JOURNAL OF BACTERIOLOGY, Sept. 1989, p. 5065–5070
0021-9193/89/095065-06$02.00/0

Vol. 171, No. 9

Cloning and Expression of Plasmid Genes Encoding Resistances to Chromate and Cobalt in *Alcaligenes eutrophus*

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Received 4 April 1989/Accepted 12 June 1989

Resistances to chromate and cobalt were cloned on a 30-kilobase-pair (kb) DNA region from the large *Alcaligenes eutrophus* plasmid pMOL28 into the broad-host-range mobilizable cosmid vector pVK102. A restriction nuclease map of the 30-kb region was generated. The resistances expressed from the hybrid plasmids after transfer back into *A. eutrophus* were inducible and conferred the same degree of resistance as the parent plasmid pMOL28. Resistances were expressed in metal-sensitive *Alcaligenes* strains and related bacteria but not in *Escherichia coli*. Resistance to chromate was further localized on a 2.6-kb *Eco*RI fragment, and resistance to cobalt was localized on an adjoining 8.5-kb *Pst*I-*Eco*RI fragment. When the 2.6-kb *Eco*RI fragment was expressed in *E. coli* under the control of a bacteriophage T7 promoter, three polypeptides with molecular masses of 31,500, 21,000, and 14,500 daltons were visible on autoradiograms. The 31,500- and 21,000-dalton polypeptides were membrane bound; the 14,500-dalton polypeptide was soluble.

Alcaligenes eutrophus CH34 contains two large plasmids which confer resistances to several metal ions (3, 11). Plasmid pMOL28 (163 kilobase pairs [kb] [11]) encodes inducible resistances to 0.2 mM CrO_4^{2-} (Chr^r), 1 mM Co^{2+} ($CobA^r$), and 1 mM Ni^{2+} (Nic^r) (11, 13). Chromate resistance results from reduced accumulation of CrO_4^{2-} (13). Cobalt and nickel resistances result from inducible, energy-dependent ion efflux (13, 16, 18, 19) and the experiments described in this and the accompanying publication (17) show that $CobA^r$ and Nic^r are determined by the same system. Resistance to 2.5 mM Zn^{2+} (Zin^r), 5 mM Co^{2+} ($CobB^r$), and 1 mM Cd^{2+} (Cad^r) is conferred by plasmid pMOL30 (238 kb [11]). These resistances also result from an inducible, energy-dependent cation efflux system (13) which has been cloned on a 9.1-kb *Eco*RI fragment of pMOL30 (12).

This report describes the molecular cloning of Chr^r and $CobA^r$ encoded by plasmid pMOL28 and expression of these resistances in a plasmid-free variant of *A. eutrophus* and related bacteria. This is a first step to compare the molecular biology of the two cobalt resistances ($CobA^r$ on pMOL28 and $CobB^r$ on pMOL30) with each other and to compare the pMOL28-encoded chromate resistance (Chr^r) with chromate resistance from *Pseudomonas* plasmids (1, 15; C. Cervantes et al., submitted for publication).

MATERIALS AND METHODS

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

Growth conditions and transport assays. Nutrient broth and Luria broth were used as complex media (2). Tris-buffered mineral salts medium (11, 12) was used for testing metal resistance and for growing *A. eutrophus* strains. Analytical-grade salts of $CoCl_2 \cdot 6H_2O$ and K_2CrO_4 were used to prepare 1.0 M stock solutions. Metal-containing medium was solidified with 20 g of Bacto-Agar (Difco Laboratories, Detroit, Mich.) per liter; other solid media contained 15 g of agar per liter. For conjugal gene transfer, overnight cultures of donor and recipient strains grown at 30°C in complex medium were mixed and plated onto nutrient broth agar. After overnight growth, the bacteria were suspended in saline (9 g of NaCl per liter), diluted, and plated onto selective media as previously described (12). Metal ion uptake and efflux experiments were performed as previously described (13). For chromate uptake experiments, however, cells were cultivated in the presence of 30 μM sulfate instead of 3 mM sulfate in order to derepress chromate uptake (14).

