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Cloning, Nucleotide Sequence, and Expression of the Chromate Resistance Determinant of *Pseudomonas aeruginosa* Plasmid pUM505

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The chromate resistance determinant of *Pseudomonas aeruginosa* plasmid pUM505 was cloned into broad-host-range vector pSUP104. The hybrid plasmid containing an 11.1-kilobase insert conferred chromate resistance and reduced uptake of chromate in *P. aeruginosa* PAO1. Resistance to chromate was not expressed in *Escherichia coli*. Contiguous 1.6- and 6.3-kilobase *Hin*dIII fragments from this plasmid hybridized to pUM505 but not to *P. aeruginosa* chromosomal DNA and only weakly to chromate resistance plasmids pLHB1 and pMG6. Further subcloning produced a plasmid with an insert of 2,145 base pairs, which was sequenced. Analysis of deletions revealed that a single open reading frame was sufficient to determine chromate resistance. This open reading frame encodes a highly hydrophobic polypeptide, ChrA, of 416 amino acid residues that appeared to be expressed in *E. coli* under control of the T7 promoter. No significant homology was found between ChrA and proteins in the amino acid sequence libraries, but 29% amino acid identity was found with the ChrA amino acid sequence for another chromate resistance determinant sequenced in this laboratory from an *Alcaligenes eutrophus* plasmid (A. Nies, D. Nies, and S. Silver, submitted for publication).

Bacterial plasmids often contain genetic determinants that confer heavy-metal resistances (26). Plasmids conferring resistance to chromate have been reported in *Pseudomonas* spp. (3, 5, 6, 13, 29), *Streptococcus lactis* (10), and *Alcaligenes eutrophus* (21). Chromate-resistant cells showed reduced accumulation of chromate (5, 21, 22). pUM505 is a 100-kilobase (kb) conjugal plasmid isolated from a *Pseudomonas aeruginosa* clinical strain (6) that confers resistance to chromate and decreased accumulation of chromate in *P. aeruginosa* PAO1 (5). This report describes the cloning and sequencing of the chromate resistance determinant of plasmid pUM505. A single hydrophobic polypeptide of 416 amino acid residues, the ChrA protein, was found to be necessary and sufficient for chromate resistance.

(The sequence reported in this paper was deposited in the EMBL/GenBank data base [accession no. M29034].)

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used are listed in Table 1. *P. aeruginosa* and *Pseudomonas fluorescens* strains were maintained on VB (3) plates supplemented with 1 g of Casamino Acids (Difco Laboratories, Detroit, Mich.) per liter. *P. aeruginosa* and *Escherichia coli* strains were routinely grown in LB broth (16). For susceptibility tests and uptake experiments, nutrient broth (Difco) was used. Antibiotic concentrations used for maintaining plasmids in *E. coli* were as previously described (16); for *P. aeruginosa*, antibiotic concentrations were as follows (μg/ml): 300, streptomycin; 270, chloramphenicol; and 500, kanamycin. Potassium chromate was added to VB Casamino Acids medium at 1 mM and to LB plates at 10 mM.

DNA isolation. Plasmid DNA from *P. aeruginosa* was isolated essentially as previously described (4). For *E. coli* plasmids, a cleared lysate procedure was used (16). Further purification of plasmids was carried out by cesium chloride-ethidium bromide density gradient centrifugation. For rapid plasmid preparation from *E. coli*, a miniprep method was used (12). Total cellular DNA was isolated as described by Marmur (17).

DNA manipulations. Standard molecular cloning, transformation, and electrophoresis procedures (16) were used. Restriction endonucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories (Gaithersburg, Md.) and used as recommended by the manufacturer. DNA polymerase I (Klenow fragment) was from United States Biochemical Corp. (Cleveland, Ohio). Transformation of *P. aeruginosa* was performed by the RbCl/MOPS method (16).

DNA hybridization. Southern blotting DNA-DNA hybridization was done according to established procedures (16). DNA fragments were purified with GeneClean (Bio101, La Jolla, Calif.). DNA probes were labeled with [^{32}P]CTP (Amersham Corp., Arlington Heights, Ill.) with a nick translation kit from Bethesda Research Laboratories. Hybridizations were carried out in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 68°C. Nitrocellulose filters (Schleicher & Schuell, Keene, N.H.) were exposed to Kodak SB5 films (Eastman Kodak Co., Rochester, N.Y.).

