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Characterization of the Inducible Nickel and Cobalt Resistance Determinant *cnr* from pMOL28 of *Alcaligenes eutrophus* CH34

HEIKO LIESEGANG, KAREN LEMKE, ROMAN A. SIDDIQUI,† AND HANS-GÜNTER SCHLEGEL*

Institut für Mikrobiologie der Universität Göttingen, Grisebachstraße 8, W-3400 Göttingen, Germany

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From pMOL28, one of the two heavy metal resistance plasmids of *Alcaligenes eutrophus* strain CH34, we cloned an *Eco*RI-*Pst*I fragment into plasmid pVDZ'2. This hybrid plasmid conferred inducible nickel and cobalt resistance (*cnr*) in two distinct plasmid-free *A. eutrophus* hosts, strains AE104 and H16. Resistances were not expressed in *Escherichia coli*. The nucleotide sequence of the 8.5-kb *Eco*RI-*Pst*I fragment (8,528 bp) revealed seven open reading frames; two of these, *cnrB* and *cnrA*, were assigned with respect to size and location to polypeptides expressed in *E. coli* under the control of the bacteriophage T7 promoter. The genes *cnrC* (44 kDa), *cnrB* (40 kDa), and *cnrA* (115.5 kDa) are probably structural genes; the gene loci *cnrH* (11.6 kDa), *cnrR* (tentatively assigned to open reading frame 1 [ORF]; 15.5 kDa), and *cnrY* (tentatively assigned to $ORF0_{ab}$; $ORF0_a$, 11.0 kDa; $ORF0_b$, 10.3 kDa) are probably involved in the regulation of expression. $ORF0_{ab}$ and ORF1 exhibit a codon usage that is not typical for *A. eutrophus*. The 8.5-kb *Eco*RI-*Pst*I fragment was mapped by Tn*5* transposon insertion mutagenesis. Among 72 insertion mutants, the majority were nickel sensitive. The mutations located upstream of *cnrC* resulted in various phenotypic changes: (i) each mutation in one of the gene loci *cnrYRH* caused constitutivity, (ii) a mutation in *cnrH* resulted in different expression of cobalt and nickel resistance in the hosts H16 and AE104, and (iii) mutations in *cnrY* resulted in two- to fivefold-increased nickel resistance in both hosts. These genes are considered to be involved in the regulation of *cnr*. Comparison of *cnr* of pMOL28 with *czc* of pMOL30, the other large plasmid of CH34, revealed that the structural genes are arranged in the same order and determine proteins of similar molecular weights. The largest protein CnrA shares 46% amino acid similarity with CzcA (the largest protein of the *czc* operon). The other putative gene products, CnrB and CnrC, share 28 and 30% similarity, respectively, with the corresponding proteins of *czc*.

Alcaligenes eutrophus CH34 is a metal-resistant bacterium that carries two plasmids; pMOL28 (163 kb) determines resistance to nickel, cobalt, mercury, and chromate, and pMOL30 (238 kb) determines resistance to cadmium, zinc, cobalt, mercury, and copper (7, 9, 16, 18). Metal-resistant bacteria isolated from low-grade ore deposits in Belgium and Zaïre (7, 14) have several traits in common with strain CH34 and thus indicate the predominance of this type among metal-resistant bacteria. Nickel and cobalt resistance (*cnr*) encoded by pMOL28 has been studied in some detail. The pMOL28-encoded nickel and cobalt resistance is inducible (31) and is due to an energy-dependent specific efflux system (20, 29, 38). In rare mutants, nickel and cobalt resistance is expressed constitutively (32). Plasmid pMOL28 has been transferred to other wild-type strains of *A. eutrophus*, such as H16, N9A, and G29, and confers the ability to tolerate 3 mM $NiCl_2$ and 5 mM $CoCl_2$ to the transconjugants when they are grown on solid or in liquid media (16). The plasmid *cnr* determinant was cloned previously (18, 30), and chimeric plasmids containing a 9.5-kb *Kpn*I fragment or a 13.5-kb *Hin*dIII fragment complemented a nickel-sensitive Tn*5* mutant (30) of *A. eutrophus* to full nickel and cobalt resistance. The hybrid plasmid containing the 13.5-kb *Hin*dIII fragment expresses full nickel resistance in all nickel-sensitive derivatives of the native host CH34 and confers partial nickel and cobalt resistance to *A. eutrophus* H16 and JMP222. A lower degree of nickel resistance also was observed in *Alcaligenes hydrogenophilus*, *Pseudomonas putida*, and *P. oleovorans*. In all transconjugants the resistance to nickel and cobalt was constitutively expressed. Resistance was not expressed in *Escherichia coli* (30).

The genes coding for chromate and cobalt-nickel resistance were cloned (18) from plasmid pMOL28, and the nucleotide sequence of the chromate resistance gene was determined (19). The genes of pMOL30, which determine resistance to cadmium, zinc, and cobalt (*czc*), have also been cloned (21) and sequenced (22). Four open reading frames (ORFs) were identified and assigned to polypeptides (CzcA, CzcB, CzcC, CzcD); the largest gene product, CzcA, was recognized to be a potential transmembrane protein (18, 20).

The present work continues the study on the genetic structure of the cobalt-nickel resistance (*cnr*) genes of pMOL28 by random Tn*5* mutagenesis of an 8.5-kb *Eco*RI-*Pst*I fragment and mapping the insertions as well as by determining the nucleotide sequences and ORFs. Characterization of the insertion mutants was aimed at identifying the structural genes and potential regulatory genes involved in cobalt-nickel resistance.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains, plasmids, and phage used in this study are listed in Table 1.

Growth conditions. Strains of *E. coli* were grown in Luria broth (LB) or in M9 medium (25) containing 0.4% (wt/vol) glucose. *A. eutrophus* strains were grown in mineral salts

* Corresponding author.
† Present address: Institut für Pflanzenphysiologie, Zellbiologie und Mikrobiologie, W-1000 Berlin 33, Germany.

TABLE 1. Bacterial strains, plasmids, and phage

Strain, plasmid, or phage	Relevant characteristics	Reference or source
A. eutrophus		
CH34	pMOL28, pMOL30, Czc$^+$ Cnr$^+$ Chr$^+$	9
AE104	Plasmid free, metal sensitive	16
AE126	pMOL28, Czc$^-$ Cnr$^+$ Chr$^+$	16
M243	pMOL28.1, Cnr^{ca}	32
H16	pHG1, metal sensitive, wild type	39
E. coli		
JM109	F′ *recA endA1 gyrA96 thi*	40
K38	pGP1-2, HfrC	1
S17-1	*thi pro hsdR hsdM*$^+$ *recA tra*$^+$	34
CSH52	*thi pro ara recA sup*^{0}, Str^r	17
Plasmids		
pVDZ′2	Tc^r, *mob*$^+$	5
pT7-5	Ap^r, ϕ10	36
pT7-6	Ap^r, ϕ10	36
pHLI1	Tc^r Cnr$^+$, *mob*$^+$, *cnrHCBA*, 12.2-kb *Eco*RI-*Xba*I fragment in pVDZ′2	This study
pHLI2	Tc^r Cnr$^+$, mob$^+$ *cnrHCBA*, 8.5-kb *Eco*RI-*Pst*I	This study
pHLI3	Tc^r Cnr, *mob*$^+$ ORF0$_{ab}$, ORF1, *cnrHCB* and part of *cnrA* from pMOL28, part of *cnrA* from pMOL28.1 in pVDZ′2	This study
pHLI4	Ap^r Cnr$^-$, ORF0$_{ab}$, ORF1, *cnrHCBA* in pT7-5	This study
pHLI5	Ap^r Cnr$^-$, ORF0$_{ab}$, ORF1, *cnrHCB*, part of *cnrA* in pT7-5	This study
pHLI6	Ap^r Cnr$^-$, ORF0$_{ab}$, ORF1, *cnrHC*, part of *cnrB* in pT7-5	This study
pHLI7	Ap^r Cnr$^-$, *cnrHC*, part of *cnrB* in pT7-5	This study
pHLI8	Ap^r Cnr$^-$, ORF0$_{ab}$, ORF1 in pT7-5	This study
pHLI9	Ap^r Cnr$^-$, *cnrC*, part of *cnrB* in pT7-6	This study
pECD315	Tc^r Cnr$^-$, ORF0$_{ab}$, ORF1, *cnrHCB*, part of *cnrA* in pSUP202	18
Phage λ467	λb221, *rex*::Tn*5*, *c*I857, *O*am29, *P*am80	4

[a] Cnr^c, constitutive Cnr phenotype.

medium as described before (16). $NiCl_2$ was added before autoclaving, and the pH was adjusted to 7.1. Selective media contained antibiotics at the following concentrations: for *E. coli*, ampicillin at 100 μg/ml, tetracycline at 25 μg/ml, and kanamycin at 50 μg/ml; for *A. eutrophus*, tetracycline at 25 μg/ml and kanamycin at 700 μg/ml. Strains of *E. coli* were grown at 37°C, and strains of *A. eutrophus* were grown at 30°C.

