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Characterization of the Nonenzymatic Chloramphenicol Resistance (*cmlA*) Gene of the In*4* Integron of Tn*1696*: Similarity of the Product to Transmembrane Transport Proteins

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Integrons constitute a novel family of DNA elements which evolved by site-specific integration of discrete units between two conserved segments. On the In*4* integron of Tn*1696*, a precisely inserted gene cassette of 1,549 bp conferring nonenzymatic chloramphenicol resistance (*cmlA*) is present between the streptomycin-spectinomycin resistance (*aadA2*) gene cassette and the 3′-conserved segment of the integron. In this study, we present the nucleotide sequence of the *cmlA* gene cassette of Tn*1696*, show its similarity to bacterial efflux systems and other transport proteins, and present evidence for alterations that its expression exerts on bacterial membranes. The *cmlA* gene cassette apparently carries its own promoter(s), a situation that has not heretofore been observed in the integrons of multiresistance plasmids and transposons of gram-negative bacteria. One or more of these promoters were shown to be functionally active in expressing a *cat* marker gene from promoter-probe vectors. The putative CmlA polypeptide appears to provoke a reduction of the content of the major porins OmpA and OmpC.

Multiresistance plasmids and transposons are actively involved in the dissemination of antibiotic resistance determinants, and evidence for their evolution by site-specific integration of antibiotic resistance genes has been reported in recent years. The characterization of multiresistance elements, plasmids and transposons, related to bacterial transposon Tn*21* revealed that rearrangements involving antibiotic resistance genes were occurring in the vicinity of the streptomycin-spectinomycin (*aadA*) and sulfonamide (*sul1*) resistance regions (20, 40, 50, 56, 62, 63). Stokes and Hall (55) have defined the elements borne on these multiresistance plasmids and transposons as integrons, a novel family of potentially mobile DNA elements which are composed of two conserved segments between which discrete units, ordinarily antibiotic resistance genes, have been integrated as gene cassettes. The 5′-conserved segment encodes a site-specific recombinase (Int) showing active-site residue similarity with the phage integrases (31, 41, 55). The 3′-conserved segment encodes a sulfonamide-resistant dihydropteroate synthase (Sul1 [56]) and two open reading frames (ORFs) of unknown phenotype, ORF4 and ORF5 (see Fig. 2A). The integrated gene cassettes encode resistance determinants, such as those for aminoglycoside acetyltransferases and adenylyltransferases, β-lactamases, trimethoprim-resistant dihydrofolate reductases, and enzymatic (acetyltransferase) and nonenzymatic resistance to chloramphenicol. Cassettes carrying ORFs of unknown function are also part of some integrons. No promoters have as yet been found on these gene cassettes, their transcription being driven by promoters located on the 5′-conserved segment (51, 58). Alternative promoters for the *sul1* gene of R46 have been located on the 3′-conserved segment (19). At the 3′ end of the gene cassettes, putative stem-loop structures potentially involved in site-specific recombination are observed (59-bp elements) (20, 30, 52, 55). The requirement for these elements in *cis* for site-specific recombination has been demonstrated by Martinez and de la Cruz (31).

Bacterial resistance to chloramphenicol, both in gram-negative and gram-positive bacteria, is usually mediated by mono- and diacetylation by chloramphenicol acetyltransferase, preventing the subsequent binding of chloramphenicol to the 50S ribosomal subunit (53, 54). A unique enzymatic mechanism has been observed in the chloramphenicol-producing actinomycete *Streptomyces venezuelae*, in which the elaboration of a chloramphenicol hydrolase accounts for the resistance mechanism (34). Another less prevalent mechanism of chloramphenicol resistance, which does not involve modification of the compound, but rather its entry into the bacterial cell, has been appropriately termed nonenzymatic and has been observed principally in gram-negative bacteria. The genetic determinants of this mechanism are usually plasmid encoded, such as in *Pseudomonas aeruginosa* and in members of the family *Enterobacteriaceae* (6, 10–12, 16, 23, 26, 35, 46); resistance determinants of chromosomal origin were also reported in *Haemophilus influenzae* (5), *Pseudomonas cepacia* (4), and *Salmonella typhi* (60). In most instances, it was demonstrated that nonenzymatic chloramphenicol resistance involves a membrane permeability barrier and that porins appear to be deficient in some of these strains. Burns et al. (6) observed the reduced expression of an outer membrane polypeptide of 50 kDa in strains containing the *cml* gene of Tn*1696*. Similar observations were also made for polypeptides of 40 kDa in *H. influenzae* (5) and of 18 kDa in *P. cepacia* (4). Toro et al. (60) recently reported a chloramphenicol-resistant strain of *S. typhi* which shows a drastic reduction in the content of the OmpF porin. Furthermore, homology between a probe derived from the Tn*1696 cml* gene and chromosomal DNA from a nonenzy-

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matic chloramphenicol-resistant *H. influenzae* strain was detected (6). The nucleotide sequence of the nonenzymatic chloramphenicol resistance gene (*cml*) of *P. aeruginosa* IncP plasmid R26 has been reported (12), and the apparent molecular mass of the gene's product (Cml) was estimated to be 31 kDa on the basis of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of ^{35}S-labeled polypeptides or 33 kDa as deduced from the *cml* gene sequence.

Tn*1696* is a 16-kb transposable element, making up part of *P. aeruginosa* IncP plasmid R1033 (22, 46). Heteroduplex analysis of IncP plasmids R1033 and R26 indicated a close relationship (61), but the presence of a transposon identical to Tn*1696* on R26 has not been clearly established. Tn*1696* carries the In*4* integron, into which four discrete units, a gentamicin acetyltransferase (*aacC1*), a DNA fragment of unknown function (X), a streptomycin-spectinomycin (*aadA2*) resistance gene, and a nonenzymatic chloramphenicol (*cmlA*) resistance gene (see Fig. 2B), are integrated.

In an effort to identify and characterize the boundaries of the *cmlA* cassette in the In*4* integron of Tn*1696*, we cloned and sequenced this gene from plasmid pCER100, a plasmid resulting from the transposition of Tn*1696* from R1033 to pMB8 (46). We found that the *cmlA* gene is part of a precise insertion of 1,549 bp between the *aadA2* gene cassette and the 3′-conserved segment of this integron. The *cmlA* gene cassette has the following interesting features: (i) it is one of two known examples of gene cassettes occurring between an *aadA* gene cassette and the 3′-conserved segment of an integron, the other being Tn*2424* (32, 33); (ii) it is the only gene cassette to mediate a nonenzymatic resistance mechanism; and (iii) it is the first gene cassette to contain its own promoter, as determined by the expression of a *cat* marker gene of promoter-probe vectors. We also evaluate the effects of *cmlA* on the protein content of inner and outer membranes of *Escherichia coli* NM522 cells containing *cmlA*-bearing plasmids.

(These results were presented in part at the 90th Annual Meeting of the American Society for Microbiology, Anaheim, Calif., 1990 [3].)

