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A New Type of *Alcaligenes eutrophus* CH34 Zinc Resistance Generated by Mutations Affecting Regulation of the *cnr* Cobalt-Nickel Resistance System

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Spontaneous mutants that were resistant to zinc were isolated from *Alcaligenes eutrophus* CH34 containing either the native plasmid pMOL28 or a derivative derepressed for its self-transfer, pMOL50. With the cured plasmid-free derivative of CH34, strain AE104, such mutants were not detected. The mutations, which were shown to be located in the plasmid, increased the level of the nickel and cobalt resistance determined by the *cnr* locus. The chromate resistance closely linked to the *cnr* locus was not affected by these mutations. In the Zn^r mutants, the resistance to zinc and nickel was constitutively expressed. Uptake studies showed that the zinc resistance in a Zn^r mutant resulted from reduced accumulation of zinc ions in comparison with that in the plasmid-free strain. Reduced accumulation of zinc was also observed to a lesser degree in the parental strain induced with nickel, suggesting that zinc interferes with the Ni^{2+} and Co^{2+} efflux system. A 12.2-kb *Eco*RI-*Xba*I restriction endonuclease fragment containing the *cnr* locus was cloned from plasmid pMOL28 harboring the mutation and shortened to an 8.5-kb *Eco*RI-*Pst*I-*Pst*I fragment conferring resistance to zinc, nickel, and cobalt. The 12.2-kb *Eco*RI-*Xba*I fragment was also reduced to a 9.7-kb *Bam*HI fragment still encoding weak resistance to nickel and cobalt but not to zinc. Complementation studies demonstrated the recessivity of the *cnr* mutations with a Zn^r phenotype. Such mutations thus allow positive selection of mutants affected in the expression of the *cnr* operon.

The aerobic gram-negative bacterium *Alcaligenes eutrophus* CH34 contains two large plasmids (pMOL30 [238 kb] and pMOL28 [163 kb]) that confer resistance to several heavy metals (8). Plasmid pMOL30 encodes resistance to Cd^{2+}, Zn^{2+} (ZinA phenotype), Co^{2+} (CobB phenotype), Cu^{2+}, Pb^{2+}, and Hg^{2+}; plasmid pMOL28 encodes resistance to Co^{2+} (CobA phenotype), Ni^{2+}, Hg^{2+}, and CrO_4^{2-} (1, 8). To facilitate the study of plasmid-determined resistance and physiological investigations, the wild-type strain CH34 was cured of plasmid pMOL30, resulting in strain AE126, which still harbors plasmid pMOL28 (8).

The Ni^{2+} and Co^{2+} resistance determinants of plasmid pMOL28 are linked and controlled by the *cnr* locus, which was cloned on an 8.5-kb *Eco*RI-*Pst*I-*Pst*I fragment (9) and on a 13.5-kb *Hin*dIII fragment (19); these fragments overlap by 6.3 kb. The resistance genes, which are inducible by nickel, encode an energy-dependent cation efflux system (14, 18). Resistance to chromate adjacent to the *cnr* locus is due to reduced cellular accumulation of CrO_4^{2-}, controlled by the *chrA* and *chrB* genes (9, 10). On the other hand, mercury resistance (determined by transposon Tn*4378*, which hybridizes with the *merRTPA* genes of Tn*501* [1, 2]) is not due to reduced uptake but rather to enzymatic detoxification of Hg^{2+} to Hg^0.

Since plasmid pMOL28 is transferred at a low frequency (10^{-7} per donor cell) to the plasmid-free *A. eutrophus* CH34 derivative AE104, we used plasmid pMOL50 (210 kb) in our mating experiments. This derivative of plasmid pMOL28 was obtained after exposure of strain CH34 to a temperature of 37°C, which induced a strong lethality (survivor frequency, 10^{-5}) as well as plasmid rearrangements (2). pMOL50, which is about 45 kb larger than pMOL28, is derepressed for its self-transfer (10^{-3} per donor cell) and mobilizes chromosomal markers at a high frequency (2). All of the resistance to heavy metal ions conferred by plasmid pMOL28 are also conferred by plasmid pMOL50.

