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Isolation of *cmr*, a Novel *Escherichia coli* Chloramphenicol Resistance Gene Encoding a Putative Efflux Pump

INGE W. NILSEN,[1]* INGRID BAKKE,[1] ANNA VADER,[2] ØRJAN OLSVIK,[3] AND M. RAAFAT EL-GEWELY[1]

Departments of Biotechnology,[1] Medical Microbiology,[3] and Cell Biology,[2] Institute of Medical Biology, University of Tromsø, N-9037 Tromsø, Norway

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A novel gene designated *cmr*, which mapped to 18.8 min of the *Escherichia coli* K-12 genome, was shown to mediate resistance to chloramphenicol when it was expressed from a multicopy vector. The accumulation of chloramphenicol was significantly less in cells overexpressing *cmr* than in control cells harboring the vector without insert. After the addition of a proton motive force blocker, the level of accumulation of chloramphenicol in the resistant cells rapidly approached the levels found in sensitive cells carrying only the chromosomal *cmr*. Northern (RNA) blot analyses revealed that the *cmr* gene is expressed as a 1.3-kb transcript. This size corresponds very well with a predicted size of 1,293 nucleotides (nt) based on the mapping of the transcription initiation site to a G residue 24 nt upstream of the start codon and the presence of a putative rho-independent terminator sequence ending 36 nt downstream of the 1,233-nt open reading frame encoding the putative Cmr protein. The 411-residue-long derived amino acid sequence contains 12 putative transmembrane segments and displays significant sequence similarities to several known drug resistance protein sequences of the major facilitator family. We provide evidence strongly suggesting that the resistance mediated by Cmr involves active exclusion of chloramphenicol.

Active efflux of antibiotics is mediated by a family of transmembrane proteins frequently referred to as drug resistance translocases. The first drug-pumping protein to be reported was the plasmid-encoded tetracycline resistance Tet protein in 1980 (27). Since then, numerous efflux systems with specificities to a wide range of substrate structures have been identified in both gram-positive and gram-negative bacteria (see references 26 and 33 for reviews). Some of these systems, such as the Tet proteins, the EmrD and Bcr proteins of *Escherichia coli* (2, 30), the ActII of *Streptomyces coelicolor* (15), and the transposon-encoded CmlA protein of *Pseudomonas aeruginosa* (3), seem to be quite specific in their choice of substrate. This is in contrast to the broad specificity found for NorA in *Staphylococcus aureus* (41) and Bmr in *Bacillus subtilis* (31). All of these proteins show significant sequence homology to each other, share a characteristic hydropathic profile predicting 12 to 14 membrane-spanning segments, and constitute closely related members of the major facilitator (MF) family of membrane drug translocases (18).

The multiresistant protein members of the resistance nodulation division (RND) family, including *E. coli* AcrB (formerly AcrE [13]) and EnvD (22) and *P. aeruginosa* MexB (34), show no significant sequence homology to members of the MF family. However, they have predicted membrane topologies and modes of action similar to those of MF drug pumps. Both MF and RND drug pump members are energized by the electrochemical gradient (35), and these transporters are classified as drug/H^+-antiporters.

A gene encoding chloramphenicol acetyltransferase (CAT) type II has been identified in *E. coli* (29). No gene for nonenzymatic chloramphenicol resistance in *E. coli* has so far been identified, except for the multiple antibiotic resistance (Mar) mediated by the *marRAB* locus (11, 17). Mar mutants show significant resistance to several antibiotic agents, including chloramphenicol (17), and this phenotype is due to mutations in *marO* or *marR* leading to *marA* transcription (11). MarA causes decreased expression of the outer membrane protein OmpF (12, 19), thus lowering the normal passive diffusion of drugs into the cells. MarA is thought to induce the expression of several genes, including genes for efflux pumps (14, 17, 21).

An active *E. coli* efflux system for chloramphenicol was recently reported (28). It was concluded that the gene responsible for the active process was unrelated to, although possibly influenced by, the *mar* locus. *mar* deletion mutants were able to produce an energy-dependent reduction of chloramphenicol accumulation, but the resistant Mar mutants had an improved net chloramphenicol efflux (28).