Tn*5* mutagenesis. Cells of *E. coli* CSH52 harboring the 8.5-kb *Eco*RI-*Pst*I fragment in the broad-host-range vector pVDZ′2 were infected with λ::Tn*5* according to the protocol of de Bruijn and Lupski (4). The plasmid DNAs of the kanamycin-resistant cells were isolated by the alkaline lysis method (25) and used to transform competent cells of *E. coli* S17-1 as described by Mandel and Higa (13). The cells were selected on LB agar plates containing tetracycline and kanamycin and purified by restreaking.

Mapping of Tn*5* insertions. The DNA of plasmid pHLI2::Tn*5* of cells of *E. coli* S17-1 was digested with various restriction endonucleases (*Bgl*II-*Bam*HI, *Hin*dIII-*Kpn*I, and *Bst*EII) as described by the manufacturer of the enzymes (GIBCO Bethesda Research Laboratories GmbH). The DNA fragments were separated in horizontal slab gels with Tris-phosphate buffer containing 1.0% (wt/vol) agarose. The lengths of the DNA bands were estimated by Southern analysis (35). Insertional sites of relevant Tn*5* mutations were sequenced with synthetic oligonucleotides from *cnr* into the Tn*5* DNA. To test the expression of the Tn*5*-carrying 8.5-kb *Eco*RI-*Pst*I fragments, the hybrid plasmids were transferred to strains AE104 and H16 by spot mating and the kanamycin-tetracycline-resistant transconjugants were selected. The mutants carrying Tn*5* within the 8.5-kb *Eco*RI-*Pst*I fragment were tested with respect to their nickel resistance, i.e., the highest tolerable nickel concentration, and the inducibility of nickel resistance.

Estimation of the highest tolerable nickel concentration. Cells of strains AE104 and H16 carrying pHLI2::Tn*5* were grown on Tris-gluconate agar and spread on agar plates containing various concentrations of nickel chloride. The plates were incubated at 30°C and inspected at intervals for up to 7 days. Instead of measuring the concentrations, which inhibit the formation of colonies completely, we estimated the maximum tolerable concentrations (MTCs) that allowed growth. This concentration is lower than the MIC (14).

Inducibility of nickel resistance genes. Precultures of *Alcaligenes* strains carrying the hybrid plasmids pHLI2::Tn*5* were grown at 30°C in Tris-gluconate medium containing kanamycin either without added $NiCl_2$ (to produce uninduced cells) or with 0.5 mM $NiCl_2$ (induced cells). Media in Klett sidearm flasks containing high nickel concentrations (1 to 5 mM $NiCl_2$) were inoculated with induced or uninduced cells. A medium without added nickel was inoculated as a positive control, and a comparable strain lacking nickel resistance genes such as strains H16 or AE104 was inoculated into a medium with a high nickel concentration as a negative control. All cultures were incubated on a rotary shaker at 30°C. Growth curves were based on turbidity measurements in 300-ml Klett sidearm flasks with a Klett-Summerson photometer.

DNA isolation and recombinant techniques. Plasmid DNA was isolated as described previously (32). Cloning was done essentially as described by Sambrook et al. (25).

Sequence analysis. For sequence analysis, three overlapping subfragments of the 8.5-kb *Eco*RI-*Pst*I fragment, the 4.3-kb *Pst*I fragment, the 5.5-kb *Eco*RI-*Sma*I fragment, and the 3.2-kb *Hin*dIII-*Sma*I fragment were cloned into the

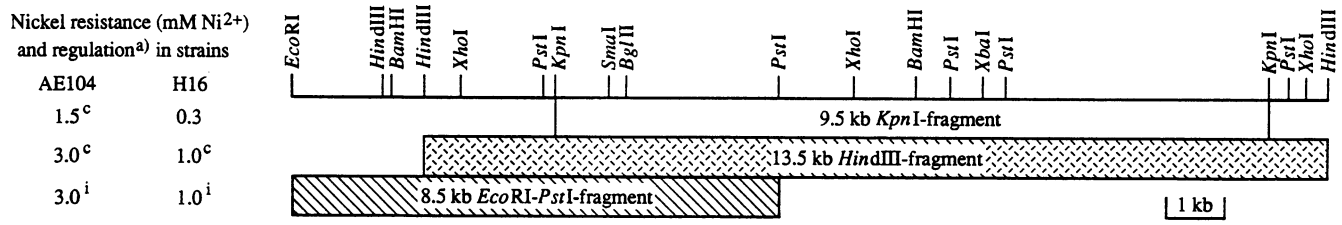

FIG. 1. Effect of DNA fragments carrying a part of the entire *cnr* determinant ligated into plasmid pVDZ'2 on the level of nickel resistance within *A. eutrophus* AE104 and H16 as hosts. The 9.5-kb *Kpn*I fragment (30) determined low (leaky) nickel resistance in *A. eutrophus* AE104 and no nickel resistance in *A. eutrophus* H16. The 13.5-kb *Hin*dIII fragment (30) expressed full, constitutive nickel resistance in strain AE104 and H16 but a lower level of nickel resistance in strain H16, and the 8.5-kb *Eco*RI-*Pst*I fragment expressed full, inducible nickel resistance in both strains. i, inducible expression; c, constitutive expression.

vector pUC19. Sequencing was done by primer walking with the universal primer and primers deduced from the nucleotide sequence by the dideoxy method of Sanger et al. (26). The complementary strand was sequenced with *cnr* internal primers. All sequencing reactions were carried out with double-stranded DNA and a T7 DNA polymerase kit from Pharmacia LKB or a Sequenase TM kit from U.S. Biochemical Corp. The nucleotide and the deduced amino acid sequences were analyzed with the Genmon program version 4.2 of the Gesellschaft für Biotechnologische Forschung mbH and the programs of the University of Wisconsin Genetics Computer Group (6).

Protein expression. Protein expression experiments were done with the coupled T7 RNA polymerase promoter system of Tabor and Richardson (36). The plasmids used are shown in Results. The expression vectors were introduced into *E. coli* K38 (1). Transformants were labelled with L-[^{35}S]methionine by the protocol of Tabor and Richardson (36, 36a). Proteins were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to autoradiography. Protein sizes were assigned to the polypeptides by comparing their electrophoretic mobilities with those of L-[^{14}C]methionine-labelled protein standards (molecular weights, approximately 14,300 to 200,000; Amersham Buchler GmbH).

Nucleotide sequence accession number. The nucleotide sequence of the *cnr* operon and flanking regions reported in this study has been submitted to the EMBL-GenBank data base under accession number M91650.

RESULTS

Construction of an 8.5-kb *Eco*RI-*Pst*I fragment. To construct a DNA fragment containing the complete region required for the inducible expression of nickel resistance (18), we combined two fragments previously cloned from plasmid pMOL28 of *A. eutrophus* CH34. From the 13.5-kb *Hin*dIII fragment of pMOL28 (previously called HKI) (Fig. 1) (30), a 7.5-kb *Sma*I-*Xba*I fragment was isolated. From plasmid pECD315 (18), the 5.5-kb *Eco*RI-*Sma*I fragment was isolated. Both fragments were cloned into the broad-host-range vector pVDZ'2, resulting in the hybrid plasmid pHLI1. The regions around the *Sma*I restriction sites within the 13.5-kb *Hin*dIII fragment and within pHLI1 were sequenced and matched perfectly. Subcloning of the combined fragment led to an 8.5-kb *Eco*RI-*Pst*I fragment (Fig. 1) within pVDZ'2, called plasmid pHLI2. When this hybrid plasmid was transferred to *A. eutrophus* H16 or to the plasmid-free, nickel-sensitive mutant AE104 of *A. eutrophus* CH34, the resulting transconjugants showed an inducible nickel resistance, indicating that all of the information for nickel resistance is encoded within the 8.5-kb *Eco*RI-*Pst*I fragment.

In addition, we used the 13.5-kb *Hin*dIII fragment derived from plasmid pMOL28.1 (formerly designated HKK; 32). This fragment is homologous to the 13.5-kb *Hin*dIII fragment from pMOL28. Both fragments confer constitutive nickel resistance to all recipients. The 13.5-kb *Hin*dIII fragment from pMOL28.1 was used to prepare a combined plasmid as described above for cloning pHLI2. When this hybrid plasmid, called pHLI3, was transferred to strains H16 and AE104, the resulting transconjugants also expressed the inducible Cnr phenotype. These results indicate that the regulatory region of the *cnr* operon is localized on the DNA fragment derived from plasmid pECD315 (18).

Mapping of the *cnr* genes by Tn*5* mutagenesis. To map the structural genes and the regulatory region of the *cnr* genes, we performed random Tn*5* mutagenesis of plasmid pHLI2 in *E. coli* CSH52. The resulting Tn*5*-mutated hybrid plasmids were transferred to *A. eutrophus* AE104 and H16 and screened for expression of nickel resistance. Among 250 Tn*5*-labelled pHLI2 plasmids, 72 carried Tn*5* insertions in the 8.5-kb *Eco*RI-*Pst*I fragment. The locations of the Tn*5* insertions within the 8.5-kb *Eco*RI-*Pst*I fragment were examined by Southern analysis (35); for the mutants listed in Table 2 (except two), the insertion sites were determined by DNA sequencing. With the help of transconjugants AE104 (pHLI2::Tn*5*) and H16(pHLI2::Tn*5*) carrying the Tn*5* insertions 1 to 8 and 67 to 72, respectively, the coding region could be restricted to about 7.2 kb (Fig. 2).

