Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 7351–7355, October 1989
Biochemistry

Expression and nucleotide sequence of a plasmid-determined divalent cation efflux system from *Alcaligenes eutrophus*

(heavy metal plasmid resistance/cation transport system)

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Communicated by Boris Magasanik, June 26, 1989

ABSTRACT **Resistance to cobalt, zinc, and cadmium specified by the *czc* determinant on plasmid pMOL30 in *Alcaligenes eutrophus* results from a cation efflux system. Five membrane-bound polypeptides that were expressed in *Escherichia coli* from this determinant under the control of a phage T7 promoter were assigned to four open reading frames identified in the nucleotide sequence of the 6881-base-pair fragment containing the *czc* putative operon. The contributions of the polypeptides to the cation efflux system were analyzed with deletion derivatives of the 6.9-kilobase fragment, constructed, and expressed in *E. coli* under the control of the phage T7 promoter and in *A. eutrophus* under the control of the *lac* promoter.**

The divalent cations Zn^{2+} and Co^{2+} are necessary as trace elements for all cells but are toxic at higher concentrations, a fact of considerable environmental importance. Cd^{2+} and Hg^{2+} are toxic and have no physiological functions. Resistance to Hg^{2+}, based on reduction of Hg^{2+} to metallic mercury, which evaporates out of the cell, is widespread among bacteria and a well-investigated system (1). In contrast, understanding of resistance to Zn^{2+}, Co^{2+}, and Cd^{2+} is limited (1). The Gram-negative soil and water bacterium *Alcaligenes eutrophus* strain CH34 contains two large plasmids that encode unique heavy metal resistances (2). Resistances to Co^{2+}, Zn^{2+}, and Cd^{2+} are encoded by plasmid pMOL30 [238 kilobase pairs (kb)] and were cloned together on a 9.1-kb *Eco*RI fragment (3). The resistances to Co^{2+}, Zn^{2+}, and Cd^{2+} are inducible and are based on a cation efflux system (4).

We have determined the nucleotide sequence† of the 6.9-kb region necessary for the expression of Co^{2+}, Zn^{2+}, and Cd^{2+} resistances and carried out transport studies with strains carrying specific mutations in this DNA region. The data are summarized in a model that proposes distinct roles for different polypeptides in the cation export process.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Those used in this study are listed in Table 1. Metal ion resistances, reduced accumulation of metal cations, and metal ion efflux rates were tested as described (3, 4). However, cells were incubated in the presence of 1 μM radioactive cation instead of 200 μM (4) in order to measure the efflux process more sensitively with mutant strains. The calculated efflux constants (k) were calculated, which are the initial efflux velocity (in μmol/min per g dry weight) divided by the initial cellular cation content (in μmol/g dry weight).

Molecular Genetics Techniques. These were performed as described by Nies *et al.* (3) and by Maniatis *et al.* (9). For construction of phage M13 derivatives containing the *czc* region, restriction endonuclease subfragments of the 9.1-kb *Eco*RI fragment (3) were cloned from plasmid pECD11 (called pEC11 in ref. 3) into phage mTM0l0 (8) in both orientations. Nested deletions were made with BAL-31 exonuclease, and the DNA sequence was determined from both strands as described (8). The polypeptides expressed by the *czc* region were analyzed as described (10, 11).

Table 1. Bacterial strains and plasmids

Strain	Plasmid	Relevant markers	Ref./ origin
Strain			
A. eutrophus	AE104	Metal-sensitive	2
E. coli			
S17-1		RP4 *tra* genes	5
JM83		Δ*lacZM15*	6
JM83	pVDZ'2	Tet^r, IncP1, lacZ', Mob$^+$, Tra$^-$	7
S17-1	pT7-3		S. Tabor*
S17-1	pT7-5		S. Tabor*
K38	pGP1-2	T7 RNA polymerase	S. Tabor*
S17-1	pECD11	9.1-kb *Eco*RI fragment	3
Phage mTM010			8
Plasmid†	(vector)		
pECD107	pT7-3		This study
pECD108	pT7-3‡		This study
pECD109	pT7-5		This study
pECD110	pT7-5‡		This study
pDNA130	pVDZ'2		This study

*Personal communication.
†*czc*-containing hybrid plasmid containing the 6.7-kb *Eco*RI–*Bam*HI fragment that was cloned into the pT7-3, pT7-5, and pVDZ'2 vectors.
‡Opposite orientation.