DNA sequencing. The 3.8-kb *Pst*I-*Bam*HI fragment from pCRO616 (Fig. 1) was cloned into the phage M13 derivative vector mTM010 (18) in both orientations. Ordered deletions were generated as previously described (18). The chain termination method (24) as modified by Johnston-Dow et al. (14) was used for sequencing reactions with Klenow DNA polymerase and [^{35}S]dATP (Amersham). For most reac-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a] (plasmid)	Reference or source
P. aeruginosa		
PAO1	Prototroph	11
PAO1161	*leu hsdR hsdM* (FP2)	9
P. fluorescens		
LB300	Cr^r (pLHB1)	3
LB303	Spontaneous Cr^s segregant of LB300	3
E. coli		
S17-1	RP4 *tra* functions, *pro recA hsdR*	28
DH1	*recA endA hsdR supE relA*	16
K38	Km^r (pGP1-2), host for T7 expression system	S. Tabor
Plasmids		
pUM505	Cr^r Hg^r	5
pSUP104	Cm^r Tc^r *mob*$^+$	27
pKT230	Sm^r Km^r *mob*$^+$	2
pLHB1	Cr^r	3
pMG6	Cr^r Cm^r Gm^r Km^r Sm^r	29
pCRO1	Cm^r Cr^r, 11.1-kb insert	This study
pCRO115	Sm^r Cr^r, 7.9-kb insert	This study
pCRO616	Sm^r Cr^r, 2.7-kb insert	This study
pT7-3	Ap^r, T7 promoter vector	S. Tabor
pT7-4	Ap^r, T7 promoter vector	S. Tabor
pT7-5	Ap^r, T7 promoter vector	S. Tabor
pT7-6	Ap^r, T7 promoter vector	S. Tabor

[a] r and s superscripts indicate resistance or sensitivity. Ap, ampicillin; Cm, chloramphenicol; Cr, chromate; Gm, gentamicin; Hg, mercuric ions; Km, kanamycin; Sm, streptomycin; Tc, tetracycline.

tions, 7-deaza-dGTP was substituted for dGTP to reduce compressions (19). Both strands were sequenced. Preparation of sequencing gels, electrophoresis conditions, and autoradiography were as previously described (18).

Deletion analysis. Deletions generated for sequencing were subcloned into broad-host-range vector pKT230. After transformation of *E. coli* DH1, streptomycin-resistant (Sm^r) clones were screened by agarose gel electrophoresis for plasmids with the predicted inserts. Hybrid plasmids were then transformed into *P. aeruginosa* PAO1, and Sm^r transformants were scored for chromate resistance.

Expression studies. The inserts from Bal 31 deletions were cloned into a series of pT7 plasmids which possess a T7 RNA polymerase-specific promoter upstream of a polycloning site (30). Recombinant plasmids were transformed into *E. coli* K38 containing plasmid pGP1-2, which expresses T7 RNA polymerase under the control of a temperature-sensitive lambda phage promoter (30). For expression experiments, a procedure (20) provided by S. Tabor (personal communication) was used.

RESULTS

Cloning the chromate resistance determinant. Purified plasmid pUM505 DNA was partially digested with nuclease *Sau*3AI, and the fragments were ligated with *Bam*HI-digested broad-host-range vector pSUP104. After transformation of the *E. coli*-mobilizing strain S17-1, approximately 5,000 Cm^r clones were obtained. Five hundred randomly chosen Cm^r Tc^s colonies were transferred to LB Plates containing 10 mM chromate. None of the colonies grew, which suggested that chromate resistance was not expressed in *E. coli*. The 500 colonies were replica plated on LB, pooled, mixed with *P. aeruginosa* PAO1161, and plated on LB containing 270 μg of chloramphenicol per ml. One of the Cm^r *P. aeruginosa* transconjugants possessed a recombinant plasmid, designated pCRO1, which contained an 11.1-kb insert that conferred resistance to chromate to *P. aeruginosa* PAO1. Resistance to chromate and reduced uptake of $^{51}CrO_4^{2-}$ by PAO1(pCRO1) were similar to those of PAO1(pUM505) (Fig. 2). When plasmid pCRO1 was transferred back to *E. coli* S17-1, chromate resistance was not expressed (data not shown).