Genetic techniques. For isolation of plasmid pMOL28 DNA, a method (12) based on the alkaline lysis procedure (7) was used. Isolation of plasmids smaller than 50 kb, agarose gel electrophoresis, and standard molecular genetic manipulations were performed as described by Maniatis et al. (10). Restriction endonucleases and T4 DNA ligase were obtained from Bethesda Research Laboratories, Gaithersburg, Md. Transformation of *Escherichia coli* S17-1 was conducted as previously described (12). Cosmid DNA was packaged and used for infection of *E. coli* S17-1 according to the protocol provided by the manufacturer of the DNA-packaging kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

Construction of plasmid pDNA121. Plasmid pDNA121 (Kan^r Tet^s) was constructed by cloning the kanamycin resistance determinant isolated from plasmid pRME1 into the single *Sal*I site of plasmid pRK290 (4), which results in interruption of the pRK290 tetracycline resistance gene. When a pSUP202-based hybrid plasmid (which is not able to replicate in *A. eutrophus* [21]) was transferred into strain AE104(pDNA121) by conjugation, the plasmids recombined via the IncP1 *mob* region present in both vectors and selection on Tet^r led to the isolation of transconjugants carrying a pDNA121::pSUP202 cointegrate plasmid (D. H. Nies, unpublished results).

Radioactive labeling of the polypeptides encoded by the Chr^r resistance determinant. The polypeptides expressed by the Chr^r resistance region were analyzed by using the bacteriophage T7 RNA polymerase-promoter system (22). Expression experiments were carried out by following a

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant phenotype	Source or reference[a]
A. eutrophus		
AE126(pMOL28)	$CobA^r$ Nic^r Chr^r	11
AE104	$CobA^s$ Chr^s	11
H16(pHG1)	Cob^s Chr^s	DSM 428, ATCC 17699
JMP222	Cob^s Chr^s	5
A. hydrogenophilus		
M50	Cob^s Chr^s	H. G. Schlegel
Pseudomonas putida	Cob^s Chr^s	ATCC 12633
P. stutzeri	Cob^s Chr^s	ATCC 17588
P. aeruginosa		
PAO1	Cob^s Chr^s	8
PAO1(pCRO616)	Cob^s Chr^r	C. Cervantes
S. marcescens	Cob^s Chr^s	ATCC 13880
E. coli K-12	Cob^s Chr^s	DSM 498
S17-1	RP4-Tra functions	20
RP4-derived plasmids		
pRK290	Tet^r	4
pVK102	Tet^r Kan^r	9
pDNA121	Tet^s Kan^r	Kanamycin resistance determinant in pRK290
pDNA206	Tet^r Chr^r $CobA^r$	30-kb DNA region from plasmid pMOL28 in pVK102
pDNA219	Tet^r Chr^s $CobA^r$	Deletion of 2.6-kb *Eco*RI fragment from pDNA206
ColE1-derived plasmids		
pSUP202	Cam^r Tet^r Amp^r	21; contains IncP1 *mob* region
pGP1-2	Kan^r	S. Tabor; contains T7 RNA polymerase gene
pT7-5	Amp^r	S. Tabor, contains T7 RNA polymerase promoter
pRME1	Amp^r Kan^r	W. Messer; contains kanamycin resistance determinant
pUC19	Amp^r	23
pECD300	Tet^r Amp^r Chr^r	2.6-kb *Eco*RI fragment in pSUP202
pECD311	Amp^r	2.6-kb *Eco*RI fragment in pT7-5 (correct orientation)
pECD310	Amp^r	2.6-kb *Eco*RI fragment in pT7-5 (opposite orientation)
pECD313	Tet^r $CobA^r$	12-kb *Xba*I-*Eco*RI fragment in pSUP202
pECD314	Tet^r $CobA^s$	3.7-kb *Kpn*I-*Eco*RI fragment in pSUP202
pECD315	Tet^r $CobA^s$	4.3-kb *Sma*I-*Eco*RI fragment in pSUP202
pECD326	Tet^r $CobA^r$	8.5-kb *Pst*I-*Pst*I-*Eco*RI fragment in pSUP202

[a] ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen.