Although the majority of the Tn*5* insertion mutants were nickel sensitive (MTC, ≤0.5 mM Ni^{2+}), a few mutants were nickel resistant (MTC, between 0.6 and 3 mM Ni^{2+}) and some even showed enhanced nickel resistance (MTC, up to 14 mM Ni^{2+}). The nickel sensitivity of the Tn*5* insertion mutants of AE104 (MTC, ≤0.5 mM Ni^{2+}) indicates the location of at least two structural genes essential for expression of nickel resistance.

Enhanced nickel resistance, i.e., resistance to nickel concentrations significantly higher than those tolerated by the control cells carrying the nonmutated 8.5-kb *Eco*RI-*Pst*I fragment was found in four insertion mutants (Tn*5* insertions 10 to 13) of AE104 (Table 2). Mutations by Tn*5* insertions 10 and 11 resulted in resistance to 14 and 8 mM Ni^{2+}, respectively, which correspond to the 5 and 3 times the control MTC. Furthermore, the respective mutants of AE104 (number 10 to 13) expressed nickel resistance constitutively. This was determined by comparing the growth rates of the mutants with that of the wild type in the presence of 3 mM Ni^{2+} (Fig. 3). The strains with Tn*5* insertions downstream of insertion 13 tolerated only 3 mM Ni^{2+} or less and expressed

TABLE 2. Expression of nickel resistance by transconjugants of *A. eutrophus* AE104 and H16 carrying Tn*5*-mutated plasmid pHLI2 derived from pMOL28

Plasmid or no. of Tn*5* insertion[a]	Site of Tn*5* insertion (bp)[b]	Regulation[c]	MTC[d]		
			Ni^{2+} (mM) in strains:		Co^{2+} (mM) in strain AE104
			AE104	H16	
No plasmid	Without Tn*5*	NR	0.5	0.3	0.5
pVDZ'2	Without Tn*5*	NR	0.5	0.3	0.5
pMOL28	Without Tn*5*	i	3	1	5
pHLI2	Without Tn*5*	i	3	1	5
2	u *cnr*, 505	i	3	1	5
6	u *cnr*, 656	i	3	1	5
7	u *cnr*, 750	ND	3	1	ND
9	u $ORF0_{ab}$, 874	i	*3*	*1*	5
10	$ORF0_{ab}$, 1061	c	*14*	5	10
11	$ORF0_{ab}$, 1120	c	8	2	*10*
12	$ORF0_{ab}$, 1150	c	6	1	8
13	$ORF0_{ab}$, 1247	c	6	1	*10*
15	ORF1, 1555	c	3	1	6
19	d ORF1, 1871	c	*0.6*	0.3	*1*
20	*cnrH*, 2174	ND	*0.6*	0.3	ND
21	*cnrH*, 2203	ND	*0.6*	0.3	ND
22	*cnrH*, 2209	ND	*0.6*	0.3	ND
24	d *cnrH*, 2346	c	*1*	0.3	1

[a] Tn*5* insertions are numbered as in Fig. 2.
[b] The sites of Tn*5* insertions were located on the molecular level by sequencing with appropriate primers of the *cnr* fragment into the Tn*5* sequence. Insertions 7 and 12 were estimated by Southern analysis (35). u, location upstream of postulated gene, ORF, or operon; d, location downstream of postulated gene or ORF.
[c] i, inducible expression; c, constitutive expression; ND, not determined; NR, not relevant.
[d] Inspected after 5 days of growth on Tris-gluconate mineral agar containing nickel salt at 30°C. Slow growth at the defined nickel concentration is indicated by italic type.

constitutive nickel resistance. The strains carrying the Tn*5* insertion upstream of insertion 10 exhibited inducible nickel resistance like the parental plasmid.

The determination of the MTC of nickel showed that in some mutants the degrees of nickel resistance expression in AE104 and H16 are different. These differences indicate host specificity. Only two mutants of AE104 carrying the Tn*5* insertions 24 and 25 tolerated Ni^{2+} up to about 1 mM. Moreover, the Tn*5* insertions 16 to 23, 26, and 30 to 32 resulted in poor nickel resistance (MTC, about 0.6 mM Ni^{2+}) in strain AE104, but no resistance was detectable in strain H16. The Tn*5* mutations showed qualitatively similar phenotypes in the backgrounds of *A. eutrophus* H16 and AE104. However, transconjugants of strain H16 expressed a degree of resistance that was significantly lower than that of the AE104 derivatives. These differences may indicate that the interactions between *cnr* genes and chromosomal genes in strains H16 and CH34 are different. Among the H16 (pHLI2::Tn*5*) mutants, only two showed enhanced nickel resistance. The MTC of nickel for the strain carrying Tn*5* insertion 10 was 5 mM Ni^{2+}, and that for the strain carrying Tn*5* insertion 11 was 2 mM Ni^{2+}; the MTC for the control H16(pHLI2) cells was 1 mM Ni^{2+}. In contrast to the transconjugants of AE104, all H16(pHLI2::Tn*5*) strains carrying Tn*5* insertions 16 to 66 (except Tn*5* insertion 39) were nickel sensitive. Various phenotypic changes, such as slow growth on nickel or cobalt, constitutivity, decreased resistance to nickel and cobalt, increased resistance to nickel and cobalt, and increased resistance to zinc, induced by Tn*5* insertions were only observed from insertion 9 up to insertion 66. Therefore, a corresponding genotype, *cnr*, is proposed for all the insertions 9 to 66. Since Tn*5* insertion 67 is located within the very end of the *cnrA* locus, this insertion is considered to belong to the *cnr* operon, although it did not

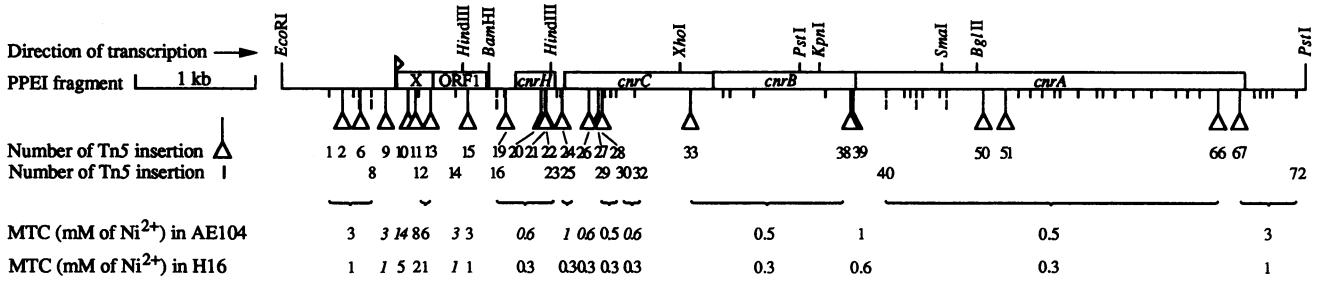

FIG. 2. Effect of Tn*5* insertions on the level of nickel resistance encoded by hybrid plasmid pHLI2 within *A. eutrophus* AE104 and H16. The numbers 1 through 72 designate the Tn*5* insertions. Tn*5* insertions localized by DNA sequencing are marked by triangles. The locations of other Tn*5* insertions are marked by short vertical lines. The numbers in the lower two lines represent the MTCs of Ni^{2+} on solid media; numbers in italic type indicate slow growth. PPEI fragment, 8.5-kb *Eco*RI-*Pst*I fragment; X, two possible ORFs, $ORF0_a$ and $ORF0_b$; ▷, potential σ^{70} promoter.