Subcloning. Various restriction nuclease fragments of pCRO1 were subcloned into the broad-host-range vector pKT230. The hybrid plasmids were then transformed into *P. aeruginosa* PAO1, and Sm^r or Km^r transformants were scored for chromate resistance. One subclone, pCRO115 (Km^s Sm^r), containing two contiguous *Hin*dIII fragments of 1.6 and 6.3 kb conferred resistance to chromate in PAO1. pCRO115 was linearized at the *Sma*I site of pKT230 (near the *Hin*dIII site indicated in Fig. 1; in the Km^r gene of pKT230) and treated with Bal 31 nuclease to generate sequential deletions, and the resulting fragments were self-ligated. Sm^r Km^s transformants of *E. coli* DH1 were screened for smaller plasmids, and these were transformed back into *P. aeruginosa* PAO1. Sm^r transformants were then

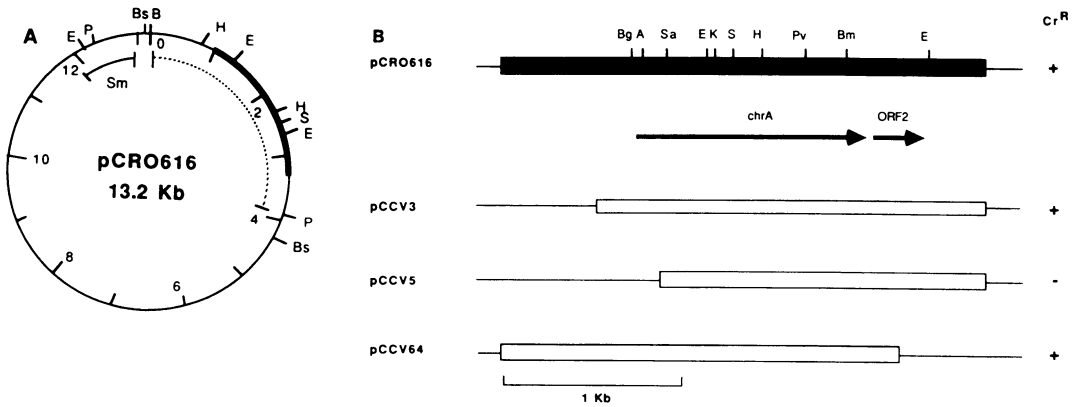

FIG. 1. Restriction nuclease site maps of plasmid pCRO616 and deletion derivatives of it. (A) Thin lines, vector pKT230 DNA; heavier line, DNA insert from pUM505; dashed line in pCRO616, the 3.8-kb *Pst*I-*Bam*HI fragment that was sequenced. Sm refers to the position of the streptomycin resistance determinant. Restriction enzyme site abbreviations are: A, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; Bm, *Bsm*I; Bs, *Bst*EII; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*I; S, *Sal*I; Sa, *Sac*I. The positions and orientations of *chrA* and ORF2 are indicated by arrows. (B) Inserts of deletion subclones pCCV3, pCCV5, and pCCV64 are shown by open boxes. The ability to confer chromate resistance in *P. aeruginosa* PAO1 is indicated to the right.

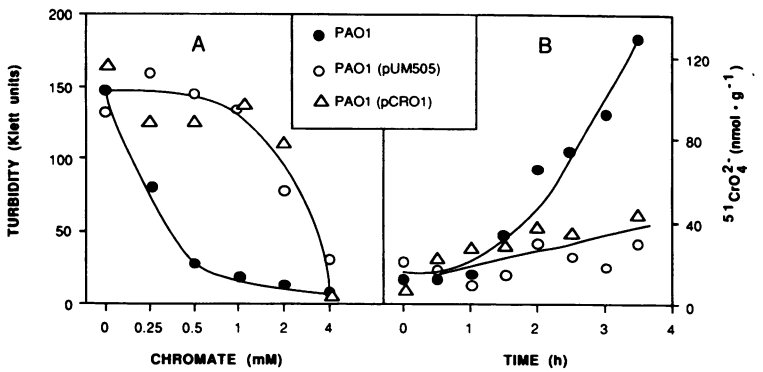

FIG. 2. Susceptibility to chromate and chromate uptake. (A) *P. aeruginosa* cells were grown in nutrient broth at 37°C for 16 h with the indicated concentrations of chromate. Culture turbidity was measured in a Klett colorimeter. (B) Overnight cultures were diluted 1 to 50 in nutrient broth containing 100 μM chromate (1 μCi of $^{51}CrO_4^{2-}$) and incubated at 37°C with aeration. Samples were filtered, washed, and counted in a scintillation counter. Symbols: ●, PAO1; ○, PAO1(pUM505); △, PAO1(pCRO1).

tested for chromate resistance. Plasmid pCRO616 (Fig. 1), with an insert of 2.7 kb, appeared to contain the smallest fragment still capable of conferring resistance to chromate.