Isolation of Mutants. DNA fragments with BAL-31-generated deletions in the 6.7-kb *Eco*RI-*Bam*HI fragment (which were used for the DNA sequence analysis) were cloned from the corresponding phage mTM0l0 hybrids into plasmids pT7-5 and pVDZ'2 using *Eco*RI and *Xba* I. To introduce small internal deletions into specific *czc* open reading frames, plasmid pECD110 was digested with *Xho* I, *Nsi* I, *Apa* I, or *Ban* II, respectively. The digested plasmids were treated briefly with BAL-31, "polished" with Klenow DNA polymerase I, ligated, and transformed into *Escherichia coli* S17-1. Plasmids from the transformants were isolated and screened for the absence of the particular restriction nuclease site. Plasmids carrying small deletions were expressed in *E. coli* strain K38(pGP1-2) to establish the altered mutant polypeptide patterns. Finally, the deletion derivatives were sub-

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Abbreviation: MIC, minimal inhibitory concentration.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M26073).

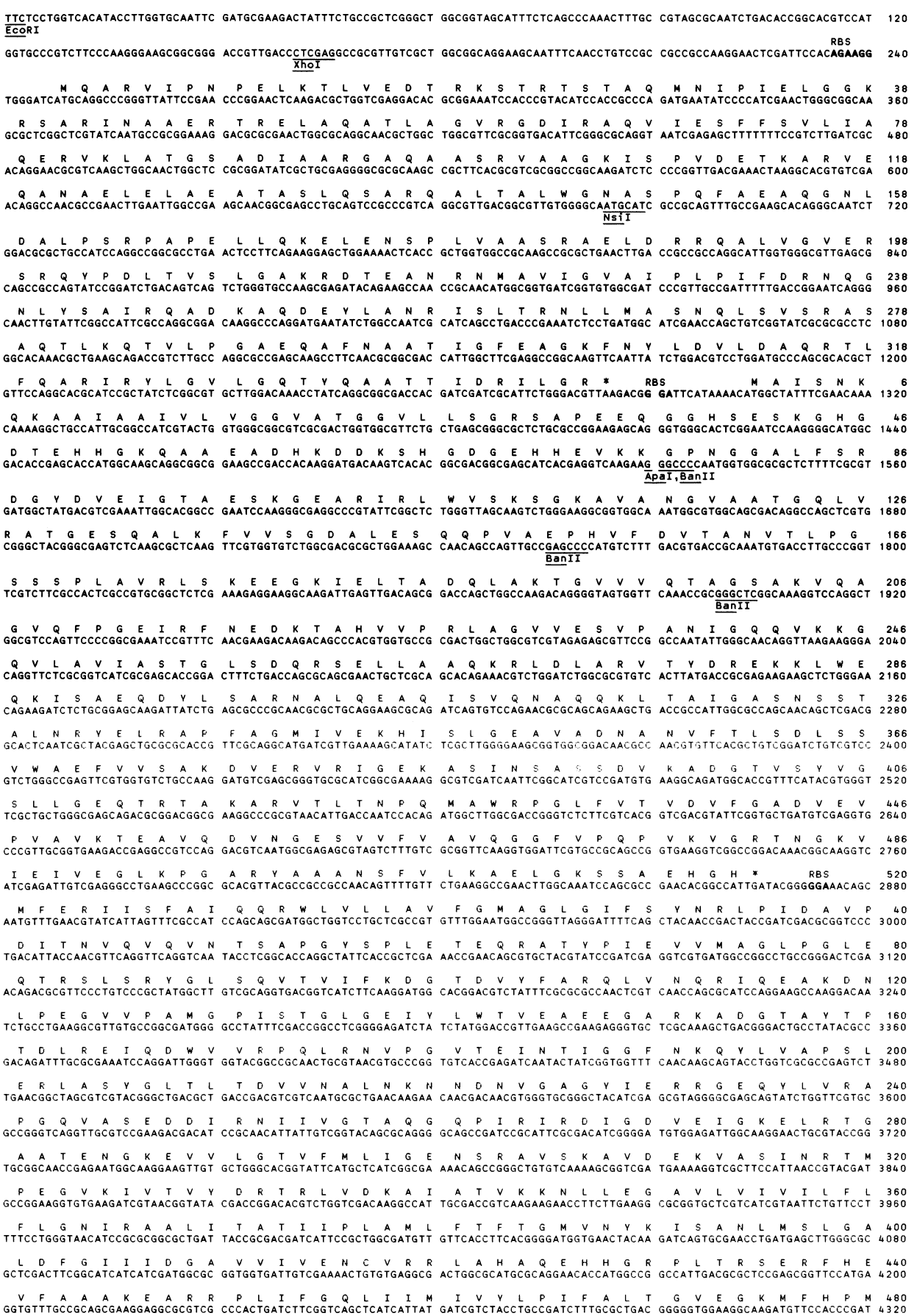

FIG. 1. (*Figure continues on the opposite page.*)

