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Plasmid-Determined Inducible Efflux Is Responsible for Resistance to Cadmium, Zinc, and Cobalt in *Alcaligenes eutrophus*

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In *Alcaligenes eutrophus* CH34, resistance to chromate is plasmid determined, inducible, and based on decreased net accumulation of the metal anion. Plasmid-encoded resistances to zinc, cadmium, cobalt, and nickel are resulting from inducible, energy-dependent cation efflux systems.

Alcaligenes eutrophus CH34 is a facultative chemolithotrophic bacterium that displays transposon-governed resistance to mercury (Mer^r) (3) and plasmid-mediated resistance to cadmium, zinc, cobalt, and nickel (7). Resistance to 1 mM $NiCl_2$ (Nic^r) and 1 mM $CoCl_2$ ($CobA^r$) is encoded by plasmid pMOL28 (163 kilobases [kb]). Nickel resistance is inducible and results from reduced accumulation of Ni^{2+} catalyzed by an efflux system (11a, 12, 13). Resistance to 2.5 mM $ZnCl_2$ (Zin^r), 5 mM $CoCl_2$ ($CobB^r$), and 1 mM $CdCl_2$ (Cad^r) is conferred by plasmid pMOL30 (238 kb; 7). A 9.1-kb *Eco*RI fragment of pMOL30 expresses Cad^r, $CobB^r$, and Zin^r in a plasmid-free derivative of strain CH34 and in other metal-sensitive *Alcaligenes* strains, but increases in the MICs of Co^{2+}, Cd^{2+}, and Zn^{2+} were lower in these strains than in the derivative of strain CH34 (8).

This paper demonstrates that resistances to Cd^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, and CrO_4^{2-} (Chr^r) in strain CH34 are based on decreased net accumulation of the metal ions, resulting from plasmid-determined, inducible ion efflux systems.

MATERIALS AND METHODS

Bacterial strains. *A. eutrophus* CH34 (pMOL30, pMOL28) (Mer^r Cad^r Zin^r $CobB^r$ $CobA^r$ Nic^r Chr^r), its mutant derivatives strains AE128(pMOL30) (Mer^r Cad^r Zin^r $CobB^r$), AE126(pMOL28) (Mer^r Chr^r Nic^r $CobA^r$), and AE104 (metal sensitive, plasmid free), and plasmid pDN7 (Cad^r Zin^r $CobB^r$) were described previously (7, 8).

Media and growth experiments. Cells were grown at 30°C in a Tris-buffered minimal salts medium (7) with 2 g of sodium gluconate per liter as the carbon source. This medium does not complex heavy-metal cations. If not otherwise stated, metal resistance was induced in resistant cells by overnight cultivation in the presence of 100 μM $ZnCl_2$, 100 μM $CoCl_2$, 100 μM $NiCl_2$, 50 μM $CdCl_2$, or 10 μM K_2CrO_4. Turbidity measurements were done with a Klett-Summerson photoelectric colorimeter (no. 54 Kodak Wratten filter; Eastman Kodak Co., Rochester, N.Y.).

Transport assays. Cells were incubated after 10-fold dilution into fresh medium for 18 h with shaking at 30°C. Cells were harvested by centrifugation at 12,000 × *g* for 5 min at 4°C and suspended in the same volume of 10 mM Tris hydrochloride buffer (pH 7.0). Cell density during the transport assays was 650 μg (dry weight) per ml. Transport assays were conducted in 10 mM Tris hydrochloride buffer (pH 7.0) at 30°C with aeration by shaking. Samples (500 μl) were filtered through 0.45-μm-pore-size filters (Nuclepore Corp., Pleasanton, Calif.) and rinsed twice with 5-ml volumes of buffer. For efflux assays, cells were incubated with 200 μM $^{60}Co^{2+}$, $^{65}Zn^{2+}$, $^{109}Cd^{2+}$, or $^{63}Ni^{2+}$ in Tris hydrochloride buffer (pH 7.0) for 20 min at 4°C. Sodium gluconate was added to a final concentration of 2 g/liter, and the cells were incubated for additional 20 min at 4°C. The zero-time sample was taken, and the cells were shifted to 30°C. The cells were incubated with aeration by shaking. Samples (0.5 ml) were filtered and treated as outlined above.