Spontaneous mutations increasing the level of resistance to zinc were found in strains carrying plasmid pMOL28 (such as AE126) or its derivative pMOL50 (such as AE245). In this report we present the characterization of these mutants and the cloning of the corresponding genetic determinants.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains, mutant derivatives, and plasmids used in the present study are listed in Table 1.

Media and growth conditions. Tris-buffered mineral salt medium (8) containing 0.2% (wt/vol) sodium gluconate as a carbon source was used for testing resistance to heavy metals and for growing *A. eutrophus* strains at 30°C. For plating, growth media were solidified with 15 g of agar per liter. Analytical-grade salts of $ZnCl_2$, $NiCl_2 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, and K_2CrO_4 were prepared as 1 M stock solutions, sterilized by autoclaving, and added to partially cooled agar medium after the medium was autoclaved. To determine resistance, the following concentrations of metal salts in solidified Tris-gluconate medium were chosen: 0.8 mM Zn^{2+}, 0.8 mM Ni^{2+}, 1 mM Co^{2+}, and 0.26 mM CrO_4^{2-}. *Escherichia coli* strains were grown at 37°C in LB medium (17). For maintenance of plasmid markers, growth of auxotrophic strains, and isolation of transconjugants and transformants, the media were supplemented with the appropriate concentration of a filter-sterilized solution of the indicated

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TABLE 1. Bacterial strains, mutants, and plasmids

Strain or plasmid	Relevant characteristics	Origin or reference
A. eutrophus		
AE104	Sensitive; plasmid-free derivative of CH34	8
AE126	pMOL28 (Ni^r Co^r Zn^s Cr^r)	8
AE245	pMOL50 (Ni^r Co^r Zn^s Cr^r), *met-81 his-245*	This study
AE963	pMOL29 (Ni^r Co^r Zn^r Cr^r)	This study
AE1013 through AE1016	pMOL55 (Ni^r Co^r Zn^r Cr^r), *met-81 his-245*	This study
E. coli		
S17/1	RP4-2 (*tetA*::Mu) (*kan*::Tn7) *recA pro thi*	22
DH5α	F^- Δ(*lacZYA-argF*)*U169* (ϕ80 *lacZΔM15*) *recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1*	GIBCO BRL
CM890	pMOL154 in S17/1	This study
Plasmids		
pRK415	*tetA*, contains *lac* promoter and the LacZα-peptide-coding region	5
pMOL154	pRK415 with 12.2-kb *Eco*RI-*Xba*I fragment of pMOL29	This study
pMOL157	pRK415 with 9.7-kb *Bam*HI fragment of pMOL29	This study
pMOL159	pRK415 with 8.5-kb *Eco*RI-*Pst*I fragment of pMOL29	This study
pMOL166	pRK415 with 4.3-kb *Pst*I fragment of pMOL29	This study
pMOL169	pRK415 with 5.4-kb *Xho*I-*Pst*I fragment of pMOL29	This study
pMOL28	Plasmid conferring inducible resistance to nickel, cobalt, and chromate in AE126	8
pMOL29	pMOL28 *cnr-963* (Zn^r)	This study
pMOL50	Derivative of pMOL28, Cma^+	2
pMOL55	pMOL50 *cnr-1013* through *-1016* (Zn^r)	This study

antibiotic or amino acid. Clones of *E. coli* DH5α harboring recombinant pRK415 plasmids were identified on LB agar plates, which contained 20 μg of tetracycline per ml, 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml, and 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Sources of restriction enzymes, antibiotics, and other chemicals. Restriction endonucleases, calf intestinal phosphatase, T4 DNA ligase, lambda DNA, 1-kb DNA ladder, IPTG and agar were obtained from GIBCO BRL, Gaithersburg, Md. X-Gal was obtained from Serva, Heidelberg, Germany. Antibiotics, amino acids, and ethidium bromide were obtained from Sigma Chemical Co., St. Louis, Mo. $^{65}ZnCl_2$ (10 mCi/ml; 1.37 mCi/mg of Zn) was purchased from DuPont de Nemours, Dreieich, Germany. All other chemicals of analytical grade were obtained from E. Merck AG, Darmstadt, Germany.

Matings. Conjugations were carried out by biparental matings. Donor strains were grown at 30°C in liquid nutrient broth (8 g/liter; Difco Laboratories) to the late-logarithmic phase, and recipient strains were grown to the stationary phase. Agar matings were performed as described previously (6).

