too low for isolation purposes. To improve yields, CnrX was cloned and overproduced as a histidine-tagged fusion (pRSETCnrX), purified by use of Ni-NTA IMAC followed by gel filtration chromatography (Fig. 2B). The structural integrity of the purified protein was examined by secondary structure determination using circular dichroism (unpublished data), which revealed a high alpha-helical content (37.3%) and a relatively high content of antiparallel beta-sheets (21.4%), features which indicate the correct folding of the protein.

Purification and characterization of CnrH. CnrH was overproduced in *E. coli*(pRSETCnrH) with an N-terminal histidine tag (Fig. 3), and the resulting inclusion bodies were purified under denaturing conditions by Ni-NTA IMAC, with protein renaturation prior to elution (Fig. 3, lane 5). Gel filtration was

used as a final purification step (Fig. 3, lane 6). The purity of the protein was quantified to be greater than 95% (as determined by reverse-phase high-pressure liquid chromatography) (data not shown). The structural integrity of the purified protein was verified by the determination of its secondary structure, which was found to include an alpha-helical content exceeding 40% (unpublished data).

CnrH is thought to be a member of the ECF σ^{70} family. As such, CnrH should interact with a specific DNA target in the presence of core RNA polymerase. The close similarity with RpoE of *E. coli* prompted us to test the DNA-binding activity of His-CnrH using the core RNA polymerase of *E. coli*. Possible DNA targets were identified following a systematic analysis of the *cnr* sequence, both for regions homologous to the presumed *cnrYp* promoter sequence (13) and based on the consensus sequence of RpoE promoters (14). Three fragments were identified for further studies: the P_Y fragment (containing the putative *cnrYp* σ^{70} -like promoter), the P_H fragment (containing the putative *cnrHp* RpoE-like promoter), and the intervening region between *cnrH* and *cnrC* (*ivrHC*), showing similarity to the sequence around the presumed *cnrYp* promoter (6). The three potential targets were used for in vitro gel shift mobility assays. As shown in Fig. 4, His-CnrH could retard the P_Y and P_H DNA fragments, but only when the core RNA polymerase enzyme of *E. coli* was included in the reactions. In the absence of the core RNA polymerase enzyme, no retardation was observed (results not shown). The addition of His-CnrX in the gel retardation reactions did not interrupt the interaction of His-CnrH with DNA (Fig. 4).

Metal-responsive transcription from the regulatory locus, *cnrYXHC'*. The transcriptional fusion of plasmid pMOL1551 was constructed by cloning the *cnr* regulatory locus, *cnrYXHC'* (positions 1 to 2418 according to the numbering of Liesegang et al. [13]), upstream of the *luxCDABE* genes in pMOL877 (30). The transcriptional responses to nickel and cobalt were tested in the CH34 as well as the AE104 background. In the latter, no inducible bioluminescent response was observed. This indicates that additional regulatory functions are present

core	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HisH, μ M	0.6	0.3	0.6	1.2	0	0.15	0.3	0.6	1.2	0	1.2	0.6	0.3	0
HisX, μ M	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-

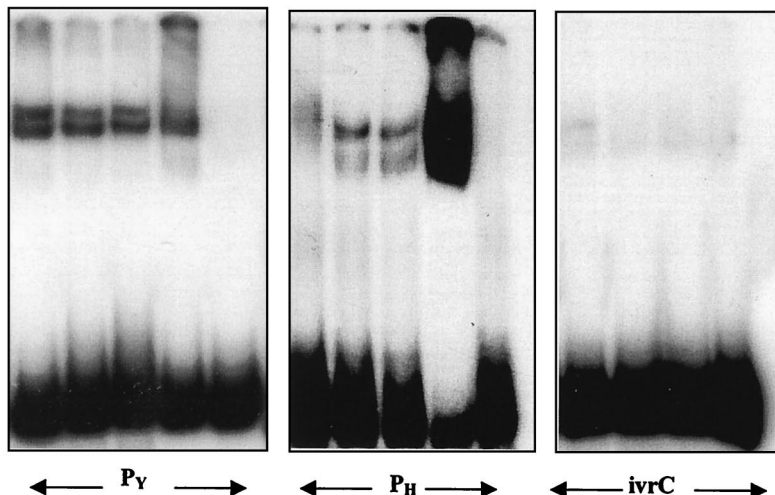


FIG. 4. Gel shift mobility assays. Purified His-CnrH was tested for its DNA-binding activity in vitro in the presence of core RNA polymerase of *E. coli*. The interaction with P_Y, P_H, and *ivrHC* with the indicated concentrations of His-CnrH, His-CnrX, and 50 nM core enzyme is shown.