Reagents and chemicals. Reagent-grade chemicals and deionized water were used in all experiments. $^{54}Mn^{2+}$, $^{60}Co^{2+}$, $^{65}Zn^{2+}$, $^{109}Cd^{2+}$, and scintillation counting fluid were obtained from Amersham Corp. (Arlington Heights,

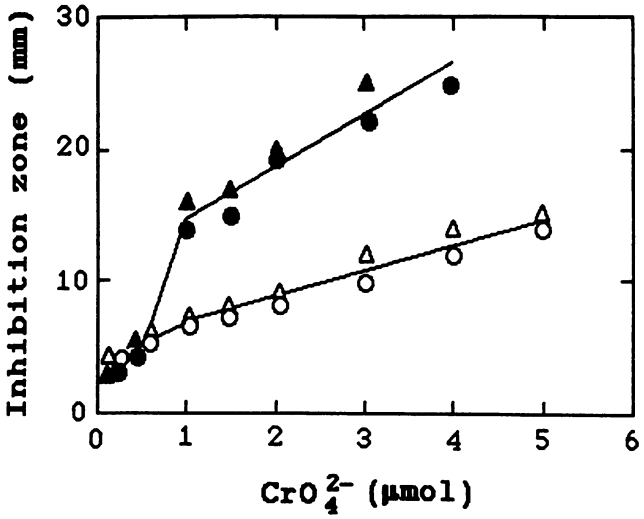

FIG. 1. Chromate resistance in strain CH34. Overnight cultures of strains CH34 (○; carrying both plasmids), AE128 (▲; carrying pMOL30 only), AE126 (△; carrying pMOL28 only), and AE104 (●; carrying no plasmids) were diluted into top agar (8 g of nutrient broth per liter of 0.7% agar) and layered onto nutrient broth plates (8 g of nutrient broth per liter of 1.5% agar). Filter disks (6.5-mm diameter) were placed on top of the solidified agar, and K_2CrO_4 was applied to the filter disks. After overnight incubation at 30°C, the diameters of the inhibition zones (minus the 6.5-mm diameter of the disk) were measured.