protocol provided by Stanley Tabor (personal communication) with slight modifications. The 2.6-kb *Eco*RI fragment carrying the Chr^r region was subcloned in both orientations from plasmid pECD300 into plasmid pT7-5. The resulting hybrid plasmids were transformed into *E. coli* K-38(pGP1-2) (22). Isolated Amp^r transformant strains were grown overnight at 30°C in Luria broth containing 50 μg of ampicillin and 50 μg of kanamycin per ml. The cultures were diluted 40-fold into fresh Luria broth and incubated for 3 h with shaking at 30°C. A 0.5-ml volume of the cell suspension was harvested by centrifugation, washed with 4 ml of M9 medium (2), and suspended in 1 ml of M9 medium containing 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 2 mg of glucose, 20 μg of thiamine, and 0.1 mg each of 18 amino acids (excluding methionine and cysteine). The cells were incubated for 1 h with shaking at 30°C and for 15 min with shaking at 42°C (to induce synthesis of bacteriophage T7 RNA polymerase). Rifampin (Sigma Biochemical Co., St. Louis, Mo.) was added to a final concentration of 200 μg/ml, and incubation at 42°C was continued with shaking for 10 min.

The cells were incubated for a further 20 min with shaking at 30°C, and 30 μCi of ^{35}S-labeled methionine (800 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was added. Incubation continued with shaking for 5 min at 30°C, and the cells were rapidly centrifuged with a microcentrifuge. The cell pellet was suspended in denaturing buffer (containing, per liter of 50 mM Tris hydrochloride [pH 6.7], 10 g of sodium dodecyl sulfate, 10 ml of β-mercaptoethanol, 0.2 g of bromophenol blue, 100 g of sucrose, and 2 mM EDTA) and incubated for 3 min at 95°C. Polyacrylamide gel electrophoresis was carried out in the presence of sodium dodecyl sulfate on a gradient gel (3 to 19% polyacrylamide [2]). Autoradiography was performed by using Kodak SB5 film (Eastman Kodak Company, Rochester, N.Y.).

Intracellular localization of polypeptides. To identify the location of the radioactive polypeptides produced by the 2.6-kb *Eco*RI fragment, an expression experiment was scaled up 10-fold. After the cells were harvested by using the microcentrifuge, the cells were suspended in 1 ml of 10 mM Tris hydrochloride buffer (pH 7.0) and disrupted on ice by using a sonicator (W225 R; Ultrasonics Inc.) at a continuous setting output of 10 for 15 min. Cell debris was removed by using the microcentrifuge. After ultracentrifugation (45 min, 100,000 × *g*; DuPont Sorvall ultracentrifuge) of the micro-

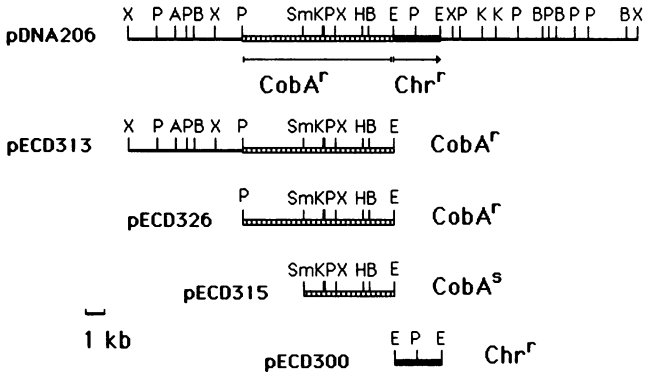

FIG. 1. Structure of the DNA region encoding resistances to chromate and cobalt. The restriction endonuclease sites of the original 30-kb insert in plasmid pDNA206 and four key subclones (see Table 1) are shown. Restriction endonuclease sites (E, *Eco*RI; X, *Xho*I; K, *Kpn*I; P, *Pst*I; A, *Xba*I; Sm, *Sma*I; B, *Bam*HI; and H, *Hin*dIII) were determined by enzyme digestion. The arrow indicates the maximum length and direction of transcription of the chromate resistance determinant (Chr^r); the bar indicates the maximum length of the cobalt resistance determinant (CobA^r). The internal 2.6-kb *Eco*RI fragment encoding Chr^r (■) and the *PSt*I-*Pst*I-*Eco*RI fragment responsible for CobA^r (▥) are indicated.

centrifuge supernatant fluid, the supernatant fluid was mixed with 0.5 volume of threefold denaturing buffer and incubated for 3 min at 95°C. The membranes were suspended in 1 ml of 10 mM Tris hydrochloride buffer (pH 7.0), again subjected to ultracentrifugation, suspended in denaturing buffer, and incubated for 3 min at 95°C. The same amount of protein from each fraction was subjected to gel electrophoresis and autoradiography as described above.