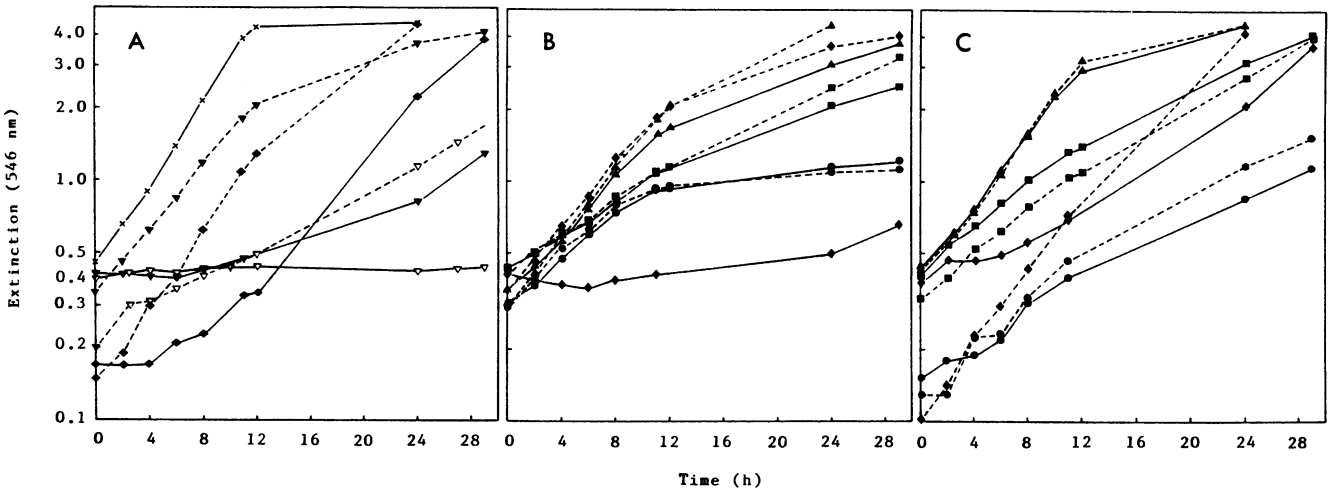

FIG. 3. Growth of *A. eutrophus* strains and transconjugants harboring plasmids or hybrid plasmids that confer inducible or constitutive nickel resistance. The cells were grown as described in Materials and Methods. Precultures grown with or without 0.5 mM $NiCl_2$ were used as inocula (induced and uninduced cells). Cells were grown in Klett sidearm flasks in Tris-gluconate medium containing 3 mM $NiCl_2$ (if not otherwise stated) at 30°C. Tn*5* insertion numbers are those in Fig. 2 and Table 2. Symbols: – – –, inoculated with induced cells; ——, inoculated with uninduced cells. (A) Controls: ▼, AE104(pHLI2); ▽, H16(pHLI2); ◆, AE126; ×, M243; all growth curves monitored at 3 mM Ni^{2+}. (B) Strain AE104 harboring different Tn*5*-mutated pHLI2; ◆, insertion 9, 3 mM Ni^{2+}; ●, insertion 10, 10 mM Ni^{2+}; ▲, insertion 11, 4 mM Ni^{2+}; ■, insertion 15, 3 mM Ni^{2+}. (C) Strain H16 harboring different Tn*5*-mutated pHLI2 plasmids: ◆, insertion 9, 1 mM Ni^{2+}; ●, insertion 10, 5 mM Ni^{2+}; ▲, insertion 11, 1 mM Ni^{2+}; ■, insertion 15, 0.8 mM Ni^{2+}.

show a phenotypic change. No genotype was assigned for the other Tn*5* insertions. The slight differences with respect to the degree of expression of the *cnr* nickel resistance genes in strains AE104 and H16 are certainly not caused by differences in the copy number of *cnr* genes. The MTCs of nickel for strains AE104(pHLI2), AE126, and AE126 (pHLI2) were identical, although the last contained two sets of *cnr* genes, one on the native plasmid pMOL28 and the other on the medium-copy-number chimeric plasmid.

The detection of a cluster of strains exhibiting constitutive as well as enhanced expression of nickel resistance among Tn*5*-mutated strains provides evidence for the location of the regulatory region upstream of the first *Hin*dIII restriction site (Fig. 2).

Nucleotide sequence of the *cnr* region. The nucleotide sequence of the 8,528-bp *Eco*RI-*Pst*I fragment (Fig. 4) revealed seven ORFs in an orientation that resulted in three observed protein bands in the T7 protein expression experiments. Three ORFs (*cnrC*, encoding a 44-kDa protein; *cnrB*, encoding a 40-kDa protein; and *cnrA*, encoding a 115.5-kDa protein) showed significant homology to the structural genes *czcC*, *czcB*, and *czcA*, respectively, from pMOL30 (18). The designation of the *cnr* genes in the order *C*, *B*, *A* was chosen in consideration of the high homology to previously described *czcCBA* genes (18). Statistical analysis on the basis of the strong third-position GC bias and the codon usage of the *czc* genes from pMOL30 (22) and the *chr* genes from pMOL28 (19) agree with the assumption that these three ORFs are coding regions (2). Nucleotide sequences and phenotypic analysis allowed us to define different subloci upstream from the structural genes *cnrCBA*. In this region four small ORFs were detected: $ORF0_a$ (encoding a 11.0-kDa protein), $ORF0_b$ (encoding a 10.3-kDa protein), ORF1 (encoding a 15.5-kDa protein), and ORF2 (encoding a 11.6-kDa protein). Tn*5* insertions 20 to 22 in the N-terminal coding region of ORF2 resulted in a sensitive phenotype for strain H16 and strongly diminished resistance of AE104. This suggests that ORF2, called *cnrH*, has an activating effect on the expression of nickel resistance. Gene *cnrH* has the same third-position GC content as *cnrCBA*, in contrast to ORF1, which has only 57% GC in the third position. Tn*5* insertions 14 and 15 are located in the putative ORF1, display a different phenotype (Table 2), and might therefore be designated *cnrR*. Tn*5* insertions 10 to 13, which display a constitutive phenotype and an increased resistance to cobalt, nickel, and zinc, map inside the putative ORFs, $ORF0_a$ (from bp 970 to 1267) and $ORF0_b$ (from bp 982 to 1270), located at the extreme left of the *cnr* locus. One of these two ORFs might therefore be denominated as sublocus *cnrY*. $ORF0_{ab}$ and ORF1 exhibit a codon usage that is not typical for *A. eutrophus*. We did not find any sequence similar to these ORFs and *cnrH* in the EMBL-GenBank data base.

Immediately upstream of $ORF0_{ab}$, a potential σ^{70} promoter with TATAAG in the −10 consensus sequence and TTCAAG in the −35 consensus sequence was found at positions 922 to 955. The −10 sequence of this potential promoter is identical to that of the *fnr*-controlled *adh* gene of strain H16 (10, 11). All Tn*5* insertions that caused constitutive nickel resistance were mapped downstream of this potential promoter sequence, and all Tn*5* insertions that mapped upstream exhibited inducible nickel resistance.

Heterologous expression studies. The 8.5-kb *Eco*RI-*Pst*I fragment and some of its subfragments (Fig. 5) were cloned into the T7 RNA polymerase promoter plasmids pT7-5 and pT7-6. The fragments were cloned in both orientations toward the ϕ10 promoter. All resulting plasmids were introduced into *E. coli* K38 containing the heat-inducible T7 RNA polymerase gene on plasmid pGP1-2. Plasmid-encoded proteins were labelled with L-[^{35}S]methionine (36), separated by SDS-PAGE, and autoradiographed (Fig. 5).

Expression studies with plasmid pHLI4, which carries the 8.5-kb *Eco*RI-*Pst*I fragment with the complete *cnr* operon, resulted in four labelled protein bands. The molecular masses were determined with approximately 125, 44, 14, and