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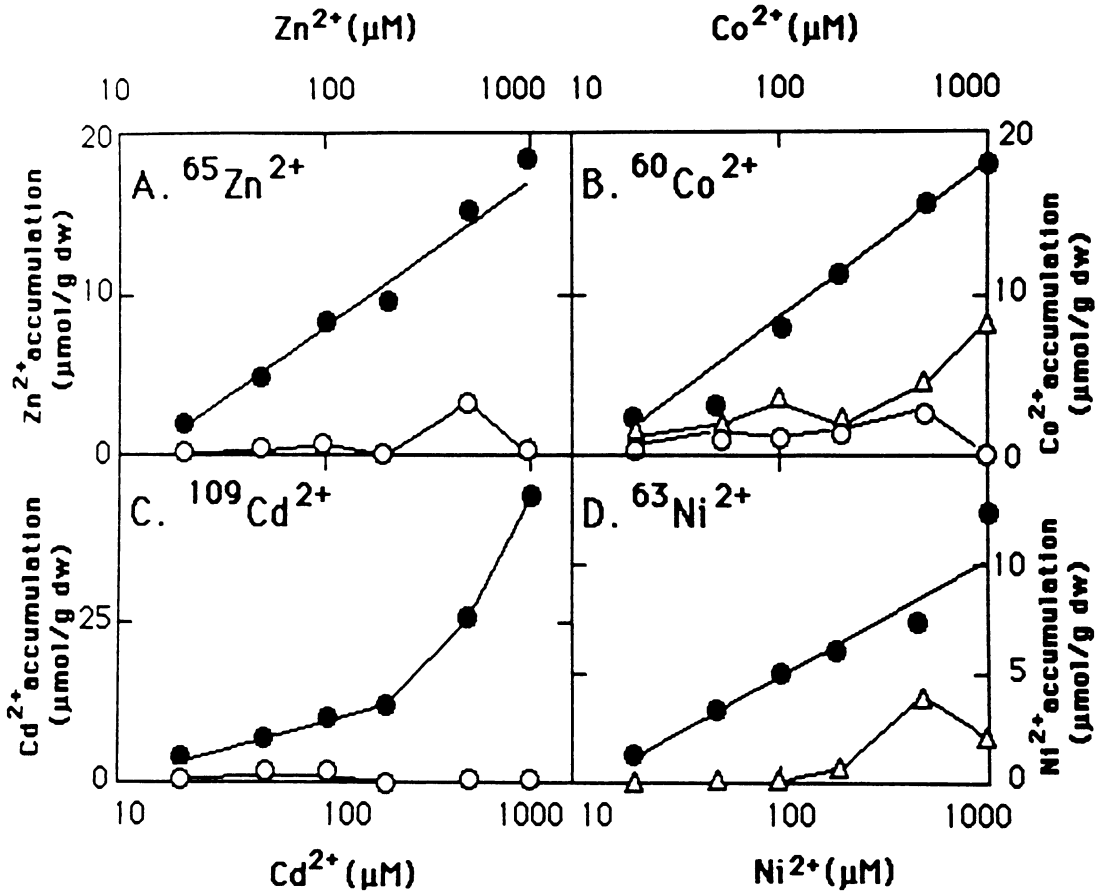

FIG. 2. Reduced accumulation of cadmium, zinc, and cobalt by strain AE128 and of cobalt and nickel by strain AE126. Uptake assays were performed at 20 μM to 1 mM $^{65}Zn^{2+}$ (A), $^{60}Co^{2+}$ (B), $^{109}Cd^{2+}$ (C), or $^{63}Ni^{2+}$ (D) for strains AE128 (○) and AE126 (△) and for the metal-sensitive strain AE104 as a control (●). Strain AE128(pMOL30) was grown and induced overnight on 50 μM $CdCl_2$, 100 μM $ZnCl_2$, or 100 μM $CoCl_2$, and strain AE126(pMOL28) was grown and induced on 100 μM $NiCl_2$ or $CoCl_2$. Accumulated cation contents at 15 min were measured and plotted against the corresponding concentrations of added cation. dw, Dry weight.

Ill.), $^{51}CrO_4^{2-}$ was purchased from ICN Pharmaceuticals Inc. (Irvine, Calif.), and $^{35}SO_4^{2-}$ and $^{63}Ni^{2+}$ were obtained from Dupont, NEN Research Products (Boston, Mass.). All samples were counted in a liquid scintillation spectrometer (Tri-Carb 3375; Packard Instrument Co., Downers Grove, Ill.). *N,N'*-dicyclohexylcarbodiimide, 2,4-dinitrophenol, and EDTA were obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Plasmid-determined resistances are inducible. Plasmid pMOL28 was found to carry Chr^r (Fig. 1) in addition to the previously known Nic^r and CobA^r.

The three pMOL28-encoded resistances showed induction in growth experiments. Cells of strain AE126(pMOL28) (Nic^r CobA^r Chr^r) induced by overnight growth on 100 μM Ni^{2+}, 100 μM Co^{2+}, or 50 μM CrO_4^{2-} started to grow after a shorter lag phase on 1 mM Ni^{2+} or 1 mM Co^{2+} than did control cells (data not shown). Surprisingly, chromate was an inducer of nickel resistance. However, resistance to 200 μM chromate was induced only by precultivation with chromate. All three resistances were also induced when cells of strain AE126 were incubated for 1 h in the presence of 100 μM $NiCl_2$, 100 μM $CoCl_2$, or 50 μM K_2CrO_4 (data not shown).