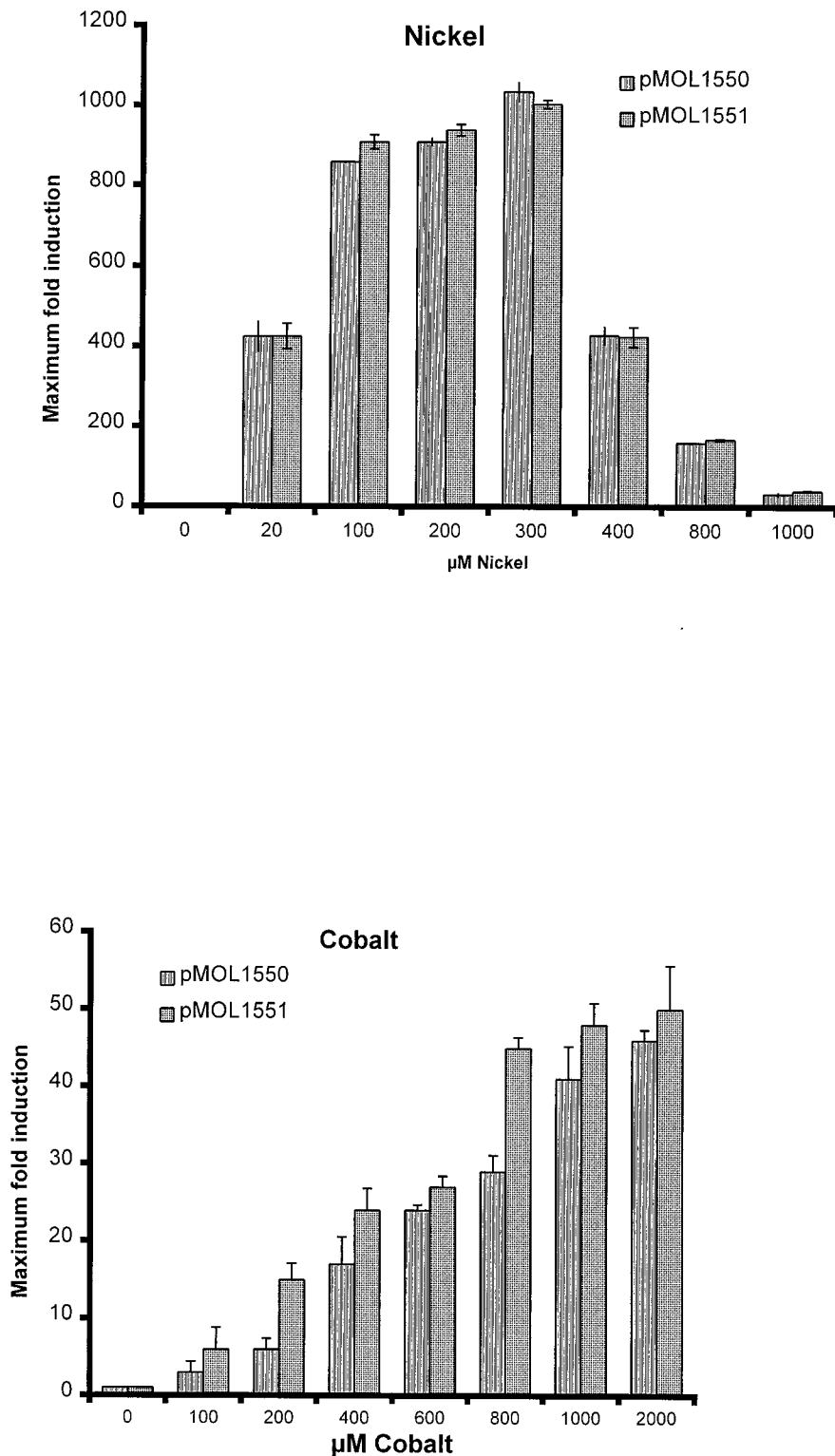


FIG. 5. Metal-inducible transcription from *cnrYXH* in the presence of nickel and cobalt. CH34 constructs bearing pMOL1550 and pMOL1551 were tested for their light production in the presence of various concentrations of nickel and cobalt at 23°C. The fold induction was calculated as the signal-to-noise ratio as a function of the biomass (OD_{660}). The data presented are averages and standard deviations from three independently performed experiments.

