JOURNAL OF BACTERIOLOGY, Nov. 1994, p. 7045–7054
0021-9193/94/$04.00+0

Vol. 176, No. 22

Combined Nickel-Cobalt-Cadmium Resistance Encoded by the *ncc* Locus of *Alcaligenes xylosoxidans* 31A

THOMAS SCHMIDT AND HANS GÜNTER SCHLEGEL*

Institut für Mikrobiologie, Georg-August-Universität, 37077 Göttingen, Germany

Received 17 June 1994/Accepted 15 September 1994

The nickel-cobalt-cadmium resistance genes carried by plasmid pTOM9 of *Alcaligenes xylosoxidans* 31A are located on a 14.5-kb *Bam*HI fragment. By random Tn*5* insertion mutagenesis, the fragment was shown to contain two distinct nickel resistance loci, *ncc* and *nre*. The *ncc* locus causes a high-level combined nickel, cobalt, and cadmium resistance in strain AE104, which is a cured derivative of the metal-resistant bacterium *Alcaligenes eutrophus* CH34. *ncc* is not expressed in *Escherichia coli*. The *nre* locus causes low-level nickel resistance in both *Alcaligenes* and *E. coli* strains. The nucleotide sequence of the *ncc* locus revealed seven open reading frames designated *nccYXHCBAN*. The corresponding predicted proteins share strong similarities with proteins encoded by the metal resistance loci *cnr* (*cnrYXHCBA*) and *czc* (*czcRCBAD*) of *A. eutrophus* CH34. When different DNA fragments carrying *ncc* genes were heterologously expressed under the control of the bacteriophage T7 promoter, five protein bands representing NccA (116 kDa), NccB (40 kDa), NccC (46 kDa), NccN (23.5 kDa), and NccX (16.5 kDa) were detected.

Among the bacterial nickel resistance genes, those of *Alcaligenes eutrophus* CH34, a hydrogen-oxidizing bacterium containing two metal resistance plasmids, are the most studied (17). One plasmid, pMOL28 (163 kb), determines resistance to nickel, cobalt, chromate, and mercury; the other plasmid, pMOL30 (238 kb), determines resistance to cobalt, zinc, and cadmium (9, 21, 26). The mechanism of pMOL28-encoded nickel and cobalt resistance was shown to involve an energy-dependent efflux system for the metal ions (38, 46). This Cnr system enables *A. eutrophus* CH34 to grow in the presence of 3 mM nickel and 5 mM cobalt ions. The 7.1-kb *cnr* sequence revealed six potential genes, *cnrYXHCBA*. The genes *cnrYXH* encode proteins involved in the regulation of the *cnr* operon (15). Recently CnrH was suggested to be an alternative sigma factor belonging to the new ECF subfamily of σ^{70} factors (16). CnrH controls the transcription of the structural genes *cnrCBA* encoding the subunits of the efflux pump (15). Interestingly, mutations in the *cnr* operon affecting its regulation also resulted in zinc resistance (4). The structural protein CnrA shows similarities to a set of three proteins (NolG, NolH, and NolI) that play a role in the nodulation of legumes, as well as to the protein EnvD, which functions in cell division (30).

Other metal-resistant bacteria have been isolated, some of which tolerate up to 40 mM nickel (14, 34, 35). Two of these high-level nickel-resistant bacteria, *Alcaligenes xylosoxidans* 31A and *A. eutrophus* KT02, turned out to be closely related. Strain 31A contains two plasmids, and strain KT02 contains three plasmids. Strain 31A, initially isolated from a copper galvanization tank, tolerates relatively high concentrations of nickel (40 mM), cobalt (20 mM), zinc (10 mM), cadmium (1 mM), and copper (1 mM). Its metal resistance genes are located on two large plasmids, pTOM8 (340 kb) and pTOM9 (200 kb), which are transferable to *A. eutrophus* strains. In *A. eutrophus* AE104, a metal-sensitive cured mutant of *A. eutrophus* CH34, each plasmid confers resistance to nickel, cobalt, and cadmium, whereas the presence of both plasmids causes in addition copper and zinc resistance (36). The other strain, *A. eutrophus* KT02, shows similar metal resistances. They are also encoded by megaplasmids. With respect to the restriction nuclease patterns, one of these plasmids, pGOE2, strongly resembles plasmid pTOM9 of *A. xylosoxidans* 31A. The nickel-cobalt-cadmium resistance genes of pTOM8, pTOM9, and pGOE2 are located on 14.5-kb *Bam*HI fragments which are apparently identical. The *Bam*HI fragments ligated in a wide-host-range vector are well expressed in strain AE104 but cause only low-level nickel resistance in *Escherichia coli*. In contrast to the expression of nickel resistance in AE104, the expression in *E. coli* depends on a small subfragment (4.3 kb) (36).

In the present study, we describe the genetic analysis of the cloned nickel resistance genes from *A. xylosoxidans* 31A. We have evidence that its plasmid, pTOM9, contains two nickel resistance systems. (i) The Ncc system confers high-level nickel resistance as well as cobalt and cadmium resistance on *A. eutrophus* AE104 but not on *E. coli*. In the corresponding DNA sequence, seven genes, *nccYXHCBAN*, were identified. (ii) The Nre system confers low-level nickel resistance on both *A. eutrophus* and *E. coli*. The predicted Ncc polypeptides reveal strong similarities to the metal resistance proteins encoded by megaplasmids pMOL28 (*cnr* operon) and pMOL30 (*czc* operon) from *A. eutrophus* CH34 (15, 27).

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are summarized in Table 1.

Growth conditions. *Alcaligenes* strains were grown at 30°C in Tris-buffered mineral medium (17) supplemented with gluconate. Heavy-metal chlorides were added before autoclaving, and the pH was adjusted to 7.0. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium in the presence of the appropriate antibiotics (32). The metal resistance of recombinant *E. coli* clones was tested in *N*-tris(hydroxmethyl)methyl-2-aminoethanesulfonic acid (TES)-buffered (50 mM) mineral medium (36) supplemented with gluconate.

Estimation of MTCs. Cultures to be screened were grown for 15 to 20 h in Tris-gluconate medium at 30°C (*Alcaligenes* strains) or TES-gluconate medium at 37°C (*E. coli* strains).

* Corresponding author. Mailing address: Institut für Mikrobiologie, Georg-August-Universität, Grisebachstrasse 8, 37077 Göttingen, Germany. Phone: 49 551 393771. Fax: 49 551 393793. Electronic mail address: hschleg1@gwdg.de.