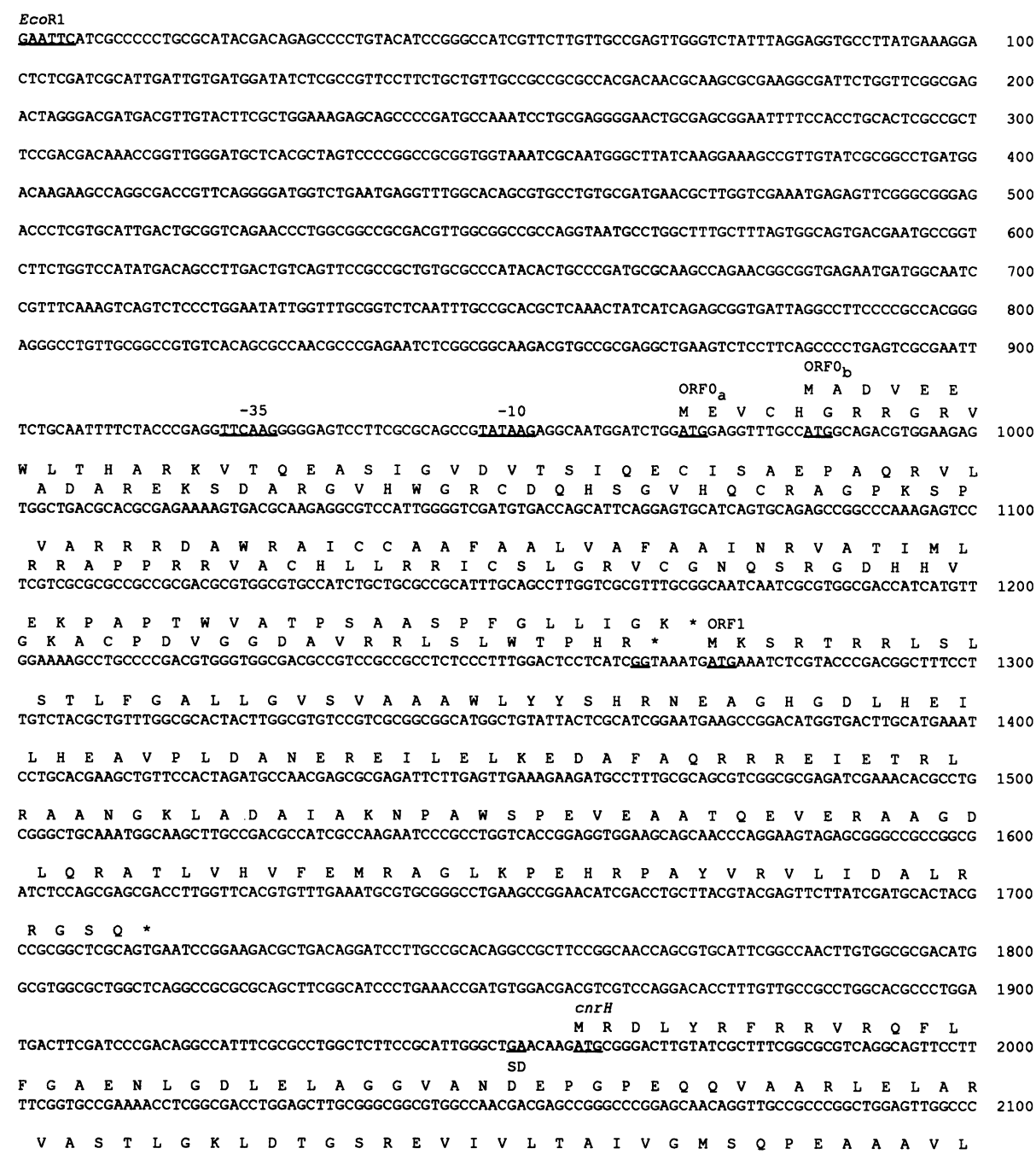

FIG. 4. Nucleotide sequence of the 8.5 kb EcoRI-PstI fragment containing the *cnr* operon and flanking regions. The postulated genes are indicated by their names. The Shine-Dalgarno sequences, the potential σ^{70} promoter, and the start codons are underlined, the stop codons are marked by asterisks.

12.5 kDa. No labelled bands were detectable with the 8.5-kb *Eco*RI-*Pst*I fragment and its subfragments in the opposite orientation. With respect to their molecular weights, the protein bands correlated with the predicted gene products of the putative genes $ORF0_{ab}$, ORF1, *cnrH*, *cnrB*, and *cnrA*. Expression experiments with pHLI4, pHLI5, pHLI6, pHLI7, and pHLI8 showed that the protein bands of the putative gene products of *cnrB* and *cnrA* were expressed

```
  V G S A I W L L P V A L S H A A E A P P F P N L L Q Q S L A L A P
TCGTCGGCTCCGCAATATGGCTGCTGCCCGTTGCGTTGAGCCACGCTGCCGAGGCGCCTCCTTTTCCGAACCTGTTGCAGCAATCGCTTGCACTCGCTCC  2500

 A M V A Q A A N V R A A G A D A A Q A Q A W L N P R I D T V L E N
GGCGATGGTCGCGCAGGCCGCCAATGTTCGTGCGGCTGGCGCCGATGCTGCTCAGGCCCAAGCCTGGCTGAATCCGCGAATCGATACGGTGCTTGAAAAC  2600

L G A P S S D G L S Q R Q N T Y S I T Q P F E L G G K R G A R I E V
CTTGGCGCTCCCAGTAGTGATGGGCTGAGTCAGCGCCAGAATACCTATTCGATCACGCAGCCTTTCGAGCTAGGAGGTAAGCGCGGCGCACGGATCGAAG  2700

  G E R N F A A A Q A R E R Q A Q V A Y A A E L A V A Y A T A E A A
TTGGCGAACGCAACTTCGCTGCGGCGCAAGCACGGGAGCGTCAGGCACAGGTAGCCTACGCCGCCGAACTTGCAGTAGCCTATGCAACGGCTGAAGCGGC  2800

 L G R K I L A T E N L A R A N E E L A A A A R A L V D S G K E A S L
GCTCGGACGCAAGATACTGGCCACGGAAAACCTGGCGCGTGCCAATGAAGAGTTGGCGGCCGCGCGTGCGTTGGTCGATTCCGGGAAAGAAGCCAGCTTG  2900

R S A Q A K A S V A A A Q A A E A A A T N D A T Q A L A R L S A M S
CGCAGCGCGCAGGCCAAGGCCAGTGTGGCGGCGGCGCAGGCCGCGGAGGCGGCAGCCACCAACGATGCCACCCAGGCTCTGGCGAGATTGTCCGCGATGT  3000

  G A S E P Y T A V T S S L L T T Q A V V P N A P A A L A E S P S V
CCGGCGCCTCCGAACCCTATACGGCCGTCACGAGTTCCTTGCTGACGACCCAGGCCGTCGTCCCGAATGCCCCGGCAGCACTCGCCGAGTCTCCGTCAGT  3100

 R A A E A E R N A L D A Q V D V E R K R W I P D V G V S A G V R R
GCGGGCCGCCGAAGCGGAACGCAATGCGCTGGATGCTCAAGTTGACGTCGAGAGGAAGCGCTGGATTCCTGATGTTGGCGTCAGTGCGGGCGTCCGCCGC  3200

Y G W T N S S G Y V V G V T A S I P L F D Q N R N G I N A A V E R V
TATGGCTGGACCAATTCCAGTGGCTATGTGGTTGGGGTCACGGCCTCCATTCCATTGTTCGATCAGAACCGGAACGGCATCAATGCCGCGGTTGAGCGGG  3300

  A A A Q A R L D S V R L E A N V A R Q S A I S Q V A T A D K Q L A
TTGCAGCCGCCCAAGCGCGGCTTGACAGCGTCCGGCTCGAGGCCAACGTGGCGCGCCAATCGGCCATATCCCAGGTAGCGACCGCCGACAAGCAGCTCGC  3400

 A A S E G E Q A A A E A Y R M G R I G Y E S G K T P L M E L L A V
TGCTGCCAGCGAAGGAGAGCAGGCGGCGGCAGAAGCCTATCGCATGGGGCGCATTGGCTATGAATCCGGCAAGACGCCACTGATGGAATTGCTAGCGGTG  3500

R R A L V D A R Q L T I D A R L A R V R A L A A L A Q A D G R L A F
CGGCGAGCGCTCGTCGACGCCCGGCAACTGACGATCGATGCGCGCCTGGCGCGCGTGCGCGCCCTGGCGGCGCTGGCACAGGCGGACGGCCGCCTAGCCT  3600
                cnrB
  E E S R M* M K N E R R S V N W P M I A G V A A V A A A V G F G A A
TTGAGGAATCACGATGATGAAGAACGAGCGCCGGTCGGTCAACTGGCCGATGATCGCAGGGGTCGCCGCCGTCGCAGCGGCAGTAGGTTTTGGCGCGGCG  3700
   SD
H L P V S E K S P A S T Q A P E A Q K P Q S A P V K P G L K E V K I
CATCTCCCAGTGTCGGAGAAGTCCCCCGCATCTACACAGGCCCCGGAAGCACAGAAGCCACAATCCGCCCCAGTAAAGCCCGGTCTGAAGGAGGTTAAGA  3800

  P A T Y L A A A N I A V E P V A S A A V G T E I L A P A T V A A L
TCCCAGCAACCTATCTTGCCGCCGCAAACATTGCCGTAGAACCTGTGGCGAGCGCCGCCGTCGGAACGGAAATACTTGCGCCCGCCACGGTTGCTGCGCT  3900

 P G S E A V I V S R A A G A V Q R V Q R R L G D V V K A G D V L A
GCCCGGTAGCGAAGCCGTGATCGTATCGCGTGCGGCCGGCGCCGTACAACGCGTCCAGCGCCGGCTTGGCGACGTCGTGAAGGCTGGCGATGTACTCGCC  4000

L V D S P E A A G M A A E R K V A Q A K A D L A R K T Y E R E A S L
CTGGTCGATAGCCCCGAGGCCGCCGGCATGGCAGCGGAGCGGAAAGTGGCCCAGGCCAAGGCCGATCTCGCGCGCAAGACCTACGAGCGCGAGGCGAGCC  4100

  F Q Q G V T P R Q E M E A A K A A L D V A Q A E A L R A A T V A Q
TGTTTCAGCAAGGCGTGACACCGCGCCAGGAAATGGAGGCGGCAAAGGCTGCTCTGGATGTTGCGCAAGCCGAGGCGCTGCGCGCTGCTACGGTGGCCCA  4200

 S A H L A S D G R S V A V V S P I A G K I T A Q S V T L G A F V A
GTCCGCTCACCTTGCGTCCGACGGGCGCTCGGTCGCCGTGGTCAGCCCGATTGCAGGAAAGATCACCGCCCAGTCCGTCACCCTGGGTGCATTTGTGGCG  4300

P Q A E L F R V A G T G A V Q V E A A V T A A D T S R I V A G S E A
CCACAAGCCGAACTCTTTCGGGTAGCCGGAACTGGCGCCGTACAGGTAGAAGCCGCCGTGACGGCTGCAGATACCAGTCGCATCGTCGCTGGGAGCGAAG  4400

  T I L L A N G S P L S A R V Q A V T P T V T G S A R V A T V V V V
CCACCATTCTGTTGGCCAATGGATCGCCATTGTCAGCGCGCGTGCAGGCGGTGACGCCAACCGTGACGGGCAGCGCTCGCGTTGCAACCGTGGTGGTGGT  4500

 P A Q P T D R L V V G E G V Q V R L R T A V A D A A A L S V P E D
ACCAGCACAGCCTACAGATCGACTTGTCGTTGGCGAGGGTGTGCAGGTGCGTCTGCGTACAGCGGTGGCTGATGCCGCCGCCCTGTCCGTGCCGGAAGAC  4600

A V Q N L D G R D V L F V R T Q E G F R P M P V L V G T R S G G S A
GCGGTGCAGAACCTCGACGGCCGTGACGTCCTATTCGTCCGCACGCAGGAAGGCTTCCGCCCTATGCCCGTCCTCGTCGGCACCCGCAGCGGCGGCTCGG  4700
```