in CH34 that are lacking in AE104. Also in *E. coli*, no transcription from the *cnr* regulatory locus was observed (data not shown). As shown in Fig. 5, transcription from *cnrYXH* was basal in the absence of Ni^{2+} and Co^{2+} . The maximal transcrip-

tional response was observed in RM medium with 0.3 mM nickel. At between 0 and 0.3 mM nickel (low levels), transcription was positively induced with increasing nickel concentrations. At higher concentrations (>0.4 mM), transcription be-

came repressed with increasing metal concentration, and toxicity effects of the metal on the growth became apparent. The maximal transcription response was attained as a first peak during the mid-log phase and a second peak in the early stationary phase. The induction of *cnr* was also observed with cobalt, but the sensitivity differed from that of nickel. The maximum level of transcription was observed with cobalt concentrations of up to 2 mM.

Role of the intervening region between *cnrH* and *cnrC* (ivrHC) in metal-responsive transcription. In order to examine the effect of the intervening region between *cnrH* and *cnrC* (ivrHC), plasmid pMOL1550 was constructed. It contains the fusion *cnrYXH-luxCDABE* and completely lacks the ivrHC region. The transcriptional responses of pMOL1550 and pMOL1551 were compared in strain CH34(pMOL28, pMOL30). The results are presented in Fig. 5. Nickel-dependent induction of *lux* in pMOL1550 and pMOL1551 showed similar kinetics. Therefore, it can be concluded that the ivrHC region is not essential for Ni-inducible regulation of transcription from the *cnr* regulatory locus. Neither Zn(II) nor Cr(VI) was able to induce *cnrYXH* transcription (data not shown). However, induction of *cnr*-derived transcription induced by low levels of Co^{2+} was more efficient with pMOL1551 than with pMOL1550, indicating that the ivrHC region might play a role in low-level Co^{2+} induction of *cnrCBA* transcription. However, with pMOL1596, which contains an ivrHC-*lux* fusion, no transcription of *lux* was observed in either the presence or the absence of Ni^{2+} and Co^{2+} (see Table 2). Thus, the ivrHC region is unlikely to contain a metal-inducible promoter.

Probing promoters and regulatory elements within the *cnrYXH* regulatory locus. Transcriptional fusions between the *cnr* regulatory locus and *luxCDABE* were constructed as shown in Table 2. The selection of putative promoter fragments was based on the gel retardation data with CnrH. The metal responsiveness of the fusions was tested in the CH34 background, to ensure the presence of the *cnrYXH* genes in *trans*, and in the presence of 0.3 mM nickel. For comparison, transcription in the CH34 construct containing pMOL1550 was considered maximal (100% strength). As shown in Table 2, the promoter regions P_Y (in pMOL1583) and P_H (in pMOL1587) were transcriptionally active, with relative signal strengths of 13 and 17%, respectively, compared to that of the complete *cnrYXH* fusion in pMOL1550. However, despite the presence of the CnrY, CnrX, and CnrH proteins in *trans*, transcription from these promoters was no longer Ni^{2+} inducible. Interestingly, the strain containing pMOL1586 (*cnrYp-cnrYX-cnrHp-luxCDABE*) showed metal induction kinetics for Lux comparable to that of pMOL1550. However, the maximum signal/noise ratio was approximately one-fifth of that attained with pMOL1550, probably due to a CnrH titration effect. In pMOL1588, where *cnrH* is transcribed from *cnrHp*, transcription was maintained for 20 h (until stationary phase) at a constant, high, constitutive level (maximum signal strength, 58%). Thus, despite the presence of CnrY and CnrX in *trans*, transcription from *cnrHp* was no longer metal regulated, indicating that an additional function was required in *cis*. Since *cnrY* and *cnrX*, together with the promoters *cnrYp* and *cnrHp*, were able to promote an Ni^{2+} -responsive transcription, transcriptional coupling derived from *cnrYp* and *cnrHp* might additionally be necessary for metal-dependent regulation of the *cnr* operon. To test this hypothesis, the *cnrYp-cnrY* region was oriented in the opposite direction to the *cnrX-cnrHp* region (Table 2). Induction studies showed that the transcriptional response was no longer metal inducible (compare pMOL1586 and pMOL1593). These data indicate that both promoters *cnrYp* and *cnrHp* are required in *cis* and must be aligned in the