Southern DNA-DNA hybridization. The 1.6- and 6.3-kb *Hin*dIII fragments of pCRO115 were used as probes for Southern blotting DNA-DNA hybridizations with *Hin*dIII-digested plasmid and chromosomal DNA. The 6.3-kb *Hin*dIII fragment of pCRO115 hybridized with a band of the same size from *Hin*dIII-digested pUM505 (Fig. 3A). The 1.6-kb fragment from pCRO115 hybridized with a band of about 15 kb of pUM505 (Fig. 3A); hybridization was also observed in the high-molecular-weight region, probably due to incompletely digested pUM505 (Fig. 3). As expected, the probes hybridized with DNA from plasmid pSUP104 but not with DNA from plasmid pKT230 (Fig. 3A). No hybridization was observed with chromosomal DNA of *P. aeruginosa* or *P. fluorescens* strains (Fig. 3B). Only weak hybridization was seen with total DNA from chromate-resistant strains LB300(pLHB1) and PAO1(pMG6) (Fig. 3B).

DNA sequencing. A 3.8-kb *Pst*I-*Bam*HI fragment from pCRO616 (Fig. 1), containing the 2.7-kb insert and vector DNA, was cloned in M13 and completely sequenced in both orientations. Two open reading frames (ORF), *chrA* and ORF2, in the nucleotide sequence could encode putative polypeptides of 416 and 86 amino acid residues, respectively (Figs. 1 and 4).

Deletion analysis. Bal 31 nuclease deletions generated for sequencing were recloned into pKT230, transferred to *P. aeruginosa* PAO1, and tested for chromate resistance. Plasmid pCCV3 (Fig. 1), with an insert starting 220 base pairs upstream of the potential initiation codon of *chrA*, and plasmid pCCV64, with a deletion removing 123 base pairs from the 3′ end of ORF2, still conferred resistance to chromate. Plasmid pCCV5, with a deletion removing 141 base pairs from the 5′ end of *chrA* (Fig. 1), did not confer chromate resistance. It was concluded that *chrA* is necessary and sufficient to determine chromate resistance in *P. aeruginosa*.

The nucleotide sequence and the deduced amino acid sequence of the two ORFs are shown in Figure 4. Two additional ORFs corresponding to polypeptides of 153 and 263 amino acid residues were found in the opposite orientation to *chrA*, but these lacked reasonable ribosomal binding sites. The G + C content of the sequence is 61.4%.

The *chrA* gene. *chrA* potentially encodes a polypeptide with a molecular weight of 44,060. A potential ribosomal binding site (25) occurs seven base pairs upstream of the initiation codon of *chrA* (Fig. 4). Upstream of *chrA* is a promoter-like region which contains a symmetry dyad flanked by consensus-like −35 and −10 regions (Fig. 4). The putative product of *chrA* is very hydrophobic, with nine hydrophobic regions suitable as potential membrane spans (not shown) distributed along the polypeptide chain.

Expression of ChrA. The ChrA polypeptide was tentatively identified in *E. coli* by using the T7 RNA polymerase-promoter system (30). Cells containing the insert of pCCV3 (Fig. 1) cloned in plasmids pT7-3 or pT7-5 synthesized a protein of apparent molecular weight 34,000 that was not found in cells containing only the vector plasmid (Fig. 5;