Isolation of plasmid DNA. Small-scale and large-scale plasmid DNA preparations from *E. coli* were done by the alkaline lysis procedure (17). Small-scale isolation of plasmid DNA from *A. eutrophus* was performed as described by Kado and Liu (4); large-scale isolation of plasmids was performed as described by Prakash et al. (15) for *Rhizobium* spp. with slight modifications. After banded plasmid DNA was recovered from the ethidium bromide-CsCl gradient, ethidium bromide was removed by three extractions with *n*-butyl alcohol saturated with water. After a 1:4 dilution with sterile TE buffer (10 mM Tris hydrochloride [pH 8.0], 1 mM disodium EDTA), plasmid DNA was precipitated with 2 volumes of ethanol at −20°C for 16 h, pelleted, washed twice with 70% ethanol, vacuum dried, and dissolved in sterile TE buffer.

DNA ligation. To prevent religation, vector DNA was dephosphorylated with alkaline phosphatase as recommended by the manufacturer. DNA fragments with sticky ends were ligated with 1 U of T4 DNA ligase to vector DNA at 14°C for 16 h in the buffer supplied by the manufacturer.

Manipulation of restriction nuclease sites by T4 DNA polymerase. The *Eco*RI- and *Xho*I-generated 5′-protuding ends were filled in the presence of the deoxynucleoside triphosphates dATP, dCTP, dGTP, and dTTP by the 5′-3′ polymerase activity of T4 DNA polymerase (17).

Electrotransformation. *E. coli* S17/1 or DH5α cells were prepared for electrotransformation as described previously (3). High-voltage pulses (2.5 kV) were delivered with a Gene-Pulser apparatus (Bio-Rad) with a 25-μF capacitor.

Analysis of plasmid DNA. Isolated plasmid DNA was digested with various restriction endonucleases as described by the manufacturer of the enzymes. DNA fragments from bacteriophage lambda digested by *Hin*dIII and from the 1-kb DNA ladder served as standards to estimate the molecular weights of unknown DNA fragments.

Assay of $^{65}Zn^{2+}$ uptake. The cells were grown in Tris-mineral-gluconate medium until an optical density at 660 nm of 0.8 was reached; the cells were washed once with sterile 10 mM Tris hydrochloride buffer (pH 7.0) and then resuspended in the same buffer at an optical density of 0.6. Then they were incubated for 15 min at 30°C with shaking. Radioactive $^{65}ZnCl_2$ diluted with appropriate concentrations of unlabeled salt was added. Samples (1 ml) withdrawn at 30 min were filtered through 0.45-μm-pore-size filters (Millipore, Molsheim, France), rinsed twice with 10-ml volumes of 10 mM Tris hydrochloride buffer (pH 7.0), dried, and dissolved in a Packard emulsifier scintillator; the radioactivity was counted in a Packard liquid scintillation spectrophotometer.

RESULTS

Isolation of Zn^r clones. When strains containing plasmid pMOL28 or its derivative pMOL50 (2) were plated on mineral medium containing gluconate as a carbon source and zinc at concentrations from 0.6 to 1 mM, resistant colonies arose at frequencies from 2×10^{-7} to 1×10^{-6}. Fifty such colonies derived from strain AE126 (containing pMOL28) and 50 colonies derived from strain AE245 (containing

TABLE 2. Sensitivity of the Zn^r mutants and strains AE126 and AE245 to Zn^{2+}, Ni^{2+}, Co^{2+}, and CrO_4^{2-} [a]

Metal and concn (mM)	Sensitivity of:	
	Zn^r mutants	AE126 and AE245
Zn^{2+}		
0.5	++	(a)
0.75	++	(a)
1	(+)	(a)
1.25	−	−
Ni^{2+}		
1	++	++
2	++	(+)
4	+	−
6	−	−
Co^{2+}		
1	++	++
2	++	++
4	++	(a)
6	+	−
8	(+)	−
10	−	−
CrO_4^{2-}		
0.156	++	++
0.27	++	++
0.364	−	−

[a] Sensitivity was estimated from growth tests after replica plating on agar media. ++, normal growth, as on the control plate; +, slow growth rate; (+), important reduction of growth and increased mortality; (a), some spontaneous mutants; −, no growth.

pMOL50) were analyzed for sensitivity to zinc, nickel, cobalt, and chromate (Table 2). The sensitivities of these mutants were estimated from growth tests after replica plating on agar media containing various concentrations of the metal salts. The 100 Zn^r mutants isolated displayed the same stable resistance to zinc. However, the levels of nickel and cobalt resistance in the Zn^r mutant strains were increased in comparison with those of the parental strains, whereas the chromate resistance level remained constant. It was not possible to isolate a mutant that was resistant to 0.6 mM Zn^{2+} from strain AE104, which is plasmid free.