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* JC2926 containing pCER100 was a kind gift of C. E. Rubens. pCER100 was isolated by the method of Beaulieu et al. (1) and purified by isopycnic ultracentrifugation on a cesium chloride-ethidium bromide gradient. *E. coli* NM522 [*hsdΔ5Δ*(*lac-pro*) (F′*pro*$^+$ *lacI*^{q}*ZΔM15*)] served as host strain for cloning. The plasmids described in this study are listed in Table 1.

Microbiological media and antibiotics. All microbiological media (Difco) were obtained from BDH. Ampicillin sulfate, chloramphenicol, and kanamycin sulfate were obtained from ICN Biochemicals. Sulfamethoxazole was purchased from Sigma. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was purchased from Boehringer Mannheim Canada Ltée.

Enzymes. Restriction endonucleases were purchased from Boehringer Mannheim Canada Ltée, Bethesda Research Laboratories, or New England BioLabs and used according to the manufacturers' conditions. T4 DNA ligase was purchased from Bethesda Research Laboratories.

Transformation and screening of recombinant plasmids. Transformation of $CaCl_2$-treated *E. coli* strains was performed by the method of Maniatis et al. (29). Selection of desired clones was done on solid media containing ampicillin (50 μg/ml), either alone or in combination with chloramphenicol (25 μg/ml), and X-Gal. Screening of recombinant plasmids was performed by the miniscale method of Birnboim and Doly (2).

TABLE 1. Plasmids used in this study

Plasmid	Characteristic (phenotype[a])	Reference or source
pCER100	pMB8::Tn*1696*	46
pTZ18R-19R	Cloning vectors, 2.9 kb (Ap^r)	Pharmacia Canada Ltée
M13KO7	Helper phage (Km^r)	Pharmacia Canada Ltée
pT7-1, pT7-2	Cloning vectors (Ap^r)	Boehringer Mannheim Canada Ltée
pBR328	Cloning vector (Ap^r Cm^r Tc^r)	Boehringer Mannheim Canada Ltée
pLQ820	7-kb *Eco*RI fragment of pCER100 cloned in pTZ18R (Ap^r Cm^r Su^r)	This study
pLQ821	3.1-kb *Eco*RI-*Bgl*II fragment of pLQ820 cloned in pTZ18R (Ap^r Cm^r)	This study
pLQ822	3.1-kb *Eco*RI-*Bgl*II fragment of pLQ820 cloned in pTZ19R (Ap^r Cm^r)	This study
pLQ894	pT7-1 containing the polylinker of pPR510 (Ap^r)	This study
pLQ895	pT7-2 containing the polylinker of pPR510 (Ap^r)	This study
pLQ896	Promoter-probe vector (Ap^r Cm^s)	This study
pLQ897	Promoter-probe vector (Ap^r Cm^s)	This study
pLQ850	375-bp *Hin*dIII-*Sst*I fragment of pLQ821 cloned in pLQ896 (Ap^r Cm^s)	This study
pLQ851	375-bp *Hin*dIII-*Sst*I fragment of pLQ821 cloned in pLQ897 (Ap^r Cm^r)	This study
pLQ854	1,330-bp *Sst*I fragment of pLQ821 cloned in pTZ18R; sense clone (Ap^r Cm^s)	This study

[a] Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol; Su, sulfamethoxazole.

Large-scale isolation of plasmid DNA. Plasmid DNA isolation from recombinant clones was performed by a modified version of a Triton lysis method, originally obtained from J. D. Friesen (University of Toronto, Ontario, Canada). This method permits rapid isolation of supercoiled plasmid DNA of up to 20 kb. Cells from a 1-liter 2× YT medium (20 g of Bacto-tryptone, 10 g of yeast extract, 10 g of NaCl, 2 g of glucose per liter) overnight culture are pelleted at 3,000 × *g* and resuspended in 4 ml of sterile STE buffer (15% sucrose, 50 mM Tris [pH 8.0], 50 mM EDTA) in a polycarbonate tube. Lysozyme (20 to 25 mg; Sigma) is added and gently mixed with the cell homogenate, which is incubated for 20 min at room temperature. SDS is added to 0.25%, well mixed, and followed by the addition of 5 ml of Triton solution (0.4% Triton X-100, 50 mM Tris [pH 8.0], 50 mM EDTA). After gentle mixing, the tube is left to stand at room temperature for 10 more min. Chromosomal DNA, cell

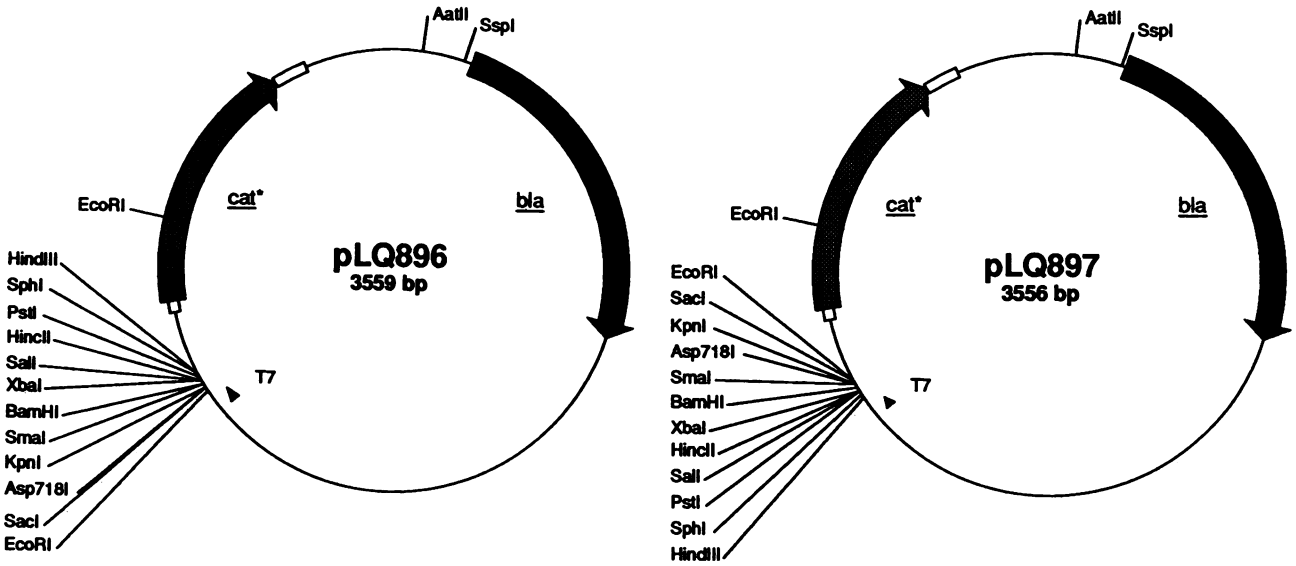

FIG. 1. Structure of the promoter-probe vectors pLQ896 and pLQ897 used to verify the functionality of the promoter regions found on the *cmlA* cassette of In*4*. *cat** is the promoterless chloramphenicol acetyltransferase gene of pBR328. *bla*, β-lactamase.

debris, and protein aggregates are pelleted at 40,000 × *g* for 35 min at 4°C, and the supernatant, transferred to fresh tubes, is phenol extracted twice and chloroform extracted once. The plasmid DNA is precipitated with 2 to 3 volumes of cold 99% ethanol and then subjected to an isopycnic ultracentrifugation on a cesium chloride-ethidium bromide gradient.