TABLE 1. Bacterial strains, plasmids, and phage

Strain, plasmid, or phage	Relevant characteristic(s)	Reference(s) or source
Strains		
A. xylosoxidans 31A	Ni^r Co^r Cd^r Cu^r Zn^r (pTOM8, pTOM9)	35
A. eutrophus AE104	Plasmid free, metal sensitive	17
E. coli		
JM109	(Δ*lac-proAB*) [F′ *traD36 proAB, lacI*^{q}ZΔM15] *recA endA1 gyrA96 thi-1*	49
CSH52	*thi-1 proA ara recA* Str^r Su^0	18
S17-1	*thi-1 proA hsdR hsdM*$^+$ *recA tra*$^+$	40
K38	HfrC *thi-1 leuB6 thr-1 supE44 tonA21* (pGP1-2)	2
Plasmids		
pTOM8	Ni^r Co^r Cd^r	35, 36
pTOM9	Ni^r Co^r Cd^r	35, 36
pTOM8 + pTOM9	Ni^r Co^r Cd^r Cn^r Zn^r	35, 36
pVDZ′2	Tc^r *mob*$^+$	6
pGP1-2	Km^r, T7 RNA polymerase	45
pT7-5	Ap^r, ϕ10	44
pT7-6	Ap^r, ϕ10	44
pBluescript	Ap^r	Stratagene
pTV1	*nccYXHCBAN*, *nre*, 14.5-kb *Bam*HI fragment in pVDZ′2	This study
pTB1	*nccYXHCBAN*, *nre*, 14.5-kb *Bam*HI fragment in pBluescript	This study
pTT1, pTT2	*nccYXHCBAN*, *nre*, 14.5-kb *Bam*HI fragment in pT7-5 (both orientations)	This study
pTT3, pTT4	*nccYXHCBAN*, 8.5-kb *Bam*HI-*Pst*I fragment in pT7-5 and pT7-6	This study
pTT5, pTT6	Part of *nccB*, *nccAN*, 5.8-kb *Eco*RI fragment in pT7-5 (both orientations)	This study
pTT7, pTT8	*nccYXHCB*, part of *nccA*, 5.2-kb *Bam*HI-*Hin*dIII fragment in pT7-5 and pT7-6	This study
pTT9, pTT10	*nccYXHC*, part of *nccB*, 3.2-kb *Bam*HI-*Eco*RI fragment in pT7-5 and pT7-6	This study
pTT11, pTT12	*nccYXH*, part of *nccC*, 2.2-kb *Bam*HI-*Sst*I fragment in pT7-5 and pT7-6	This study
Phage λ467	λb221, *rex*::Tn*5*, *c*I857, *O*am29, *P*am80	5

The cells were streaked onto agar plates containing various concentrations of metal chlorides. The plates were incubated at 30 and 37°C, respectively, and inspected at intervals for up to 5 days. The maximum tolerable concentration (MTC) of metal chloride was designated as the highest concentration of metal chloride which allows confluent growth after 5 days (*Alcaligenes* strains) or 2 days (*E. coli*).

Tn*5* mutagenesis of cloned fragments. Cells of *E. coli* CSH52 harboring the 14.5-kb *Bam*HI fragment in vector pVDZ′2 (pTV1) were infected with λ467 as described by de Bruijn and Lupski (5). Tn*5* mutants were selected on LB agar containing 20 μg of kanamycin per ml (about 150 mutants per plate). The Km^r colonies were washed off the plate, and the plasmid DNAs of these mutants were used to transform *E. coli* S17-1. Tn*5*-carrying plasmids were identified by selecting Km^r Tc^r transformants.

DNA sequence analysis. DNA sequencing was performed by the method of Sanger et al. (33) as specified by the manufacturer of the Sequenase kit (United States Biochemical Corp., Cleveland, Ohio). For determining the nucleotide sequence of the *ncc* operon, initially the corresponding Tn*5* insertion mutants were used. The relevant plasmids were digested with *Bam*HI-*Eco*RI, and subfragments containing part of Tn*5* and the adjacent *ncc* sequence were cloned into the Bluescript vector. Double-stranded DNA sequencing was performed with a primer derived from IS*50*L. It is important to consider that Tn*5* insertion mutations are accompanied by duplication of a short target DNA sequence (9 bp). Distances of more than 0.6 kb between adjacent transposons required the application of additional synthetic primers.

The opposite DNA strand was sequenced by using single-stranded template DNA (pTB1) and primers synthesized on the basis of the sequence data obtained. The nucleotide sequence data were compiled and analyzed with Genmon program 4.3 of the Gesellschaft für Biotechnologische Forschung mbH, Braunschweig, Germany, and the programs of the University of Wisconsin Genetics Computer Group (8). Sequence similarity searches in the current NCBI nucleic acid databases were performed by using the BLAST network service (1).

Identification of proteins involved in nickel resistance. The T7 RNA polymerase/promoter system was used as previously described (45). The genes to be expressed were cloned into a T7 promoter-containing vector, pT7-5 or pT7-6 (44, 45), and the resulting plasmids were transformed into *E. coli* K38(pGP1-2). The transformants were induced at 42°C in the presence of rifampin (400 μg/ml) and pulsed with L-[^{35}S]methionine (10 μCi/ml) for 5 min at 30°C. To analyze the induced proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, the cells were resuspended in SDS sample buffer (60 mM Tris HCl [pH 6.8], 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.01% bromphenol blue) and heated for 5 min at 100°C immediately before electrophoresis. Sizes of radioactively labelled proteins were estimated by comparing their electrophoretic mobilities with those of appropriate standards (molecular weight kits SDS-6H and SDS-7 manufactured by Sigma Chemie GmbH, Deisenhofen, Germany).

Nucleotide sequence accession number. Accession number L31363 (EMBL and NCBI databases) has been assigned to the nucleotide sequence of the *ncc* operon and adjacent regions.

RESULTS

Tn*5* mutagenesis of cloned metal resistance genes. It had been shown previously that the nickel resistance genes of *A. xylosoxidans* 31A are located on a 14.5-kb *Bam*HI fragment (36). This fragment in vector pVDZ′2 (called pTV1) confers high-level nickel resistance (MTC of 30 mM) together with cobalt and cadmium resistance (MTCs of 15 and 0.8 mM, respectively) on the plasmid-free, metal-sensitive derivative AE104 of CH34. In contrast, in *E. coli* S17-1, only low-level nickel resistance (MTC of 3 mM) was observed. This observation as well as expression studies with the subfragments derived from the 14.5-kb *Bam*HI fragment suggested the presence of at least two separate gene clusters (36). We isolated Tn*5*-induced mutants of the hybrid plasmid pTV1. Among 170 Tn*5*-carrying derivatives of pTV1, 54 different Tn*5* insertions in the 14.5-kb *Bam*HI fragment were found. The insertions were localized by analyzing *Bam*HI-*Eco*RI digests and *Sal*I digests, and about half of them were also localized by

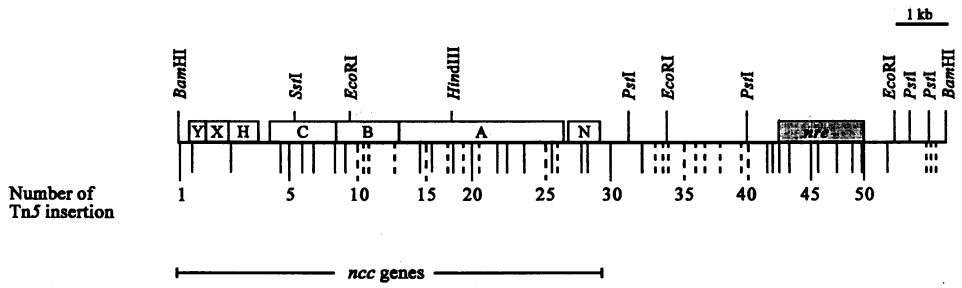

FIG. 1. Sites of Tn*5* insertions in the 14.5-kb *Bam*HI fragment. Fifty-four Tn*5* insertions localized by DNA sequencing or by restriction analysis are shown as lines or dashed lines, respectively. The *ncc* genes and the *nre* locus are also indicated.

DNA sequencing (Fig. 1). Fragments carrying Tn*5* insertions were transferred to *A. eutrophus* AE104 by conjugation. The effects of the insertions on the degree of metal resistance in *A. eutrophus* and *E. coli* S17-1 are summarized in Table 2. There were three types of Tn*5* insertional mutants.