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  A  F  T  V  V  L  A  L  L  G   A  M  I  L  S  V  T  F  V  P   A  A  V  A  L  F  I  G  E  R   V  A  E  K  E  N  R  L  M  L  520
GGCGTTCACGGTCGTCCTGGCGCTGCTGGG CGCGATGATTCTGTCCGTGACGTTCGTTCC GGCTGCGGTCGCCTTGTTCATCGGCGAACG GGTGGCCGAGAAAGAAAATCGTCTCATGCT 4440

  W  A  K  R  R  Y  E  P  L  L   E  K  S  L  A  N  T  A  V  V   L  T  F  A  A  V  S  I  V  L   C  V  A  I  A  A  R  L  G  S  560
CTGGGCGAAGCGTCGCTACGAGCCGCTGCT GGAAAAGTCGCTCGCGAACACGGCCGTTGT ATTGACGTTTGCCGCGGTGTCAATTGTTCT GTGCGTGGCCATTGCGGCCCGCCTGGGCAG 4560

  E  F  I  P  N  L  N  E  G  D   I  A  I  Q  A  L  R  I  P  G   T  S  L  S  Q  S  V  E  M  Q   K  T  I  E  T  T  L  K  A  K  600
CGAGTTCATCCCCAATCTGAACGAAGGCGA CATTGCCATCCAGGCGCTGCGCATTCCTGG CACGAGCCTGTCGCAGTCCGTGGAGATGCA GAAGACGATCGAGACGACCCTCAAGGCAAA 4680

  F  P  E  I  E  R  V  F  A  R   T  G  T  A  E  I  A  S  D  L   M  P  P  N  I  S  D  G  Y  I   M  L  K  P  E  K  D  W  P  E  640
ATTCCCCGAAATCGAGCGCGTGTTTGCGCG GACAGGTACGGCGGAGATTGCATCCGATCT GATGCCGCCGAATATTTCGGATGGCTACAT CATGCTCAAGCCTGAGAAGGATTGGCCAGA 4800

  P  K  K  T  H  A  E  L  L  S   A  I  Q  E  E  A  G  K  I  P   G  N  N  Y  E  F  S  Q  P  I   Q  L  R  F  N  E  L  I  S  G  680
GCCGAAGAAAACACATGCCGAACTGCTGTC CGCCATCCAGGAGGAAGCCGGCAAGATCCC CGGGAACAACTACGAGTTCTCCCAACCGAT CCAGCTGCGGTTCAACGAGCTGATCTCCGG 4920

  V  R  S  D  V  A  V  K  I  F   G  D  D  N  N  V  L  S  E  T   A  K  K  V  S  A  V  L  Q  G   I  P  G  A  Q  E  V  K  V  E  720
GGTCCGCTCGGACGTCGCAGTCAAGATCTT CGGCGATGACAACAACGTGCTCAGCGAGAC GGCGAAGAAGGTATCGGCCGTGCTGCAGGG CATCCCCGGCGCGCAGGAGGTGAAGGTAGA 5040

  Q  T  T  G  L  P  M  L  T  V   K  I  D  R  E  K  A  A  R  Y   G  L  N  M  S  D  V  Q  D  A   V  A  T  G  V  G  G  R  D  S  760
ACAGACCACCGGCTTGCCGATGCTGACGGT CAAGATCGATCGGGAGAAGGCGGCGCGATA CGGGCTTAACATGAGCGACGTGCAAGACGC GGTGGCAACGGGCGTCGGAGGCCGTGATTC 5160

  G  T  F  F  Q  G  D  R  R  F   D  I  V  V  R  L  P  E  A  V   R  G  E  V  E  A  L  R  R  L   P  I  P  L  P  K  G  V  D  A  800
CGGAACCTTCTTCCAGGGCGATCGTCGTTT CGATATCGTGGTCCGCCTGCCCGAAGCTGT GCGCGGCGAGGTCGAGGCTCTGCGCCGATT GCCGATTCCGTTGCCAAAAGGAGTGGACGC 5280

  R  T  T  F  I  P  L  S  E  V   A  T  L  E  M  A  P  G  P  N   Q  I  S  R  E  N  G  K  R  R   I  V  I  S  A  N  V  R  G  R  840
GAGAACGACGTTTATCCCATTGAGCGAGGT GGCGACGCTGGAAATGGCGCCCGGCCCGAA CCAGATCTCGCGCGAGAACGGCAAGCGCCG CATCGTGATCAGTGCCAACGTTCGTGGACG 5400

  D  I  G  S  F  V  P  E  A  E   A  A  I  Q  S  Q  V  K  I  P   A  G  Y  W  M  T  W  G  G  T   F  E  Q  L  Q  S  A  T  T  R  880
TGATATTGGTTCATTCGTGCCCGAGGCGGA AGCGGCTATCCAAAGCCAGGTCAAGATCCC GGCTGGCTACTGGATGACATGGGGTGGCAC CTTTGAGCAACTGCAGTCCGCCACCACCCG 5520