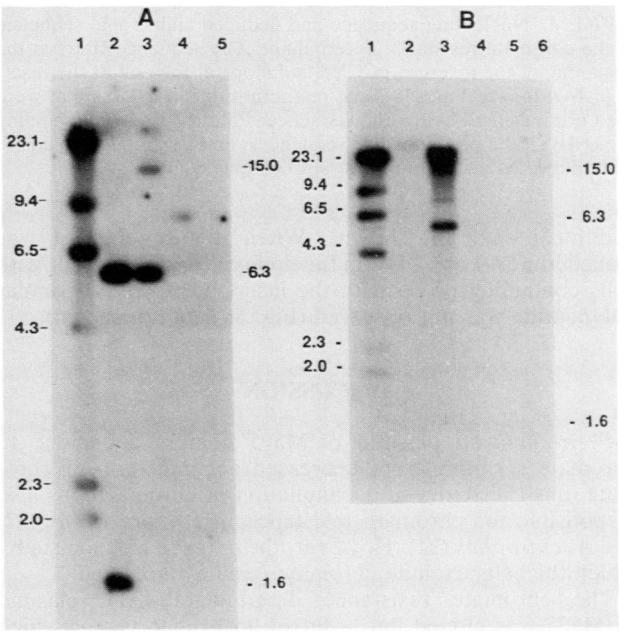

FIG. 3. Autoradiograms of Southern blotting DNA hybridization of ^{32}P-labeled 1.6- and 6.3-kb *Hin*dIII fragments of pCRO115 as probes. (A) Lane 1, phage lambda DNA *Hin*dIII-digest size markers; lanes 2 through 5 contain the following *Hin*dIII-digested plasmid DNAs: pCRO115, pUM505, pSUP104, and pKT230, respectively. (B) Lane 1, lambda *Hin*dIII markers; lanes 2 through 6 contain *Hin*dIII-digested total cellular DNA from the following strains: LB300(pLHB1), PAO1(pUM505), PAO1(pMG6), PAO1, and LB303, respectively. ^{32}P-labeled lambda DNA was mixed with the probe. The sizes of the lambda markers are given to the left in kilobase pairs. Numbers to the right indicate the sizes in kilobases of the probe and of the bands hybridizing to pUM505.

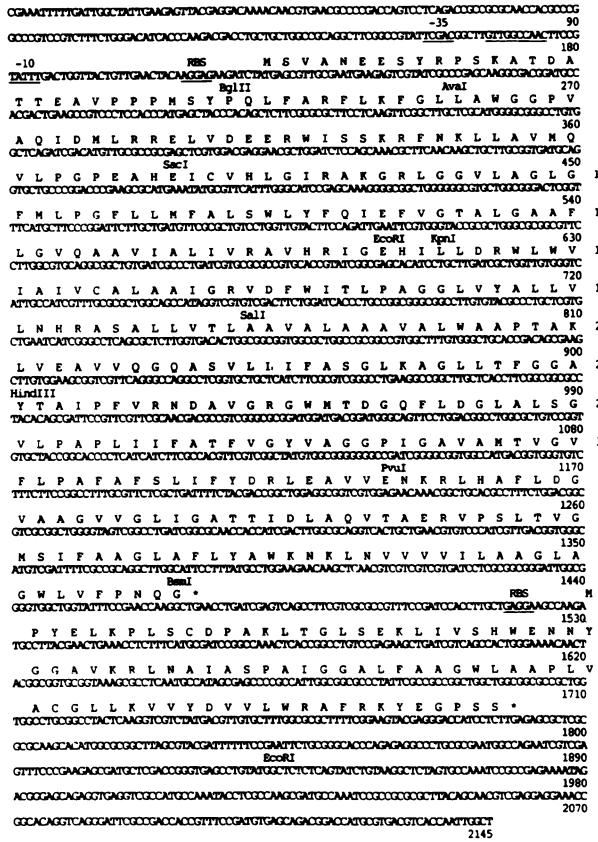

FIG. 4. Nucleotide sequence and deduced amino acid sequence of the chromate resistance determinant. The sequence starts at the beginning of the insert in pCCV3 and stops at the end of the insert in pCRO616 (see Fig. 1). Some restriction nuclease sites that were used are indicated below the sequence. Putative ribosomal binding sites (RBS), the promoter-like sequences, and a symmetry dyad are underlined. Asterisks represent stop codons.

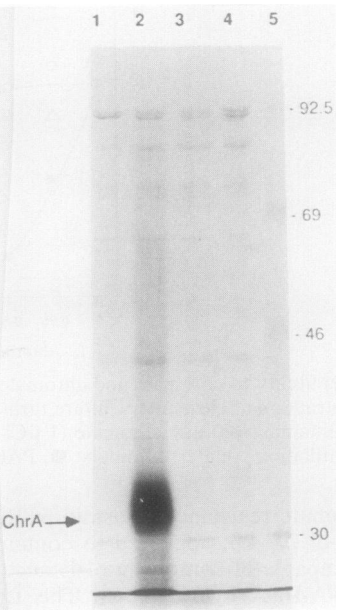

FIG. 5. Expression of the chromate resistance determinant. Inserts from deletions shown in Fig. 1 were subcloned in the pT7 series of plasmids and transformed into *E. coli* K38. Cells were heat induced and treated with rifampin, and the proteins were labeled with [^{35}S]methionine. After sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis, radioactive polypeptides were identified by autoradiography. Lanes: 1, pT7-5 vector with no insert; 2, insert from pCCV3 in pT7-5; 3, insert from pCCV3 in pT7-6 (opposite orientation); 4, insert from pCCV5 in pT7-5; 5, molecular weight markers indicated in kilodaltons. The position of ChrA is indicated by the arrow.

additional data not shown). When the same insert was cloned in pT7-4 or pT7-6 (in the opposite orientation) or with cells containing pT7-5 with the insert of pCCV5, a similar polypeptide was not observed (Fig. 5; data not shown).

