JOURNAL OF BACTERIOLOGY, Mar. 2000, p. 1390–1398 Vol. 182, No. 5
0021-9193/00/$04.00+0

Regulation of the *cnr* Cobalt and Nickel Resistance Determinant from *Ralstonia* sp. Strain CH34†

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Received 19 July 1999/Accepted 16 November 1999

***Ralstonia* sp. strain CH34 is resistant to nickel and cobalt cations. Resistance is mediated by the *cnr* determinant located on plasmid pMOL28. The *cnr* genes are organized in two clusters, *cnrYXH* and *cnrCBA*. As revealed by reverse transcriptase PCR and primer extension, transcription from these operons is initiated from promoters located upstream of the *cnrY* and *cnrC* genes. These two promoters exhibit conserved sequences at the −10 (CCGTATA) and −35 (CRAGGGGRAG) regions. The CnrH gene product, which is required for expression of both operons, is a sigma factor belonging to the sigma L family, whose activity seems to be governed by the membrane-bound CnrY and CnrX gene products in response to Ni^{2+}. Half-maximal activation from the *cnrCBA* operon was determined by using appropriate *lacZ* gene fusions and was shown to occur at an Ni^{2+} concentration of about 50 μM.**

Ralstonia sp. strain CH34 (formerly *Alcaligenes eutrophus* strain CH34 [3]) contains at least seven determinants encoding resistances to toxic heavy metals, located either on the bacterial chromosome or on one of the two indigenous plasmids pMOL28 (180 kb [37]) and pMOL30 (238 kb [7, 20]). The *cnr* determinant of plasmid pMOL28 mediates inducible resistance to Co^{2+} and Ni^{2+} in *Ralstonia* sp. strain CH34 (15). The *cnr* determinant is similar to *ncc* (nickel-cobalt-cadmium resistance) of *Alcaligenes xylosoxidans* 31A (34) and *czc* (cobalt-zinc-cadmium resistance) on plasmid pMOL30 of *Ralstonia* sp. strain CH34 (28). All three resistances are based on cation efflux, which is best characterized for Czc (13, 24, 29). In analogy to Czc (9, 25, 28, 32), the products of the genes *cnrA*, *cnrB*, and *cnrC* are likely to form a membrane-bound protein complex catalyzing an energy-dependent efflux of Ni^{2+} and Co^{2+}, and the mechanism of action of the CnrCBA complex may be that of a proton/cation antiporter.

Three regulators seem to control Cnr, an extracellular function (ECF) sigma factor (CnrH) and the products of two additional genes with unknown precise functions (CnrX and CnrY products, respectively) (15, 16). The genes *cnrYXH* are located upstream of *cnrCBA* and have the same direction of transcription. Transposon Tn*5* insertion upstream of *cnrH* led to a constitutive expression of nickel resistance and to low zinc resistance as well (4, 15). However, this may be a polar effect and, additionally, a readthrough from a transposon promoter. As shown with Tn*5*-*lacZ* fusions (31), *cnr* is best induced by 128 μM Ni^{2+}. Other metals serve as less efficient inducers; however, this experiment was done with nickel-sensitive *cnr-lacZ* transposon-insertion mutants. In this study an improved *cnrCBA-lacZ* operon fusion was constructed, which mediates full nickel resistance and which was used to evaluate the physiology of *cnr* regulation.

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† This publication is dedicated to Hans G. Schlegel, who started the *cnr* work, on his 75th birthday.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Tris-buffered mineral salts medium (20) containing 2 g of sodium gluconate/liter was used to cultivate *Ralstonia* strains (Table 1). Solid Tris-buffered medium contained 20 g of agar/liter. β-Galactosidase activity in permeabilized cells was determined as published previously (26), with 1 U defined as the activity forming 1 nmol of *o*-nitrophenol per min at 30°C.

Genetic techniques. Standard molecular genetic techniques were used (24, 33). For conjugative gene transfer, overnight cultures of donor strain *Escherichia coli* S17/1 (35) and of the *Ralstonia* recipient strains grown at 30°C in complex medium were mixed (1:1) and plated onto nutrient broth agar. After overnight growth, the bacteria were suspended in saline (9 g of NaCl/liter), diluted, and plated onto selective medium as previously described (24). DNA sequences were obtained with an A.L.F. sequencer (Pharmacia, Uppsala, Sweden). Total RNA of *Ralstonia* strains was isolated as described previously (11, 30).

Reporter protein fusions. The complete *cnrY* and *cnrX* genes were cloned into fusion vector pECD500 (*phoA* fusions) (32) in *E. coli* CC118 (17). Specific activity of alkaline phosphatase (17) was determined in triplicate as described previously (27).

Construction of a Φ(*cnrCBA-lacZ*) transcriptional fusion. To construct the *lacZ* reporter strain DN177(pMOL28-2), the 300 bp upstream of the *cnrA* stop codon were PCR amplified as a *Pst*I-*Xba*I fragment from megaplasmid DNA of *Ralstonia* sp. strain AE126(pMOL28). Similarly, the 300 bp downstream of the *cnrA* stop codon were amplified as an *Xba*I-*Pst*I fragment. These fragments were digested with *Xba*I, but not with *Pst*I, and both fragments were cloned into vector plasmid pGEM T-Easy (Promega, Madison, Wis.) in one step. As confirmed by control sequencing, this led to a plasmid harboring a 600-bp *cnr* fragment with an *Xba*I site located directly downstream of the stop codon of *cnrA*, mutating the sequence $\mathbf{TGA}_{8025}$GTTTGCGA (the TGA stop codon of *cnrA* is in boldface) to **TGA**GTTTCTAGA (numbering is according to reference 15). The promoterless *lacZ* gene of plasmid pMC1871 (Pharmacia, Freiburg, Germany) was inserted into the *Xba*I site of this plasmid, and the fragment containing *cnr-lacZ* was cloned as a *Pst*I fragment into plasmid pLO2 (14). Finally, the pLO2 hybrid plasmid with Φ(*cnrCBA-lacZ*) was used in a double-recombination event to insert the *lacZ* gene downstream of *cnrA* on megaplasmid pMOL28 as described previously (11). The correct insertion and orientation of *lacZ* in strain DN177(pMOL28-2) was verified by PCR and restriction endonuclease digestion (Fig. 1).

Construction of other bacterial strains. The control region *cnrYXH* was deleted from megaplasmid pMOL28-2 as previously described (11), leading to strain DN190(pMOL28-3), which is Φ(*cnrCBA-lacZ*) Δ*cnrYXH* (Fig. 1). Briefly, PCR was used to amplify a 600-bp fragment from plasmid pMOL28. This fragment contains the 300 bp upstream of *cnrY*, including the first 24 bases of this gene (up to position G_{1006}), and the 300 bp downstream of *cnrH* starting from C_{2261}, with the last 24 bases of that gene. Both fragments were joined using a *Mun*I site. With double recombination using a pLO2 derivative, the wild-type fragment on plasmid pMOL28-2 was exchanged for this mutated fragment.