  L  Q  V  V  V  P  V  A  L  L   L  V  F  V  L  L  F  A  M  F   N  N  I  K  D  G  L  L  V  F   T  G  I  P  F  A  L  T  G  G  920
CCTGCAGGTGGTAGTGCCGGTGGCGCTGTT GCTGGTCTTCGTACTGTTGTTTGCGATGTT CAACAACATCAAGGATGGCTTGCTAGTCTT CACGGGCATTCCCTTTGCGCTGACTGGCGG 5640

  I  L  A  L  W  I  R  G  I  P   M  S  I  T  A  A  V  G  F  I   A  L  C  G  V  A  V  L  N  G   L  V  M  L  S  F  I  R  S  L  960
GATTCTTGCCCTGTGGATACGCGGCATTCC GATGTCCATTACTGCAGCGGTGGGCTTCAT CGCGCTGTGCGGGGTGGCGGTGCTCAATGG TCTGGTGATGCTGTCGTTTATCCGATCGCT 5760

  R  E  E  G  H  S  L  D  S  A   V  R  V  G  A  L  T  R  L  R   P  V  L  M  T  A  L  V  A  S   L  G  F  V  P  M  A  I  A  T  1000
GCGCGAAGAAGGGCATTCCCTCGACAGCGC GGTCCGAGTTGGCGCCCTGACGCGACTGCG TCCGGTGCTGATGACGGCCCTGGTGGCATC CCTGGGTTTCGTGCCGATGGCCATCGCCAC 5880

  G  T  G  A  E  V  Q  R  P  L   A  T  V  V  I  G  G  I  L  S   S  T  A  L  T  L  L  V  L  P   V  L  Y  R  L  A  H  R  K  D  1040
CGGTACGGGCGCTGAGGTGCAACGTCCCCT CGCAACGGTGGTAATCGGTGGCATCTTGTC GTCCACGGCGCTGACCCTACTGGTGTTGCC GGTGCTCTATCGACTTGCTCACCGCAAGGA 6000

  E  D  A  E  D  T  R  E  P  V   T  Q  T  H  Q  P  D  Q  G  R   Q  P  A  *                                                    RBS      106
TGAGGACGCGGAAGATACTCGCGAGCCAGT CACTCAGACGCATCAACCGGATCAAGGCCG CCAGCCTGCATGACGTAAATCCTTGGGCGG TCATCGTACCGCCCAATTTTTATAGGAGTT 6120

    M  G  A  G  H  S  H  D  H   P  G  G  N  E  R  S  L  K  I   A  L  A  L  T  G  T  F  L  I   A  E  V  V  G  G  V  M  T  K   39
TCTATGGGCGCAGGTCACTCACACGACCAT CCCGGTGGCAACGAGCGATCGCTCAAGATC GCCCTTGCGCTGACCGGTACGTTCCTGATT GCCGAAGTGGTCGGTGGTGTCATGACGAAG 6240

  S  L  A  L  I  S  D  A  A  H   M  L  T  D  T  V  A  L  A  I   A  L  A  A  I  A  I  A  K  R   P  A  D  K  K  R  T  F  G  Y   79
AGCCTGGCGTTGATCTCCGACGCCGCGCAC ATGCTCACGGACACCGTCGCACTGGCCATC GCACTGGCTGCTATTGCGATCGCCAAGCGA CCCGCGGACAAGAAGCGGACATTTGGCTAC 6360

  Y  R  F  E  I  L  A  A  A  F   N  A  L  L  L  F  G  V  A  I   Y  I  L  Y  E  A  Y  L  R  L   K  S  P  P  Q  I  E  S  T  G   119
TACCGTTTTGAGATTCTTGCCGCGGCCTTT AACGCATTGCTGCTGTTCGGTGTGGCTATC TACATCCTGTACGAAGCCTACCTGCGGCTG AAATCGCCACCTCAGATTGAGTCAACCGGC 6480

  M  F  V  V  A  V  L  G  L  I   I  N  L  I  S  M  R  M  L  S   S  G  Q  S  S  S  L  N  V  K   G  A  Y  L  E  V  W  S  D  L   159
ATGTTCGTCGTGGCTGTGCTGGGCCTGATC ATCAATCTCATCAGCATGCGCATGCTGTCC TCCGGGCAAAGCAGCAGCCTGAACGTGAAG GGTGCTTATCTGGAAGTCTGGAGCGATCTG 6600