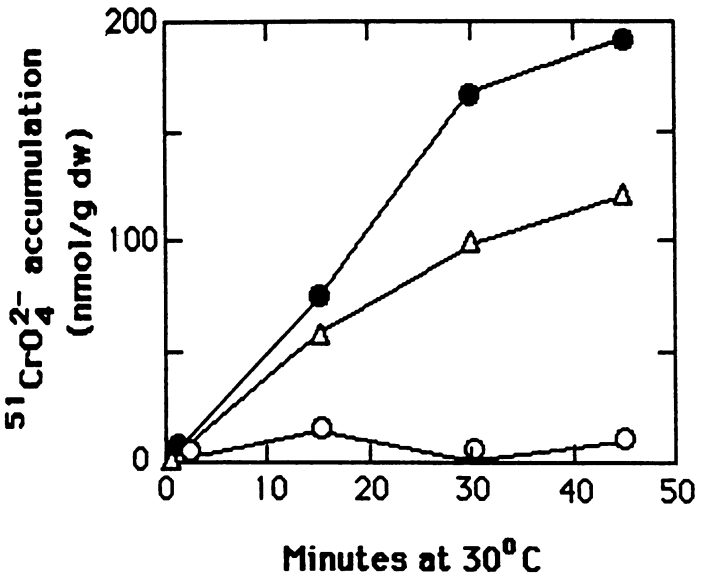

FIG. 3. Reduced accumulation of chromate by strain AE126. Accumulations of 20 μM $^{51}CrO_4^{2-}$ by chromate-induced cells of strain AE126 (○), uninduced cells of strain AE126 (△), and cells of the metal-sensitive strain AE104 (●) were compared. dw, Dry weight.

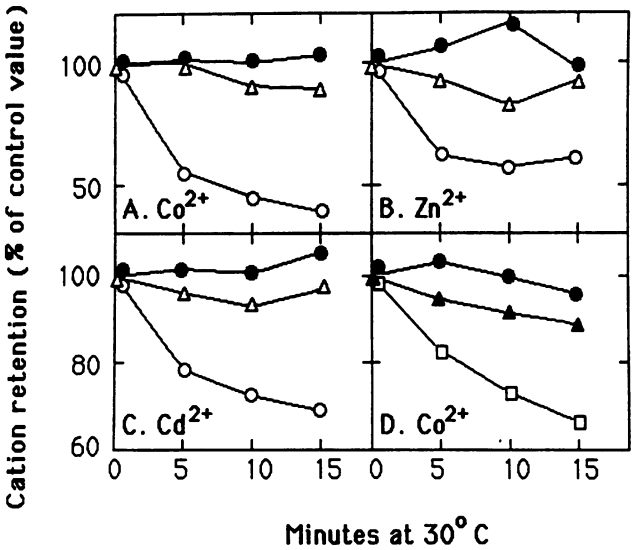

FIG. 4. Cation efflux. Cells of strains AE128 and AE104 were incubated with 200 μM $^{60}Co^{2+}$ (A), $^{65}Zn^{2+}$ (B), or $^{109}Cd^{2+}$ (C) and cells of strains AE126 and AE104 were incubated with $^{60}Co^{2+}$ (D) for 20 min at 4°C. Gluconate was added, and the cells were incubated for additional 20 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 percentage of the zero-time sample. Strain AE128 was grown overnight without inducer (△) and in the presence of 50 μM $CdCl_2$ (○). Strain AE126 was grown overnight without inducer (▲) and in the presence of 100 μM $NiCl_2$ (□). Strain AE104 (●) was grown overnight without inducer.