FIG. 4—*Continued.*

only from DNA fragments carrying the corresponding ORFs. Since plasmids pHLI4, pHLI5, pHLI6, and pHLI8 contain ORF1, $ORF0_a$, and $ORF0_b$, it is not possible to assign the 14-kDa protein product to one of them. Because of degradation products of the partial gene *cnrB* in plasmids pHLI6, pHLI7, and pHLI9, a correlation between a 12.5-kDa labelled protein and *cnrH* is not certain. We did not detect any protein that correlated in molecular weight or

```
                                                                    cnrA
  Q  I  L  S  G  V  Q  A  G  E  Q  V  A  T  R  N  A  F  L  V  K  A  E  M  N  K  G  G  G  D  E  E M*
CACAGATTCTGTCAGGCGTGCAGGCTGGAGAACAGGTGGCCACCCGTAATGCCTTCCTGGTTAAGGCCGAGATGAACAAAGGCGGCGGGGGACGAGGAATG  4800
                                                                                                SD
  I  E  S  I  L  S  G  S  V  R  Y  R  W  L  V  L  F  L  T  A  V  V  A  V  I  G  A  W  Q  L  N  L  L  P
ATCGAGAGCATTCTCAGCGGCTCAGTCCGCTATCGCTGGCTGGTCCTGTTTCTGACAGCAGTTGTCGCCGTAATTGGGGCCTGGCAGCTCAACCTGCTCC  4900

  I  D  V  T  P  D  I  T  N  K  Q  V  Q  I  N  S  V  V  P  T  M  S  P  V  E  V  E  K  R  V  T  Y  P
CCATCGACGTCACACCGGACATTACCAACAAGCAGGTCCAGATCAACTCCGTGGTGCCAACGATGAGCCCCGTGGAGGTAGAAAAGCGTGTCACGTATCC  5000

  I  E  T  A  I  A  G  L  N  G  V  E  S  T  R  S  M  S  R  N  G  F  S  Q  V  T  V  I  F  K  E  S  A
AATCGAGACGGCGATTGCTGGTCTGAACGGGGTGGAAAGCACGCGATCGATGTCGCGCAACGGCTTCAGCCAGGTGACGGTGATCTTCAAGGAGAGTGCC  5100

  N  L  Y  F  M  R  H  E  V  S  E  R  L  A  Q  A  R  P  N  L  P  E  N  V  E  P  Q  M  G  P  V  S  T  G
AACCTGTACTTCATGCGCCACGAGGTGTCAGAACGACTGGCACAGGCGCGGCCGAATCTGCCAGAAAACGTCGAGCCACAGATGGGGCCTGTTTCGACGG  5200

  L  G  E  V  F  H  Y  S  V  E  Y  Q  Y  P  D  G  T  G  A  S  I  K  D  G  E  P  G  W  Q  S  D  G  S
GGCTGGGGGAAGTGTTCCACTACAGCGTGGAATACCAGTATCCGGACGGCACAGGGGCCTCCATCAAGGACGGCGAGCCCGGATGGCAGAGCGACGGCAG  5300

  F  L  T  E  R  G  E  R  L  D  D  R  V  S  R  L  A  Y  L  R  T  V  Q  D  W  I  I  R  P  Q  L  R  T  T
CTTTCTCACCGAGCGTGGCGAGAGGTTGGACGATCGTGTGTCACGACTGGCCTATTTGCGTACGGTGCAAGACTGGATCATCCGCCCGCAATTGCGCACC  5400

  T  P  G  V  A  D  V  D  S  L  G  G  Y  V  K  Q  F  V  V  E  P  D  T  G  K  M  A  A  Y  G  V  S  Y  A
ACGCCTGGTGTCGCCGATGTCGATTCCCTAGGGGGCTATGTCAAGCAATTTGTGGTGGAGCCGGACACGGGGAAGATGGCAGCATACGGAGTTTCCTACG  5500

  D  L  A  R  A  L  E  D  T  N  L  S  V  G  A  N  F  I  R  R  S  G  E  S  Y  L  V  R  A  D  A  R  I
CGGACCTAGCCCGGGCGCTCGAAGATACCAACCTTTCTGTCGGCGCGAACTTTATTCGGCGCTCAGGCGAGTCGTATCTGGTCCGCGCCGACGCGCGTAT  5600

  K  S  A  D  E  I  S  R  A  V  I  A  H  G  K  M  S  H  H  V  G  Q  V  A  R  V  K  I  G  G  E  L  R
CAAATCTGCGGACGAGATATCCCGTGCCGTGATTGCGCACGGCAAAATGTCCCATCACGTCGGGCAGGTAGCGCGCGTCAAGATTGGTGGTGAATTGCGA  5700

  S  G  A  A  S  R  N  G  N  E  T  V  V  G  S  A  L  M  L  V  G  A  N  S  R  T  V  A  Q  A  V  G  D  K
TCAGGCGCCGCCAGCCGGAATGGCAATGAAACGGTGGTGGGCAGTGCGCTGATGCTGGTCGGTGCCAACAGCCGGACGGTGGCGCAAGCCGTGGGTGACA  5800

  L  E  Q  I  S  K  T  L  P  P  G  V  V  I  V  P  T  L  N  R  S  Q  L  V  I  A  T  I  E  T  V  A  K
AGCTCGAACAGATCTCGAAGACGCTGCCACCAGGCGTGGTGATTGTCCCGACGCTGAATCGTTCGCAACTGGTGATTGCAACCATCGAAACGGTGGCAAA  5900

  N  L  I  E  G  A  L  L  V  V  A  I  L  F  A  L  L  G  N  W  R  A  A  T  I  A  A  L  V  I  P  L  S
AAACCTGATCGAAGGCGCGCTGCTCGTGGTGGCGATTCTGTTCGCGCTGCTGGGTAACTGGCGTGCCGCCACCATTGCCGCGCTAGTCATTCCGTTGTCC  6000

  L  L  V  S  A  I  G  M  N  Q  F  H  I  S  G  N  L  M  S  L  G  A  L  D  F  G  L  I  I  D  G  A  V  I
CTGCTGGTCAGCGCAATCGGCATGAATCAGTTCCACATCTCGGGCAACCTGATGAGCCTGGGTGCGCTCGACTTCGGCCTGATCATTGACGGCGCGGTCA  6100

  I  V  E  N  S  L  R  R  L  A  E  R  Q  H  R  E  G  R  L  L  T  L  D  D  R  L  Q  E  V  V  Q  S  S
TCATCGTCGAGAACTCCCTCAGGCGGCTGGCCGAGCGCCAGCACCGCGAAGGGCGCCTGCTGACGCTCGATGACCGTTTGCAGGAAGTTGTCCAATCGTC  6200

  R  E  M  V  R  P  T  V  Y  G  Q  L  V  I  F  M  V  F  L  P  S  L  T  F  Q  G  V  E  G  K  M  F  S
ACGTGAAATGGTGCGTCCGACGGTCTACGGCCAGCTTGTGATCTTCATGGTCTTCCTGCCATCTCTGACATTCCAGGGCGTGGAAGGCAAGATGTTCTCC  6300

  P  M  V  I  T  L  M  L  A  L  A  S  A  F  V  L  S  L  T  F  V  P  A  M  V  A  V  M  L  R  K  K  V  A
CCGATGGTAATCACGCTGATGCTGGCGCTTGCCTCTGCCTTCGTGCTGTCGCTCACCTTCGTGCCGGCCATGGTCGCCGTGATGCTCCGGAAAAAGGTTG  6400

  E  T  E  V  R  V  I  V  A  T  K  E  S  Y  R  P  W  L  E  H  A  V  A  R  P  M  P  F  I  G  A  G  I
CAGAGACAGAAGTGCGCGTCATCGTGGCGACCAAAGAAAGTTACCGGCCATGGCTGGAGCATGCCGTGGCACGCCCAATGCCCTTCATCGGCGCAGGCAT  6500

  A  T  V  A  V  A  T  V  A  F  T  F  V  G  R  E  F  M  P  T  L  D  E  L  N  L  N  L  S  S  V  R  I
CGCGACAGTCGCTGTGGCTACAGTGGCGTTCACATTTGTTGGTCGCGAGTTTATGCCCACGCTGGATGAGTTGAACCTGAACCTGTCGTCGGTTCGGATT  6600

  P  S  T  S  I  D  Q  S  V  A  I  D  L  P  L  E  R  A  V  L  S  L  P  E  V  Q  T  V  Y  S  K  A  G  T
CCGTCTACGTCGATTGACCAGTCGGTTGCCATAGACCTGCCGCTCGAACGCGCCGTCTTGTCGCTGCCGGAAGTGCAGACTGTGTATTCGAAGGCGGGTA  6700

  A  S  L  A  A  D  P  M  P  P  N  A  S  D  N  Y  I  I  L  K  P  K  S  E  W  P  E  G  V  T  T  K  E
CGGCCAGCCTCGCCGCCGACCCCATGCCGCCCAATGCATCGGATAACTACATCATTCTGAAACCGAAAAGCGAATGGCCGGAGGGCGTGACGACCAAGGA  6800

  Q  V  I  E  R  I  R  E  K  T  A  P  M  V  G  N  N  Y  D  V  T  Q  P  I  E  M  R  F  N  E  L  I  G
CCAGGTGATTGAGCGTATCCGCGAGAAGACAGCACCGATGGTCGGCAACAACTACGACGTGACGCAACCGATCGAGATGCGCTTTAACGAGCTGATCGGT  6900

  G  V  R  S  D  V  A  V  K  V  Y  G  E  N  L  D  E  L  A  A  T  A  Q  R  I  A  A  V  L  K  K  T  P  G
GGGGTCCGAAGCGACGTGGCCGTAAAGGTGTACGGAGAGAATCTCGACGAGTTGGCGGCCACAGCGCAACGCATTGCCGCAGTCTTGAAGAAGACGCCGG  7000
```