  L  G  S  V  G  V  I  A  G  A   I  I  I  R  F  T  G  W  A  W   V  D  S  A  I  A  V  L  I  G   L  W  V  L  P  R  T  W  I  L   199
CTCGGGTCGGTTGGCGTCATCGCCGGTGCG ATCATCATCCGCTTCACGGGCTGGGCGTGG GTCGACTCCGCCATTGCGGTGCTGATCGGC CTCTGGGTACTGCCTCGCACGTGGATCCTC 6720
                                                                                                                    BamHI
  *
TGAAGTCGAGCCTGAATGTGCTGCTCGAAG GCGTACCCGATGACGTGGATCTGGCAGAGG TTGAGAAGCAAATTCTCCGACGCCCGGGGT AAAAAGCTTCCATGAACCTCCACATCTGGG 6840
                                                                                                  HindIII
CCACTTACCAACGGCCAGGCGAGCTTGACG GTTCAGTCTGT 6881
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FIG. 1. Nucleotide sequence of the 6881-bp DNA fragment containing the *czc* determinant. The strand equivalent to the mRNA is shown from the *Eco*RI site to 146 bp beyond the *Bam*HI site (see Fig. 2). Proposed ribosome binding sites (RBS), the predicted polypeptides, and restriction endonuclease sites used for mutagenesis are indicated.

cloned into plasmid pVDZ'2 by using *Eco*RI and *Xba* I, leading to the plasmids pDNA145 (*Xho* I-generated, no CzcC protein), pDNA146 (*Nsi* I-generated, a 25-kDa truncated CzcC; $CzcC^-$), pDNA147 (*Apa* I-generated, no CzcB protein), and pDNA148 (*Ban* II-generated, a CzcC-CzcB fusion protein; $CzcC^+$ $CzcB^-$).

RESULTS AND DISCUSSION

Nucleotide Sequence of the *czc* Determinant. Fig. 1 shows the nucleotide sequence of the 6881 base pairs (bp) containing the putative *czc* operon and the amino acid translation products of the four major open reading frames. The sequence starts with the last three nucleotides of the left *Eco*RI site of the 9.1-kb *Eco*RI fragment (3) and ends 146 bp after the *Bam*HI site (Fig. 1). Besides the four open reading frames summarized in Table 2, one additional open reading frame was detected, which is oriented in the opposite direction, compared with the four open reading frames shown in Fig. 2. It would start at position 2336, end at nucleotide 1495, and potentially correspond to a 35-kDa polypeptide (data not shown). However, no consensus ribosome binding site was found upstream of the proposed start codon, and the T7 polymerase experiments (see Fig. 3) did not indicate that a polypeptide was expressed from the opposite strand. Proposed start and stop codons and ribosomal binding sites for the four major open reading frames are summarized in Table 2.

Table 2. Proposed genes (from open reading frames) of the *czc* nucleotide sequence

Proposed gene	Start codon, bp	Stop codon, bp	Length, a.a.	Size,* kDa
czcC	248	1285	345	37.3
czcB	1303	2865	520	54.5
czcA	2881	6073	1063	116.0
czcD	6124	6723	199	21.2

a.a., amino acids.
*Size of the predicted polypeptide in kilodaltons.

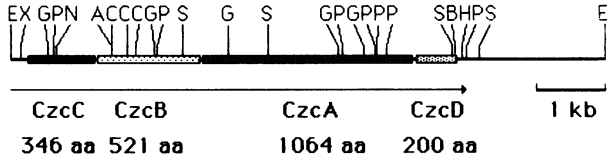

FIG. 2. Structure of the DNA region containing the *czc* determinant. The restriction endonuclease sites of the original 9.1-kb *Eco*RI fragment (4) shown (E, *Eco*RI; X, *Xho* I; G, *Bgl* II; P, *Pst* I; N, *Nsi* I; A, *Apa* I; C, *Ban* II; S, *Sal* I; B, *Bam*HI; and H, *Hin*dIII) were determined by enzyme digestion and predicted from the DNA sequence in Fig. 1. The arrow gives the predicted length and direction of transcription of the *czc* determinant. The sizes of the predicted gene products in amino acids (aa) are indicated.

The 6.7-kb *Eco*RI–*Bam*HI Fragment Encodes Five Membrane-Bound Polypeptides. The 6.7-kb *Eco*RI–*Bam*HI fragment (Fig. 2) was cloned in both orientations into plasmids pT7-3 and pT7-5. Both vector plasmids carry a phage T7 RNA polymerase promoter downstream of a multilinker restriction nuclease site. In plasmid pT7-3, the *bla* gene encoding ampicillin resistance can be transcribed from the phage T7 promoter; in plasmid pT7-5, the *bla* gene is oriented in the opposite direction, β-Lactamase appeared as three bands corresponding to sizes of about 27 kDa (Fig. 3; ref. 10). When plasmids pECD108 and pECD110 (Table 1) were expressed in *E. coli* strain K38(pGP1-2), five radioactive polypeptide bands corresponding to sizes of about 120 (CzcA), 66 (CzcB), 44 (CzcC1), 42 (CzcC2; tentatively hypothesized to originate from the same gene), and 21 (CzcD) kDa appeared (Fig. 3*A*). No extra radioactive polypeptide was visible after expression of plasmids pECD107 and pECD109 (Fig. 3*A*). [Two polypeptides with the same sizes as CzcC1 and CzcC2 appeared after expression of plasmids pECD107, pECD109, pT7-3 and pT7-5 (Fig. 3*A*). However, these bands were faint compared with the bands appearing after expression of plasmids pECD108 and pECD110 (Fig. 3*A*).] In plasmids pECD108 and pECD110, parts of the polylinker region downstream of the *Bam*HI site (which is different in the two plasmids because of different ways of construction) were fused and incorporated into the CzcD open reading frame (Fig. 2). Therefore, the two CzcD polypeptides seen had slightly different sizes (Fig. 3*A*). The CzcA polypeptide appeared as a broader band rather than a sharp band (Fig. 3). This is characteristic for membrane proteins on polyacrylamide gels (12).