The first type is represented by Tn*5* insertions 2 to 29, which affected the expression of nickel resistance in *A. eutrophus* AE104 but not in *E. coli* S17-1. Most of these mutations (numbers 3 to 27) caused a strong reduction of nickel resistance (MTCs of 1 to 2 mM) and also the loss of cobalt and cadmium resistance in strain AE104. The corresponding DNA region comprised about 8 kb. In contrast, mutants 28 and 29 were impaired only in nickel resistance, thus indicating special functions of the gene(s) affected. Their MTCs were reduced to 10 mM nickel (Table 1). Moreover, Tn*5* insertion 2 caused a remarkable effect, i.e., an enhancement of nickel-cobalt-cadmium resistance in *A. eutrophus* AE104, thus indicating a gene possibly involved in the regulation of a putative nickel-cobalt-cadmium (*ncc*) resistance operon.

TABLE 2. MTCs of metal ions of mutants carrying Tn*5* insertions in the 14.5-kb *Bam*HI fragment derived from plasmid pTOM9 of *A. xylosoxidans* 31A

Insertion no.[a]	Metal resistance of *A. eutrophus* AE104 (mM)[b,c,d]			Nickel resistance of *E. coli* S17-1 (mM)[c,d]
	Nickel	Cobalt	Cadmium	
1	30	15	0.8	3
2	40	25	1	3
3	2	2	0.2	3
4–5	1	<1	0.2	3
6	(1)	<1	0.2	3
7	1	<1	0.2	3
8	2	<1	0.2	3
9–11	(1)	<1	0.2	3
12	1	<1	0.2	3
13–14	(1)	<1	0.2	3
15	1	<1	0.2	3
16–27	(1)	<1	0.2	3
28–29	10	15	0.8	3
30–42	30	15	0.8	3
43	25	15	0.8	1
44	25	15	0.8	3
45–46	(20)	15	0.8	<1
47–48	20	15	0.8	<1
49	30	15	0.8	3
50	30	15	0.8	(2)
51–54	30	15	0.8	3
pTV1	30	15	0.8	3
pVDZ'2	<1	<1	0.2	<1

[a] See Fig. 1.
[b] Inspected after 4 days of growth at 30°C on Tris-gluconate mineral agar containing metal salt (metal concentrations used were 1, 2, 10, 20, 25, 30, and 40 mM nickel, 1, 2, 15, 20, and 25 mM cobalt, and 0, 0.4, 0.6, 0.8, 1, and 1.2 mM cadmium).
[c] Inspected after 2 days of growth on TES-gluconate mineral agar containing nickel salt at 37°C (nickel concentrations used were 0, 1, 2, 3, and 4 mM).
[d] Slow growth at the defined metal concentration is indicated by parentheses.

The second type of Tn*5* insertions represented by mutations 43, 45 to 48, and 50 affected nickel resistance in *A. eutrophus* AE104 as well as in *E. coli* S17-1. In strain AE104, a slight reduction of nickel resistance was observed (MTCs from 20 to 25 mM). In contrast, in *E. coli* S17-1, Tn*5* insertions 45 to 48 caused a complete loss of nickel resistance, and Tn*5* insertions 43 and 50 resulted in a reduction of nickel resistance to MTCs of 1 and 2 mM, respectively. These results suggested the existence of a second metal resistance determinant, different from the putative *ncc* operon and responsible for low-level nickel resistance. This was tentatively called the *nre* operon.

The third type of Tn*5* insertions included mutations 1, 30 to 42, and 51 to 54; they turned out to have no effects on metal resistance. Interestingly, mutations 30 to 42 are located between the *ncc* and *nre* genes, thus indicating that the two putative resistance operons are separated by a large interspace fragment.

Nucleotide sequence of the *ncc* genes. All Tn*5* insertions in the 14.5-kb *Bam*HI fragment affecting the nickel-cobalt-cadmium resistance in *A. eutrophus* AE104 mapped in a DNA region of about 8 kb. Although the Tn*5* insertions were localized in one of the *ncc* genes, it should be noted that downstream genes were usually affected. The Tn*5* derivatives were used for sequencing of the putative *ncc* operon (see Materials and Methods).

The nucleotide sequence of the entire DNA region is shown in Fig. 2. Seven open reading frames (ORFs) designated *nccYXHCBAN* were identified (Fig. 1). They show significant similarities to the metal resistance operons (*cnr* and *czc*) of *A. eutrophus* CH34 (15, 27). The nucleotide sequences of *nccYXH* and *nccCBA* overlap by one codon.

The first ORF (*nccY*) extends from nucleotides 223 to 507, encoding a possible protein of 95 amino acid residues (10.5 kDa). Interestingly, Tn*5* insertion 2, located in *nccY*, caused an increased nickel-cobalt-cadmium resistance in *A. eutrophus* AE104 (Fig. 1; Table 2). The second ORF (*nccX*), which encodes 148 amino acids (16.5 kDa), starts at nucleotide 507 and continues to nucleotide 950. NccX contains many histidine residues, possibly forming metal-binding sites. The third ORF (*nccH*) begins with a GTG codon at nucleotide 950 and continues to nucleotide 1507. The corresponding protein consists of 186 amino acids (21 kDa). A Tn*5* insertion in *nccH* (insertion 3) caused a strong decrease of nickel-cobalt-cadmium resistance in strain AE104. The predicted amino acid sequences of *nccY*, *nccX*, and *nccH* share strong amino acid identities with proteins encoded by the *cnr* operon (*nccY* and