RESULTS

Cloning of Chr^r and CobA^r. Plasmid pMOL28 DNA (163 kb) was purified from strain AE126, partially digested with *Xho*I, and cloned into the *Xho*I site of the broad-host-range cosmid vector pVK102 (9). After ligation, the DNA was packaged into lambda phage particles which were used to infect *E. coli* S17-1. Independent Tet^r Kan^s transfectants (541) of strain S17-1 were isolated. To study expression of resistances in *A. eutrophus*, each transfectant was used as the donor in a mating with plasmid-free *A. eutrophus* AE104. Tet^r transconjugants of strain AE104 were isolated and tested for metal resistance by replica plating to medium containing 1 mM Co^{2+} or 0.2 mM CrO_4^{2-}. AE104 transconjugants with the phenotype CobA^r Chr^r (11 transconjugants), CobA^r Chr^s (3 transconjugants), or CobA^s Chr^r (3 transconjugants) were isolated. All CobA^r transconjugants were also Nic^r. Therefore, CobA^r and Chr^r are encoded by different genetic loci, but CobA^r and Nic^r are probably encoded by the same determinant.

Physical map of the region coding for chromate and cobalt resistances. A restriction endonuclease site map of the region of pMOL28 governing chromate and cobalt resistance is shown in Fig. 1. Plasmid DNA of the 17 hybrid plasmids was isolated from *E. coli* host strains and subjected to *Xho*I digestion. Plasmids encoding CobA^r and Chr^r always had two approximately 7-kb *Xho*I fragments in common. Chr^r was lost together with only one of the two 7-kb *Xho*I fragments; CobA^r was lost with either of the two 7-kb *Xho*I fragments. Therefore, Chr^r was encoded by a single 7-kb *Xho*I fragment, and CobA^r required both 7-kb *Xho*I fragments.

TABLE 2. MICs of cobalt and chromate for pDNA206-containing transconjugants

Strain	MIC (mM)[a] of:	
	Co^{2+}	CrO_4^{2-}
A. eutrophus		
AE104	0.2	0.1
AE126(pMOL28)	5.0	0.2
AE104(pDNA206)	5.0	0.2
AE104(pDNA219)	5.0	0.1
AE104(pDNA121::pECD326)	5.0	NT[b]
AE104(pDNA121::pECD300)	0.2	0.2
H16	0.05	0.05
H16(pDNA206)	1.0	0.2
JMP222	0.5	0.05
JMP222(pDNA206)	0.5	0.2
A. hydrogenophilus		
M50	0.05	0.05
M50(pDNA206)	0.5	0.2
P. stutzeri	0.1	0.05
P. stutzeri (pDNA206)	0.2	0.1
P. aeruginosa		
pAO1	1.0	0.05
pAO1(pDNA206)	1.0	0.1
pAO1(pCRO616)	1.0	0.2

[a] Conditions, 4 days of growth at 30°C on mineral salts agar containing metal salts.
[b] NT, Not tested.

One hybrid plasmid (pDNA206), containing two additional 11- and 5-kb *Xho*I fragments flanking the two 7-kb *Xho*I fragments, was chosen for detailed studies. When subcloned separately into plasmid pVK102, the 11- and the 5-kb *Xho*I fragments did not encode Chr^r or CobA^r. Plasmid pDNA206 (which confers Chr^r and CobA^r on *A. eutrophus* AE104) was isolated from the *E. coli* host strain and characterized by restriction endonuclease digestion with various enzymes. The physical map of the insert region of plasmid pDNA206 is shown in Fig. 1.