Location of the mutations leading to a Zn^r phenotype. To determine whether these mutations were located in the plasmid, four Zn^r mutants (strains AE1013 to AE1016) derived from strain AE245 (His^- Met^-) were crossed with the plasmid-free strain AE104. Transconjugants were selected on minimal medium containing either Zn^{2+}, Ni^{2+}, Co^{2+}, or CrO_4^{2-} at the concentrations indicated in Materials and Methods. The transfer frequencies per donor of the Zn marker varied from 2×10^{-2} to 1.8×10^{-4}, which is approximately the transfer frequency observed for plasmid pMOL50 (2). In each cross, the transfer frequencies of the four markers were almost the same, except for the chromate marker, which was transferred at lower frequencies (1.8×10^{-3} to 6×10^{-6}). This was probably due to the fact that the concentration of chromate used for selection is close to the MIC. Sixty transconjugants isolated on each metal were tested for their resistance to the three other metals. Each transconjugant showed resistance to all four metals tested, indicating 100% cotransfer of resistance to all four metals (Zn, Ni, Co, and Cr).

Regulatory phenotype in Zn^r mutants. The inducible or constitutive character of zinc resistance was studied in two Zn^r mutants: AE963 (derived from strain AE126; Fig. 1a) and AE452 (derived from strain AE245; data not shown). In a liquid mineral medium containing 1 or 1.5 mM Zn^{2+}, cells induced by 0.15 mM Zn for 14 h and uninduced cells grew without a lag phase. This suggests that zinc resistance is constitutively expressed in the Zn^r mutants. Since the levels of nickel and cobalt resistance were also increased in the Zn^r mutants (Table 2) and since *cnr* is induced effectively only by nickel (12), the regulation of the nickel resistance was studied in the two Zn^r strains (Fig. 1c) as well as in the parental strains AE126 (Fig. 1b) and AE245 (data not shown) after induction with Ni^{2+}. For strain AE126, cells induced by overnight growth in the presence of 0.2 mM Ni^{2+} began to grow immediately, whereas uninduced cells showed a lag phase of about 15 h. On the other hand, no difference between the growth rate of the induced cells and that of the uninduced cells of the Zn^r strain AE963 was observed, indicating that the nickel resistance had become constitutive in the Zn^r mutants. It was confirmed that the levels of the