same direction of transcription for a metal induction response of *cnr*. Therefore, *cnr* induction by nickel (and also cobalt) is achieved by the combined activities of these two promoters. In pMOL1561, point mutations were incorporated in the putative -35 region of *cnrHp*; this changed the CnrX codon usage without disturbing the CnrX protein sequence, while a wild-type copy was present in addition in *trans* on pMOL28. The Lux phenotype became constitutive and dropped to the same level as that with pMOL1593, where transcription is directed only from the *cnrYp* promoter. This suggests the removal of a promoter region, as compared to the case with pMOL1550.

Genetic analysis of the ZinB phenotype. To complete the characterization of mutant phenotypes affected in *cnr* regulation, the genetic basis of the ZinB phenotype (1) was investigated. In strain AE963, nickel and cobalt resistances are constitutive and accompanied by a low-level zinc resistance that is probably due to overexpression of the *cnr* structural resistance proteins (1). Comparison of the *Eco*RI digests of pMOL28 from AE126 and pMOL29 from AE963 showed that in the latter, the *Eco*RI fragment bearing the *cnr* operon was approximately 1 kb larger than in the original pMOL28 (unpublished data). Further restriction analyses showed that an insertion in the *cnrY* gene was responsible for the observed ZinB mutant phenotype. Sequencing analysis revealed the presence of an IS2-type insertion element, which was designated IS1087.

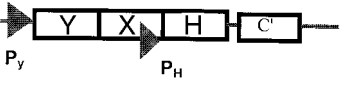
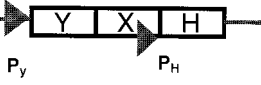
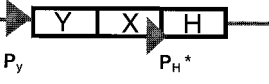
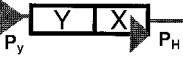
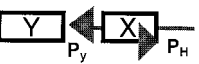
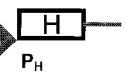

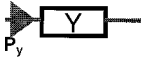


Transcription start sites. The transcription start sites in *cnr* mRNA on induction with nickel were determined by primer extension. As shown in Fig. 6A, extension products were obtained in AE126 with both the *cnrYp*- and *cnrHp*-specific primers. No extension product with the ivrHC primer (corresponding to the presumed *cnrCp* promoter [6]) was observed. The *cnrYp* extension product corresponded to a distance of 133 nucleotides from the site where the pUC18 universal primer hybridized to its template. The deduced transcription start is therefore 5'-T₉₄₉ATAAGAGGCAATGGATCTGGATG (the +1 nucleotide A₉₅₉ is underlined). The *cnrHp* extension product corresponded to a distance of 101 nucleotides from the site where the primer hybridized, with the deduced start site being 5'-T₁₆₈₃CTTATCGATGC (the +1 G₁₆₉₃ is underlined). In AE963 (*cnrY963::IS1087*), no extension product was obtained with either the *cnrYp* or the ivrHC primers, while a strong signal was observed at the same position with the *cnrHp* primer as in AE126. An additional extension product was detected which would correspond to a transcription start 5'-T₁₆₈₃CTTATCGATGCACTACG. Since the *cnrYp* promoter is located upstream of the IS1087 insertion point in *cnrY* (position 1036), this also proves that no promoter from which transcription of the *cnr* structural genes occurs is present on IS1087. Therefore, the constitutive *cnr* expression in AE963 is due to transcription from the *cnrHp* promoter. No signals were obtained with the plasmid-free control strain, AE104.

DISCUSSION

Since the early report on the nucleotide sequence of the *cnr* cobalt and nickel resistance operon of the *R. eutropha*-like strain CH34 by Liesegang et al. (13), the regulation of this resistance determinant has remained a mystery. In addition, the novelty of this regulatory locus, compared to well-characterized systems, at the genetic and protein levels has contributed to the slow advances made. In this paper, the roles of the individual components of the *cnr* regulatory region are examined.