Single-stranded pTZ template preparation and DNA sequencing. The isolation of pTZ single-stranded templates, suitable for the dideoxynucleotide chain termination sequencing procedure (49) was performed according to the manufacturers' conditions (Pharmacia Canada Ltée), with some modifications. First, the day before template isolation, clones are streaked on minimal (M9) agar (29) supplemented with 1 mM $MgSO_4$, 0.2 mM $CaCl_2$, 2.5 μg of thiamine per ml, and 0.02 mg of leucine per ml and containing antibiotics (ampicillin and/or other selective antibiotics). Second, the phage resuspension after polyethylene glycol precipitation is subjected to two phenol, up to four phenol-chloroform (50:50), and two chloroform extractions. The single-stranded DNA template is precipitated with ethanol and resuspended in 35 μl of water or TE buffer. Nucleotide sequencing with these templates was performed with the Sequenase DNA sequencing kit of United States Biochemicals Corp. using α^{35}S-dATP (NEN-Dupont). G+C compressions were resolved by using dITP. Electrophoresis of 6% polyacrylamide-urea gels was performed with the LKB Macrophor Sequencing System, and the autoradiography was done on X-Omat XAR-5 films (Eastman Kodak). Nucleotide sequence data were analyzed by the Genetics Computer Group (GCG) software (8).

Construction of promoter-probe vectors derived from pT7-1 and pT7-2. In order to verify the activity of promoterlike sequences on the *cmlA* cassette, we cloned specific fragments of the upstream region of *cmlA* into new promoter-probe vectors pLQ896 and pLQ897 (Fig. 1). We first cloned the pUC18-like polylinker of pPR510 (44) into plasmids pT7-1 and pT7-2. The resulting plasmids, pLQ894 and pLQ895, were checked by restriction endonuclease digestion, especially with *Asp*718I, which does not cut pT7-1 and pT7-2. We then cloned a promoterless *cat* gene (*cat**) from pBR328 on a 780-bp *Taq*I fragment into the unique *Nar*I site of pLQ894 and pLQ895, to yield pLQ896 and pLQ897. These promoter-probe vectors were checked by restriction endonuclease digestion and by their inability to allow growth of transformed *E. coli* cells on chloramphenicol (25 μg/ml) plates.

Functional assay for transcription-promoting regions of the *cmlA* cassette. Two experiments were designed to check the functionality of transcription-promoting regions of the *cmlA* cassette: (i) cloning of specific fragments of pLQ821 into promoter-probe vectors pLQ896 and pLQ897, and (ii) cloning of a 1,330-bp *Sst*I fragment containing the *cmlA* ORF without the p1-p2-p3 region of pLQ821, in phagemid vector pTZ18R. A 375-bp *Hin*dIII-*Sst*I fragment from pLQ821 which contains putative promoter-like sequences was cloned in pLQ896 and pLQ897, in both orientations. Recombinant plasmids were checked by restriction endonuclease digestions, and selection for promoter activity was done by using chloramphenicol (25 μg/ml) plates.

Membrane isolation and fractionation. Bacterial membranes were isolated by a modification of the method of Godfrey et al. (17). Selected recombinant, vector-containing or plasmidless *E. coli* cells grown in 1 liter of 2× YT media with appropriate selective pressure (50 μg of ampicillin per ml and 25 μg of chloramphenicol per ml, if needed) were pelleted, resuspended in 20 ml of TD buffer (50 mM Tris-HCl [pH 7.9], 0.2 mM dithiothreitol) containing 20% sucrose, and lysed by sonication (four times, 30 s each) with a model W-375 sonicator (Heat Systems-Ultrasonics Inc.). Cell debris and unbroken cells were pelleted at 2,000 × *g* for 10 min, and membranes were differentially sedimented by a 1-h centrifugation at 225,000 × *g* in a Beckman 50.2 Ti rotor and then resuspended in 3.0 ml of TD buffer containing 20% sucrose. Of this resuspension, 2.5 ml was layered on a discontinuous sucrose gradient (58–64–70% sucrose, made in TD buffer) and centrifuged for 16 h at 100,000 × *g* in a Beckman TY65 rotor. Inner and outer membranes were collected above the 58% phase and at the 64–70% interface, respectively, washed once with TD buffer, pelleted by a 1-h

centrifugation at 225,000 × *g*, and resuspended in 350 μl of TD buffer. Protein aliquots were then subjected to electrophoresis on a SDS-PAGE gel (27) and visualized by Coomassie brilliant blue staining. Low-molecular-weight markers (Bio-Rad) were used for molecular weight determination.

Nucleotide sequence accession number. The sequence described in this paper has been assigned GenBank accession number M64556.

RESULTS

Molecular cloning of the *cmlA* gene from plasmid pCER100. Burns et al. (6) have shown that the nonenzymatic chloramphenicol resistance gene from plasmid pCER100 could be cloned on a 2.0-kb *Hin*dIII fragment, the expression apparently being driven by a promoter on the vector. In order to study the entire *cmlA* gene cassette, we cloned a 7-kb *Eco*RI fragment of pCER100 into pTZ18R to yield pLQ820. We then subcloned a 3.1-kb *Eco*RI-*Bgl*II fragment of pLQ820 into pTZ18R and pTZ19R, generating pLQ821 and pLQ822. This *Eco*RI-*Bgl*II fragment contains the carboxy-terminal region of the pSa-type streptomycin-spectinomycin resistance gene (*aadA2*) (57), the *cmlA* gene cassette, and most of the sulfonamide resistance gene (*sul1*) (56) (Fig. 2).

Nucleotide sequencing and determination of the precise point of insertion of the *cmlA* gene into Tn*1696*. The nucleotide sequence of the 2.1-kb region between the *Eco*RI site in the *aadA2* gene and the first of the two closely spaced *Hin*dIII sites of the 3′-conserved segment (Fig. 2A), downstream of the *cmlA* cassette, was determined (Fig. 3). The first 396 nucleotides correspond to the 3′-end of the *aadA2* gene cassette which encodes the streptomycin-spectinomycin adenylyltransferase found on plasmid pSa (57). The 3′-conserved segment of In*4* commences at base 1946 and is similar to the 3′ segment of the In*1* integron of plasmid R46 (55). At the 3′ ends of both the *aadA2* and the *cmlA* gene cassettes, structures resembling the consensus 59-bp elements (20, 55) are present (Fig. 4).