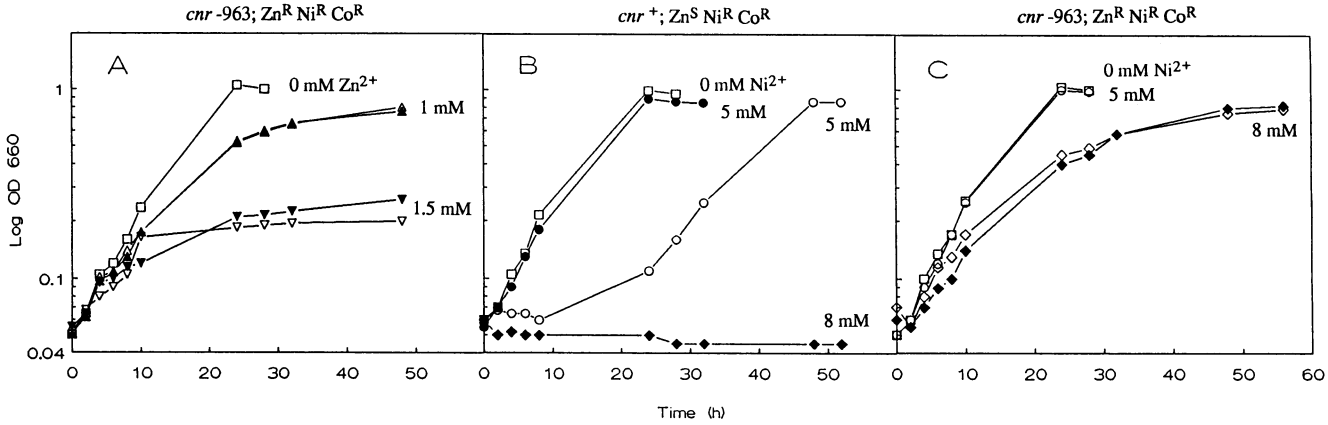

FIG. 1. Growth of strain AE963 (*cnr-963*; Zn^r Ni^r Co^r) (A and C) and parental strain AE126 (*cnr*$^+$; Zn^s Ni^r Co^r) (B) in the presence of Zn^{2+} or Ni^{2+}. Uninduced (□, △, ▽, ○, ◇) or induced (■, ▲, ▼, ●, ◆) cells were inoculated into mineral medium containing gluconate as a carbon source and the metal salt (zero time). Symbols: □, no metal; △, 1 mM Zn^{2+}; ▽, 1.5 mM Zn^{2+}; ○, 5 mM Ni^{2+}; ◇, 8 mM Ni^{2+}. The induction was realized by overnight growth in the presence of 0.15 mM Zn^{2+} (A) or 0.2 mM Ni^{2+} (B and C).

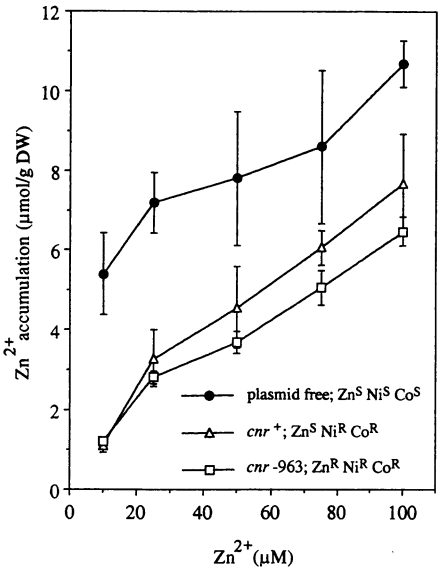

FIG. 2. Accumulation of zinc by the AE104 (plasmid-free), AE126 (*cnr*$^+$), and AE963 (*cnr-963*) strains. Uptake assays were performed at various $^{65}Zn^{2+}$ concentrations. Strain AE126 was induced by 0.2 mM $NiCl_2$ before the uptake experiment. The ^{65}Zn content at 30 min of uptake was measured and plotted against the corresponding concentrations of added cation. DW, dry weight. Each point represents the mean value of three samples.

nickel resistance in the Zn^r mutants were increased in comparison with those of the parental strains. In contrast, the chromate resistance (encoded by the *chr* locus, which is adjacent to *cnr*) remained inducible and its resistance level was not changed in the Zn^r strains.

Accumulation of Zn^{2+} and zinc resistance in pMOL28-containing strains induced by nickel. The accumulation of $^{65}Zn^{2+}$ by strains AE963 (Zn^r Ni^r Co^r), AE126 (the parental strain of AE963; Zn^s Ni^r Co^r), and AE104 (the plasmid-free strain; Zn^s Ni^s Co^s) was measured as described in Materials and Methods. Expression of the *cnr* operon in AE126 was induced by 0.2 mM Ni^{2+} to test the ability of the wild-type strain to mediate Zn efflux. Strain AE104 accumulated considerable amounts of Zn^{2+} at concentrations ranging from 10 to 100 μM (Fig. 2). In contrast, strains AE126 and AE963 accumulated much smaller amounts of Zn^{2+}. At Zn^{2+} concentrations higher than 50 μM, the accumulation of Zn^{2+} was lowest in strain AE963, which displayed the Zn^r phenotype. Therefore, zinc resistance resulted in reduced accumulation of the zinc ions; however, a reduced net accumulation was also observed with Ni^{2+}-induced cells of the parental strain AE126. This result suggests that the induction of the strain AE126 with Ni^{2+} confers a weak resistance to zinc. This was confirmed by measuring the effect of induction by various concentrations of Ni^{2+} on the cell doubling time in media containing different concentrations of zinc (Table 3). Induction by 0.2 mM Ni^{2+} allowed strain AE126 to grow in the presence of 0.1 mM Zn^{2+} with a doubling time essentially the same as that of the control cells, whereas uninduced cultures showed a twofold increase of the cell doubling time. The best induction was observed at 0.8 mM Ni^{2+}.