To separate *cnrYXH* from *cnrCBA*, the *cnr* region from bp 2100 to 2400 (15) was PCR amplified and cloned as an *Xba*I-*Pst*I fragment into pLO2 (14). The resulting plasmid, pECD581, was integrated into plasmid pMOL28-2, leading to plasmid pMOL28-5 in strain DN410(pMOL28-5) Ω(2100 bp::pECD581) Φ(*cnrCBA-lacZ*) (Fig. 1). (Note that the region between bp 2100 and 2400 is

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype	Source or reference
A. xylosoxidans 31A	*ncc* determinant	34
E. coli strains		
CC118	*phoA*	17
S17/1	RP4 mobilization system	35
Ralstonia sp. strains		
AE104	No megaplasmid, metal sensitive	20
AE128(pMOL28)	*cnr* on megaplasmid pMOL28	20
DN176(pMOL28-1)	*cnrY1*(Con), constitutive expression of *cnr*	This publication
DN177(pMOL28-2)	Φ(*cnrCBA-lacZ*)	This publication
DN190(pMOL28-3)	Φ(*cnrCBA-lacZ*) Δ*cnrYXH*	This publication
DN195(pMOL28-4)	*cnrY1*(Con) Φ(*cnrCBA-lacZ*)	This publication
DN410(pMOL28-5)	pMOL28-2 Ω(2100 bp::pECD581) Φ(*cnrCBA-lacZ*)	This publication
Plasmids		
pDNA291	*cnrYXH* in pVDZ′2	This publication
pDNA298	Φ(*cnrHp-lacZ*) in pVDZ′2	This publication
pDNA299	Φ(*cnrCp-lacZ*) in pVDZ′2	This publication
pDNA300	Φ(*cnrYp-lacZ*) in pVDZ′2	This publication
pECD500	*phoA* fusion vector	32
pECD581	pLO2 with bp 2100–2400 of *cnrH′C′*	This publication
pGEM T-Easy	PCR cloning vector	Promega
pLO2	Broad-host-range vector for recombination in *Ralstonia*	14
pMC1871	*lacZ* gene without promoter	Pharmacia, Freiburg, Germany
pVDZ′2	IncP1, broad-host-range *Ralstonia* vector	6

duplicated and flanks the integrated vector plasmid pLO2 on megaplasmid pMOL28-5 [Fig. 1].)

When plasmids used for complementation assays were generated, the respective *cnr* genes were amplified from plasmid pMOL28 by PCR (which introduced suitable restriction sites), cloned into pGEM T-Easy (Promega), sequenced, and subcloned into the broad-host-range vector pVDZ′2 (6). To measure the activity of the *cnr* promoters, *cnrYp* (bp 698 to 1284), *cnrHp* (bp 1516 to 1740), and *cnrCp* (bp 2250 to 2360) were fused upstream to a promoterless *lacZ* gene and into plasmid pVDZ′2 in the orientation opposite to that of the *lac* promoter located on this vector plasmid (Table 1).

Primer extension and RT-PCR experiments. Primer extension analysis was performed with a modification of a standard protocol (33) using fluorescein-labeled oligonucleotides and an automated A.L.F. DNA Sequencer (Pharmacia, Uppsala, Sweden) as described previously (11, 39). The fluorescein-labeled 3′ antisense primers (5′-GGCGCAGCAGATGGCACG for *cnrYp* and 5′-GGCT CAACGCAACGGG for *cnrCp*) were complementary to the corresponding gene regions. After phenol-chloroform extraction, the cDNA was precipitated with ethanol, vacuum dried, and suspended in 4 μl of H_2O and 4 μl of A.L.F. stop solution (Pharmacia, Uppsala, Sweden). Following heat denaturation, the sample was loaded on a 7% polyacrylamide sequencing gel. In parallel, a sequencing reaction was performed with the same fluorescein-labeled primer and a DNA fragment containing the respective gene region. The transcription start site was determined by comparison of the retention time of the primer extension reaction with that of the sequencing reaction. Primer extension experiments with total RNA from plasmid-free AE104 cells and a control reaction carried out without reverse transcriptase were used as negative controls. Reverse transcriptase PCR (RT-PCR) was carried out as described previously (11) using various primer pairs (Fig. 1) and a variety of negative control reactions, such as those with RNA isolated from the plasmid-free strain AE104, with no RNA template, and with no reverse transcriptase, as described previously (11).

RESULTS

Structures of the *cnr* mRNAs and the *cnr* promoters. After induction with 300 μM Ni^{2+} (10 min, 30°C, Tris-buffered mineral salts medium), total RNA was isolated from cells of strain AE126(pMOL28). Northern mRNA-DNA hybridization experiments with *cnr*-specific probes did not yield clear signals, probably due to highly unstable *cnr* messages (data not shown). Using RT-PCR, however, signals in the regions of *cnrA* (the RT-PCR product contained bp 7734 to 8028 of *cnr* [Fig. 1]), *cnrC* (bp 2360 to 2610 [Fig. 2]), and *cnrH* (bp 2086 to 2393 and 1934 to 2393 [Fig. 1]) indicated the presence of transcripts. Additionally, RT-PCR revealed continuous transcripts from *cnrA* into the incomplete *orf104ff* following *cnrA* (bp 7736 to 8333) but no continuous transcript upstream of *cnrY* (bp 698 to 1059 and 698 to 1204 [Fig. 1]). There were no signals in the negative controls (RNA from the plasmid-free strain AE104, given for *cnrC* in Fig. 2, lane B).

The start points of the *cnrYXH* and the *cnrCBA* mRNAs were studied with primer extension (Fig. 3). Both mRNAs started about 20 bp upstream of the respective ATG start codons (Fig. 4). Regions which were highly conserved between *cnrYp* and *cnrCp* as well as between the respective promoters of the nickel-cobalt-cadmium resistance determinant, *nccYp* and *nccCp*, were found upstream of these start sites. In contrast, no signal was found when a primer extension experiment was done with a primer located in the *cnrH* gene (Fig. 3).

Activities of the *cnr* promoters *cnrYp* and *cnrCp*. The *lacZ* gene was cloned together with the respective *cnr* promoter regions upstream into plasmid pVDZ′2 (6), leading to plasmid pDNA300 Φ(*cnrYp-lacZ*), pDNA299 Φ(*cnrCp-lacZ*), or pDNA298 Φ(*cnrHp-lacZ*). The last plasmid contained a possible ECF sigma factor recognition site upstream of *cnrH*, but no nickel induction of β-galactosidase activity could be observed in strain AE126(pMOL28) with plasmid pDNA298 Φ(*cnrHp-lacZ*) in *trans* (Table 2; Fig. 5). None of the three plasmids expressed nickel-inducible β-galactosidase activity in the megaplasmid-free strain AE104 (data not shown). However, the plasmids with the *cnrYp* and *cnrCp* promoters were nickel inducible in AE126(pMOL28) (Fig. 5). The activity of *cnrYp* was about twofold that of *cnrCp*, and there was also some induction when the cells started to grow in fresh medium without nickel (Table 2).