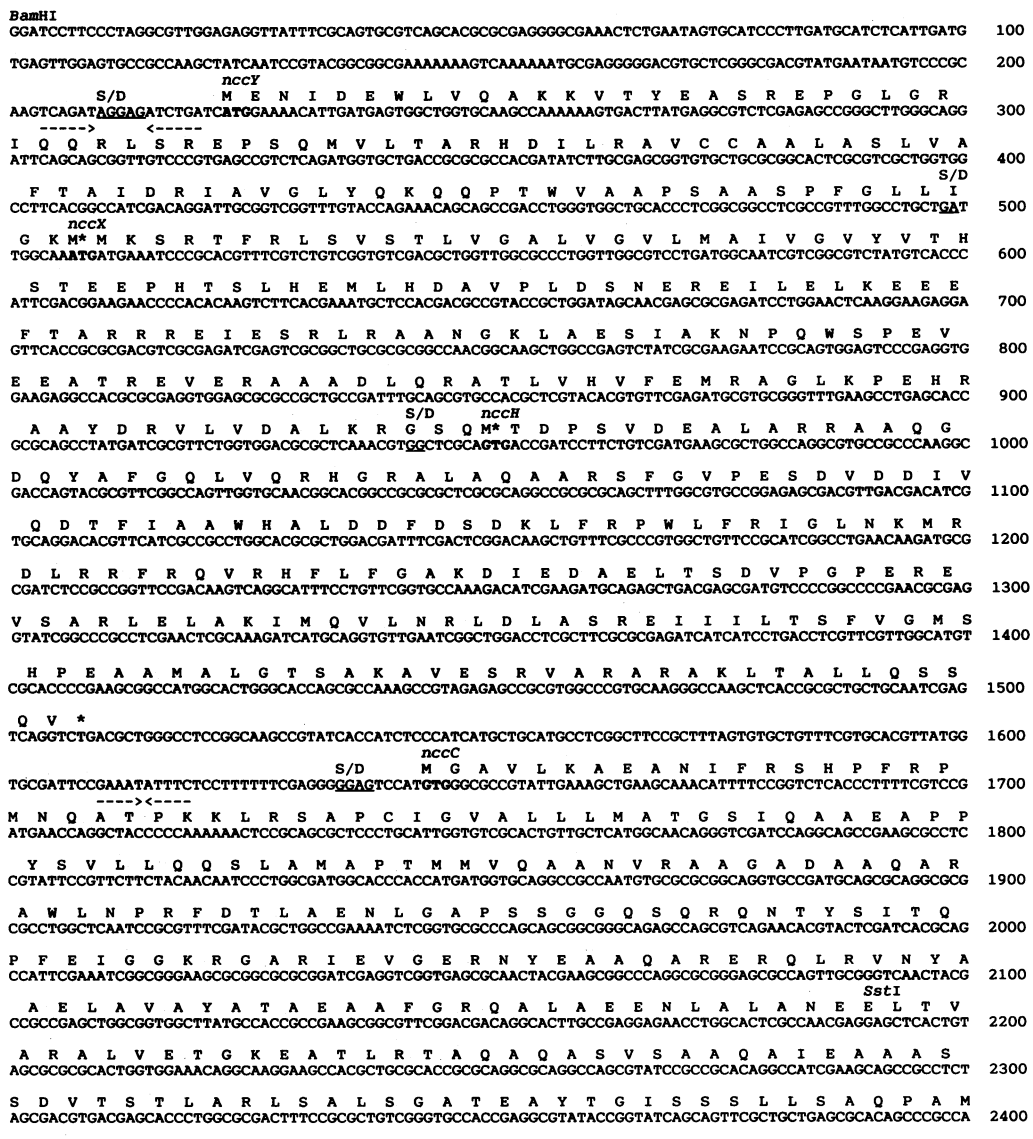

FIG. 2. Nucleotide sequence and predicted translation products of the *ncc* operon. The Shine-Dalgarno (S/D) sequences and restriction endonuclease recognition sites are underlined and labeled above the sequence. Start codons are printed in boldface type; stop codons are marked by asterisks. Regions of dyad symmetry are marked by arrows.

cnrY, 59%; *nccX* and *cnrX* [ORF1], 76%; and *nccH* and *cnrH*, 67%; Fig. 3). Additionally NccH shows significant similarities to a new subfamily of σ^{70} factors, the ECF subfamily (16).

More than 100 bp downstream from *nccH*, three other transcriptional ORFs were identified (*nccCBA*). The corresponding gene products show strong similarities to proteins CnrC, CnrB, CnrA, CzcC, CzcB, and CzcA of *A. eutrophus* CH34 (Fig. 3). Probably *nccC* begins with a GTG codon at nucleotide 1644, because it is the only possible start codon preceded by a ribosome-binding-site-like sequence. The product of this gene comprises 437 amino acids (46 kDa) and revealed 75% identity with CnrC and 29% identity with CzcC. The second ORF (*nccB*) begins at nucleotide 2954 and should encode a protein of 397 amino acids (40 kDa). This protein shares 75% identity with CnrB and 31% identity with CzcB. The third ORF (*nccA*) starts at nucleotide 4144 and continues to nucleotide 7371. The product of *nccA* consists of 1,076 amino acids (116 kDa), sharing 89% identity with CnrA and 49% identity with CzcA. All Tn*5* insertions located in *nccC*, *nccB*, or *nccA* caused a strong decrease of nickel-cobalt-cadmium resistance in *A. eutrophus* AE104.

About 70 bp downstream from *nccA*, the last ORF (*nccN*)

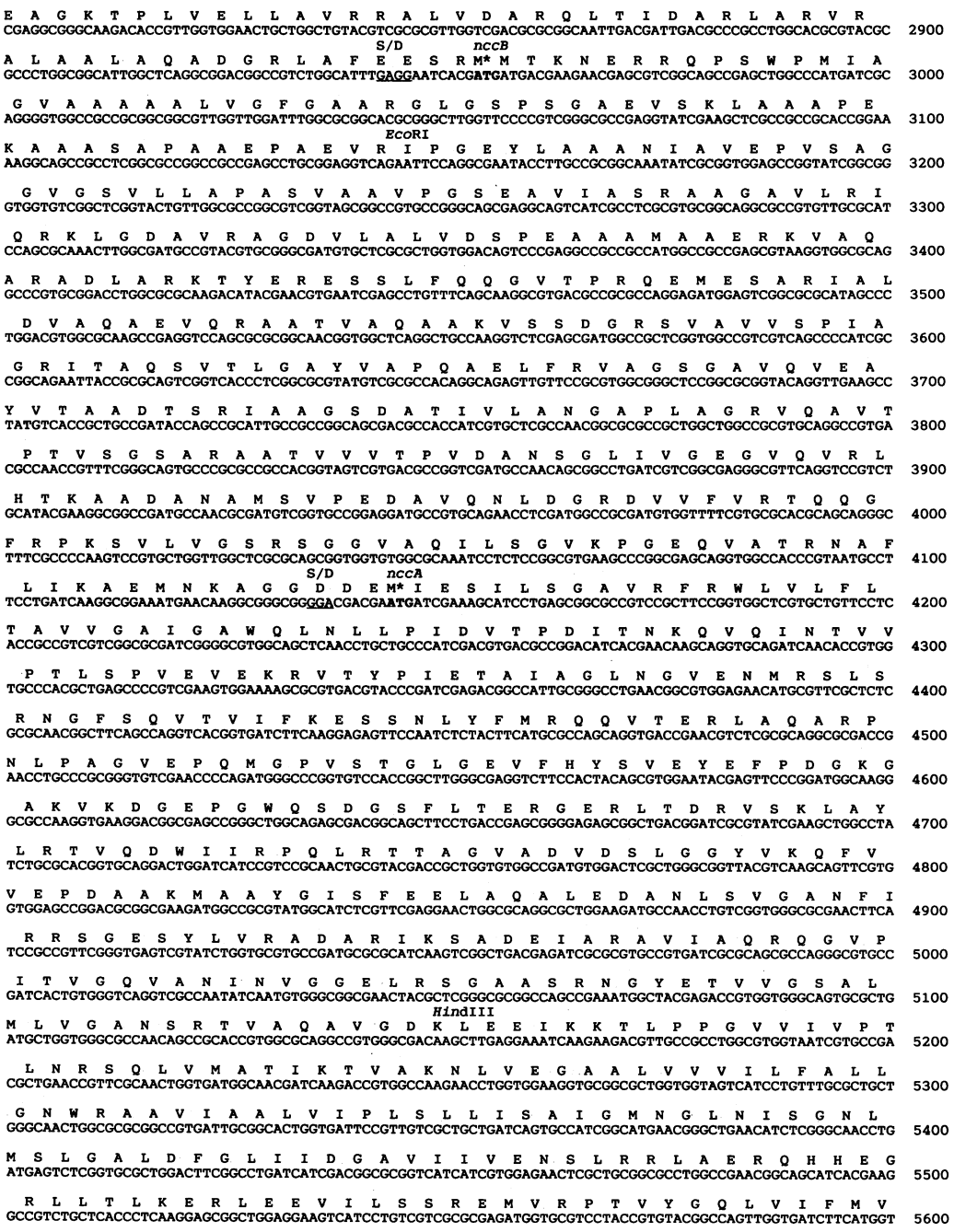

FIG. 2—*Continued.*

belonging to the *ncc* operon was identified. The hydrophobic protein NccN consists of 213 amino acids (23.5 kDa) and shows strong similarities to a new possible Czc protein (216 amino acids, 66% identity). The corresponding ORF (*czcN*) is located in the same DNA region as *czcR*. *czcN* would be transcribed in the same orientation as the *czcCBA* operon, while *czcR* is transcribed in the opposite direction (11) (Fig. 3). No equivalent protein was found in the Cnr resistance system. This result is remarkable, because Tn*5* mutations in *nccN* cause only a reduction of nickel resistance which is not encoded by the *czc* operon.