Cells of strain AE128(pMOL30) (Cad^r Zin^r CobB^r) grown overnight in the presence of 100 μM Zn^{2+}, Cd^{2+}, or Co^{2+} started to grow immediately upon inoculation into fresh medium containing 2.5 mM Zn^{2+}, 1 mM Cd^{2+}, or 1 mM Co^{2+}, respectively. In contrast, control cells grown overnight without added Zn^{2+}, Cd^{2+}, or Co^{2+} exhibited significant lag phases of 1.1 h (1 mM Co^{2+}), 1.5 h (2.5 mM Zn^{2+}), or more than 8 h (1 mM Cd^{2+}) (data not shown). The resistances showed cross-induction; cells induced with one of the three metals were resistant to all three heavy-metal ions (data not shown). Cadmium resistance was also induced when cells were incubated for 1 h with 20, 50, or 100 μM $CdCl_2$ in Tris minimal salts medium before addition of 1 mM $CdCl_2$ (data not shown).

Cad^r, Zin^r, CobB^r, CobA^r, Chr^r, and Nic^r are based on reduced accumulation of the toxic ions. Resting cells of the sensitive strain AE104 accumulated considerable amounts of $^{60}Co^{2+}$, $^{65}Zn^{2+}$, $^{109}Cd^{2+}$, and $^{63}Ni^{2+}$ at concentrations of 20 μM to 1 mM as well as $^{51}CrO_4^{2-}$ at 20 μM. The cellular cation content at 15 min was an exponential function of the cation concentration except for $^{109}Cd^{2+}$ at concentrations of ≥200 μM (Fig. 2). In contrast, cells of strain AE128 induced with cobalt, cadmium, or zinc accumulated lesser amounts of $^{60}Co^{2+}$, $^{109}Cd^{2+}$, or $^{65}Zn^{2+}$, respectively, and cells of strain AE126 induced with cobalt, nickel, or chromate accumulated lesser amounts of these ions (Fig. 2 and 3). Therefore, resistance to these ions resulted from reduced accumulation.

To demonstrate the induction of reduced accumulation, strains AE128 and AE126 were grown overnight without inducers and incubated for 2 h in the presence of 0, 1, 10, or 100 μM Co^{2+}, Zn^{2+}, or Cd^{2+} (strain AE128) or Co^{2+} or Ni^{2+} (strain AE126). Cells that were incubated in the presence of radioactive cations showed less accumulation than did control cells (data not shown); 100 μM ion was always the optimal inducer concentration. At least 1 h of incubation at 30°C was necessary for full expression of the decreased accumulation (data not shown). Uninduced cells of strains AE128 and AE126 always showed less accumulation than did sensitive AE104 cells. Inducibility of Chr^r in strain AE126 was demonstrated by overnight induction of this resistance (Fig. 3). Therefore, the reduced accumulation of all six metal ions was inducible.

Uptake of $^{65}Zn^{2+}$ by the sensitive strain AE104 was partially inhibited at 4°C compared with 30°C (data not shown). In contrast, zinc-induced AE128 cells accumulated only small amounts of $^{65}Zn^{2+}$ at 30°C, and at 4°C they started to accumulate $^{65}Zn^{2+}$ after a delay of 5 min (data not shown). Accumulation of $^{109}Cd^{2+}$ and $^{60}Co^{2+}$ by induced AE128 cells and accumulation of $^{60}Co^{2+}$ and $^{63}Ni^{2+}$ by induced AE126 cells were also greater (after a delay) at 4°C than at 30°C (data not shown); chromate accumulation was not tested.