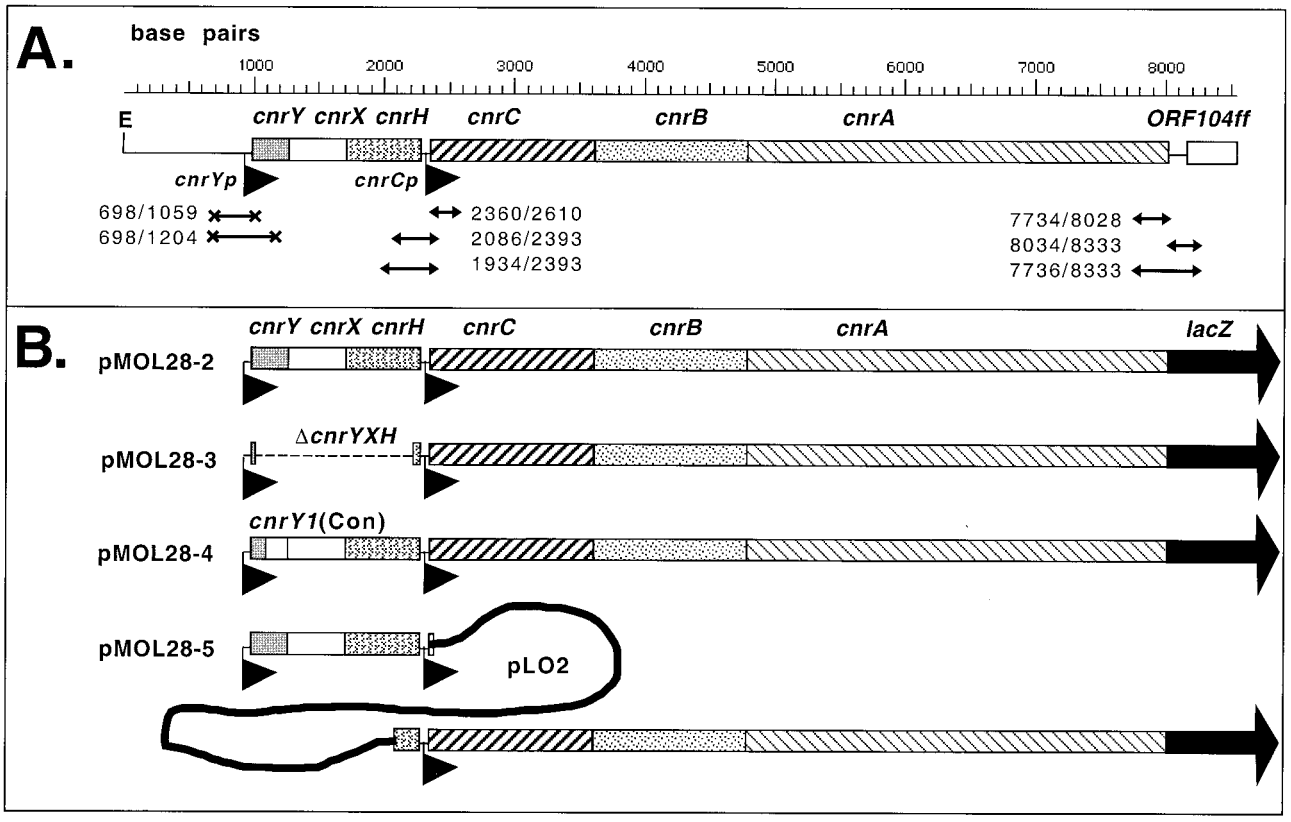

FIG. 1. RT-PCR experiments and physical map of the *cnr* region. (A) Physical map of the *cnr* region, with rectangles indicating the genes. The size marker is in base pairs (15). Triangles mark the two promoters *cnrYp* and *cnrCp*; double-headed arrows indicate positive RT-PCR results, with the 5′ positions of the primer pair given adjacently in base pairs. The crossed arrows indicate negative RT-PCR results, with the primer positions given on the left. E, *Eco*RI restriction site between *cnr* and the adjacent chromate resistance region *chr* (22, 23), which would be left of *cnr* in this figure. (B) Physical map of derivatives of the *cnr* region on plasmid pMOL28. In all plasmids, the *lacZ* gene (large black arrows) was inserted between *cnrA* and *orf104ff*. In plasmid pMOL28-3, *cnrYXH* were additionally deleted in frame. In plasmid pMOL28-4, the *cnrY1*(Con) gene carries a 14-bp insertion after position 1111; the open reading frame continues out of frame thereafter (white box following the truncated *cnrY* symbol). This *cnrY1* frameshift mutation leads to constitutive expression of Cnr. In plasmid pMOL28-5, insertion of plasmid pECD581 leads to a separation of *cnrYXH* and *cnrCBA*. The vector plasmid of pECD581, pLO2 (14), is shown as a thick line.

Induction of a Φ(*cnrCBA-lacZ*) operon fusion by heavy metal cations. To study induction of *cnr* in a fully nickel-resistant bacterial cell, the *lacZ* gene was inserted immediately downstream of *cnrCBA* on plasmid pMOL28, leading to *lacZ* reporter strain DN177(pMOL28-2). The metal resistance of this strain was identical to the resistance of AE126(pMOL28) as shown by determination of the MICs of Ni^{2+} and Co^{2+} on agar plates (Table 3) and in liquid medium (data not shown).

Nickel (0.5 mM Ni^{2+}) was the best inducer of β-galactosidase activity in DN177(pMOL28-2). Co^{2+} (0.5 mM), Zn^{2+} (0.3 mM), and chromate (0.1 mM) induced only slightly (Fig. 6); due to the toxic effects on AE126 strains, zinc and chromate concentrations lower than the nickel concentration had to be used for induction. The increase in β-galactosidase activity after nickel induction was linear for at least 4 h. At up to 0.5 mM Ni^{2+}, the slope of this increase was a function of the nickel concentration (data not shown). The increase in β-galactosidase activity could be described using saturation kinetics in a Lineweaver-Burk plot (data not shown). The regression coefficient was 1.00, the maximum increase in β-galactosidase activity after nickel induction was 94.8 U/h · mg (dry weight), and the nickel concentration required for half-maximal induction was 49 μM. Induction of Φ(*cnr-lacZ*) expression with 2 mM Ni^{2+}, however, was significantly lower than induction with all of the other Ni^{2+} concentrations used, probably due to the toxic effect of a nickel concentration close to the MIC.