Gene expression directed by the T7 promoter. The 14.5-kb *Bam*HI fragment and different subfragments were cloned in both orientations into expression vector pT7-5 or pT7-6. The corresponding plasmids were transferred to *E. coli* K38, harboring a heat-inducible T7 RNA polymerase gene on plasmid pGP1-2. In the presence of rifampin, this strain enables the exclusive expression of genes located downstream from the T7 RNA polymerase promoter (ϕ10). The resulting proteins were labeled with L-[^{35}S]methionine and identified by autoradiography after SDS-polyacrylamide gel electrophoresis (Fig. 4). In expression experiments performed with plasmids pTT1 to pTT12, the ^{35}S-labeled proteins were detectable only if the ϕ10-dependent transcription proceeded in the direction indicated in Fig. 4. Expression of pTT1, carrying both the *ncc* locus

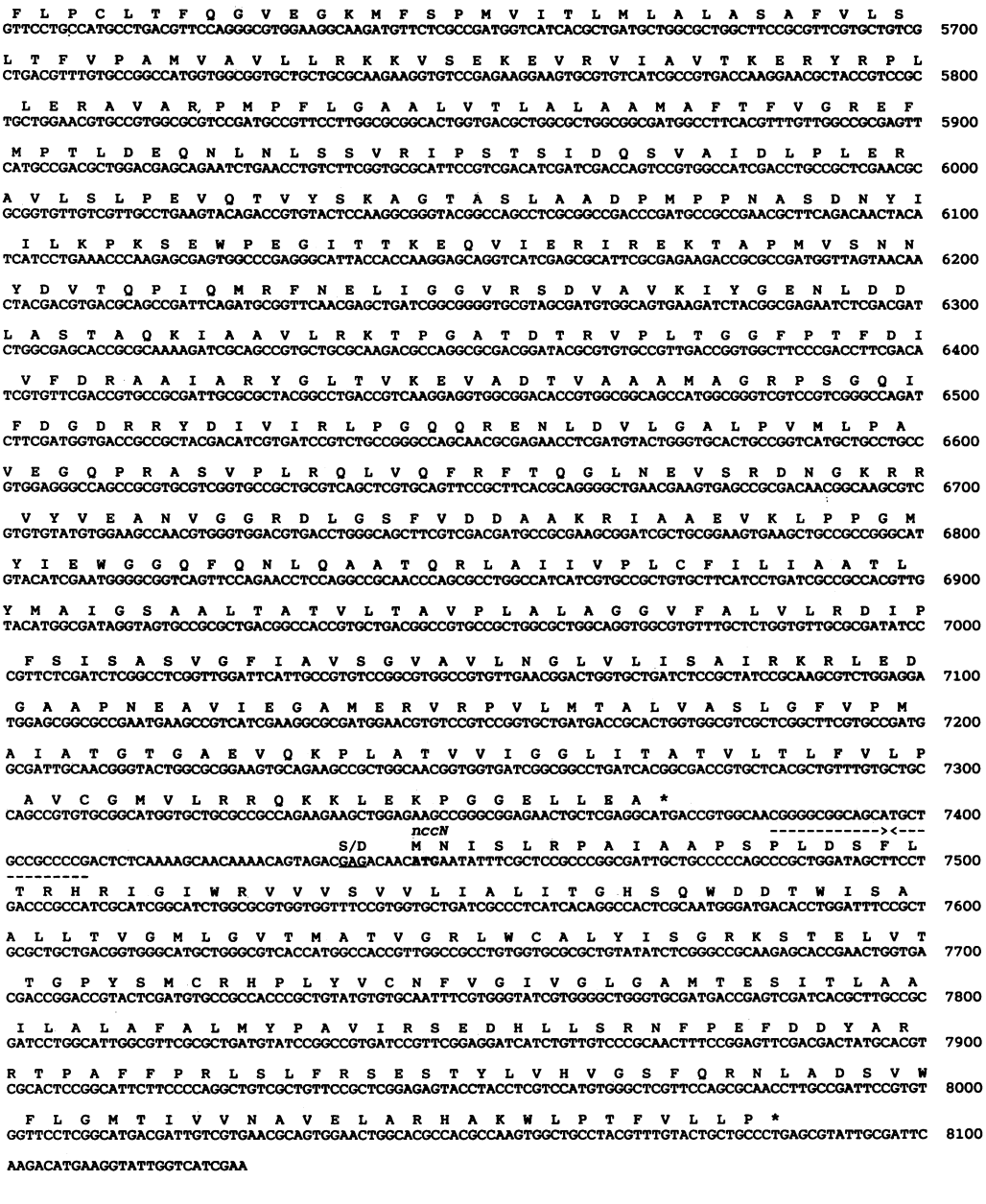

FIG. 2—*Continued.*

and the *nre* locus, resulted in several protein bands (Fig. 4, lane 1), five of which could be related to *ncc* genes. The apparent molecular mass of the largest protein (114 kDa) corresponds to the calculated value (116 kDa) derived from gene *nccA*. Moreover, the synthesis of the 114-kDa protein appears to be correlated exclusively with the presence of *nccA* (Fig. 4, plasmids pTT1, pTT3, and pTT5; lanes 1 to 3). The 43-kDa protein is encoded by plasmids pTT1, pTT3, pTT7, and pTT10 (Fig. 4, lanes 1, 2, 4, and 5). This band probably represents NccC, a gene product with a calculated molecular mass of 46 kDa. Plasmids pTT1, pTT3, and pTT7 also produce a 41-kDa protein, which seemed to be NccB (calculated molecular mass, 40 kDa). The synthesis of the 21-kDa protein depends on the presence of *nccN* (Fig. 4, lanes 1 to 3). The calculated molecular mass amounts to 23.5 kDa, also confirming the assignment of this protein as NccN. Plasmids pTT1, pTT3, pTT7, pTT10, and pTT12 directed the synthesis of an 18-kDa protein, which seemed to be either NccX or NccH (calculated molecular mass, 16.5 or 21 kDa, respectively). Presumedly the 18-kDa protein represents NccX; many RNA polymerase sigma factors like NccH show anomalous mobilities on SDS-polyacrylamide gels; i.e., they run larger than their calculated masses (16). A gene product corresponding to NccY was not detectable.