Cation efflux. Cation efflux was demonstrated by incubating resistant induced AE128 and AE126 cells with radioactive cations at 4°C, adding sodium gluconate as the energy source, and shifting the temperature to 30°C. In all such experiments, a decrease of accumulated metal cations was observed in the induced resistant strains relative to strain AE104 (sensitive cells; Fig. 4). Compared with efflux of $^{60}Co^{2+}$ in strain AE126, efflux of $^{63}Ni^{2+}$ was slow and followed by $^{63}Ni^{2+}$ reuptake after 5 min (data not shown). Cation efflux was inducible (Fig. 4). The initial efflux velocity was a linear function of the cellular cation content (Fig. 5), with slopes of 0.015 (AE128, $^{60}Co^{2+}$ efflux), 0.023 (AE128, $^{65}Zn^{2+}$ efflux), 0.023 (AE126, $^{60}Co^{2+}$ efflux), or 0.036 (AE128, $^{109}Cd^{2+}$ efflux) per min. However, the initial velocity of $^{109}Cd^{2+}$ efflux dropped above cellular Cd^{2+} content of more than 20 μmol/g (dry weight) (Fig. 5), probably because of Cd^{2+} toxicity.

Without the temperature shift to 30°C, no efflux was observed (data not shown). Cation efflux was not inhibited by 50 μM *N,N'*-dicyclohexylcarbodiimide but was inhibited by 1 mM 2,4-dinitrophenol and was therefore energy depen-

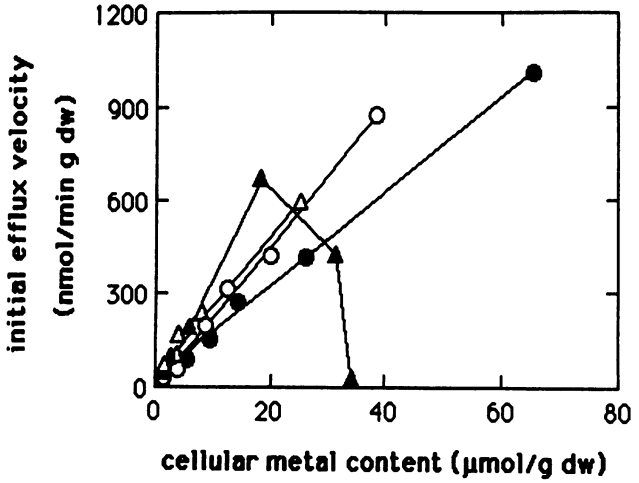

FIG. 5. Dependence of initial efflux velocity on cellular cation content. Cells of strain AE128 were incubated with $^{60}Co^{2+}$ (●), $^{65}Zn^{2+}$ (△), or $^{109}Cd^{2+}$ (▲) and cells of strain AE126 were incubated with $^{60}Co^{2+}$ (○) at various concentrations (range, 20 μM to 1 mM). The initial efflux velocity was plotted against the cellular cation content calculated from the zero-time sample. dw, Dry weight.

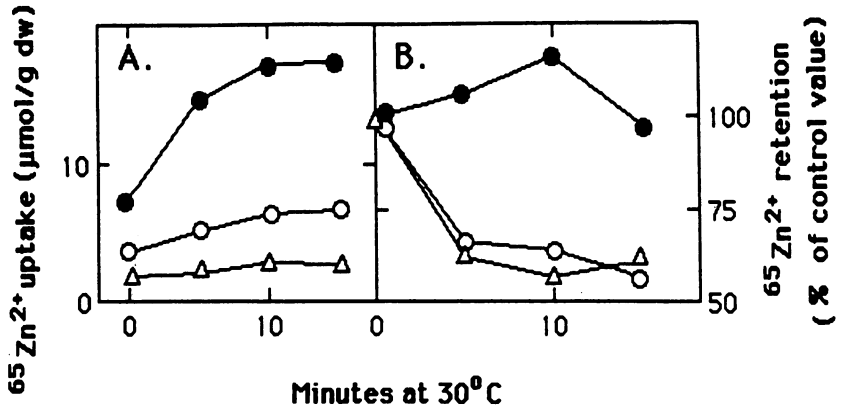

FIG. 6. Reduced accumulation of zinc and accelerated zinc efflux by strain AE104(pDN7). Uptake of 200 µM $^{65}Zn^{2+}$ (A) and $^{65}Zn^{2+}$ efflux (B) after incubation with 200 µM $^{65}Zn^{2+}$ by strains AE104 (●), AE104(pDN7) (○), and AE128(pMOL30) (△) was measured as described in the legends to Fig. 2 and 4. Strain AE104(pDN7) was induced by overnight growth in the presence of 100 µM $ZnCl_2$; strain AE128 was induced with 50 µM $CdCl_2$.

dent (data not shown). No efflux of chromate was observed with strain AE126 because the uptake of chromate was inhibited at 4°C (data not shown).