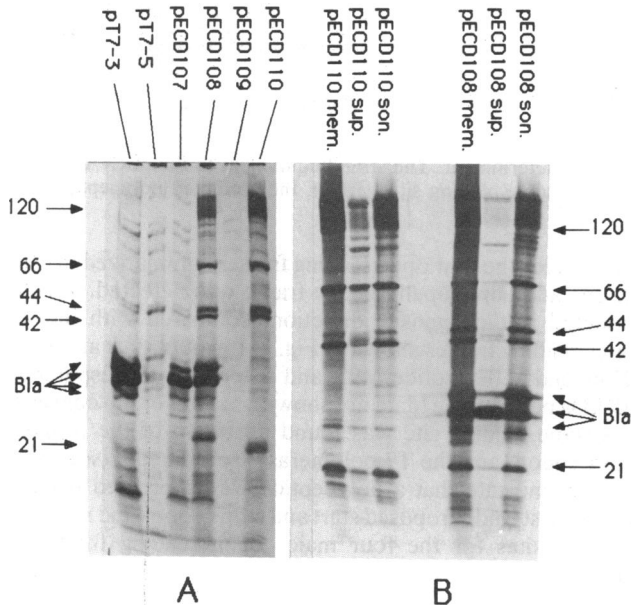

FIG. 3. Polypeptides produced by the *czc* determinant. The 6.7-kb *Eco*RI–*Bam*HI fragment was cloned in both orientations into plasmids pT7-3 and pT7-5, leading to plasmids pECD107 (vector pT7-3, opposite orientation), pECD108 (vector pT7-3, correct orientation), pECD109 (vector pT7-5, opposite orientation), and pECD110 (vector pT7-5, correct orientation). In vector plasmid pT7-3, the *bla* gene products (Bla, 25.0, 26.5, 27.5, and 29.0 kDa; ref. 10) are also expressed under the control of phage T7 promoter. (*A*) Five ^{35}S-labeled polypeptides with sizes corresponding to 21, 42, 44, 66, and 120 kDa are marked as expressed by plasmids pECD108 and pECD110. The approximate sizes were assigned to these polypeptides by comparison to the electrophoretic mobilities of radioactive protein standards. (*B*) Polypeptides in the total sonicate extract (son.) of the cells with plasmids pECD108 and pECD110 or membrane (mem.) and supernatant (sup.) fractions after ultracentrifugation.

Since the *bla* gene products were strongly expressed from plasmid pECD108 (Fig. 3*A*), transcription was not interrupted within the 6.7-kb fragment. Therefore, the five polypeptides are all gene products encoded by this fragment. No polypeptides were expressed from the opposite strand with plasmids pECD107 and pECD109 (Fig. 3*A*). Therefore, the direction of transcription of the *czc* operon is from the *Eco*RI site (the left end in Fig. 2) toward the *Bam* HI site. When the *E. coli* cells containing plasmids pECD108 and pECD110 were disrupted, the major fraction of each of the five *czc*-encoded polypeptides appeared in the washed membranes (Fig. 3*B*), while only a small amount was visible as contamination in the supernatant fractions. Therefore, the *czc* polypeptides are probably membrane-bound.

Assignment of the Open Reading Frames and Polypeptides. Derivatives of the 6.7-kb *Eco*RI–*Bam*HI fragment carrying various deletions were subcloned into plasmid pT7-5 and expressed in *E. coli* strain K38(pGP1-2) (Table 3). Thus, the polypeptides expressed from the 6.7-kb fragment could be assigned to the different open reading frames: the 21-kDa polypeptide corresponds to CzcD; the 120-kDa polypeptide, to CzcA; the 66-kDa polypeptide, to CzcB; and the 42- and the 44-kDa polypeptides, both to CzcC (Table 2). Note that two polypeptides differing in size by 2 kDa are tentatively assigned to *czcC*, although there is no evidence as to whether this results from two start sites or proteolytic processing.