Cloning of the region responsible for the Zn^r phenotype. Since the mutations that resulted in a Zn^r phenotype altered the level of expression of the *cnr* operon, we decided to clone the 12.2-kb *Eco*RI-*Xba*I fragment (Fig. 3) that confers resistance to nickel and cobalt (10, 20) and to study whether

TABLE 3. Effect of Ni^{2+} induction on the tolerance to Zn^{2+} in AE126[a]

Ni^{2+} concn (mM) in preculture	Doubling time (h) with the following concn of Zn^{2+} (mM):			
	0	0.1	0.25	0.5
0	2.17	4.17	6.25	10
0.2	2.22	2.61	4.17	6.66
0.4	2.27	2.41	3.55	5.63
0.8	2.29	2.29	3.33	5.48

[a] Precultures of AE126 (*cnr*$^+$) cultivated in the presence of nickel were washed and transferred to fresh minimal medium with various concentrations of Zn^{2+}. Doubling times were measured during the first 8 h after the transfer.

this fragment also conferred the Zn^r phenotype. The DNA of pMOL29 was purified from the strain AE963, digested by *Xba*I and *Eco*RI, and cloned into the multiple cloning site of the broad-host-range plasmid pRK415 (5). After ligation, the DNA was used to electrotransform *E. coli* S17/1. Tc^r transformants of strain S17/1 were amplified in liquid medium containing 20 μg of tetracycline per ml and then used as donors in a mating with AE104. Ten transconjugants isolated on media containing 0.6 mM Zn^{2+}, 0.6 mM Ni^{2+}, or 0.6 mM Co^{2+} were checked for tetracycline resistance. All transconjugants selected on one of the three metals were shown to be resistant to tetracycline and also to the two other metals. All were chromate sensitive. The fragment cloned in pRK415 did not hybridize (data not shown) with plasmid pMOL30 carrying the *czcCBAD* operon (11, 13), which is responsible for efflux of zinc, cobalt, and cadmium (14).

Physical mapping and subcloning of the region conferring the Zn^r phenotype. Figure 3 shows the restriction endonuclease site map of the 12.2-kb *Eco*RI-*Xba*I fragment of pMOL29 conferring the Zn^r phenotype as well as resistance to Ni^{2+} and Co^{2+}. The smallest fragment cloned by Nies et al. (10) that restored inducible and full resistance to cobalt and nickel was an 8.5-kb *Eco*RI-*Pst*I-*Pst*I fragment (Fig. 3). In contrast, the 13.5-kb *Hin*dIII fragment cloned by Siddiqui et al. (20) restored only constitutive resistance. These two fragments overlap by 6.3 kb.

To delete the right 3.7-kb *Pst*I-*Pst*I-*Xba*I-*Pst*I fragment (Fig. 3) and to obtain an 8.5-kb *Eco*RI-*Pst*I-*Pst*I fragment, plasmid pMOL154 was partially digested by *Pst*I. The partially digested plasmids were ligated on themselves and used for electrotransformation of *E. coli* S17/1. Plasmid pMOL154, having lost the 3.6-kb *Pst*I fragment, was screened from among 20 Tc^r transformants after plasmid extraction and digestion. This plasmid was designated pMOL159. A clone containing pMOL159 was then crossed with AE104, and the transconjugants were analyzed for resistance to Zn^{2+}, Ni^{2+}, and Co^{2+}. The *A. eutrophus* transconjugants containing pMOL159 all showed resistance to Zn^{2+}, Ni^{2+}, and Co^{2+}.