FIG. 4—*Continued.*

position to the putative gene for *cnrC*. Considering the background bands in the region of the predicted protein size, a weak protein band of *cnrC* might be hidden in the background (Fig. 5).

Homology of Cnr and Czc. The derived amino acid sequences of the *cnr* and the *czc* structural genes share significant homologies, and the genes are arranged in the same order. We compared the hydrophobicity patterns (data

```
  A T D V R V P L T S G F P T F D I V F D R A A I A R Y G L T V K E
GAGCGACGGACGTCCGTGTGCCTTTGACTAGCGGCTTTCCGACCTTTGACATCGTGTTCGACCGCGCCGCAATCGCCCGATATGGACTCACGGTCAAGGA  7100

 V A D T I S T A M A G R P A G Q I F D G D R R F D I V I R L P G E
AGTCGCCGATACTATTTCCACCGCCATGGCGGGCCGACCTGCCGGGCAGATTTTCGATGGTGACCGCCGCTTCGATATTGTGATCCGCTTGCCCGGTGAG  7200

Q R E N L D V L G A L P V M L P L S E G Q A R A S V P L R Q L V Q F
CAGCGGGAAAACCTCGACGTCCTCGGCGCGCTTCCCGTCATGTTGCCGCTATCGGAAGGCCAGGCCCGCGCCTCGGTGCCTTTGCGCCAACTGGTACAGT  7300

  R F T Q G L N E V S R D N G K R R V Y V E A N V G G R D L G S F V
TCCGGTTCACGCAGGGGCTCAACGAAGTAAGCCGCGACAACGGAAAGCGGCGCGTCTACGTGGAGGCCAATGTCGGCGGCCGTGACCTGGGCAGTTTTGT  7400

 D D A A A R I A K E V K L P P G M Y I E W G G Q F Q N L Q A A T K
GGACGATGCCGCGGCGCGGATCGCCAAGGAAGTGAAGTTGCCGCCGGGTATGTACATCGAATGGGGTGGCCAATTTCAGAATCTTCAGGCGGCTACGAAG  7500

R L A I I V P L C F I L I A A T L Y M A I G S A A L T A T V L T A S
CGCCTGGCCATCATTGTCCCGCTGTGCTTTATTTGATTGCGGCGACGCTGTACATGGCGATCGGCAGCGCGGCGCTGACGGCGACCGTGCTGACCGCCT  7600

  P L A L A G G V F A L L L R G I P F S I S A A V G F I A V S G V A
CGCCGCTGGCACTTGCGGGGGGCGTGTTTGCGCTGCTACTGCGCGGGATTCCGTTCTCTATTTCGGCGGCAGTTGGCTTCATTGCGGTATCCGGTGTGGC  7700

 V L N G L V L I S A I R K R L D D G M A P D A A V I E G A M E R V
GGTGCTGAACGGACTGGTGCTGATTTCCGCTATCCGAAAACGCCTGGACGATGGGATGGCGCCTGACGCTGCGGTGATTGAAGGTGCGATGGAACGCGTG  7800

R P V L M T A L V A S L G F V P M A I A T G T G A E V Q K P L A T V
CGCCCGGTACTGATGACGGCTCTGGTGGCCTCTCTGGGCTTCGTGCCGATGGCGATTGCTACAGGTACAGGTGCCGAAGTCCAGAAACCACTAGCTACGG  7900

  V I G G L V T A T V L T L F V L P A L C G I V L K R R T A G R P E
TCGTCATCGGTGGACTCGTCACCGCAACTGTGCTGACACTGTTTGTACTGCCGGCCCTTTGCGGCATAGTACTGAAGCGCCGGACCGCTGGCCGACCGGA  8000

 A Q A A L E A *
AGCGCAAGCAGCGCTGGAAGCATGAGTTTGCGAGTCCACGCTCCCACTGCGCCTTGATCCTTGATTTCCCATGTCTATTCGTACCGCATCTCCGGCATTA  8100

CTGGCGCTCTGCTTTTCGGAGCTAGCACGCCGCTGGCCAAGGCGCTAACCGGTTCCGTTGCCCCACTCCTGTTGGCGGGCTTGCTCTATCTTGGCAGTGG  8200

CATCGGACTGGCGCTTGTGCTCGCAATCCGAAGAGCCTGGATCACGCCTGACCCAGGAAATGATGACGTGGGTATTCCGCGAGGGGAAGTGCCGTGGCTG  8300

ATCGGCGCAATCCTCGCTGGCGGCGTAGCGGGGCCAGCGTTGCTGATGACCGGGCTCGTCTCAACCAATGCCGCTTCGGCATCCCTGCTGCTGAACGTGG  8400

AGGGTGTGTTCACGGCCGTCATCGCCTGGGTGGTGTTCAAGGAGAATGCTGACCGCCAGATTGTCCTGGGGATGATCGCGATCGTGGCCGGTGGCCTTCT  8500
                    PstI
GCTGTCTTGGCAGCCGGGCAGCCTGCAG                                                                            8528
```

FIG. 4—*Continued.*

not shown) and amino acid sequences (6) of the *cnr* structural genes with those of the *czc* structural genes.

CnrC (418 amino acids) and CzcC (346 amino acids) share 30% identity on the amino acid level, and both are hydrophilic proteins. CnrB (395 amino acids) and CzcB (521 amino acids) share 28.5% identity. According to computer analysis, neither of these gene products contains a pronounced hydrophobic region, but each carries a short hydrophobic peptide of about 25 amino acids at its N terminus. This peptides do not show similarity to known signal sequences. The *cnrB* gene product is essential for nickel resistance, since the Tn*5* insertions in this gene (except Tn*5* insertion 39 in the C terminus; Fig. 2) resulted in a nickel-sensitive phenotype.

CnrA (1,076 amino acids) and CzcA (1,064 amino acids) share 45.8% amino acid identity. A comparison of CzcA with CnrA on the basis of related amino acids revealed even greater similarity. The hydropathy patterns of the deduced amino acid sequences revealed two strongly hydrophobic regions and two hydrophilic regions in both proteins and a striking similarity of the hydrophobic and hydrophilic patterns in these regions (20; data not shown). Both proteins start with a hydrophobic peptide with simmilarity to known common leader peptides (24). Secondary structure analysis for CnrA predicted six hydrophobic α-helices. These are probably transmembrane helices, as predicted for CzcA (20). These findings indicate that CnrA represents the internal membrane protein of the *cnr* resistance system, just as CzcA is postulated to be in the *czc* resistance system. In SDS-PAGE gels, the protein band that correlated with the *cnrA* gene product had the fuzzy shape that has been described as typical for membrane proteins (8).

Surprisingly, we did not encounter any frame within the *cnr* nucleotide sequence that is homologous to the regulatory gene *czcD* (20, 22), and we did not find any frame in the *czc* nucleotide sequence that is homologous to *cnrH* and ORF1. This indicated that the *cnr* and *czc* resistance systems export the toxic divalent cations by a similar mechanism but that they are regulated in a different manner.