Expression of Chr^r and CobA^r in other metal-sensitive bacteria. *E. coli* S17-1(pDNA206) was used as the donor strain in matings with a variety of metal-sensitive recipients. Plasmid pDNA206 conferred chromate and cobalt resistance to *A. eutrophus* H16, *Alcaligenes hydrogenophilus* M50, and *Pseudomonas stutzeri* (Table 2) but did not confer resistance to *E. coli* K-12 or *Serratia marcescens* (data not shown). Plasmid pDNA206 conferred Chr^r but not CobA^r to *A. eutrophus* JMP222 and *Pseudomonas aeruginosa* PAO1 (Table 2).

Subcloning of Chr^r. The 2.6-kb *Eco*RI fragment of plasmid pDNA206 (Fig. 1) was deleted, yielding plasmid pDNA219. Strain AE104(pDNA219) was CobA^r but Chr^s. Thus, the 2.6-kb internal *Eco*RI fragment of plasmid pDNA206 was involved in the expression of Chr^r but not CobA^r. The 2.6-kb *Eco*RI fragment was subcloned into the single *Eco*RI site of plasmid pSUP202, yielding plasmid pECD300. Plasmid pSUP202 can be mobilized into *A. eutrophus* but is not able to replicate in this host (21). However, plasmid pSUP202 and its derivatives can survive in strain AE104 as cointegrates with plasmid pDNA121. When transferred into strain AE104 (pDNA121), the resulting transconjugant strain carrying the plasmid pDNA121::pECD300 was Chr^r. Therefore, Chr^r is encoded by the 2.6-kb *Eco*RI fragment of pDNA206.

Subcloning of CobA^r. The 12-kb *Xba*-I-*Eco*RI fragment (Fig. 1) was cloned from plasmid pDNA219 into plasmid pUC19 (23). The kanamycin resistance determinant from plasmid pRME1 was cloned into the single *Xba*I site of the resulting pUC19 hybrid plasmid. Since the kanamycin resistance cassette is flanked by multilinker restriction endonuclease sites, the 12-kb *Xba*I-*Eco*RI fragment was cloned as an *Eco*RI fragment into plasmid pSUP202, leading to plasmid pECD313 (Fig. 1). When transferred into strain AE104 (pDNA121), the resulting transconjugant strain carrying cointegrate plasmid pDNA121::pECD313 was CobA^r (data not shown). Therefore, the 12-kb *Xba*I-*Eco*RI fragment (Fig. 1) was sufficient for the expression of CobA^r.

Since the multilinker site attached to the *Xba*I restriction nuclease site of the 12-kb fragment contained *Kpn*I and *Sma*I restriction nuclease sites, an 8.3-kb *Kpn*I fragment and a 7.7-kb *Sma*I fragment could be deleted from plasmid pECD313, leading to plasmids pECD314 (containing the 3.7-kb *Kpn*I-*Eco*RI fragment [Fig. 1]) and pECD315 (containing the 4.3-kb *Sma*I-*Eco*RI fragment [Fig. 1]), respectively. When transferred into strain AE104(pDNA121), the resulting transconjugant strains carrying plasmids pDNA 121::pECD314 and pDNA121::pECD315 both were CobA^s. Therefore, the DNA region necessary for expression of CobA^r must extend to the left of the *Sma*I site shown in Fig. 1.

Plasmid pECD313 was partially digested with *Pst*I, ligated, and transformed into *E. coli* S17-1. Plasmid DNA of tetracycline-resistant *E. coli* transformants which were able to transfer CobA resistance to *A. eutrophus* AE104(pDNA 121) were isolated and digested with *Pst*I. The smallest plasmid conferring CobA^r, plasmid pECD326, contained the 8.5-kb *Pst*I-*Pst*I-*Eco*RI fragment marked in Fig. 1. Strains AE104(pDNA121::pECD313) and AE104(pDNA121::pECD 326) were Nic^r, but strains AE104(pDNA121::pECD314) and AE104(pDNA121::pECD315) were Nic^s (data not shown). Thus, CobA^r and Nic^r were located on the 8.5-kb *Pst*I-*Eco*RI fragment.