DISCUSSION

P. aeruginosa plasmid pUM505 confers resistance to chromate resulting from decreased accumulation of chromate ions (5). Reduced accumulation of chromate was also responsible for chromate resistance in *P. fluorescens* (22) and *A. eutrophus* (21). However, the detailed mechanism by which the cells exclude chromate remains unknown.

The chromate resistance determinant from plasmid pUM505 was cloned into a broad-host-range vector which conferred resistance to chromate in *P. aeruginosa* (Fig. 2A) but not in *E. coli*. It is not known if this failure is due to a transcriptional barrier or an inability of the protein product(s) to function in the *E. coli* membrane. Decreased accumulation of chromate was demonstrated in *P. aeruginosa* (Fig. 2B).

DNA-DNA hybridization experiments identified the cloned chromate resistance determinant within plasmid pUM505 (Fig. 3A). A weak hybridization of the UM505 probe was found with DNA from chromate-resistant *P. fluorescens* LB300(pLHB1) and *P. aeruginosa* PAO1 (pMG6) (Fig. 3B), which mediate chromate resistance by a similar mechanism (3; unpublished data).

By sequencing and deletion analysis, an ORF designated *chrA*, which encoded a putative polypeptide of 416 amino acid residues, was identified. It is surprising that the chromate resistance determinant of pUM505 comprises a single gene. This result may be related to the fact that chromate resistance in *P. aeruginosa* appears to be constitutive (5). In the other plasmid-determined anion resistance systems thus far studied (namely those for arsenate, arsenite, and tellurite), the resistance phenotypes are inducible (26). From the sequence of the *ars* operon from *E. coli* plasmid R773, four genes have been identified (7, 26; B. P. Rosen, personal communication). The *arsA* gene encodes an ATPase that energizes an efflux system (7). *arsB* encodes a very hydrophobic integral inner membrane protein (23) believed to contain the ion translocation channel responsible for the efflux of arsenic ions. A third gene, *arsC*, codes for a small polypeptide involved in determining substrate specificity (7). The fourth gene, *arsR*, is involved in the regulation of the *ars* operon (26; B. P. Rosen, personal communication).

Comparison of the amino acid sequence of ChrA with protein sequences in the Swissprot data base (15) showed only weak homologies with restricted regions of some membrane proteins. The most extended homology was found between ChrA and the *E. coli* MalG protein, an inner membrane protein involved in the transport of maltose (8). ChrA and MalG are 18% identical over a 154 amino acid range when optimally aligned (data not shown).

ChrA appeared to be expressed in *E. coli* under the T7 RNA polymerase-promoter system (30) as a protein of molecular weight 34,000 (Fig. 5). The apparent molecular

weight of this protein is smaller than the predicted size of ChrA, based on the amino acid sequence (44,060). This discrepancy may be attributed to the abnormal migration behavior of membrane proteins in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1). Similar, anomalously small apparent sizes on gel electrophoresis have been found with the *Alcaligenes* chromate resistance determinant (20) and with the arsenic resistance determinant (23).

Although we have not been able to demonstrate efflux of chromate ions by chromate-resistant cells, the involvement of a transport mechanism as the basis for the resistance to chromate by the pUM505 determinant seems likely (Fig. 2) (5). It can be hypothesized that ChrA is the inner-membrane protein responsible for the translocation of chromate ions. Chromate resistance requires solely the *chrA* plasmid gene. Any additional requirements for the resistance mechanism would need to be provided by host cell genes.

The nucleotide sequence of an inducible chromate resistance determinant cloned from *A. eutrophus* plasmid pMOL28 (20, 21) has recently been determined in our laboratory (A. Nies, D. H. Nies, and S. Silver, submitted for publication). This chromate resistance determinant did not hybridize with a probe prepared from pCRO616 in Southern blotting DNA hybridization experiments (data not shown). A significant homology was found between the ChrA proteins from pUM505 and pMOL28 (29% amino acid identities; data not shown). The overall hydrophobic character and hydropathy profiles (not shown) were very similar. Although the promoter-distal ORF2 from pUM505 and the comparable ORF from pMOL28 are not necessary for chromate resistance, they are highly related (53% identities at the protein level; 35 of the first 41 amino acids are identical). An explanation for this close homology of currently cryptic ORFs is not available.

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