The *chrH′C′* region (bp 2100 to 2400 [Fig. 1]) was cloned into plasmid pLO2 (14), leading to plasmid pECD581. Integration of this plasmid into plasmid pMOL28-2 led to segregation of the *cnrCBA* operon from the *cnrYXH* operon on the

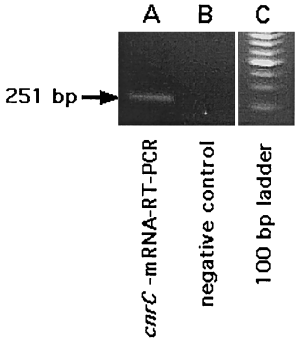

FIG. 2. RT-PCR of the *cnrC* region. After induction with 300 μM Ni^{2+}, total RNA was isolated from cells of strain AE126(pMOL28). RT-PCR experiments were performed, and the products were separated on an agarose gel which was stained with ethidium bromide. Lane A, *cnrC* mRNA RT-PCR product (positions 2360 to 2610 [Fig. 1]); lanes B, respective negative control (RNA from the plasmid-free strain AE104); lane C, 100-bp ladder starting at bp 200. The original photograph was scanned using Ofoto 2.0 (Light Source Computer Images, Inc.) and processed using Photoshop 3.0 (Adobe Systems, Inc.).

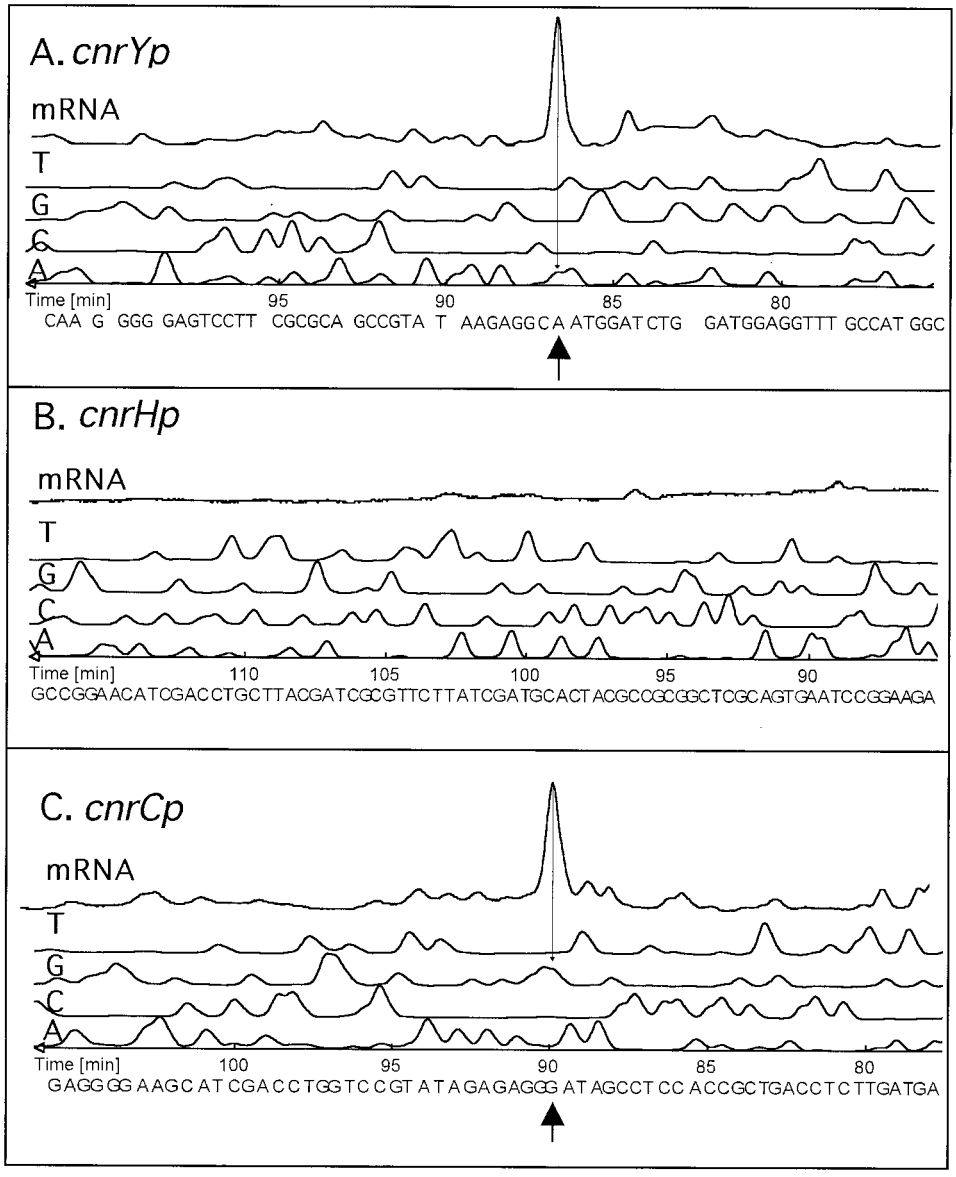

FIG. 3. Primer extension experiments. After induction with 300 μM Ni^{2+}, total RNA was isolated from cells of strain AE126(pMOL28). Primer extension analyses were performed on an automated fluorescent DNA sequencer using this total RNA and fluorescently labeled oligonucleotides as primers (mRNA traces). The primers were located in the 5′ end of *cnrY* (A), *cnrH* (B), or *cnrC* (C). Dideoxy sequencing was run as a size marker with the same primer. The raw data output is shown below the retention time (in minutes), with traces A, C, G, and T representing the successively detected dideoxy nucleotide sequencing reaction products. This sequence corresponds to bp 924 to 987 (A), 1653 to 1726 (B), and 2302 to 2364 (C) (base numbering is according to reference 15). The arrows give the initiation sites for *cnrY* and *cnrC*.

resulting plasmid, pMOL28-5 (Fig. 1). The β-galactosidase level before induction was slightly higher in DN410(pMOL28-5) (103 U/mg [dry weight]) than in the control DN177 (pMOL28-2) (64 U/mg [dry weight]), probably due to some initiation of transcription from unknown promoters located on plasmid pLO2, e.g., that of the kanamycin resistance gene. After induction with 0.5 mM Ni^{2+}, the increase in the β-galactosidase activity of Φ(*cnrCBA-lacZ*) in pMOL28-5 was 90.7 U/h · mg (dry weight), which was similar to the control value with plasmid pMOL28-2 (86.4 U/h · mg [dry weight] [Table 2]). Thus, the activity of *cnrCp* is sufficient to explain the observed induction of Φ(*cnrCBA-lacZ*) by nickel.