The 1,549-bp *cmlA* cassette extends from bases 397 to 1945 and contains a long ORF of 1,257 nucleotides starting at a GTG codon (position 601), near a consensus ribosome binding site (AGGAG). This is in contrast with that of the ATG codon situated 87 bp downstream (GGCA). This ORF encodes a polypeptide (CmlA) of 419 amino acids with an expected molecular mass of 44,228 Da. Two smaller ORFs overlapping the *cmlA* ORF are also found: *orf1* (1461 to 1808), which could encode a polypeptide of 116 amino acids (12,767 Da), and *orf2* (1811 to 2119), a polypeptide of 103 amino acids (12,229 Da). *orf1* is contained within *cmlA*, while *orf2* overlaps the C-terminal region of *cmlA* and the junction between the *cmlA* cassette and the N-terminal region of *orf4*. ORF4 is characteristic of the 3′-conserved segment (55). Sequencing of the *sul1* gene region of Tn*1696* (data not shown) indicates 100% identity with the *sul1* gene of plasmid R388 (56).

Transcription-promoting regions on the *cmlA* cassette. The gene cassettes of characterized integrons are usually of sufficient length to encode one polypeptide and do not contain putative promoter-like sequences. By using Tn*5* mutagenesis, Dorman and Foster (11) have shown that at least 1.4 kb of the *cmlA* cassette is essential for high-level chloramphenicol resistance. This region extends from approximately 300 bp downstream of a *Hin*dIII site located in *aadA2* (base 233) to the *Pst*I site located at the 3′ end of the *cmlA* unit (base 1893). One insertion mutant downstream of the *Pst*I site resulted in a low-level resistance phenotype. Therefore, the *cmlA* gene might be expressed from a promoter(s) located on the gene cassette. We cloned a region which contains adequately spaced sequences similar to the −35 and −10 regions of the *E. coli* consensus promoter (Table 2) into promoter-probe vectors pLQ896 and pLQ897 (Fig. 1), and this region acts as a promoter for a *cat* marker gene when cloned in the sense orientation relative to the *cmlA* gene. The p1-p2-p3 cluster of putative promoters was cloned on a 375-bp *Hin*dIII-*Sst*I fragment, into pLQ897; the resulting plasmid, pLQ851, is chloramphenicol resistant. A recombinant clone in the opposite orientation (in pLQ896) was sensitive to chloramphenicol, supporting the hypothesis that one or more of these promoters might be functional in vivo. From transcriptional fusion results, Dorman and Foster (11) classified the *cml* promoter as a weak one.

We also cloned a 1,330-bp *Sst*I fragment of pLQ821 into pTZ18R. This fragment, cloned in the sense orientation relative to the *lacZ′* promoter of pTZ18R, was sensitive to chloramphenicol (25 μg/ml) and, therefore, indicated use of the GTG initiation codon at base 601 rather than the ATG at base 688 for expression of the resistance mechanism.

Sequence similarity of the CmlA polypeptide to bacterial transport proteins. The Pearson programs FASTA and TFASTA and the GCG program PROFILESEARCH were used to compare the predicted CmlA polypeptide to the protein and nucleic acid data bases. Significant similarity to the family of efflux proteins and other bacterial transport proteins including both gram-positive and gram-negative tetracycline resistance, Mmr methylenomycin resistance from *Streptomyces coelicolor*, QacA antiseptic resistance from *Staphylococcus aureus*, and the *E. coli* arabinose transporter AraE, was observed. This family was recently described by Rouch et al. (45). Figure 5 shows the alignment of a 200-amino-acid region in the amino half of these proteins. CmlA has its greatest overall similarity with the gram-positive tetracycline genes and with Mmr, but shows some strong local similarities with gram-negative Tet proteins and with AraE.

The CmlA protein has grouped charged residues separated by hydrophobic regions. Through analysis of CmlA, of QacA (45), and of the *Staphylococcus aureus* NorA quinolone resistance protein (64) with the GCG programs PEPPLOT and PEPTIDESTRUCTURE/PLOTSTRUCTURE and taking into account the authors' assignments of transmembrane domains, 12 transmembrane domains can be assigned for CmlA (Fig. 6), with the basic residues predominantly on the cytoplasmic side.

Electrophoresis of bacterial membrane proteins. The alternation of hydrophobic domains and β-turns predicted for CmlA by the program PEPPLOT (8) suggests that it is a membrane-associated polypeptide (14, 24). We therefore isolated inner and outer membranes of *E. coli* NM522 and of cells carrying pTZ19R or pLQ822 by sucrose gradient fractionation. The electrophoretograph of an SDS–PAGE gel reveals extensive modifications of the membranes' protein content (Fig. 7). Important variations of the outer membrane, including a diminution of the major porins, OmpC (preferentially expressed in high-osmolarity media [39]), and OmpA, and of some minor porins (22 and 20.5 kDa) and the appearance of a relatively abundant polypeptide of molecular mass 32,000 Da and of additional polypeptides ranging from 16.5 to 100 kDa, were observed.

The inner membrane also revealed other subtle modifications. First, we observed the appearance of a polypeptide of