Cloned cobalt resistance was inducible. On Tris gluconate mineral salts agar, the MIC of Co^{2+} for the plasmid-free strain AE104 was 0.2 mM, and for strains AE126 (pMOL28), AE104(pDNA206), AE104(pDNA219), and AE104(pDNA121::pECD326) it was 5 mM (Table 2) (11). For strain AE126(pMOL28), CobA^r and Nic^r are inducible by overnight cultivation in the presence of 100 μM Ni^{2+} or 100 μM Co^{2+} (13, 18). Comparable to strain AE126, cells of strains AE104(pDNA206), AE104(pDNA219), and AE104 (pDNA121::pECD326) induced by overnight growth on 100 μM Ni^{2+} or 100 μM Co^{2+} started to grow after a shorter lag phase on 1 mM Co^{2+} or 1 mM Ni^{2+} than did uninduced cells (data not shown). Strain AE104(pDNA206) also exhibited decreased accumulation of 0.2 mM $^{60}Co^{2+}$ or 0.2 mM $^{63}Ni^{2+}$ (data not shown) and stimulated Co^{2+} efflux (Fig. 2A), all comparable to strain AE126. Therefore, the complete inducible CobA and Nic resistance determinants were cloned in plasmid pDNA206 and were encoded by the 8.5-kb *Pst*I-*Eco*RI fragment.

Plasmids pDNA206 and pECD300 encode full and inducible resistance to chromate. On Tris gluconate mineral salts agar, strains AE104 and AE104(pDNA219) had a CrO_4^{2-} MIC of 0.1 mM, and strains AE126, AE104(pDNA206), and AE104 (pDNA121::pECD300) had a CrO_4^{2-} MIC of 0.2 mM (Table 2). Resistance to CrO_4^{2-} encoded by plasmid pDNA206 was inducible by overnight cultivation in 20 μM CrO_4^{2-} (data not shown). Strains AE126, AE104(pDNA206), and AE104 (pDNA121::pECD300) exhibited decreased accumulation of chromate compared with strains AE104 and AE104 (pDNA219) (Fig. 2B). Strain AE104(pDNA219) accumulated less chromate compared with strain AE104 (Fig. 2B) but did not exhibit any residual chromate resistance in growth experiments (data not shown). Reduced accumulation of chromate was inducible in all three strains (data not shown). However, uninduced cells of strains AE104(pDNA206) and AE104(pDNA121::pECD300) also showed somewhat decreased accumulation of chromate when compared with Chr^s strains and with uninduced cells of strain AE126 (data not shown). This result may be due to a gene dosage effect caused by a higher copy number of the IncP1 hybrid plasmids compared with plasmid pMOL28 in strain AE126.

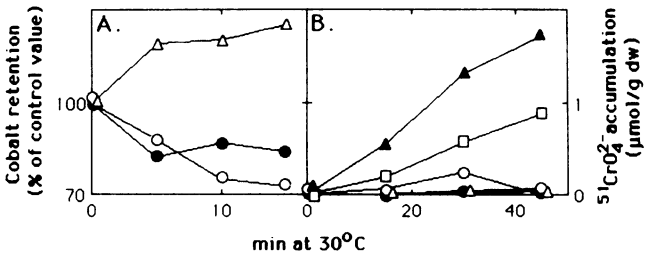

FIG. 2. Efflux of cobalt and reduced accumulation of chromate. (A) Efflux of cobalt. Cells of strains AE126(pMOL28) (○), AE104(pDNA206) (●), and AE104 (△) were incubated with 0.2 mM $^{60}Co^{2+}$ for 20 min at 4°C. Gluconate was added, and the cells were incubated for an additional 10 min at 4°C. The zero-time sample was taken, and the cells were shifted to 30°C. Cation retention was measured and plotted as a percentage of the zero-time sample radioactivity (AE104, 10,900 cpm/mg [dry weight]; AE126, 18,390 cpm/mg [dry weight]; AE104(pDNA206), 9,300 cpm/mg [dry weight]). Strain AE104 was grown overnight without inducer, and the strains AE126 and AE104(pDNA206) were grown in the presence of 100 μM Co^{2+}. (B) Reduced accumulation of chromate. Accumulation of 20 μM $^{51}CrO_4^{2-}$ by strains AE126(pMOL28) (○), AE104(pDNA206) (●), AE104 (▲), AE104(pDNA219) (□), and AE104(pDNA121::pECD300) (△) was compared. Strains AE104 and AE104(pDNA219) were cultivated overnight without inducer, and strains AE126, AE104(pDNA206), and AE104(pDNA121::pECD300) were cultivated overnight in the presence of 10 μM CrO_4^{2-}. dw, Dry weight.