Function of the *czc* Gene Products. The deletion derivatives of the 6.7-kb *Eco*RI–*Bam*HI fragment were subcloned from the pT7-5 hybrid plasmids into plasmid pVDZ'2. When transferred into *A. eutrophus* strain AE104, the deletion derivatives were constitutively expressed under the control of *E. coli lac* promoter (since there is no *lac* repressor gene present; ref. 7), and the effect of each deletion on the expression of metal resistance was determined.

Compared with the expression of the complete *czc* determinant (plasmid pDNA130), deletion of the carboxyl-terminal 62 amino acids of the putative CzcD protein (plasmid pDNA135; Table 3) had no effect on the minimal inhibitory concentration (MIC) for Co^{2+}, Zn^{2+}, and Cd^{2+} and no effect on zinc and cobalt efflux (Fig. 4). Cd^{2+} efflux in strains AE104(pDNA135) and AE104(pDNA130) was slightly different (Fig. 4), but these strains showed similar low levels of

Table 3. Deletion derivatives of the 6881-bp fragment cloned into plasmids pT7-5 and pVDZ'2

Plasmid*	Mutation	Deletion	Ccz polypeptides formed
pDNA130	None	None	A, B, C, D
pDNA135	*czcD*	Δbp 6536–6713	A, B, C, ΔD = 15 kDa
pDNA132	Δ*czcAD*	Δbp 5896–6713	B, C, ΔA = 110 kDa
pDNA137	Δ*czcAD*	Δbp 5108–6713	B, C, ΔA = 72 kDa
pDNA138	Δ*czcAD*	Δbp 4016–6713	B, C, ΔA nv.
pDNA139	Δ*czcAD*	Δbp 2890–6713	B, C, ΔA nv.
pDNA140	Δ*czcBAD*	Δbp 2646–6713	C, ΔB = 59 kDa
pDNA141	Δ*czcBAD*	Δbp 2106–6713	C, ΔB nv.
pDNA142	Δ*czcBAD*	Δbp 1508–6713	C, ΔB = 15 kDa
pDNA148	*czcB*	*Ban* II sd.	A, D, C::B = 80 kDa†
pDNA147	*czcB*	*Apa* I sd.	A, C, D, ΔB nv.
pDNA146	*czcC*	*Nsi* I sd.	A, B, D, ΔC = 25 kDa
pDNA145	*czcC*	*Xho* I sd.	A, B, D, ΔC nv.

The deletion derivatives of the 6.7-kb fragment were cloned into plasmid pT7-5 and expressed from the phage T7 promoter to establish the altered mutant polypeptide pattern (the names of these hybrid plasmids are not given). The sizes of deletion derivatives of the Czc proteins (ΔA, ΔB, or ΔD) seen in *E. coli* under T7 promoter function are given in kDa. The deletion derivatives were then subcloned into plasmid pVDZ'2 and expressed in *A. eutrophus* strain AE104. nv., Not visible; sd., enzyme-promoted small deletion.
*Names of the pVDZ'2-derived hybrid plasmids.
†Fusion between CzcC and CzcB.

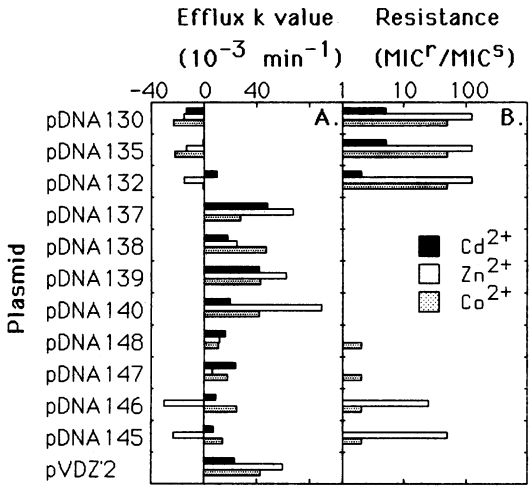

FIG. 4. Effect of deletion mutations in the *czc* determinant on expression of cation resistances. Plasmid pDNA130 (Table 3) contains the complete *czc* determinant; plasmids pDNA135, pDNA132, pDNA137, pDNA138, pDNA139, and pDNA140 contain progessively larger deletions of the *czc* determinant from the distal 3′ end as indicated (Table 3). Plasmids pDNA148, pDNA147, pDNA146, and pDNA145 have small deletions in either *czcC* (pDNA145 and pDNA146) or *czcB* (pDNA147 or pDNA148) (Table 3). All *czc* fragments were cloned in plasmids pVDZ'2 and transferred into *A. eutrophus* strain AE104 for MIC and transport studies. (*A*) Efflux (negative values) or net uptake of Cd^{2+} (black bars), Zn^{2+} (white bars), and Co^{2+} (shaded bars) by strains with mutant plasmids. (*B*) The MICs of strain AE104 containing a mutant plasmid divided by the MIC of the metal-sensitive strain AE104(pVDZ'2) for Cd^{2+} (black bars), Zn^{2+} (white bars), or Co^{2+} (shaded bars).