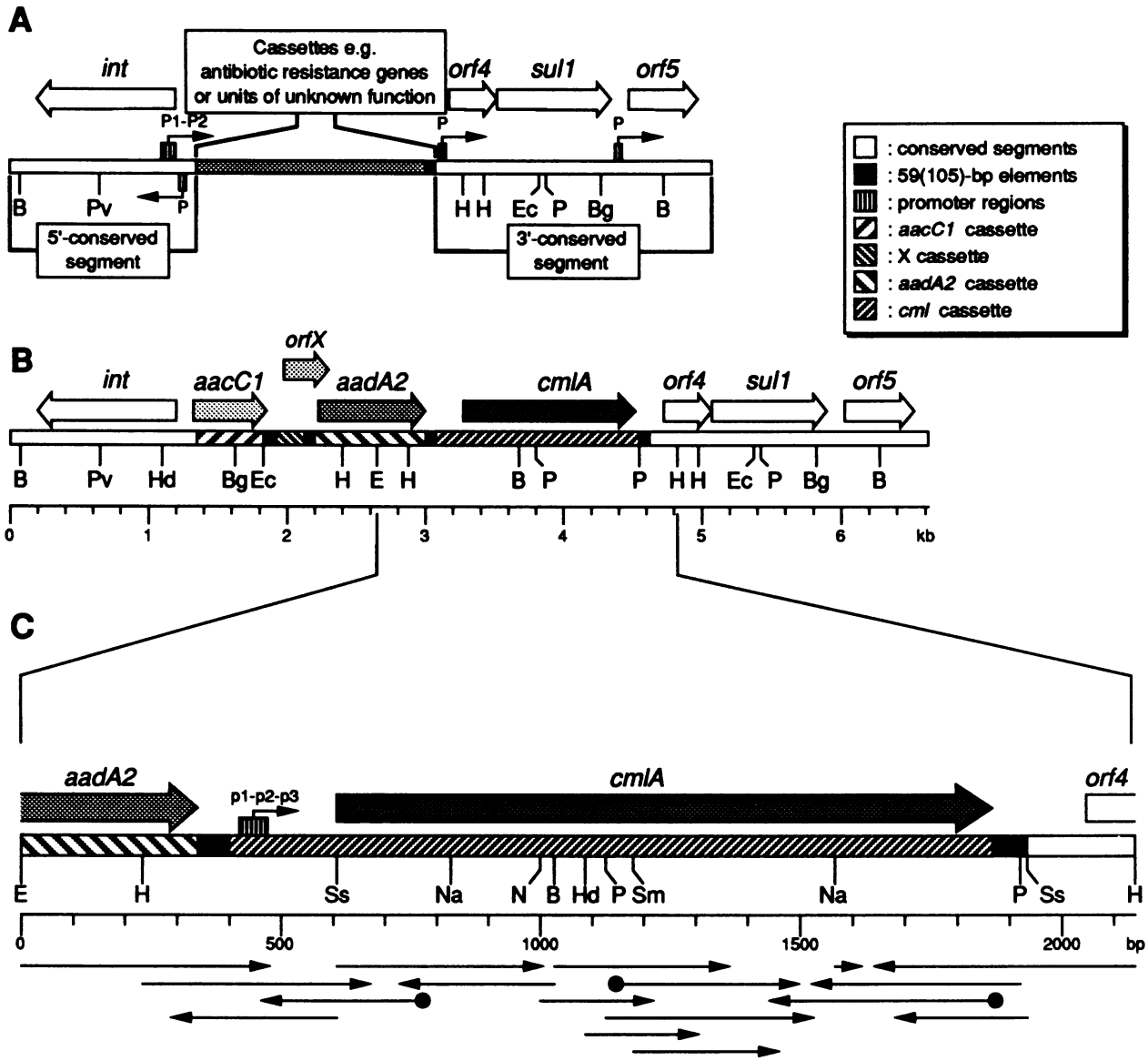

FIG. 2. (A) General structure: integrons evolved by site-specific integration of discrete units (cassettes) between two conserved segments (55). Promoters of the conserved segments are shown. (B) Structure of the In*4* integron on Tn*1696*. The *aacC1* and X cassettes are described elsewhere (63). (C) Restriction map of the sequenced region of recombinant plasmids pLQ821 and pLQ822 and sequencing strategy. *cmlA* and other ORFs are indicated. The location and orientation of the putative promoters p1, p2, and p3 are shown. The length and direction of thin arrows indicate the extent of sequencing reactions. Thin arrows starting with a dot indicate sequencing data obtained from custom-made oligonucleotides. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; Ec, *Eco*RV; H, *Hin*dIII, Hd, *Hin*dII; N, *Nru*I; Na, *Nar*I; P, *Pst*I; Pv, *Pvu*II; Sm, *Sma*I; Ss, *Sst*I.

approximately 27 kDa in the inner membrane fraction of NM522 carrying *cmlA* and the apparent overexpression of polypeptides of molecular masses 13,000 and 56,000 Da. Second, extensive variations were observed for polypeptides of molecular masses 50,000, 59,000, and 79,000 Da, whose quantity is reduced in cells expressing *cmlA*.

DISCUSSION

The multiresistance integrons have evolved by site-specific integration of discrete units, principally containing antibiotic resistance genes, between two conserved segments (55). The characterization of these cassettes and the genes they harbor is important for the determination of the origin of these units and the reconstitution of the recombinational events involved in their integration into an ancestral structure. The In*4* integron of Tn*1696* is made up of four integrated gene cassettes. Between the 5′-conserved segment and the *aadA2* gene cassette, two cassettes are present: an *aacC1* gene cassette and a cassette of unknown phenotype (X), which are described elsewhere (59, 63). In this report, we characterized the nonenzymatic chloramphenicol resistance (*cmlA*) gene cassette. This 1,549-bp cassette is the longest characterized to date, and it is one of the rare cassettes present between an *aadA* gene cassette and the 3′-conserved segment, which includes the sulfona-

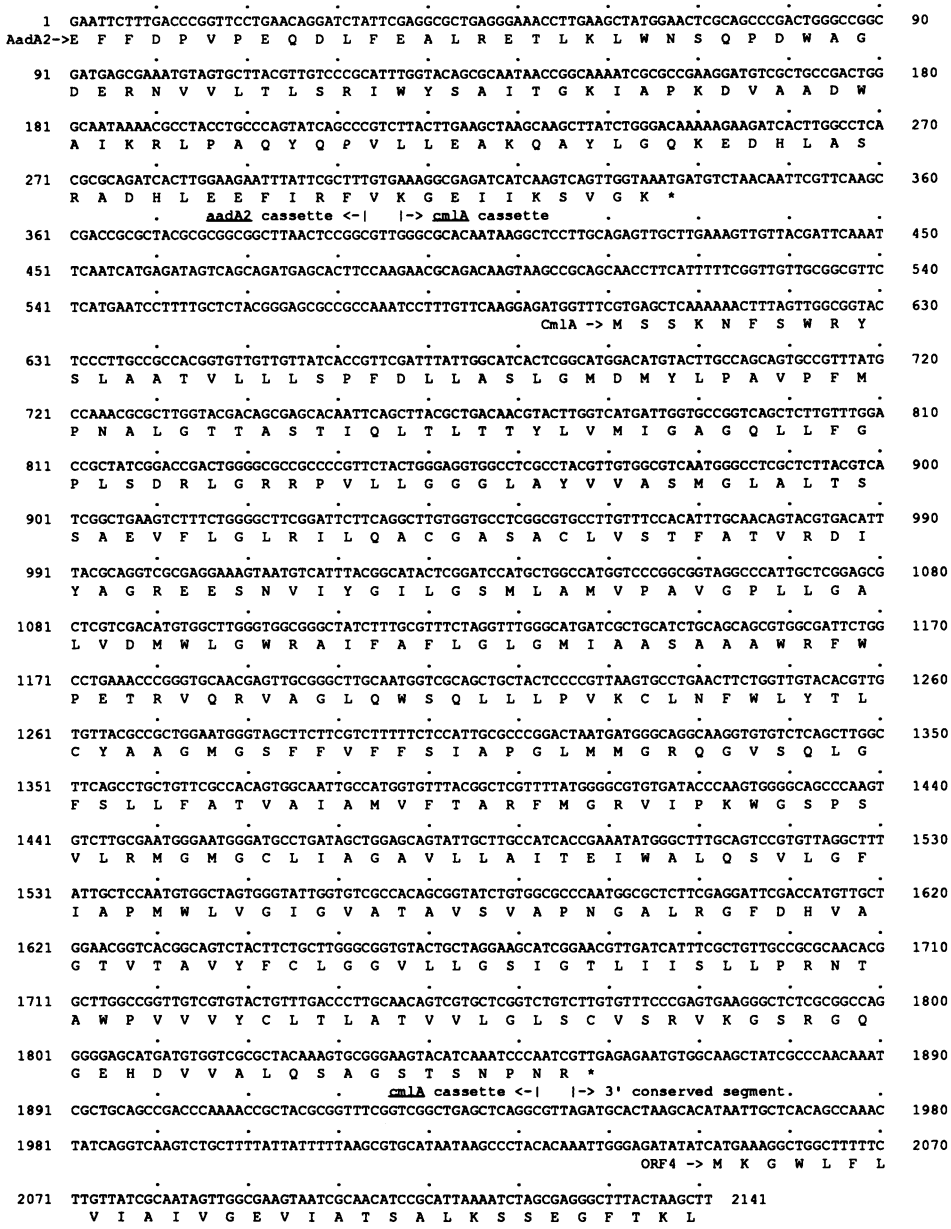

FIG. 3. Nucleotide sequence of the *cmlA* cassette and flanking regions on the In*4* integron of Tn*1696*. The nucleotide sequence of the 2.1-kb *Eco*RI-*Hin*dIII region of plasmid pCER100 encoding *cmlA* and the deduced primary structure of the CmlA protein are shown. The limits between the *aadA2* and *cmlA* cassettes and between *cmlA* and the 3′-conserved segment are indicated.