Deletion of *cnrYXH* almost abolishes *cnr* induction by nickel. To study the influence of *cnrXYH* on *cnr* induction, these three genes were deleted from pMOL28-2, leading to strain DN190(pMOL28-3) Φ(*cnrCBA-lacZ*) Δ*cnrYXH* (Fig. 1). Both promoters, *cnrYp* and *cnrCp*, were still present on plasmid pMOL28-3. Strain DN190(pMOL28-3) showed a drastic reduction in nickel resistance when compared to strain DN177(pMOL28-2) and to strain AE126(pMOL28); however, DN190(pMOL28-3) was slightly more nickel resistant than the plasmid-free strain AE104 (Table 3).

There was some slight induction of β-galactosidase activity in the Δ*cnrYXH* deletion strain DN190(pMOL28-3) (Fig. 7) by 0.5 mM Ni^{2+}, a level similar to the induction level reached by DN177(pMOL28-2) after induction with low concentrations of Co^{2+} (Fig. 6). To test if cobalt resistance encoded by *cnr* is limited by insufficient induction of *cnrCBA*, strain DN177

A. Structure of *cnr* promoters.

```
                        -35                          -10      +1          RBS
cnrYp:  tctacccgaggttcaaggggagtccttcgcgcagccgtataagaggcAatggatctggatggaggtttgccatg
cnrCp:  agtttggattcaccgaggggaagcatcgacctggtccgtatagagaggGatagcctccaccgctgacctcttgatg
consensus cnr:          craggggrag            ccgtata      Rat
```

B. Predicted structure of *ncc* promoters.

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nccYp:  gtcaaaaaatgcgaggggacg-tgctcgggcgacgtatgaataatgtcccgcaagtcagataggagatctgatcatg
nccCp:tttctccttttttcgaggggagtccatgtgggcgccgtattgaaagctgaagcaaacattttccggtctcacccttttcgtccgatg
consensus ncc:      cgagggggas            ccgtatwr