Polypeptides expressed from the 2.6-kb *Eco*RI fragment in *E. coli*. The 2.6-kb *Eco*RI fragment which encodes inducible resistance to chromate was cloned in both orientations into plasmid pT7-5 (leading to plasmids pECD310 and pECD311) and expressed in *E. coli* from the phage T7 promoter. After sodium dodecyl sulfate-gel electrophoresis and autoradiography, three radioactive polypeptide bands were visible from expression of the 2.6-kb *Eco*RI fragment in one orientation (plasmid pECD311) but not in the other orientation (plasmid pECD310) nor in the control with plasmid pT7-5 alone (Fig. 3A). The bands correspond to radioactive polypeptides with molecular masses of 31,500, 21,000, and 14,500 daltons, respectively. The band corresponding to the 21,000-dalton polypeptide was weaker than the two other bands (Fig. 3).

The 31,500-dalton polypeptide appeared as a broad band rather than as a sharp band (Fig. 3). This is characteristic for membrane proteins on polyacrylamide gels (6). When [^{35}S]methionine-labeled *E. coli* cells containing plasmid pECD311 were disrupted, the major fractions of the 31,500- and the 21,000-dalton polypeptides appeared in the washed membranes (Fig. 3B), although some proteins were also visible in the supernatant fluid. In contrast, most of the 14,500-dalton polypeptide appeared in the supernatant fluid (Fig. 3B). Therefore, the 31,500- and the 21,000-dalton

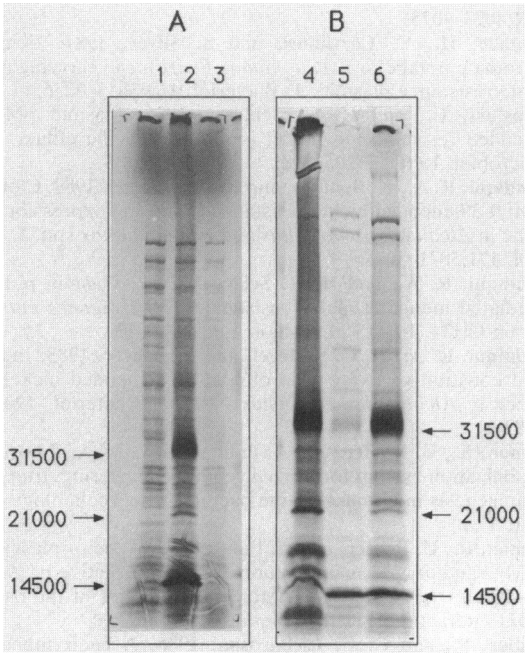

FIG. 3. Synthesis of [^{35}S]methionine-labeled polypeptides determined by the chromate resistance region. The 2.6-kb *Eco*RI fragment containing the Chr^r region was cloned under control of the phage T7 promoter and expressed in *E. coli* K38(pGP1-2). (A) [^{35}S]methionine-labeled polypeptides expressed by pT7-5 alone (lane 3) or with the Chr^r determinant cloned either in the correct (lane 2) or the opposite (lane 1) orientation. (B) [^{35}S]methionine-labeled polypeptides in the total sonic extract (lane 6) of cells with plasmid pECD311 or in membrane (lane 4) or supernatant (lane 5) fractions after ultracentrifugation. The three insert-specific radioactive polypeptides are marked by arrows with the appropriate molecular weights.

polypeptides are probably membrane bound, while the 14,500-dalton polypeptide is soluble. Comparable T7 promoter expression studies of the CobA^r determinant were not done.