mide resistance gene. In Tn*2424*, tandem cassettes coding for an *aacA1a*-type aminoglycoside acetyltransferase and for a novel *cat* gene are similarly located relative to the *aadA* gene (32, 33, 42, 43). Furthermore, Tn*1696 cmlA* is the first described gene cassette that contains transcription-promoting regions. Moreover, *cmlA* is also the first gene inserted into a multiresistance integron which encodes a nonenzymatic resistance mechanism, supporting the assumption of Stokes and Hall (55) that genes of functions(s) other than those of antibiotic resistance could have been inserted into a genetic environment that would facilitate their dissemination. Such genes would not be selected for as easily as were the determinants for β-lactamases, aminoglycoside-modifying enzymes, trimethoprim-resistant dihydrofolate reduc-

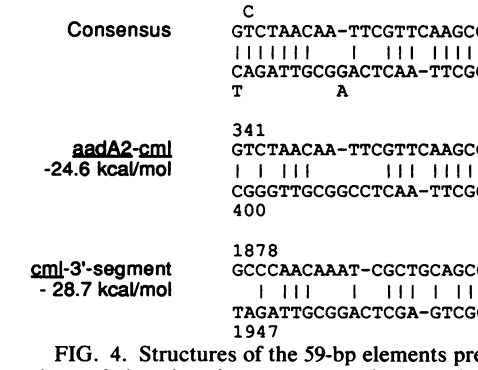

FIG. 4. Structures of the 59-bp elements present at the 3′ end of the *aadA2* and *cmlA* cassettes and comparison to the consensus elements described by Stokes and Hall (55).

TABLE 2. Putative promoters in the *cmlA* cassette of In4 aligned with the consensus promoter of Harley and Reynolds (21)

Promoter	−35 region			Spacing[c]	−10 region		
	Base[a]	Sequence	Match[b]		Sequence	Match[b]	Base[a]
Consensus		TTGACA		16–18	TATAAT		
p1	416	TTGcag	3	17	TAcgAT	4	444
p2	428	TTGAaA	5	16	TtcAAT	4	455
p3	435	TTGtta	4	17	TgagAT	3	463

[a] The numbers of the leftmost and rightmost bases in the −35 and −10 regions, respectively, are given.
[b] The number of bases in each promoter sequence matching the 6-base consensus sequence in the respective regions.
[c] Spacing indicates the number of bases separating the −35 and −10 region sequences.

tases, sulfonamide-resistant dihydropteroate synthase, and nonenzymatic chloramphenicol resistance.

The nucleotide sequence of the nonenzymatic chloramphenicol resistance determinant (*cml*) of *P. aeruginosa* IncP plasmid R26 was reported by Dorman et al. (12). The *cmlA* gene of Tn*1696* and *cml* of R26 should be very similar, since the relationship between these plasmids has been demonstrated by heteroduplex analysis (61). The alignment between the Tn*1696 cmlA*- and R26 *cml*-encoding and flanking regions reveals a high level of similarity but exhibits important differences: mismatches and gaps of up to 13 nucleotides in length. While some of these differences are probably real, others may result from sequencing errors. We resolved localized G+C compressions, a common source of sequencing errors, by using dITP. A sequence identical to ours has been obtained independently (19a).

```
          1                                                  50
QacA      LAVSLFVVTM DMTILIMALP ELVRELEPSG TQQLWIVDIY SLVL....AG
Mmr       LATGFVMATL DVTVVNVAGA TIQESLDTTL TQLTWIVDGY VLTF....AS
CmlA      VLLLSPFDLL ASLGMDMYLP AVPFMPNALG TTASTIQLTL TTYLVMIGAG
Tet181    LCILSFFSVL NEMVLNVSLP DIANHFNTTP GITNWVNTAY MLTFSIGTAV
Tet15     LCILSFFSVL NEMVLNVSLP DIANDFNKPP ASTNWVNTAF MLTFSIGTAV
Tet908    LCVLSFFSVL NEMVLNVSLP DIANEFNKLP ASANWVNTAF MLTFSIGTAL
TetB      L.VITLLDAM GIGLIMPVLP TLLREFIASE DIANHFGVLL AL.YALMQVI
TetC      ILGTVTLDAV GIGLVMPVLP GLLRDIVHSD SIASHYGVLL AL.YALMQFL
TetA      ILSTVALDAV GIGLIMPVLP GLLRDLVHSN DVTAHYGILL AL.YALVQFA
NorA      LYFNIFLIFL GIGLVIPVLP VYLKDLGLTG SDLGLLVAAF AL....SQMI
Bmr       LLTNLFIAFL GIGLVIPVTP TIMNELHLSG TAVGYMVACF AI....TQLI
AraE      AAVAGLLFGL DIGVIAGALP FITDHFVLTS RLQEWVVSSM MLGAAIGA..

          51                                                 100
QacA      FIIPLSAFAD KWGRKKALLT GFALFGLVSL AIFFAESAE. ...FVIAIRF
Mmr       LLMLAGGLAN RIGAKTVYLW GMGVFFLASL ACALAPTAE. ...TLIAARL
CmlA      QLLF.GPLSD RLGRRPVLLG GGLAYVVASM GLALTSSAE. ...VFLGLRI
Tet181    Y....GKLSD YINIKKLLII GISLSCLGSL IAFI...GHN HFFILIFGRL
Tet15     Y....GKLSD QLGIKRLLLF GIIINCFGSV IGFV...GHS FFSLLIMARF
Tet908    Y....GKLSD QLGIKNLLLF GIMVNGLGSI IGFV...GHS FFPILILARF
TetB      FAPWLGKMSD RFGRRPVLLL SL....IGAS LDYLLLAFSS ALWMLYLGRL
TetC      CAPVLGALSD RFGRRPVLLA SL....LGAT IDYAIMATTP VLWILYAGRI
TetA      CAPVLGALSD RFGRRPILLV SLA....GAT VDYAIMATAP FLWVLYIGRI
NorA      ISPFGGTLAD KLGKKLIICI GLILF...SV SEFMFAVGHN .FSVLMLSRV
Bmr       VSPIAGRWVD RFGRKIMIVI GLLFF...SV SEFLFGIGKT .VEMLFITRM
AraE      ..LFNGWLSF RLGRKYSLMA GAILFVLGSI GSAFATSVE. ...MLIAARV

          101                                                150
QacA      LLGIAGA.LI MPTTLSMIRV IFENPKERAT ALAVWSIASS IGAVFGPIIG
Mmr       VQG.AGAALF MPSSLSLLVF SFPEKRQRTR MLGLWSAIVA TSSGLGPTVG
CmlA      LQA.CGASAC LVSTFATVRD IYAGREESNV IYGILGSMLA MVPAVGPLLG
Tet181    VQG.VGSAAF .PSLIMVVVA RNITRKKQGK AFGFIGSIVA LGEGLGPSIG
Tet15     IQG.AGAAAF .PALVMVVVA RYIPKENRGK AFGLIGSIVA MGEGVGPAIG
Tet908    IQG.IGAAAF .PALVMVVVA RYIPKENRGK AFGLIGSLVA MGEGVGPAIG
TetB      LSGITGATGA VA...ASVIA DTTSASQRVK WFGWLGASFG LGLIAGPIIG
TetC      VAGITGATGA VA...GAYIA DITDGEDRAR HFGLMSACFG VGMVAGPVAG
TetA      VAGITGATGA VA...GAYIA DITDGDERAR HFGFMSACFG FGMVAGPVLG
NorA      IGG..MSAGM VMPGVTGLIA DISPSHQKAK NFGYMSAIIN SGFILGPGIG
Bmr       LGG..ISAPF IMPGVTAFIA DITTIKTRPK ALGYMSAAIS TGFIIGPGIG
AraE      VLGI..AVGI ASYTAPLYLS EMASENVRGK MISMYQLMVT LGIVLAFLSD

          151                                                200
QacA      GALLEQFSWH SAFLINVPFA IIAVVAGLFL LPESKLSKEK SHSWDIPSTI
Mmr       GLMVSAFGWE S..IFLLNLP .IGAIGMAMT YRYIAATESR ATRLAVPGHL
CmlA      ALVDMWLGWR AIFAF.LGLG MIAASAAAWR FWPETRVQRV AGLQWSQLLL
Tet181    G.IIAHY.IH WSYL..LILP MITIVTIPFL IKVMVPGKST KNTLDIVGIV
Tet15     G.MIAHY.IH WSYL..LLIP MITIITVPFL MKLLKKEVRI KGHFDIKGII
Tet908    G.MVAHY.IH WSYL..LLIP TATIITVPFL IKLLKKEERI RGHIDMAGII
TetB      GFAGE.ISPH SPFFIAALLN IVTFLVVMFW FRETKNTRDN TDT..EVGVE
TetC      GLLGA.ISLH APFLAAAVLN GLNLLLGCFL MQESHKGERR PMPLRAFNPV
TetA      GLMGG.FSPH APFFAAAALN GLNFLTGCFL LPESHKGERR PLRREALNPL
NorA      GFMAE.VSHR MPFYFAGALG ILAFIMSIVL IHDPKKSTTS GFQKLEPQLL
Bmr       GFLAE.VHSR LPFFFAAAFA LLAAILSILT LREPERNPEN QEIKGQKTGF
AraE      TAFSYSGNWR AMLGVLALPA VLLIILVVFL PNSPRWLAEK GRHIEAEEVL
```