The first determinant examined was *cnrY*, which is immediately preceded by the *cnrYp* promoter. Our findings point to ORFb (13) as being the correct ORF for *cnrY*, and this is

TABLE 2. Probing promoters *cnrYXH*^a

Plasmid	Cloned fragment	Maximum fold induction with nickel ^{b,d}	Transcription	Relative signal strength (%) ^{c,d}
pMOL1551 (1–2418)		1,027 (11)	Inducible	100 (0.7)
pMOL1550 (1–2298)		1,036 (25)	Inducible	100
pMOL1561 (1–2298)		<2	Constitutive	13 (2.0)
pMOL1586 (870–1720)		210 (12)	Inducible	23 (3.5)
pMOL1593 (843–1256), (1256–1720)		<2	Constitutive	15 (3.8)
pMOL1588 (1579–2298)		<2	Constitutive	58 (9.2)
pMOL1591 (1253–1720)		<2	Constitutive	17 (2.8)
pMOL1592 (870–1280)		<2	Constitutive	11 (0.7)
pMOL1583 (870–1000)		<2	Constitutive	13 (2.8)
pMOL1587 (1579–1720)		<2	Constitutive	17 (3.5)
pMOL1596 (2280–2418)	—	<2	Constitutive	0.4 (0.07)
pMOL877		0	Inactive	0.0

^a Transcriptional fusions between different regions of the *cnr* regulatory locus and the *luxCDABE* genes were tested for their metal-responsive activity in vivo in CH34.

^b The metal response to 0.3 mM nickel was quantitated as the fold induction compared to noninduced cells and is expressed as the signal-to-noise ratio. A value of <2 is considered constitutive transcription.

^c Maximum light production (in ALU) divided by the OD_{660nm} of the culture, as compared to the transcription of the complete *cnr* regulatory locus from pMOL1550.

^d Standard deviations are indicated in parentheses and are based on the results from three independent experiments.

further supported by the strong similarity of ORFb with *nccY* (where only one ORF exists). Furthermore, the ATG translation start of the putative protein ORFb is 6 nucleotides from the proposed ribosome-binding site, while no ribosome-binding site for ORFa translation could be identified (13). The CnrY protein is thought to have a downregulating role, based on *cnrY963::IS1087*, displaying a zinc-resistant (ZinB) phenotype. In this mutant, *IS1087* is inserted at position 1036 in *cnrY* and disrupts the CnrY protein close to the N terminus. The resulting phenotype is low-level zinc resistance and elevated constitutive resistance to both cobalt and nickel, suggesting the inactivation of a repressor (1, 13). In primer extension assays with the *cnrYp* primer, no fragment was obtained with mRNA of strain AE963, showing that there is no promoter activity

from *IS1087* in the direction of *cnrXH*. It was not possible to overproduce the CnrY protein in vivo in *E. coli*; this is commonly encountered for negative regulators, which can confer toxicity to host cells on overproduction. CnrY, a 95-amino-acid protein (13) (for the revised sequence, see reference 6), shows no homology to well-characterized proteins in the database, except the homologous NccY. There are no obvious motifs in the primary amino acid structure of CnrY. However, the predicted secondary structure suggests two potential transmembrane domains towards the C terminus of the protein, a feature which was confirmed by use of *phoA* fusions (6). It is therefore possible that CnrY could be a negative regulator acting at the level of the cytoplasmic membrane. There are no obvious DNA-binding motifs in CnrY or obvious operator regions