DISCUSSION

Resistance to the toxic divalent heavy-metal cations of cobalt, nickel, cadmium, and zinc is present in many bacteria that have been isolated after enrichment culture with any one of these metals as a selective agent (7, 15, 16, 27, 37). In the case of *A. eutrophus* CH34, resistance to cobalt, zinc, and cadmium is determined by the *czc* operon and resistance to cobalt and nickel is determined by the *cnr* operon, which can mutate to additional zinc resistance (3). The extensive homologies of the *cnr* and *czc* structural genes and the ranges of the exported cations suggest that the two operons have evolved from a common ancestor operon. Both operons code for cobalt efflux. Mutants containing only a functional *czcA* gene confer a low-level cobalt resistance and no

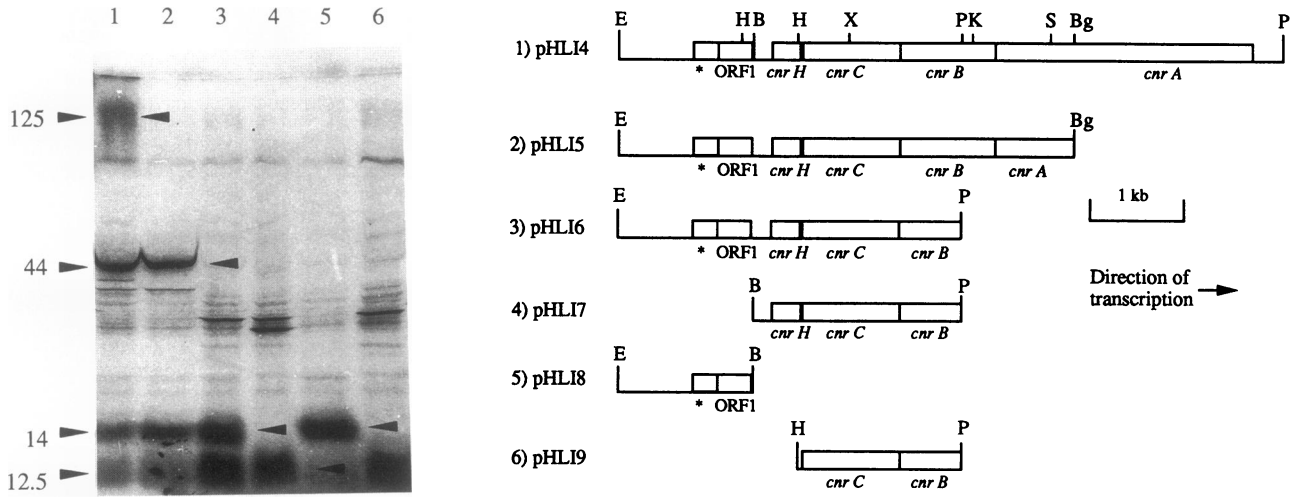

FIG. 5. Heterologous protein expression of the *cnr* determinant. The 8.5-kb *Eco*RI-*Pst*I fragment and some of its subfragments were cloned in pT7-5 or pT7-6, resulting in the depicted plasmids (the vector parts of the plasmids are not shown). Only the fragments with correct orientation toward the T7-RNA polymerase promoter of the vector resulted in protein formation. Plasmid pT7-5, without an insert, did not show protein bands. The polypeptides formed by the heterologous expression correlated with respect to their molecular mass to $ORF0_a$, $ORF0_b$, ORF1, *cnrH*, *cnrB*, and *cnrA* and are marked with arrows. For comparison, the proposed *cnr* genes are displayed as boxes in the plasmids. The approximate sizes were assigned to the polypeptides by comparison with the electrophoretic mobilities of radioactive protein standards. In the case of the protein band with a molecular mass of 12.5 kDa (lanes 1 and 2), a degradation product of the partial *cnrB* gene product of the same size is visible in lanes 3, 4, and 6. This product therefore may hide a product of the *cnrH* gene, which should code for a protein of comparable size. A protein band correlating to *cnrC* was not detected. Lanes: 1, pHLI4 containing the complete *cnr* operon; 2, pHLI5 ($ORF0_{ab}$, ORF1, *cnrHCB*, part of *cnrA*); 3, pHLI6 ($ORF0_{ab}$, ORF1, *cnrHC*, part of *cnrB*); 4, pHLI7 (*cnrHC*, part of *cnrB*); 5, pHLI8 ($ORF0_{ab}$, ORF1); 6, pHLI9 (*cnrC*, part of *cnrB*). Restriction endonuclease sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sma*I; X, *Xho*I. *, $ORF0_{ab}$.

resistance to cadmium and zinc (20). Spontaneous mutations of the regulatory region of the *cnr* operon lead to a zinc-resistant Zn^r phenotype (3). Thus these data support the idea of an ancestral cobalt or cobalt-zinc resistance operon that evolved divergently by duplication and by acquiring additional specificities to become *cnr* and *czc*.

A previous study (28) reports on DNA-DNA hybridization signals between the low-level metal resistance determinants of pMOL28 from *A. eutrophus* CH34 and the high-level metal resistance determinants of pTOM8 and pTOM9 from *Alcaligenes xylosoxydans* 31A, pGOE2 from *A. eutrophus* KT02, and chromosomally encoded cobalt-nickel resistance determinants from *A. denitrificans* 4a-2 (12). In the case of pTOM9, the significant DNA homology between pMOL28 and pTOM9 is restricted to a 3.6-kb fragment encoding nickel resistance and to the potential structural gene *cnrA*. Tn*5* insertions in the regulatory region of the *cnr* operon resulted in an increased nickel resistance up to 14 mM $NiCl_2$. These findings suggest that the differences between low-level and high-level resistance are due to regulatory functions. Possibly the resistance operons present in pTOM9 and in pMOL28 belong to one family of divalent cation transporters.

Tn*5* insertions in the region upstream from the *Hin*dIII restriction site at position 2250 and the constitutive expression of the 13.5-kb *Hin*dIII fragment (30) suggest that the regulation of expression of the *cnr* genes is coded downstream from Tn*5* insertion 9 and upstream from the gene *cnrC*. Considerations on the mechanism of the *cnr* regulatory system revealed at least three regulatory elements: one involved in inducibility of nickel resistance, one involved in the increase of nickel resistance in strain H16, and one setting the upper limit of cobalt and nickel resistance. All Tn*5* insertions downstream from the Tn*5* insertion 9 confer constitutive nickel resistance to H16 and AE104. A fragment containing the regulatory region of *cnr*, including *cnrH*, turned out to complement the 13.5-kb *Hin*dIII fragment to full but still constitutive expression in H16 (12a). This indicates that upstream from *cnrH*, one locus, possibly an operator connected to the potential σ^{70} promoter, is required to induce the *cnr* operon. Furthermore this experiment shows that *cnrH* can activate the *cnr* structural genes in *trans*. In the plasmid-free strain AE104, the 13.5-kb *Hin*dIII fragment containing only the three structural genes *cnrCBA* is sufficient to express resistance to 3 mM $NiCl_2$, whereas the strain *A. eutrophus* H16, in addition, *cnrH* is needed for wild-type-level resistance. This host-dependent different expression of the *cnr* structural genes in *A. eutrophus* H16 and AE104 is perhaps due to a *cnrH*-like chromosomal gene of AE104. The Tn*5* insertions in the gene locus *cnrY*, which lead to increased nickel resistance, indicate that the potential capacity of the *cnr* system is higher than that expressed in the wild type. Since the corresponding mutants of Tn*5* insertions 10 to 13 express different levels of nickel resistance, the effect of *cnrY* might not necessarily be mediated by a protein. This points to an additional mechanism of control of the *cnr* operon that is different from that of *cnrH*, which sets the upper limit of the expression of nickel resistance. Such a mechanism of downregulation makes sense insofar as *A. eutrophus* requires nickel as a trace element for the synthesis of hydrogenase and thus depends on the maintenance of a certain intracellular nickel concentration.

Concerning the mechanism of metal transport, we did not encounter any amino acid sequence motifs resembling known functional sites, especially an ATP-binding site, in the sequences of *cnrC*, *cnrB*, and *cnrA*. This is in accordance with results of studies on the efflux of nickel ions in

pMOL28-harboring strains of *A. eutrophus*. The efflux of $^{63}Ni^{2+}$ is highly sensitive to protonophores such as carbonyl cyanide *m*-chlorophenylhydrazone, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, and tetrachlorosalicylanilide, which dissipate the electrochemical potential, but insensitive to *N,N'*-dicyclohexyl carbodiimide, which is an inhibitor of the F_o channel of ATPase. These results have already suggested that the pMOL28-encoded nickel efflux is dependent on chemiosmotic potential rather than on ATP (38). Only the unexpected finding that the cadmium efflux genes, previously assumed to code for Cd^{2+}-H^+ antiporters (23), exhibited a high homology with cation transporting ATPases (33) made us hesitant to conclude that nickel efflux is due to a Ni^{2+}-H^+ antiporter. Further investigations ought to result in more molecular data to explain the kind of regulation, the cellular localization of the *cnr*-encoded proteins, the molecular mechanism of cation efflux, and potential interactions of determinants linked to the *hox* genes and the *cnr* efflux systems.

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