Additionally we found a 28-kDa protein, which was expressed by pTT1 but not by pTT3, carrying also the complete *ncc* operon (Fig. 4). Therefore, the corresponding gene seemed to

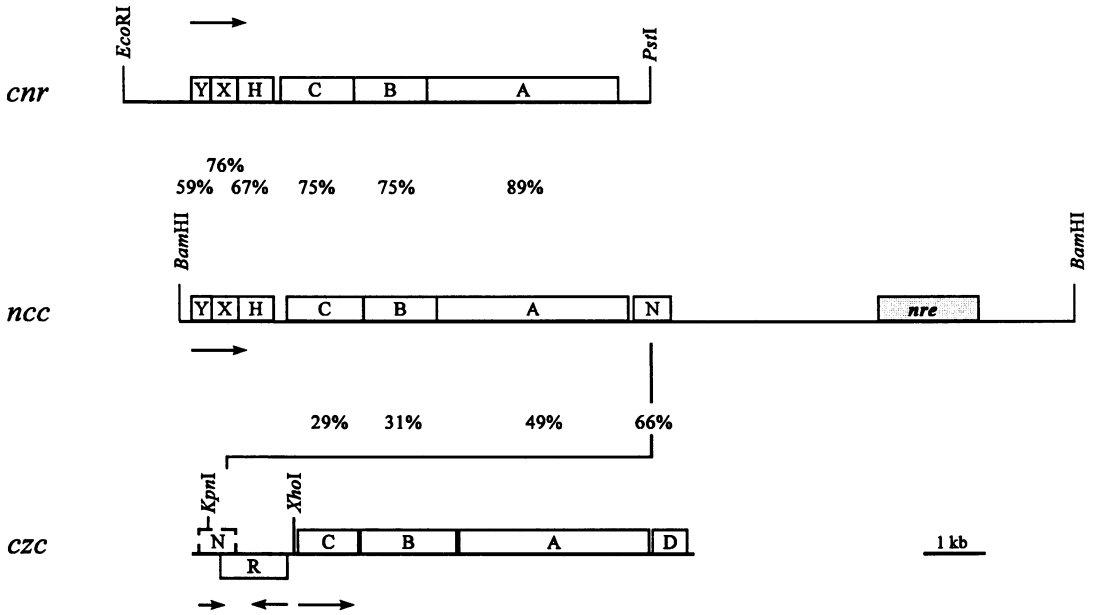

FIG. 3. Organization of the metal resistance operons *ncc*, *cnr*, and *czc*. The putative gene *czcN* (11) is marked by a dashed frame, and its orientation in the direction opposite to *czcR* is indicated with arrows. *czcN* starts 147 nucleotides upstream of the *Kpn*I site of the DNA sequence published by Nies (23) and continues to nucleotide 876. The percent homologies of the predicted gene products are indicated. The direction of transcription is marked by arrows.

be located downstream from the DNA region which we have sequenced.

DISCUSSION

Like many metal-resistant bacteria, *A. xylosoxidans* 31A carries resistance determinants located on plasmids. These plasmids, pTOM8 and pTOM9, are transferable to the metal-sensitive strain *A. eutrophus* AE104 and cause a combined nickel, cobalt, and cadmium resistance (35). The corresponding genes were localized on a 14.5-kb *Bam*HI fragment. They confer nickel resistance to *A. eutrophus* AE104 and also to *E. coli* strains (36). In the present report, we demonstrate that two DNA loci, designated *ncc* and *nre*, are involved in nickel resistance of *A. xylosoxidans* 31A. The nucleotide sequence of the *ncc* locus revealed seven ORFs, showing strong homologies to the metal resistance loci *cnr* and *czc* of *A. eutrophus* CH34 (15, 23, 27).

The existence of two nickel resistance loci on the 14.5-kb *Bam*HI fragment was indicated by experiments described previously (36). Depending on the host strain, we found two levels of nickel resistance, i.e., high-level resistance (up to 30 mM $NiCl_2$) in *A. eutrophus* AE104 and low-level resistance (up to 3 mM $NiCl_2$) in *E. coli* strains. Additionally, as shown by studies with subfragments, the relevant genes comprise DNA regions of different sizes. By Tn*5* mutagenesis, we were able to characterize those gene loci more precisely. The 8-kb *ncc* locus encodes nickel-cobalt-cadmium resistance in *A. eutrophus* AE104, whereas the 1.8-kb *nre* locus encodes low-level nickel resistance in *E. coli* and *A. eutrophus* AE104. The different features of the Ncc and Nre systems suggest that the two systems evolved separately. This hypothesis has been supported by DNA-DNA hybridization experiments. Fragments carrying either the *ncc* locus or the *nre* locus were used as biotinylated probes. Target DNAs originated from various strains isolated all over the world. Although the majority of the strains are carrying resistance determinants that show homologies to both *ncc* and *nre*, some of the tested strains possess only resistance determinants homologous to *ncc*. A new strain, *Klebsiella oxytoca* 15788, carrying exclusively an *nre* equivalent, was also included (42, 43). Besides the divergent evolution of *ncc* and *nre*, these results indicate that both resistance determinants are widespread among nickel-resistant bacteria.

The mechanism of resistance conferred by the *ncc* and *nre* genes has not been characterized in detail. Previous studies on the transport of nickel ions in *A. xylosoxidans* 31A suggest that the cells contain an energy-dependent efflux system resembling the Cnr system of *A. eutrophus* CH34 (37, 38). The efflux system of strain 31A is probably encoded by the *ncc* locus, because it shares strong homologies with the *cnr* locus (15) and the *czc* locus (27). The largest of the Ncc proteins, NccA (116 kDa), contains a double set of six extended hydrophobic regions possibly spanning the membrane. Additionally, the amino-terminal part of NccA shows similarities to common leader peptides (31). NccA is thought to form a membrane tunnel, which allows ion transport across the membrane. Neither potential ATP-binding motifs (19) nor homologies to known metal ion-translocating ATPases (3, 28, 41) were found in any of the Ncc proteins. On the other hand, the NccABC complex shows strong similarities to the CzcABC complex, which seems to be a cation-proton antiporter (24, 25). Therefore, we tentatively conclude that the Ncc resistance complex works in a similar way.

NccB and NccC contain small hydrophobic regions of about 15 amino acids in their amino-terminal parts. Both proteins show significant similarities to CzcB or CzcC, which are assumed to be involved in metal cation binding or substrate (metal cation) specificity, respectively (24). NccB belongs to a novel family of extracytoplasmic proteins (MFP family). Possibly these proteins are embedded in the cytoplasmic membrane at their N termini and function in conjunction with export permeases (10).