The 12.2-kb *Eco*RI-*Xba*I fragment was also restricted to a 9.7-kb *Bam*HI fragment and a 4.3-kb *Pst*I fragment (Fig. 3). These fragments were cloned into the polylinker of plasmid pRK415, resulting in plasmids pMOL157 and pMOL166, respectively. After selection on plates containing tetracycline, X-Gal, and IPTG, white Tc^r transformants of *E. coli* DH5α were purified and their plasmids were extracted to transform S17/1. After mating with strain AE104, the Tc^r transconjugants were sensitive to the three metals for plasmid pMOL166 and sensitive to zinc but expressed reduced resistance to nickel and cobalt for plasmid pMOL157. There-

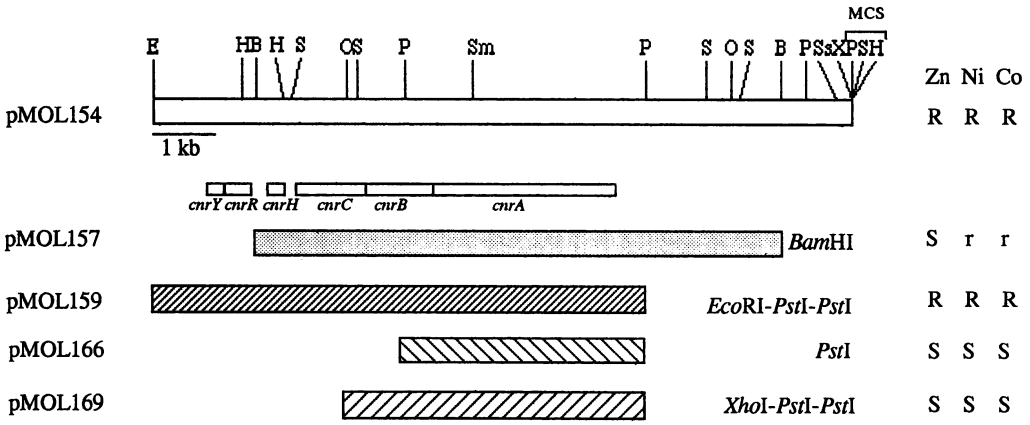

FIG. 3. Structure of the 12.2-kb *Eco*RI-*Xba*I fragment cloned from pMOL29 into pRK415. This fragment includes the mutation leading to the Zn^r phenotype and the *cnr* locus. The restriction endonuclease sites of the original 12.2-kb insert, the remaining restriction endonuclease sites of the pRK415 polylinker (MCS), and four key subclones (Table 1) are shown. The phenotypes conferred by these fragments in AE104 are also given (R, resistant; S, sensitive; r, weakly resistant). Restriction endonuclease sites (X, *Xba*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I, Sm, *Sma*I; Ss, *Sst*I; O, *Xho*I) were determined by enzyme digestion. The different loci identified by Liesegang et al. (7) are also shown.

fore, the 1.7-kb *Eco*RI-*Bam*HI fragment (Fig. 3) is necessary (i) for expression of the Zn^r phenotype and (ii) for full expression of nickel and cobalt resistance.

The 8.5-kb *Eco*RI-*Pst*I-*Pst*I fragment of plasmid pMOL 159 was also reduced to a 5.4-kb *Xho*I-*Pst*I-*Pst*I fragment by first digesting pMOL159 with *Eco*RI and *Xho*I and then transforming the sticky ends into blunt ends as described in Materials and Methods. After ligation, this resulted in plasmid pMOL169. This plasmid was used as described above to measure metal resistance in strain AE104. All Tc^r transconjugants showed a Zn^s Ni^s Co^s phenotype.

Complementation studies. Plasmid pMOL154 (plasmid pRK415 containing the 12.2-kb *Eco*RI-*Xba*I fragment) was transferred from *E. coli* CM890 to strains AE126(pMOL28) and AE245(pMOL50) (*his met*). Tc^r transconjugants were isolated on Tris-buffered minimal medium supplemented with histidine and methionine. Three transconjugants of each cross were purified and then analyzed for the presence of the two plasmids and for resistance to Zn^{2+}, Ni^{2+}, and Co^{2+}. Transconjugants containing the recombinant plasmid pMOL159 and either pMOL28 or pMOL50 displayed a Zn^s Ni^r Co^r phenotype (data not shown). The growth of AE126 (pMOL154) and AE245(pMOL154) was studied for induced (overnight culture in presence of 0.2 mM Ni^{2+}) and uninduced cells in a liquid mineral medium containing nickel. A lag phase comparable to that observed in parental strains (AE126 and AE245) was observed for uninduced cells of the transconjugant clones, demonstrating the inducibility of the nickel resistance in the merodiploids and confirming the recessive character of the mutations resulting in a Zn^r phenotype.