consensus cnr/ncc:  cgagggggag            ccgtatn
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C. Other ECF promoters:

```
sigWp:      aaaattgaaaccttttgaaacgaagctcgtatacatacaGAcc
MtP2:       gcgctggaaataacccgctggcgaacacggttgattggttC
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FIG. 4. Start sites of *cnrYp* and *cnrCp*. (A) The transcriptional start sites of the *cnrY*- and the *cnrC*-specific mRNAs were determined by primer extension analysis and are given in capital boldface letters (A and G, respectively). These are the results of several experiments. The **a** following those two sites indicates the one nonreproducible result. The predicted −35 region cragggrag and the predicted −10 region ccgtata are in boldface (consensus sequences are below), and the predicted ribosome binding sites upstream of the ATG codons of both genes are underlined. (B) Conserved sequences upstream of *nccY* and *nccC* (34) which might be NccH-dependent promoters. For optimal homology between CnrC and NccC, a start site of $A_{1701}TG$ (190 bp downstream of the 3′ end of *cnrH*) instead of $G_{1464}TG$ (in the *cnrH* gene) for *nccC* has been assumed. Both proteins have now a size of 418 aa. (C) ECF promoters *sigWp* from *Bacillus subtilis* (12) and MtP2 from *Mycobacterium smegmatis* (8).

(pMOL28-2) was incubated in the presence of Co^{2+} and inducing concentrations of Ni^{2+} (Table 3), and indeed, the MIC of cobalt increased when Ni^{2+} induced Cnr. The MICs of Zn^{2+} and Cd^{2+}, however, did not change with Ni^{2+} induction (data not shown).

To demonstrate in *trans* complementation of the *cnrYXH* deletion, *cnrYXH* were PCR cloned into plasmid pVDZ′2 (6), leading to plasmid pDNA291. Strain DN190(pMOL28-3, pDNA291) was fully resistant to Ni^{2+} (Table 3), and nickel was again able to induce the Φ(*cnrCBA-lacZ*) fusion in this strain (Fig. 7); however, the time-dependent increase in β-galactosidase activity was no longer linear. Thus, a strain carrying a deletion of *cnrYXH* had lost the nickel-specific induction of Cnr, and this mutation could be complemented in *trans*.

A variety of DN190(pMOL28-3) derivatives were constructed which contained all combinations of *cnrY*, *cnrX*, and *cnrH* in *trans* on the pVDZ′2 vector (6) (Table 2). In these strains, any complementing plasmid harboring *cnrH* (*cnrH* alone or the combination *cnrXH* or *cnrYH*) led to expression of β-galactosidase activity at a high constitutive level. Complementation with *cnrYH* yielded the highest levels of *cnrCBA-lacZ* expression observed in all experiments. All strains without *cnrH* (*cnrX* alone, *cnrY*, or *cnrYX*) were not inducible and remained at a constitutive low level of β-galactosidase expression. Thus, CnrH alone is able to activate *cnr* expression, and both CnrY and CnrX are needed for nickel control of CnrH.

Constitutive expression of *cnr*. To learn more about the interaction of the Cnr regulators, a mutant of strain AE126(pMOL28) which expressed Cnr constitutively was isolated. This strain, DN176(pMOL28-1), was isolated by a published procedure as a AE126 derivative able to grow in the presence of 1 mM Zn^{2+} (4), leading to DN176(pMOL28-1) *cnrY1*(Con). Into pMOL28-1, *lacZ* was inserted downstream of *cnrCBA*, leading to DN195(pMOL28-4) *cnrY1*(Con) Φ(*cnrCBA-lacZ*). Strain DN195(pMOL28-4) was resistant to a higher level of Ni^{2+} than DN177(pMOL28-2) and reached the same level of Co^{2+} resistance as nickel-induced cells of DN177 (pMOL28-2) (Table 3).

The Φ(*cnrCBA-lacZ*) operon was expressed at a high constitutive level in DN195(pMOL28-4) (Table 2). Again, nickel induced Φ(*cnrCBA-lacZ*) when *cnrYXH* was supplied in *trans* in strain DN195(pMOL28-4, pDNA191) (Table 2). However, this strain did not loose its higher metal resistance (Table 3). The *cnrY1*(Con) *cnrXH* region, PCR cloned from plasmid pMOL28-4, led to constitutive expression of β-galactosidase when located in *trans* to Δ*cnrYXH* in strain DN190 (pMOL28-3) (Table 2). Thus, the mutation leading to the constitutive phenotype must be located in one of the three regulator genes. When strain DN195(pMOL28-4) was complemented with single regulator genes or combinations thereof, *cnrX*, *cnrH*, or *cnrXH* did not restore nickel regulation of *cnr*, but *cnrY* did (Table 2). However, for a full complementation, *cnrH* and *cnrX* had to be present; *cnrYX* repressed induction of *cnr* but did not yield inducibility, while *cnrYH* did not complement at all.

DNA sequence analysis (data not shown) indicated an insertion of the sequence CGCGACGCGTCGCGCGC at position 1111 of the *cnr* sequence (15). Moreover, the wild-type

TABLE 2. Expression of *cnrCBA-lacZ* in various AE126 mutant strains[a]

Megaplasmid	In *trans*	0 mM Ni^{2+}		0.5 mM Ni^{2+}	
		β-Galactosidase activity[b] before induction	Increase in β-galactosidase activity[c]	β-Galactosidase activity before induction	Increase in β-galactosidase activity
pMOL28[d]	Φ(*cnrHp-lacZ*)	11.1 ± 3.7	0	10.8 ± 8.6	0
	Φ(*cnrYp-lacZ*)	6.43 ± 1.49	8.76	0.06 ± 0.01	39
	Φ(*cnrCp-lacZ*)	0.87 ± 0.52	1.26	0	15
pMOL28-2, Φ(*cnrCBA-lacZ*)	None	62.2 ± 6.8	0	64 ± 5	86.4
	nccYXH	40.0 ± 4.5	0	46.1 ± 4.7	0
pMOL28-5[e] Φ(*cnrCBA-lacZ*) Ω(*cnrHC*::pECD581)	None	121 ± 5	0	102.6 ± 0.4	90.7
pMOL28-3, Φ(*cnrCBA-lacZ*) Δ*cnrYXH*	None	28.1 ± 2.5	0	26.6 ± 1.0	0
	cnrYXH	36.0 ± 5.1	0	48.5 ± 1.8	24.8
	cnrY	39.9 ± 3.3	0	33.2 ± 3.3	0
	cnrX	34.6 ± 6.0	0	36.7 ± 3.7	0
	cnrH	937 ± 106	0	770 ± 52	0
	cnrYX	31.6 ± 3.6	0	35.0 ± 4.8	0
	cnrYH	1,650 ± 93	0	1,590 ± 140	0
	cnrXH	900 ± 195	0	1,025 ± 320	0
	cnrY1(Con) *cnrXH*	1,350 ± 60	0	1,380 ± 97	0
	nccYXH	39.9 ± 4.2	0	47.9 ± 1.3	0
pMOL28-4, Φ(*cnrCBA-lacZ*) *cnrY1*(Con)	None	1,025 ± 107	0	1,095 ± 88	0
	cnrYXH	63.1 ± 5.9	18.4	61.3 ± 1.2	44.5
	cnrY	114 ± 4	0	97 ± 2	26.0
	cnrX	687 ± 53	0	896 ± 19	0
	cnrH	678 ± 97	0	785 ± 64	0
	cnrYX	151 ± 9	0	155 ± 19	0
	cnrYH	1,010 ± 68	0	1,050 ± 176	0
	cnrXH	1,226 ± 254	0	1,190 ± 176	0
	cnrY1(Con) *cnrXH*	1,180 ± 55	0	1,450 ± 123	0
	nccYXH	53.0 ± 6.3	0	59.1 ± 5.3	0

[a] Ni^{2+} (0.5 or 0 mM) was added to cells of various *Ralstonia* strains which were growing in the early exponential phase (0 h). The β-galactosidase activity was determined for at least 3 h and plotted against time.
[b] Means ± standard deviations. Values are units per milligram (dry weight). If no increase occurred, the mean values of all data points of the respective experiment are given.
[c] Values are units per hour per milligram (dry weight).
[d] The *lacZ* gene is under control of a *cnr* promoter on a pVDZ′2 plasmid complementing in *trans*.
[e] Carries an insertion of pLO2 flanked by the duplicated region at bp 2100 to 2400. This separates *cnrYXH* from *cnrCBA* (Fig. 1).