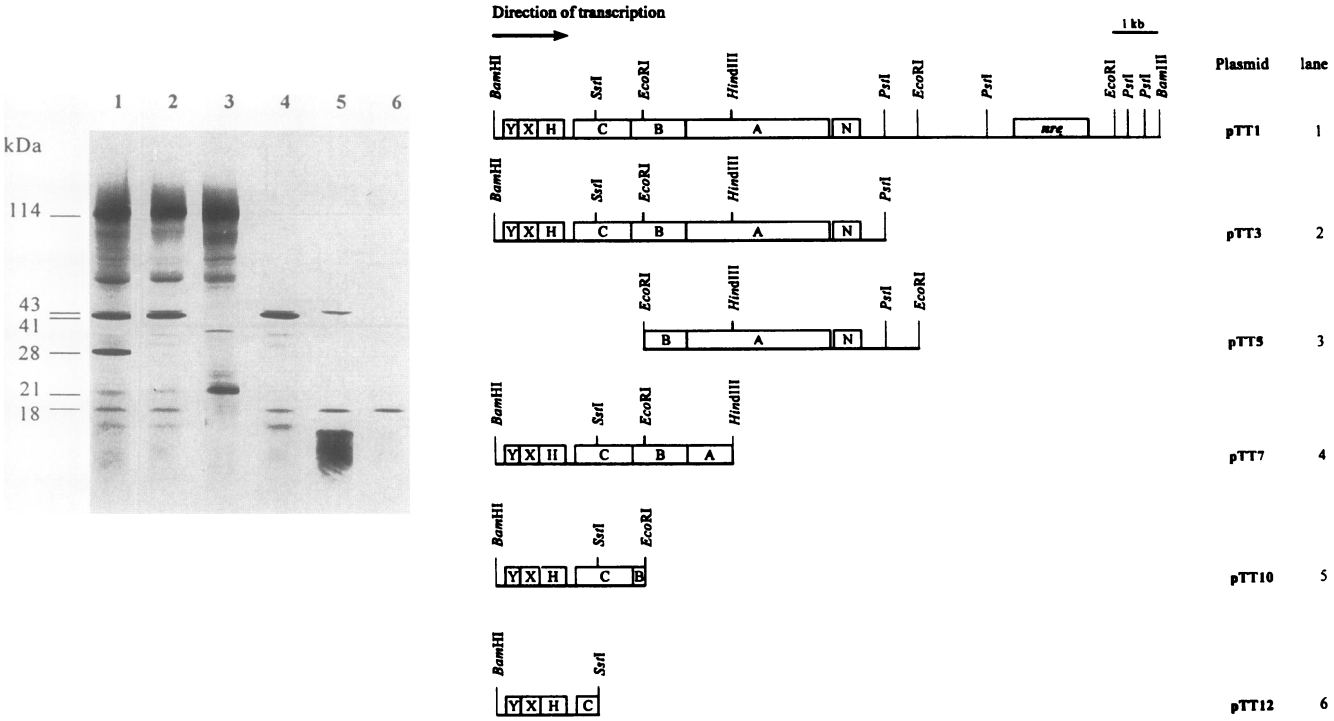

FIG. 4. Expression of proteins encoded by the *ncc* genes. The 14.5-kb *Bam*HI fragment and five different subfragments carrying *ncc* genes were cloned into vectors pT7-5 and/or pT7-6 (plasmids pTT1 to pTT12; Table 1). Gene products were expressed in *E. coli* K38(pGP1-2) and labeled with L-[^{35}S]methionine. As indicated, only one orientation of the fragments cloned behind the T7 promoter resulted in [^{35}S]methionine-labeled proteins. The second orientation of the fragments and vectors pT7-5 and pT7-6 without inserts expressed no labeled proteins (data not shown). Lanes: 1, pTT1 (*ncc* operon and *nre* locus); 2, pTT3 (*ncc* operon); 3, pTT5 (part of *nccB*, *nccAN*); 4, pTT7 (*nccYXHCB*, part of *nccA*); 5, pTT10 (*nccYXHC*, part of *nccB*); 6, pTT12 (*nccYXH*, part of *nccC*). Five potential gene products of the *ncc* operon were identified: a 114-kDa protein (NccA), a 43-kDa protein (NccC), a 41-kDa protein (NccB), a 21-kDa protein (NccN), and an 18-kDa protein (NccX). A 28-kDa protein correlating to a gene downstream from the *ncc* operon was also found. Gene products corresponding to NccY and NccH were not detectable.

NccH probably functions as a sigma factor and belongs to the ECF subfamily of σ^{70} factors (16). Like CnrH, it shows significant similarity to the conserved regions of the ECF sigma factors. These data are in accordance with previous results. First, experiments performed with DNA fragments containing *cnrCBA* or *cnrHCBA* indicate that full constitutive nickel resistance in *A. eutrophus* AE104 depends only on the genes *cnrCBA*, whereas in *A. eutrophus* H16 (wild type), *cnrH* is also essential (29, 39). Second, CnrH was shown to function as a *trans*-acting activator in strain H16 (15); in strain AE104, its function is probably complemented by a related chromosomally encoded sigma factor.

Although the data available so far confirm that NccH belongs to the ECF subfamily (σ^{E}), the promoter specificity seems to be different. In the DNA region preceding the structural genes *nccCBA*, there are no striking similarities to the σ^{E} consensus promoter (7, 16). We identified only a few single sequences resembling the −10 or −35 region.

The function of NccX has not been resolved, but some conclusions may be drawn from the comparison with the related protein CnrX (ORF1). In comparison to the other Ncc and Cnr proteins, NccX and CnrX contain many histidine residues and therefore seem to be potentially capable of binding nickel ions (13). Since CnrX is not necessary for expression of full nickel resistance, it could be involved in the regulation of the *cnr* operon (15).

The first gene of the putative *ncc* operon, *nccY*, effects a down regulation of the operon; a Tn*5* insertion in *nccY* resulted in higher levels of resistance to nickel, cobalt, and cadmium ions. Similar effects were observed with *cnrY*, the homologous gene of the *cnr* operon (15). Concerning their functions, NccY and CnrY may resemble MerD and ArsD. These *trans*-acting regulatory proteins bind to the *mer* and *ars* operators, respectively, and limit gene expression, when the corresponding operons are induced (20, 48). If resistance systems export essential metal ions like nickel and cobalt, such a down regulation is important to maintain the beneficial metal concentrations inside the cell.

The protein NccN is apparently involved in metal specificity. Whereas all of the other Ncc proteins affect nickel, cobalt, and cadmium resistance, NccN affects only nickel resistance. Together with the hydrophobic properties of NccN, these results suggest that the protein is located in the membrane, possibly involved in nickel transport. Surprisingly, NccN shows strong similarities to a putative gene product of the *czc* operon. The corresponding gene (*czcN*) is a newly recognized ORF upstream from the *czc* structural genes (11, 22, 23) and oriented in the opposite direction to the previously proposed gene *czcR*.

As shown previously, plasmid pTOM9 of *A. xylosoxidans* 31A encodes an inducible nickel resistance, whereas the loci *ncc* and *nre* located on the cloned 14.5-kb *Bam*HI fragment were expressed constitutively (36). Therefore, we assume that besides *nccY*, at least one other gene located outside the 14.5-kb *Bam*HI fragment is involved in the regulation of the nickel resistance genes. Apparently the putative NccR has a function similar to that of MerR or ArsR, namely, the repres-