DISCUSSION

Chr^r could be physically separated from CobA^r. Chr^r was subcloned on a 2.6-kb *Eco*RI fragment, while CobA^r was subcloned on an 8.5-kb *Pst*I-*Eco*RI fragment. Resistance to chromate encoded by plasmid pMOL28 was inducible and based on decreased accumulation of chromate (13). The chromate resistance determinant cloned from plasmid pMOL28 lacks detectable homology with the chromate resistance region cloned from *Pseudomonas* plasmid pUM505 in Southern DNA-DNA hybridization experiments (C. Cervantes et al., manuscript in preparation). Chromate resistance in *Pseudomonas* spp. also results from decreased accumulation of chromate (1, 15), and the Chr^r determinant has been cloned (H. Ohtake and C. Cervantes, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, H213, p. 180). Judging by the ratio of MICs of resistant strains to MICs of sensitive strains, chromate resistance was not strong compared with divalent cation resistance. Plasmid pCR0616 increased the MIC of chromate for *P. aeruginosa* fourfold; plasmid pDNA206 increased the MIC for the same strain only twofold and increased the MICs for some *Alcaligenes* strains fourfold (Table 2). In contrast, the CobA determinant increased the MIC of Co^{2+} for strain AE104 25-fold (Table 2). CobB^r of plasmid pMOL30 is even stronger (11).

Three polypeptides were expressed in *E. coli* from the 2.6-kb *Eco*RI fragment. The 31,500- and the 21,000-dalton polypeptides were membrane bound, while the 14,500 dalton polypeptide was soluble. DNA sequence analysis of the 2.6-kb *Eco*RI fragment (A. Nies et al., manuscript in preparation) revealed two open reading frames corresponding to predicted polypeptides of 401 and 196 amino acid residues which may correspond to the 31,500- and 21,000-dalton polypeptides. The 14,500-dalton polypeptide possibly is expressed from an open reading frame at the end of the 2.6-kb fragment which continues into the pT7-5 vector part of pECD311 and is not necessary for expression of chromate resistance (A. Nies et al., manuscript in preparation).

The heavy metal cations Co^{2+}, Zn^{2+}, Cd^{2+}, and Ni^{2+} are toxic to *A. eutrophus* cells at high concentrations. Except for Cd^{2+}, these cations are also nutrients essential at low concentrations as trace elements. The divalent cations are transported into *A. eutrophus* cells by the energy-dependent Mg^{2+} transport system (14), which also transports Mn^{2+}. Plasmid-encoded resistances to Co^{2+}, Zn^{2+}, Cd^{2+}, and Ni^{2+} are induced in *A. eutrophus* by low concentrations, less than those causing growth inhibition (13, 17). The mechanism of protection is energy-dependent efflux of the heavy metal cations (13, 16).

Two different efflux systems are responsible for resistance to cobaltous ions: the CobA resistance determinant located on plasmid pMOL28 and the CobB resistance determinant located on plasmid pMOL30 (11). The systems lack DNA sequence homology by Southern hybridization analysis (12) and have different substrate specificities. CobB^r is determined by the *czc* efflux system of pMOL30, which also results in resistance to Zn^{2+} and Cd^{2+} and consists of four membrane-bound polypeptides (D. H. Nies et al., submitted for publication). CobA^r is encoded by plasmid pMOL28 and is lost together with Nic^r in Tn*5* insertion mutants (17; A. Nies and D. H. Nies, unpublished data). When the plasmid pMOL28-encoded nickel resistance was cloned on a *Hin*dIII fragment, the hybrid plasmid also conferred resistance to cobalt (17), and plasmids pDNA206, pDNA219, pDNA121::pECD313, and pDNA121::pECD326 also conferred inducible resistance to nickel. The restriction maps of the *Hin*dIII fragment (17) and the 8.5-kb *Pst*I-*Eco*RI fragment (Fig. 1) are identical from the left *Xba*I site to the single *Hin*dIII site (Fig. 1). Therefore, CobA^r and Nic^r are very likely encoded by a single resistance determinant.

ACKNOWLEDGMENTS

We thank R. A. Siddiqui and H. G. Schlegel for friendly exchanges of relevant data and Stanley Tabor for providing bacterial strains and the protocol necessary for protein expression. We also thank C. Cervantes, A. Berry, H. G. Schlegel, B. Friedrich, and W. Messer for bacterial strains and plasmids.

This work was supported by National Science Foundation grant DMB86-04781.

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