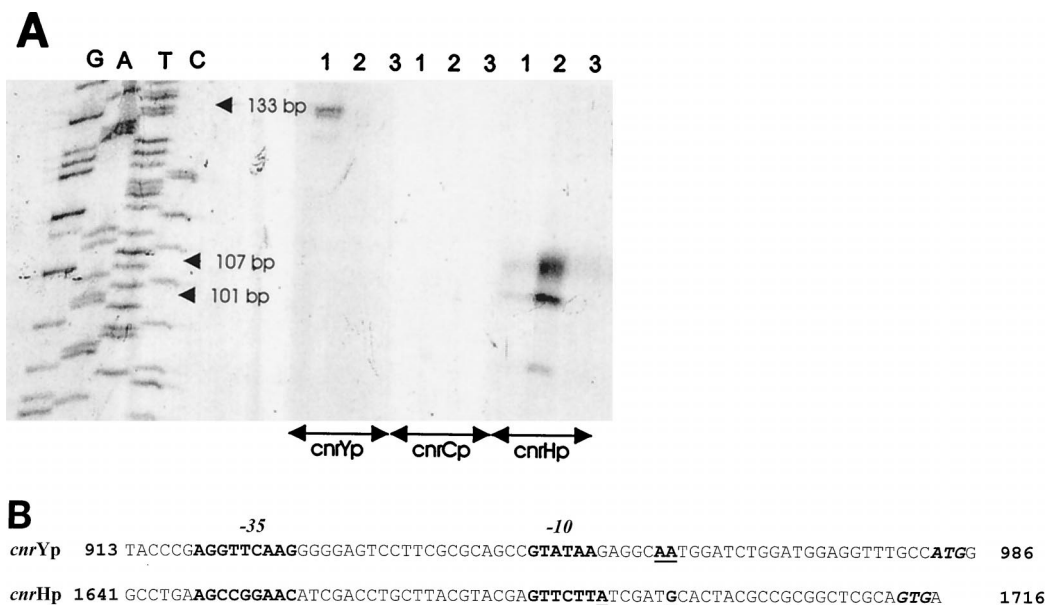


FIG. 6. Transcription start sites in the putative *cnr* promoters. Primer extension was used to determine the start sites of the potential *cnr* promoters for transcription under metal induction conditions (0.4 mM Ni²⁺). (A) A pUC18 DNA sequencing ladder was used as a reference to determine the sizes of the extension products. Lanes 1 to 3, primer extension products for mRNAs of nickel-induced cultures of AE126, AE963, and AE104, respectively. The primers used to identify transcripts from the presumed *cnrYp*, *cnrCp*, and *cnrHp* promoters are indicated under the respective lanes. The sizes of the extension products, calculated with the help of the pUC18 sequencing ladder, are also indicated. (B) Predicted structures of the *cnrYp* and *cnrHp* promoters. Based on the sizes of the primer extension products found in panel A, the transcriptional start sites of *cnrYp* and *cnrHp* were determined. The positions of the -35 and -10 regions (in boldface) and +1 transcriptional start sites (in boldface and underlined) are shown. Sequence numbering is according to Liesegang et al. (13). The translation start sites are indicated in boldface italics.

within *cnrYXH*, and therefore it is difficult to assign the protein a role as a DNA-binding repressor (13). Nevertheless, a putative role as an anti-sigma factor against CnrH can be envisaged, since many ECF sigma factors are controlled by a small, membrane-bound protein which is produced in stoichiometric proportions and which ensures a rapid on-off switch of sigma activity (10, 11). For *cnrY963::IS1087* in AE963, transcription from *cnrYp* is disrupted. Therefore, high-level constitutive transcription in ZinB mutants must be due to transcription from a promoter downstream of *cnrY*, since there are no candidate promoter sequences within *IS1087* in the direction of *cnrXH*, and no transcripts were identified by primer extension studies. In the accompanying study by Grass et al. (6), a similar (but not identical) constitutive, elevated resistance phenotype due to a frameshift mutation in *cnrY* resulting from a gene duplication of 14 bp mediated by an insertion sequence element is described. This supports the hypothesis that high-level transcription of *cnrCBA* is directed from another promoter downstream of *cnrYp* and that it is the combination of a disrupted CnrY protein and uncoupling of *cnrYp* and a downstream promoter which give rise to the constitutive, elevated nickel resistance. The localization of this promoter is discussed below.

CnrH, a 21-kDa protein, belongs to a family of ECF σ^{70} -like proteins that direct the specific transcription of signals generated outside the cytoplasmic boundaries (14). The closest homologs of CnrH are the NcH (65% identity) and RpoE (32% identity) of *E. coli*. As with other σ^{70} -like factors, there is a prominent helix-turn-helix motif at the carboxy terminus of CnrH (14). Purified His-CnrH, with an N-terminal histidine tag, in the presence of core RNA polymerase of *E. coli* could bind DNA targets containing the presumed *cnrYp* and *cnrHp* promoters. The DNA targets were confirmed to display promoter activities in *lux*-based transcription studies, and the corresponding promoter transcription start sites were identified