sequence as published (15) has to be corrected. The published sequence is 1108-CGCCGCCGC-1117, but here the sequence was determined to be only CGCCGC. The sequence of the insertion in the *cnrY1*(Con) mutant gene contains a nearly complete duplication of the sequence CGCGACGCGTgGCG tGC (nonidentical base pairs are shown in lowercase) located directly downstream of the 14-bp insertion. Although the site of this putative target duplication is different from that of the insertion of IS*1087* reported in the accompanying study (38), this duplication is a strong hint that an insertion sequence element was also responsible for the *cnrY1*(Con) mutation in strain DN195(pMOL28-4). The mutation leads to a frameshift resulting in the expression of a 134-amino-acid (aa) mutant protein which is identical in its amino-terminal 46 aa to CnrY but continues in another reading frame thereafter (15, 38). In contrast to CnrY, the mutant protein does not contain a possible transmembrane α-helix.

The carboxy-terminal parts of CnrY and CnrX are located in the periplasm. With *phoA* fusions of the genes *cnrY* and *cnrX*, specific activities of 32.4 ± 3.3 U/mg (dry weight) for *cnrY* and of 57.6 ± 2.2 U/mg (dry weight) for *cnrX* were determined. As a control, the leader sequence of the β-lactamase gene of plasmid pUC19 (40) was cloned upstream of the *phoA* gene in plasmid pECD500 (32; T. Pribyl and D. H. Nies, unpublished data), which leads to a specific PhoA activity of 41.6 ± 7.6 U/mg (dry weight). When the leader sequence of the β-lactamase gene was deleted (Pribyl and Nies, unpublished data), the specific activity decreased to 1.4 ± 1.3 U/mg (dry weight) (all data are triplicate determinations done twice independently and are not shown). Thus, since the complete genes were cloned into plasmid pECD500, the carboxy termini of both proteins are located in the periplasm.

Interaction between NccYXH and CnrYXH. To study a possible interaction between *cnr* and the highly related nickel-cobalt-cadmium resistance determinant *ncc* (34), the regulatory regions *nccYXH* and *nccN* were PCR cloned from *A. xylosoxidans* 31A into pVDZ′2 (6). In the Δ*cnrYXH* strain DN190(pMOL28-3), expression of *nccYXH* in *trans* did not mediate nickel-inducible expression of *cnr* (Table 2). Thus, NccYXH did not activate *cnr* promoters. NccN did not have any influence on nickel resistance in DN177(pMOL28-2) (data not shown).

Surprisingly, when *nccYXH* were expressed in *trans* in DN177(pMOL28-2), almost no induction of *cnr* by nickel was observed (Table 2). The same down-regulation was even obtained with the constitutive mutant strain DN195(pMOL28-4). Thus, the Ncc regulators are able to prevent induction of Cnr by its own regulators.

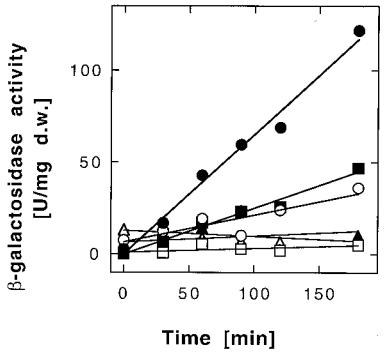

FIG. 5. Induction of β-galactosidase activity with *cnr* promoters upstream of *lacZ*. Cells of *Ralstonia* sp. strain AE126(pMOL28) with plasmid pDNA300 Φ(*cnrYp-lacZ*) (● and ○), pDNA299 Φ(*cnrCp-lacZ*) (■ and □), or pDNA298 Φ(*cnrHp-lacZ*) (38) (▲ and △) were diluted 15-fold to a cell density of 30 Klett units into fresh medium containing no added heavy metal (○, □, and △) or with 0.5 mM Ni^{2+} (●, ■, and ▲) added after 4 h. Incubation was continued with shaking at 30°C, and the specific β-galactosidase activity was measured. d.w., dry weight.

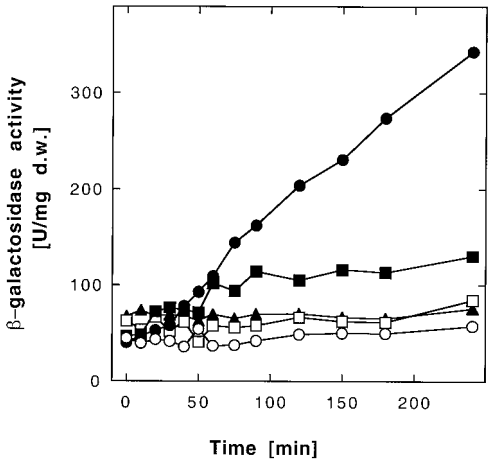

FIG. 6. Induction of β-galactosidase activity in a *cnrCBA-lacZ* strain. Cells of *Ralstonia* sp. strain DN177(pMOL28-2) containing a Φ(*cnrCBA-lacZ*) operon fusion on plasmid pMOL28-2 were diluted 15-fold to a cell density of 30 Klett units into fresh medium containing no added heavy metal (○) or were induced after 4 h with 0.5 mM Ni^{2+} (●), 0.5 mM Co^{2+} (■), 0.3 mM Zn^{2+} (□), or 0.1 mM potassium chromate (▲). Incubation was continued with shaking at 30°C, and the β-galactosidase activity was determined. d.w., dry weight.

DISCUSSION

The *cnr* and the *chr* resistance determinants on megaplasmid pMOL28 of *Ralstonia* sp. strain CH34 might be required for survival against combined nickel, cobalt, and chromate toxicities, e.g., in serpentine-like soils (1; S. Juhnke, N. Peitzsch, and D. H. Nies, unpublished data). The *cnr* determinant is composed of at least six genes, encoding products with regulatory functions (*cnrY*, *cnrX*, and *cnrH*) or the subunits of the Co^{2+}/Ni^{2+} efflux pump (*cnrC*, *cnrB*, and *cnrA*). Downstream of *cnrA* (Fig. 1) starts an open reading frame (*orf104ff*) which is not complete in the published DNA sequence (15). The predicted 104-aa product shows homology to MTH841, a 343-aa transporter-like protein from *Methanobacterium thermoautotrophicum* with unknown function (36). It is not clear if *orf104ff*, which was not disturbed by the insertion of *lacZ* downstream of *cnrA* in plasmid pMOL28-2, is involved in nickel resistance. It was not essential in the initial cloning experiments (15), but transcription from *cnrCBA* may continue into this open reading frame (Fig. 1).

Translation of *cnrYXH* and translation of *cnrCBA* seem to be closely coupled. The stop codons of the respective upstream genes overlap with the start codons of the following genes: ATGATGA for *cnrYX*, GTGA for *cnrXH*, ATGATGA for *cnrCB*, and ATGA for *cnrBA*. A comparable tight translational coupling has been shown to be important for regulation of other ECF sigma factor-controlled operons, e.g., the *car* operon from *Myxococcus xanthus* (10). This fact, the positions of the two identified promoters *cnrYp* and *cnrCp*, and the RT-PCR experiments indicate two possible tricistronic mRNAs as transcripts of *cnrYXH* and *cnrCBA*.

Deletion and complementation results indicate that CnrY, CnrX, and CnrH are essential as well as sufficient for *cnr* regulation, which is based on regulation of transcription. High-level constitutive expression of *cnr* was observed when CnrH was present in *Ralstonia* cells but CnrY and CnrX were not. Thus, nickel-dependent regulation of *cnr* depends on the presence of CnrY and CnrX. These data fit the fact that CnrH is a sigma factor (16) of the ECF family. In many examples from gram-negative and gram-positive bacteria (5, 10, 12, 18, 21), ECF sigma factors control operons encoding products which deal with environmental stimuli. Most of these ECF sigma factors are regulated by membrane-bound anti-sigma factors. If a stress condition is sensed by this anti-sigma factor, which might interact with a sensing protein, the sigma factor is released and is free to initiate transcription.

TABLE 3. MICs of nickel and cobalt in various derivatives of *Ralstonia* sp. strain AE126(pMOL28)

Bacterial strain	Relevant genotype	MIC (mM)[a]	
		Ni^{2+}	Co^{2+}
AE126(pMOL28)	Wild type	4.0	5.0
AE104	Plasmid-free control	0.3	0.3
DN177(pMOL28-2)	Φ(*cnrCBA-lacZ*)	4.0	5.0
DN177(pMOL28-2)[b]	Φ(*cnrCBA-lacZ*)		10
DN177(pMOL28-2, pDNA291)	Φ(*cnrCBA-lacZ*), *cnrYXH* in *trans*	5.0	3.0
DN190(pMOL28-3)	Δ*cnrYXH* Φ(*cnrCBA-lacZ*)	0.4	0.3
DN190(pMOL28-3, pDNA291)	Δ*cnrYXH*, *cnrYXH* in *trans*	4.0	5.0
DN195(pMOL28-4)	*cnrY1*(Con)	7.0	10
DN195(pMOL28-4, pDNA291)	*cnrY1*(Con), *cnrYXH* in *trans*	7.0	10

[a] The MIC is defined as the minimal concentration of heavy metal cation inhibiting growth at 30°C for 3 days. Occurrence of single colonies was not counted as growth. Each determination was repeated twice with identical results.

[b] Cultivated in the presence of cobalt plus 1 mM Ni^{2+} for induction.