FIG. 5. Amino acid sequence similarities between CmlA and other bacterial transport proteins, adapted from Rouch et al. (45) with the addition of CmlA, of the quinolone resistance protein NorA of *S. aureus* (64), and of the multidrug resistance protein Bmr of *Bacillus subtilis* (assigned EMBL accession number M33768 [37]).

Evolutionarily speaking, the *cmlA* gene of Tn*1696* is interesting not only because of its genetic features but also because it is the only known gene occurring in the multiresistance integrons that seems to still possess a chromosomal analog, since homology was detected by molecular hybridization between the Tn*1696 cmlA* gene and chromosomal DNA from nonenzymatic chloramphenicol-resistant *H. influenzae* isolates (6). Chromosomal analogs of integron-borne genes could serve as substrates for experimentally reproducing their introduction, by site-specific integration, into a "primitive" integron and thereby help elucidate this mechanism of antibiotic resistance gene dissemination in nature. Although R1033 was isolated from *P. aeruginosa*, the codon usage of the *cmlA* gene is not typical of *P. aeruginosa* (data not shown); *cmlA* may be of enterobacterial origin or, by its eventual position in the phylogenetic tree of Rouch et al. (45), of gram-positive origin.

On the *cmlA* unit, we found promoterlike structures (Table 2) which were functionally tested by using promoter-probe vectors. The presence of such regulatory elements had not previously been encountered with any other integron-associated gene cassette. The orientation-specific expression of a *cat* marker gene by fragments upstream of *cmlA* suggest that they contain active promoter sequences; however, no exact transcription start point has been determined. The p1-p2-p3 region, when cloned upstream of the pLQ897 *cat* gene, allowed growth of cells on plates containing 25 μg of chloramphenicol per ml.

The In*4 cmlA* cassette could encode a major hydrophobic polypeptide (CmlA) of 419 amino acids (44,228 Da) and two minor ORFs, of 116 (ORF1) and 103 (ORF2) amino acids, which are less hydrophobic than CmlA. The expected molecular mass of CmlA (44.2 kDa) of Tn*1696* is dissimilar to that of the Cml polypeptide of R26: 33 kDa as deduced from the sequence or 31 kDa when expressed in minicells (12). We have not detected expression of the additional ORFs borne on the *cmlA* gene cassette, even though ORF1 and ORF2 possess good potential ribosome binding sites (GGGA and GGAG). Tn*5* insertional mutagenesis analysis of the *cml* gene of R26 revealed that at least 1.4 kb of the cloned insert of pDU1249 was required in order to obtain a high-level chloramphenicol resistance phenotype (11). All but one of their insertion mutants within this region were chloramphen-

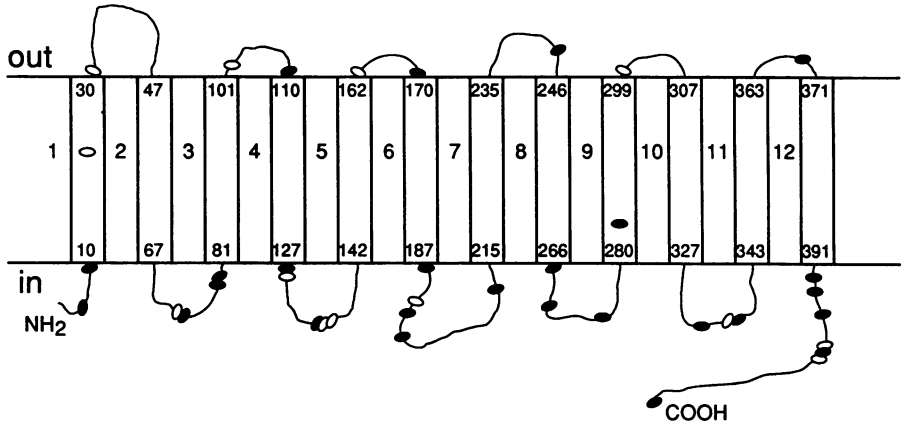

FIG. 6. Model for transmembrane domains of CmlA. The 12 predicted membrane-spanning segments are shown with residues numbered. Acidic residues (D and E) and basic residues (H, K, and R) are shown as open and filled circles, respectively.