Plasmid pDN7 conferred constitutive resistance to cadmium, zinc, and cobalt. Plasmid pDN7 contains a 9.1-kb fragment of pMOL30 with the cloned Cad^r, Zin^r, and CobB^r of this plasmid (8). Strain AE104(pDN7) showed decreased accumulation and accelerated efflux of $^{109}Cd^{2+}$, $^{65}Zn^{2+}$, and $^{60}Co^{2+}$ (Fig. 6; data for $^{60}Co^{2+}$ and $^{109}Cd^{2+}$ not shown). Therefore, the Co^{2+}, Zn^{2+}, and Cd^{2+} efflux system(s) was encoded by the 9.1-kb fragment. As judged by growth, decreased accumulation, and efflux, all three resistances were constitutively expressed by pDN7 in strains AE104 and AE128 (data not shown).

DISCUSSION

Plasmid-determined reduced accumulation of cadmium has been described in *Pseudomonas putida* (6) and in *Staphylococcus aureus* (16). The plasmid-determined *cadA* system (9) mediates cadmium resistance via energy-dependent Cd^{2+} efflux (15). DNA sequence analysis of the *cadA* determinant revealed that this system includes a cation-translocating ATPase (S. Silver, G. Nucifora, L. Chu, and T. K. Misra, Trends Biochem. Sci., in press). Metal ion efflux by CH34 cells was also energy dependent. Efflux was inhibited at 4°C and by the addition of 1 mM 2,4-dinitrophenol. However, it is not known whether the CH34 efflux systems are driven by ATP or by a transmembrane gradient.

Plasmid pMOL28 mediates chromate resistance which results from reduced accumulation of the anion. Bacterial resistance to chromate has been found in *Pseudomonas* strains (1, 1a, 2, 5, 14) and also with a plasmid in *Streptococcus lactis* (4). The basis for chromate resistance in *Pseudomonas* strains is also reduced accumulation (1a, 5, 10). However, the pMOL28-mediated chromate resistance is linked to Nic^r and CobA^r: chromate induced Nic^r and CobA^r, and all three resistances were lost together in mutants of strain AE126 (A. Nies and D. Nies, unpublished data).

The six metal ion resistances (in addition to two transposon-encoded mercury resistance determinants) that *A. eutrophus* CH34 exhibits are linked in groups of three to large plasmids; CobA^r, Nic^r, and Chr^r are encoded on plasmid pMOL28, and Cad^r, Zin^r, and CobB^r are encoded by a 9.1-kb *Eco*RI fragment of plasmid pMOL30 (7, 8). Plasmid pDN7, which contains the 9.1-kb fragment (8), mediates reduced accumulation and accelerated efflux of $^{109}Cd^{2+}$, $^{65}Zn^{2+}$, and $^{60}Co^{2+}$. Therefore, divalent ion efflux is indeed the mechanism for the Cad^r, Zin^r, and CobB^r determinants. However, all three resistances in plasmid pDN7 were expressed constitutively in strains AE104 and AE128. Thus, the regulatory control determinant was not cloned with the 9.1-kb fragment and could not be provided by plasmid pMOL30 in *trans*. Molecular genetic evidence suggests that all three resistances are encoded by one operon (D. Nies and A. Nies, manuscript in preparation). Since zinc and cadmium are chemically related and cobalt can bind to zinc-binding sites (for example, in alcohol dehydrogenase [11]), the three metal cations are probably transported by one efflux system.

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