DISCUSSION

The majority of metal resistance genes are inducible, as reported for several systems including mercury (23); arsenic and antimony (21, 24); chromate (10); copper (16); cadmium (25); cadmium, zinc, and cobalt (*czc* operon [14]); and nickel and cobalt (*cnr* operon [14, 18]). In this study, we report spontaneous mutations conferring constitutive resistance to zinc in *A. eutrophus* CH34. These mutations were shown to interact strongly with genes conferring nickel and cobalt resistance (*cnr* locus), increasing the level of the resistance and drastically altering regulation (constitutive phenotype in the Zn^r mutants). Constitutive nickel-resistant mutants of plasmid pMOL28 were also previously isolated by Siddiqui et al. (20) after treatment with nitrite or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. These mutants displayed increased nickel and cobalt resistance as well. Therefore, the isolation of Zn^r mutants allows fast positive selection of mutants affected in a regulatory function in the *cnr* operon.

The resistance phenotype in a Zn^r mutant was due to substantial reduction of the zinc accumulation in comparison with that in the plasmid-free strain AE104. However, reduced accumulation of zinc was also observed in the parental strain AE126 (induced with nickel), but to a lesser degree than in the Zn^r mutant. This suggests that the reduced accumulation of zinc in strain AE126 depends on the nickel-cobalt efflux system, which catalyzes the extrusion of nickel and cobalt ions from the cell (14, 18). This hypothesis is partially supported by the fact that overnight growth of strain AE126 in the presence of Ni induced weak resistance to zinc. In a system that overexpresses the proteins encoding nickel and cobalt efflux, as could be the case in the Zn^r mutants, zinc, which has a weak affinity for this system, would be efficiently expelled as a result of derepression. Up to now, pMOL28 mutants with increased affinity for Zn had not been found because mutants affected in the *cnr* regulation are probably more common. Mutants with increased affinity to zinc are expected to be found by screening clones of the strain AE126 displaying an increased resistance to zinc but remaining inducible by nickel.

The nickel and cobalt resistance genes were previously cloned on a 13.5-kb *Hin*dIII fragment by Siddiqui et al. (19) and on an 8.5-kb *Eco*RI-*Pst*I-*Pst*I fragment by Nies et al. (9). These fragments conferred constitutive inducible phenotypes, respectively. The constitutive phenotype may be explained by a deletion of the regulatory region, which should be located between *Eco*RI and the second *Hin*dIII site (Fig. 3). This was recently confirmed by the sequencing of this region, which shows three loci containing four small open reading frames (*cnrY*, overlapping $ORF0_a$ and $ORF0_b$; *cnrR*, ORF1; *cnrH*, ORF2) involved in the regulation of the *cnr* operon (7). By cloning a 12.2-kb *Eco*RI-*Xba*I fragment of a pMOL28 Zn^r plasmid (pMOL29) on which the cobalt and

nickel resistance genes and the regulatory region were located, we were able to confer the zinc resistance to the sensitive strain AE104. This fragment was subsequently reduced to an 8.5-kb *Eco*RI-*Pst*I-*Pst*I fragment still encoding resistance to the three metals (Zn^{2+}, Ni^{2+}, and Co^{2+}) and a 9.7-kb *Bam*HI fragment conferring reduced resistance to nickel and cobalt and a Zn^s phenotype. These results suggest that (i) a regulatory sequence required for optimal expression of *cnr* is located on the 1.7-kb *Eco*RI-*Bam*HI fragment and (ii) the locus corresponding to the Zn^r phenotype could be located in this 1.7-kb *Eco*RI-*Bam*HI fragment. Four Tn*5* insertions that were mapped in this region, specifically, inside *cnrY* ($ORF0_a$ or $ORF0_b$), display Zn resistance as well as a constitutive phenotype and increased resistance to Ni and Co (7). This would indicate that the spontaneous mutations leading to the Zn^r phenotype should be located in the *cnrY* locus as well.

Complementation studies showed that the wild-type allele acts in *trans* on the 12.2-kb *Eco*RI-*Xba*I mutated fragment by restoring the wild-type sensitivity to zinc and the inducibility of nickel resistance (recessivity of the mutations conferring the Zn^r phenotype). This suggests that the mutations might alter a *trans*-acting repressor.

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