sion of the corresponding operon at low metal concentrations (12, 47). In this connection, it is interesting to consider the *cnr* locus of *A. eutrophus* CH34, which strongly resembles the *ncc* locus. Although the two loci are very similar in organization, the *cnr* genes are inducible but the *ncc* genes are not. Therefore, future investigations should aim at completing the sequencing of the *ncc* operon and identifying the *ncc* and *cnr* regulation genes.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft. We thank M. Buttner for early information on the ECF subfamily of σ^E factors.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Appleyard, R. K.** 1954. Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from *Escherichia coli* K12. Genetics **39:**440–452.
3. **Chen, C.-M., T. Misra, S. Silver, and B. P. Rosen.** 1986. Nucleotide sequence of the structural genes for an anion pump: the plasmid-encoded arsenical resistance operon. J. Biol. Chem. **261:**15030–15038.
4. **Collard, J.-M., A Provoost, S. Taghavi, and M. Mergeay.** 1993. A new type of *Alcaligenes eutrophus* CH34 zinc resistance generated by mutations affecting regulation of the *cnr* cobalt-nickel resistance system. J. Bacteriol. **175:**779–784.
5. **De Bruijn, F. J., and J. R. Lupski.** 1984. The use of Tn5 mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids—a review. Gene **27:**131–149.
6. **Deretic, V., S. Chandrasekharappa, J. F. Gill, D. K. Chatterjee, and A. M. Chakrabarty.** 1987. A set of cassettes and improved vectors for genetic and biochemical characterization of *Pseudomonas* genes. Gene **57:**61–72.
7. **Deretic, V., M. J. Schurr, J. C. Boucher, and D. W. Martin.** 1994. Conversion of *Pseudomonas aeruginosa* to mucoidy in cystic fibrosis: environmental stress and regulation of bacterial virulence by alternative sigma factors. J. Bacteriol. **176:**2773–2780.
8. **Devereux, J., P. Haeberli, and O. Smithies.** 1986. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–396.
9. **Diels, L., and M. Mergeay.** 1990. DNA probe-mediated detection of resistance bacteria from soils highly polluted by heavy metals. Appl. Environ. Microbiol. **56:**1485–1491.
10. **Dinh, T., I. T. Paulsen, and M. H. Saier, Jr.** 1994. A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of gram-negative bacteria. J. Bacteriol. **176:**3825–3831.
11. **Dong, Q., and M. Mergeay (Mol, Belgium).** Personal communication.
12. **Hennecke, H.** 1990. Regulation of bacterial gene expression by metal-protein complexes. Mol. Microbiol. **4:**1621–1628.
13. **Hochuli, E.** 1990. Purification of recombinant proteins with metal chelate adsorbent, p. 87–98. *In* J. K. Setlow (ed.), Genetic engineering, principles and methods. Plenum Press, New York.
14. **Kaur, P., K. Ross, R. A. Siddiqui, and H. G. Schlegel.** 1990. Nickel resistance of *Alcaligenes denitrificans* strain 4a-2 is chromosomally coded. Arch. Microbiol. **154:**133–138.
15. **Liesegang, H., K. Lemke, R. A. Siddiqui, and H. G. Schlegel.** 1993. Characterization of the inducible nickel and cobalt resistance determinant *cnr* from pMOL28 of *Alcaligenes eutrophus* CH34. J. Bacteriol. **175:**767–778.
16. **Lonetto, M. A., K. L. Brown, K. E. Rudd, and M. J. Buttner.** 1994. Analysis of the *Streptomyces coelicolor* sig*F* gene reveals the existence of a subfamily of eubacterial RNA polymerase σ factors involved in the regulation of extracytoplasmic functions. Proc. Natl. Acad. Sci. USA **91:**7573–7577.
17. **Mergeay, M., D. Nies, H. G. Schlegel, J. Gerits, P. Charles, and F. Van Gijsegem.** 1985. *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J. Bacteriol. **162:**328–334.
18. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Mitchell, C., and D. Oliver.** 1993. Two distinct ATP-binding domains are needed to promote protein export by *Escherichia coli* SecA ATPase. Mol. Microbiol. **10:**483–497.
20. **Mukhopadhyay, D., Y. Hongri, G. Nucifora, and T. K. Misra.** 1991. Purification and functional characterization of MerD. J. Biol. Chem. **266:**18538–18542.
21. **Nies, A., D. H. Nies, and S. Silver.** 1989. Cloning and expression of plasmid genes encoding resistances to chromate and cobalt in *Alcaligenes eutrophus*. J. Bacteriol. **171:**5065–5070.
22. **Nies, D. H. (Halle, Germany).** Personal communication.
23. **Nies, D. H.** 1992. CzcR and CzcD, gene products affecting regulation of resistance to cobalt, zinc, and cadmium (*czc* system) in *Alcaligenes eutrophus*. J. Bacteriol. **174:**8102–8110.
24. **Nies, D. H.** 1992. Resistance to cadmium, cobalt, zinc and nickel in microbes. Plasmid **27:**17–28.
25. **Nies, D. H.** Unpublished data.
26. **Nies, D. H., M. Mergeay, B. Friedrich, and H. G. Schlegel.** 1987. Cloning of plasmid genes encoding resistance to cadmium, zinc and cobalt in *Alcaligenes eutrophus* CH34. J. Bacteriol. **169:**4865–4868.
27. **Nies, D. H., A. Nies, L. Chu, and S. Silver.** 1989. Expression and nucleotide sequence of a plasmid-determined divalent cation efflux system from *Alcaligenes eutrophus*. Proc. Natl. Acad. Sci. USA **86:**7351–7355.
28. **Nucifora, G., L. Chu, T. K. Misra, and S. Silver.** 1989. Cadmium resistance from *Staphylococcus aureus* plasmid p1258 *cadA* gene results from a cadmium efflux ATPase. Proc. Natl. Acad. Sci. USA **86:**3544–3548.
29. **Oldenburg, I.** Unpublished data.
30. **Saier, M. H., Jr., R. Tam, A. Reizer, and J. Reizer.** 1994. Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport. Mol. Microbiol. **11:**841–847.
31. **Saier, M. H., Jr., P. K. Werner, and M. Müller.** 1989. Insertion of proteins into bacterial membranes: mechanism, characteristics, and comparisons with the eucaryotic process. Microbiol. Rev. **53:**333–366.
32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5363–5467.
34. **Schlegel, H. G., J.-P. Cosson, and A. M. Baker.** 1991. Nickel-hyperaccumulating plants provide a niche for nickel-resistant bacteria. Bot. Acta **104:**18–25.
35. **Schmidt, T., and H. G. Schlegel.** 1989. Nickel and cobalt resistance of various bacteria isolated from soil and highly polluted domestic and industrial wastes. FEMS Microbiol. Ecol. **62:**315–328.
36. **Schmidt, T., R.-D. Stoppel, and H. G. Schlegel.** 1991. High-level nickel resistance in *Alcaligenes xylosoxidans* 31A and *Alcaligenes eutrophus* KTO2. Appl. Environ. Microbiol. **57:**3301–3309.
37. **Sensfuss, C.** Unpublished data.
38. **Sensfuss, C., and H. G. Schlegel.** 1988. Plasmid pMOL28-encoded resistance to nickel is due to specific efflux. FEMS Microbiol. Lett. **55:**295–298.
39. **Siddiqui, R. A., K. Benthin, and H. G. Schlegel.** 1989. Cloning of pMOL28-encoded nickel resistance genes and expression of the genes in *Alcaligenes eutrophus* and *Pseudomonas* spp. J. Bacteriol. **171:**5071–5078.
40. **Simon, R., U. Priefer, and A. Pühler.** 1983. Vector plasmids for *in vivo* and *in vitro* manipulations of Gram-negative bacteria, p. 98–106. *In* A. Pühler (ed.), Molecular genetics of bacteria-plant interactions. Springer-Verlag, Berlin.
41. **Snavely, M. D., C. G. Miller, and M. E. Maguire.** 1991. The *mgtB* Mg^{2+} transport locus of *Salmonella typhimurium* encodes a P-type ATPase. J. Biol. Chem. **266:**815–823.
42. **Stoppel, R.-D.** 1992. Charakterisierung und Klonierung der plas-

midgebundenen Nickelresistenz von *Alcaligenes eutrophus* KT02 und Untersuchungen zur Homologie und Verbreitung verschiedener Nickelresistenzgene. PhD. dissertation, Universität Göttingen, Göttingen, Germany.

43. **Stoppel, R.-D., M. Meyer, and H. G. Schlegel.** 1995. The nickel resistance determinant cloned from the enterobacterium *Klebsiella oxytoca*: conjugational transfer, expression, regulation and DNA homologies to various nickel-resistant bacteria. BioMetals **8**:121–130.
44. **Tabor, S.** 1990. Expression using the T7 RNA polymerase/promoter system, p. 16.2.1–16.2.11. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. Greene Publishing, New York.
45. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1078.
46. **Varma, A. K., C. Sensfuss, and H. G. Schlegel.** 1990. Inhibitor effects on the accumulation and efflux of nickel ions in plasmid pMOL28-harboring strains of *Alcaligenes eutrophus*. Arch. Microbiol. **154**:42–49.
47. **Wu, J., and B. P. Rosen.** 1991. The ArsR protein is a *trans*-acting regulatory protein. Mol. Microbiol. **5**:1331–1336.
48. **Wu, J., and B. P. Rosen.** 1993. The *arsD* gene encodes a second *trans*-acting regulatory protein of the plasmid-encoded arsenical resistance operon. Mol. Microbiol. **8**:615–623.
49. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.