Cd^{2+} accumulation (data not shown). Deletion of the entire *czcD* gene and of 59 amino acids of the putative CzcA protein (plasmid pDNA132; Table 3) reduced the MIC for Cd^{2+} but did not affect the MICs for Co^{2+} and Zn^{2+}. Zn^{2+} efflux was not affected, but Co^{2+} and Cd^{2+} efflux were reduced.

Further deletion of about one-third (321 of 1063 amino acids) from the carboxyl terminus of the putative CzcA polypeptide (plasmid pDNA137; Table 3) completely eliminated all three resistances, and strain AE104(pDNA137) accumulated Cd^{2+}, Zn^{2+}, and Co^{2+} at rates equivalent to the sensitive strain AE104(pVDZ'2) (Fig. 4). Larger deletions had similar effects (plasmids pDNA138, pDNA139, and pDNA140; Table 3 and Fig. 4). Therefore, the CzcA polypeptide is essential for the expression of all three resistances.

Strains AE104(pDNA145) and AE104(pDNA146), which are $CzcC^-$ (Table 3) are sensitive to Cd^{2+} and have reduced MICs for Co^{2+} but only slightly reduced MICs for Zn^{2+} (Fig. 4). Both strains showed Zn^{2+} efflux and accumulated Cd^{2+} and Co^{2+} to a lesser extent than did the sensitive strain AE104(pVDZ'2) (Fig. 4). Strains AE104(pDNA147) and AE104(pDNA148) were $CzcB^-$ (Table 3) and sensitive to Cd^{2+} and Zn^{2+} and exhibited residual Co^{2+} resistance similar to the $CzcC^-$ strains AE104(pDNA145) and AE104(pDNA146) (Table 3 and Fig. 4). In contrast to the $CzcC^-$ strains, the $CzcB^-$ strains did not show Zn^{2+} efflux or Zn^{2+} resistance (Fig. 4).

These results may be incorporated into a preliminary model with the CzcA protein alone having low cation transport activity for Co^{2+}. CzcA and CzcB together would act in Zn^{2+} efflux nearly as effectively as the complete Czc efflux system (CzcABC). Thus, the CzcB protein is thought to funnel Zn^{2+} cations to the CzcA transport protein.

When the CzcC protein was added to CzcA and CzcB, the efflux system gained specificity for Cd^{2+} and Co^{2+}. However, the CzcC and CzcA proteins apparently did not catalyze Co^{2+} or Cd^{2+} efflux when the CzcB protein was absent. Therefore, the CzcC protein appears to modify the specificity of the system, perhaps by acting on the CzcB protein.

The absence of the CzcD polypeptide did not affect expression of the *czc* efflux system from the *E. coli lac* promoter. The CzcD protein was necessary, however, for activation of the *czc* determinant in *A. eutrophus*. A $CzcD^-$ mutation in plasmid pMOL30 led to low-level expression of metal resistance. This mutation could be complemented in trans by a *czcD* gene cloned into plasmid pVCZ'2 and expressed in the mutant strain (D.H.N., unpublished data).

The *czc* determinant is the only determinant known to encode the Zn^{2+}, Co^{2+}, and Cd^{2+} resistances together. The *cadA* determinant of *Staphylococcus aureus* encodes Cd^{2+} and Zn^{2+} resistance (13) and has been cloned and sequenced (14, 15), but the sequence consists of one gene unrelated to *czc*. Although both Cd^{2+} resistances are based on energy-dependent efflux of Cd^{2+} (4, 16), the *czc* system is inducible (4), whereas the *cadA* system appears to be expressed constitutively (17). The *czc* system appears more complicated compared to the single-gene *cadA* system.

One other example of a three-polypeptide cation transport system is the well-studied Kdp potassium transport system of *E. coli* (18). KdpA, KdpB, and KdpC are membrane-bound polypeptides (18). KdpB contains ATPase activity and is related to ATPases of eukaryotic organisms (18). The Czc polypeptides showed no sequence relationships to the Kdp polypeptides and the direction of transport is opposite—efflux rather than uptake. Therefore, multiple component membrane transport systems may occur following more than a single pattern.

We are grateful to T. K. Misra for helpful discussions during the sequencing work. We thank W. Messer, S. Tabor, T. K. Misra, A. Chakrabarty, and V. Deretic for bacterial strains and plasmids. This work was supported by National Science Foundation Grant DMB86-04781 and by a fellowship from the Deutsche Forschungsgemeinschaft.

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