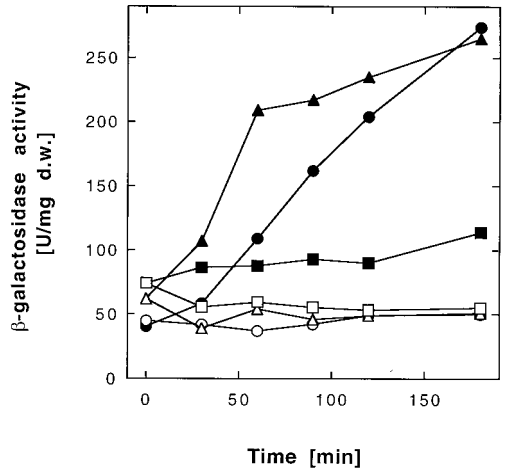

FIG. 7. Induction of β-galactosidase activity in a *cnrCBA-lacZ* Δ*cnrYXH* strain. Cells of *Ralstonia* sp. strain DN177(pMOL28-2) containing a Φ(*cnrCBA-lacZ*) operon fusion on plasmid pMOL28-2 (● and ○), of strain DN190(pMOL28-3) with an additional deletion of the *cnrYXH* regulatory genes (■ and □), and of strain DN190(pMOL28-3, pDNA291) with *cnrYXH* supplied in *trans* to this deletion (▲ and △) were diluted 15-fold to a cell density of 30 Klett units into fresh medium containing no added heavy metal (○, □, and △) or were induced after 4 h with 0.5 mM Ni^{2+} (●, ■, and ▲). Incubation was continued with shaking at 30°C, and the β-galactosidase activity was measured.

The PhoA data clearly demonstrate that the carboxy termini of CnrX and CnrY are in the periplasm. CnrH is indeed a sigma factor as shown by gel retardation assays (38) and runoff transcription (G. Grass, S. Kühnemund, and D. H. Nies, unpublished data). Thus, it is possible that CnrH is controlled by a CnrYX transmembrane anti-sigma factor complex which binds CnrH in the absence of Ni^{2+}. If Ni^{2+} appears in the periplasm, it may be bound by CnrX; the signal then would be transmitted by CnrY into the cytoplasm and CnrH would be released. Since periplasmic nickel is probably the inducer of Cnr, this explains why induction of Cnr, as judged by the increase in β-galactosidase activity, continues for at least 4 h, although the CnrCBA complex, which detoxifies the cytoplasm, should have been synthesized in the meantime.

The MIC for the *cnr*-free derivatives of *Ralstonia* sp. is 300 μM Ni^{2+}. Thus, with half-maximum induction of *cnr* at about 50 μM, *cnr* is strongly expressed at toxic Ni^{2+} concentrations. On the other hand, Ni^{2+} is an essential trace element for *Ralstonia*, at least required for synthesis of hydrogenases (2, 20) at a concentration of about 10 nM. At this concentration, synthesis of CnrCBA-LacZ is slower than dilution of these proteins by cell doubling. Thus, regulation of Cnr guarantees a sufficient supply of Ni^{2+} as a trace element and at the same time efficient detoxification at higher concentrations.

The Ncc system mediates resistance to Ni^{2+}, Co^{2+}, and Cd^{2+} and is composed of seven proteins. Three are the subunits of the efflux pump, NccCBA, and three are the regulators NccY, NccX, and NccH. NccN is related to CzcN; however, no function has been assigned to these proteins yet (11, 34). Homology between the Cnr and the Ncc proteins indicates that *ncc* is also regulated by an NccYX sensory complex and the ECF sigma factor NccH. Upstream of *nccY* and *nccC*, sequences with strong similarity to the consensus sequences of the CnrH-dependent promoters (Fig. 4B) were found. The common consensus motifs for both systems are CGAGGGG GAG (−35) and CCGTAT (−10). The −35 motifs lack an AAC motif, which was proposed to be a consensus motif for all EFC sigma factors (19). In other ECF sigma factor-dependent promoters (Fig. 4, MtP2), however, AAC is also lacking.

Despite the similarity between the two systems, the Ncc regulators were not able to complement a Δ*cnrYXH* mutant. On the contrary, NccYXH repressed induction of Cnr by Ni^{2+}, even in the constitutive mutant. One possible explanation could be complete sequestration of NccH and, if present, CnrH by a putative NccYX complex and no release of a sigma factor in the presence of Ni^{2+}. However, a better explanation of the tight repression observed is that NccH-RNA polymerase holoenzyme might form tight closed complexes at the *cnr* promoters but is not able to convert into the open complex for transcription initiation. This explanation fits the differences between the conserved motifs of the *cnr* and the *ncc* promoters; however, further studies are required to solve this question.

The assumption of the existence of the two Cnr promoters *cnrYp* and *cnrCp* is based on (i) the homology between the *cnrY-nccY* and the *cnrC-nccC* upstream regions (Fig. 4), (ii) primer extension data (Fig. 4), (iii) induction of Φ(*cnrYp-lacZ*) and Φ(*cnrCp-lacZ*) constructs by nickel (Fig. 5), and (iv) induction of Φ(*cnrCBA-lacZ*) by nickel under conditions when *cnrYXH* and Φ(*cnrCBA-lacZ*) were separated by insertion of plasmid pECD581 (Table 2). We found only evidence for the absence of the proposed promoter *cnrHp* (38); there were no results in the primer extension experiments (Fig. 3), no induction of Φ(*cnrHp-lacZ*) by nickel (Table 2), and no similarity between the upstream regions of *cnrH* and *nccH* with respect to an ECF promoter motif. The assumption of *cnrHp* is based on binding of the CnrH-containing RNA polymerase holoenzyme to a DNA sequence upstream of *cnrH* and on the loss of induction in a strain carrying a mutation in the *cnrHp* promoter region (38). However, mutation of the *cnrHp* promoter region may have changed the activity or expression level of CnrX, which would also explain the observed loss of induction. Binding of the CnrH-RNA polymerase to a region upstream of *cnrH* may be another element of Cnr regulation; e.g., it may be required to prevent too-high expression levels of CnrH.

Since the Φ(*cnrCBA-lacZ*) fusion located on plasmid pMOL28-5 can still be induced by nickel but *cnrYXH* and *cnrCBA* are separated by the insertion of plasmid pECD581 into plasmid pMOL28-5, *cnrHp* has no influence on the expression of Φ(*cnrCBA-lacZ*). Moreover, the reporter system used in this study is an insertion of *lacZ* directly downstream of *cnrA*. Thus, expression of *cnrCBA* was studied in a fully metal-resistant bacterial strain and with a *cnrCBA* copy number identical to the copy number in the wild-type situation. In contrast, the accompanying study has used the luciferase reporter system situated on vector plasmids which probably were present in higher copy numbers. Although both studies agree on the existence of promoter *cnrYp*, no induction of Φ(*cnrYp-lux*) was observed (38). Thus, the fact that no induction was measured with Φ(*cnrHp-lux*) and Φ(*cnrCp-lux*) is no evidence against the existence of *cnrHp* or *cnrCp*.

Despite these differences, both studies outline the following model of Cnr regulation. A periplasmic protein complex composed of CnrX and the carboxy-terminal part of CnrY senses nickel. This information is probably transmitted by the transmembrane protein CnrY to the ECF sigma factor CnrH, perhaps by release of CnrH from the amino-terminal part of CnrY when nickel is bound to CnrX. CnrH binds to RNA polymerase core, and transcription is initiated from the *cnrYp* promoter and at least a second promoter which is located upstream or downstream of *cnrH*.

ACKNOWLEDGMENTS

We thank Grit Schleuder and Ute Lindenstrauß for skillful technical assistance. Niels van der Lelie is acknowledged for fruitful and cooperative discussion.

This work was supported by Forschungsmittel des Landes Sachsen-Anhalt and by Fonds der Chemischen Industrie.

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