using primer extension. It is interesting that the two promoter DNA targets have quite different sequences (13). In vitro, the P_Y promoter region was retarded by purified His-CnrH. Therefore, it is expected that in vivo CnrH would also transcribe *cnr* from the *cnrYp* promoter. Interestingly, the *ivrHC* region, whose sequence includes a region similar to the -10 region of *cnrYp*, was not retarded in in vitro DNA-binding assays. Furthermore *cnr-lux* transcriptional fusions incorporating (pMOL1551) and lacking (pMOL1550) this region showed similar *lux* induction kinetics in the presence of Ni²⁺, while an *ivrHC-lux* fusion (pMOL1596) showed a background activity in strain CH34. Also, no promoter in this region could be identified by the primer extension experiments. The activity of *cnrHp* in the presence of metal was confirmed in primer extension assays, which contradicts the findings of Grass et al. (6), who were not able to detect a transcription start site from this region, although a downstream start site was reported originating from the *ivrHC* region (*cnrCp* promoter). Nonetheless, the observation of reverse transcriptase PCR products for the intervening region of *cnrH* and *cnrC*, as described by Grass et al. (6), also further supports a possible *cnrHCBA* transcript. The presumed *cnrHp* sequence resembles closely that of the RpoE promoter consensus and closely fits the common -35 AAC consensus proposed for the ECF family. The location of *cnrHp* within the *cnrX* coding region was confirmed by mutating the -35 AAC sequence without affecting the amino acid sequence of *cnrX*. A *lux* fusion of the mutated *cnrHp* no longer had a metal-inducible *Lux*⁺ phenotype, indicating that the mutation completely inactivated the promoter activity of the P_H region. Thus, the AAC sequence is indeed part of the *cnrHp* promoter. It is not unknown for ECFs to recognize different promoter sequences. Since the assembly of the RNA polymerase holoenzyme involves a complex interaction between the sigma factor, core, and DNA, it is conceivable that CnrH is recruited

by one type of core RNA polymerase to direct transcription from *cnrYp* and by another type for transcription from *cnrHp*.

The role of *cnrX* in regulation has been the most intriguing. Metal resistance in a *CnrX*⁻ mutant was constitutively expressed, without affecting the level of resistance (13). It therefore appears that *CnrX* is not essential for the resistance phenotype (13). However the transcription data suggest that this locus is required for metal-inducible transcription from *cnrYp* and *cnrHp*. It has been postulated that *CnrX* functions as an anti-sigma factor on *CnrH*. However, our results do not support this hypothesis, since the binding of *CnrH* to the *cnrYp* fragment was not affected by the addition of purified His-*CnrX*. The N-terminal domain of *CnrX* is quite hydrophobic and has features of a leader peptide. *CnrX* overproduced in *E. coli* fractionated predominantly in the membrane fractions, although in whole-cell cultures a lower-molecular-weight form was also evident. This would indicate an inefficient processing of a signal sequence. PhoA fusions confirmed that the *CnrX* N terminus would function as a leader sequence. The topological orientation of the *CnrX* C terminus would therefore be periplasmic, which is corroborated by the findings of Grass et al. (6), who found that the *CnrX* C terminus induced high alkaline phosphatase activities in *phoA* reporter fusions. *CnrX* contains six histidine residues, whose spatial arrangement in the primary amino acid sequence is identically conserved in *NccX*. The cluster of these residues together with the numerous glutamate residues may constitute important metal-binding sites in the protein. Therefore, *CnrX* may function as a periplasmic metal-sensing protein, interacting with *CnrY*, which is also localized at the membrane (6).

In conclusion, the *cnr* regulation appears to be effected by the activities of at least two promoters, *cnrYp* and *cnrHp*, whose presence was confirmed by gel retardation studies, primer extension, and transcriptional fusions. The *cnrHp* promoter, located within the *cnrX* coding region, is associated with high-level activity and is therefore likely to promote the high levels of transcription observed in the presence of metal. The *cnrYp* promoter is also transcriptionally active, but with a lower promoter strength than *cnrHp*. *CnrH* recognizes both promoter regions in complex with the core RNA polymerase, and the combined activities of both promoters are necessary for metal-responsive transcription of the *cnr* operon. *ivrHC*, which is not recognized by *CnrH* and seems indispensable for Ni-induced *cnrCBA* transcription, nevertheless seems to be required for optimal transcription when low levels of Co²⁺ are present. *CnrY* might function as a repressor or anti-sigma factor, and *CnrX* might function as a periplasmic sensor. Clearly, further studies on the *in vivo* preferences of the *cnr* promoters in the presence and absence of metal will be required if a model of regulation is to be hypothesized.

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