icol sensitive; a Tn*5* insertion just downstream of the *Pst*I site (pDU1280) at the 3′ end of the unit expressed only low-level chloramphenicol resistance. On Tn*1696*, this location corresponds to the ORF2 coding region, and the involvement of this ORF in the expression of *cmlA* is possible, but a polypeptide of the corresponding size was not observed by Dorman et al. (12) in minicells harboring pDU1249. Dorman and Foster (11) constructed transcriptional and translational fusions between R26 *cml* and a β-galactosidase (*lac*) gene and demonstrated that only *cml-lac* translational fusions were inducible by chloramphenicol. The authors concluded that regulation of *cml* was operating at the translational level. Translational regulation of resistance genes by an attenuation mechanism has been suggested for the inducible chloramphenicol acetyltransferase genes of gram-positive bacteria (28). Posttranscriptional and translational control of bacterial gene expression might involve (i) attenuation, (ii) translational coupling, (iii) secondary structures that bury the translation signals, and (iv) protein and RNA activators (18). Dick and Matzura (9) have demonstrated the implication of a stem-loop structure (ΔG = −19.9 kcal/mol) located upstream of the initiator codon of the *cat* gene of pUB112. This stem-loop is involved in the inducibility mechanism by chloramphenicol and enhances the stability of the *cat* mRNA. Using the GCG FOLD program (8), we could not find a similar conformation upstream of the *cmlA* gene of Tn*1696*. There may be a short leader peptide upstream of *cmlA* which could be involved in attenuation.

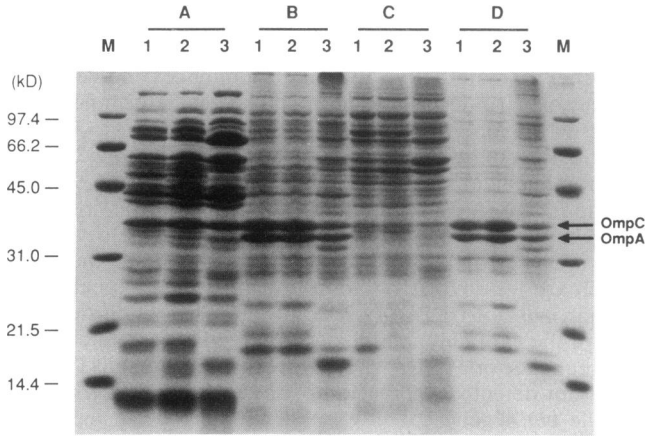

FIG. 7. Electrophoretograph of a 12% Coomassie brilliant blue-stained SDS-polyacrylamide gel of sucrose gradient-fractionated *E. coli* NM522 membranes. OmpC and OmpA, the major porins, are indicated by arrows. A: Total protein B: Total membrane fraction C: Inner membrane fraction D: Outer membrane fraction M: M. W. Markers 1: Vectorless 2: pTZ19R 3:pLQ822.

CmlA is a 44.2-kDa hydrophobic polypeptide which shows characteristics of a transmembrane protein because of the alternation of hydrophobic domains and β-turns (14, 24). The topology of CmlA was predicted by using the Kyte-Doolittle method, and the inner membrane location was confirmed by the appearance of a polypeptide with a molecular mass corresponding to that deduced from the nucleotide sequence (44.2 kDa). It has been shown that alteration of membrane polypeptides may cause cross-resistance to aminoglycosides, β-lactams, quinolones, tetracyclines, and chloramphenicol (7, 38, 48). The *cmlA* resistance mechanism is ineffective against fluorinated analogs of chloramphenicol (10) and the fluoroquinolones ciprofloxacin and temafloxacin (data not shown), revealing a high level of specificity for chloramphenicol.

A homology search, using the PROFILESEARCH program (8), revealed that CmlA shows a certain level of similarity with gram-positive and gram-negative tetracycline resistance proteins, which are known to be transmembrane polypeptides that achieve resistance by an active efflux of tetracycline (13, 47). CmlA also shows similarity to efflux systems for quaternary ammonium compounds (QacA) of *S. aureus* (45) and for norfloxacin and other hydrophilic quinolones (64). Additional similarity was also found with the *S. coelicolor* methylenomycin A resistance polypeptide (Mmr) (36). No efflux mechanism of chloramphenicol has thus far been demonstrated. No significant similarity was detected between CmlA of Tn*1696* and RomA of *Enterobacter cloacae*, an outer membrane protein which has been recently characterized and which causes a reduction of OmpC and multiple resistance of *E. cloacae* and *E. coli* (25).

The expression of CmlA induces dramatic changes in the inner and outer membrane protein content. A certain number of polypeptides are either reduced or augmented, and some polypeptides appear to be induced by the expression or presence of CmlA. Interestingly, the major porins, OmpA and OmpC, are diminished, and a polypeptide of approxi-

mately 33 kDa seems to be overexpressed. This polypeptide might be CmlA, since Shaw (54) indicated that the R26 Cml polypeptide is associated with the outer membrane and molecular weights of membrane proteins are known to be underestimated on SDS gels. Additional polypeptides, of higher and lower molecular weights, are also affected by the expression of CmlA. These polypeptides, if not degradation products, might result from a compensation mechanism that would serve to equilibrate the outer membrane and therefore substitute for the uptake mechanism of small hydrophilic molecules. The observed modifications of the outer membrane proteins are a reflection of the necessity for this structure to maintain an equilibrium in porin content; therefore, it is not unexpected that porins are overexpressed, to compensate for the loss of several components (15). Toro et al. (60) observed a drastic reduction of the major porin OmpF in the outer membrane of a nonenzymatic chloramphenicol-resistant (200 μg/ml) *S. typhi* strain. In this strain, molecular cloning of the *E. coli ompF* gene increased the sensitivity towards chloramphenicol to 40 μg/ml, while cloning of the *Salmonella typhimurium ompC* gene did not influence the resistance mechanism, and transformation with plasmids containing a *cat* gene did not result in acetylated chloramphenicol. In this resistant strain, the OmpA porin levels were unaffected. Therefore, in *S. typhi*, the entry of chloramphenicol into the cell appears to be dependent on the presence of OmpF, which might not be the case for *E. coli*. CmlA expression also induces changes in the overall protein patterns of *E. coli* NM522 (Fig. 7A), and polypeptides of cytoplasmic origin or loosely associated with the membranes might be affected. Thus, it is possible that CmlA interferes with some global regulatory network implicated in the assembly of the membranes.

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