Here, we report the molecular characterization and chromosomal location of *E. coli cmr* encoding a putative protein with sequence features characteristic of membrane translocases. Overexpression of *cmr* conferred increased resistance to chloramphenicol and was correlated with an energy-dependent efflux of the drug.

MATERIALS AND METHODS

Strains and media. *E. coli* DH5αMCR cells (Gibco) were grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with 0.2% glucose. Lambda phage preparations were made with the *E. coli* host strain Y1090r$^-$ (Stratagene). The pGEM7Zf(+) plasmid vector (Promega) was used for cloning, sequencing, and expression, for which cells were grown in the presence of ampicillin (50 μg/ml on plates or 100 μg/ml in liquid medium).

Isolation of RNA and plasmid DNA. After the addition of diethyl pyrocarbonate to the culture (5 μl/10 ml), total RNA was isolated by hot phenolacetate extraction as described elsewhere (12, 38). Ethanol-precipitated RNA was dissolved in sample buffer (50% deionized formamide, 7% formaldehyde, 0.5× electrophoresis buffer, 10% glycerol, 0.005% bromophenol blue, 0.005% xylene cyanol) or in diethyl pyrocarbonate-treated H_2O and was used in Northern (RNA) blotting or primer extension reactions. RNA markers (Promega) and the internal 23S (2.9-kb) and 16S (1.54-kb) rRNAs were used for transcript size determinations. Plasmid DNA for cloning or DNA sequencing was prepared with the Wizard Minipreps DNA system (Promega).

Southern and Northern analyses. DNA or RNA was transferred from agarose

* Corresponding author. Mailing address: Department of Biotechnology, MH Building, Institute of Medical Biology, University of Tromsø, N-9037 Tromsø, Norway. Fax: 47 77645350. Electronic mail address: ingewn@fagmed.uit.no.

gels (7% formaldehyde for RNA) to Nytran N-13 membranes (Schleicher & Schuell) by using a vacuum unit (Pharmacia) conducting a pressure of 40 to 50 cm of H_2O for 60 min in the presence of 0.4 M NaOH for DNA or H_2O for RNA. Southern blot membranes were subsequently neutralized in 50 mM Tris-HCl (pH 7.2). For Northern blots, the RNA was cross-linked to the membrane by UV irradiation. The probe used for hybridization to Northern and Southern blots was labelled by random priming with [α-^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear) by using the Ready-To-Go DNA labelling kit (Pharmacia). Hybridizations were performed at 65°C in a sodium phosphate buffer with 4% sodium dodecyl sulfate essentially as described elsewhere (9). Kodak XAR-5 films were used for autoradiography.

Cloning strategy and genomic mapping. The clone pCBD (see Fig. 1) was cut with *Rsa*I and *Bam*HI to release a 0.9-kb fragment including the 5′ part of *cmr*. After separation on a 1% agarose gel, the *Rsa*I-*Bam*HI fragment was recovered from the gel and was subsequently used to probe a Gene-Mapping Membrane (Takara Shuzo Co., Ltd.). The probe hybridized to two single lambda recombinant clones with the mini-set serial numbers 209 and 210, corresponding to the clones 5F4 and 3H12, respectively, described by Kohara et al. (23). The clone 5F4 was picked from the Kohara *E. coli* library and was used for phage production prior to DNA isolation according to the instructions of the Lambda Maxi kit (Qiagen). Lambda 5F4 DNA was subjected to restriction cutting with different enzymes before probing as described above. A hybridizing 2.8-kb *Eco*RI fragment of clone 5F4 was subcloned into the pGEM7Z vector. This clone was designated pGLambdaE. The construct pdE was made by restricting pGLambdaE with *Bam*HI (cutting once in the insert and once in the vector) and religating the plasmid, thereby removing the 5′ of the insert (see Fig. 1).

DNA sequencing. Sequencing reactions were primed by universal primers for the pGEM7Z flanking sequences or by custom-designed internal primers. Sequence data were obtained from both strands with the Sequenase kit (version 2.0; U.S. Biochemical Corporation) and [α-^{35}S]dATP (1,000 Ci/mmol; New England Nuclear), except for [α-^{32}P]dCTP which was used in the control sequencing reaction for primer extension analysis. Additional sequence data were obtained with the automated A.L.F. system (Pharmacia LKB), for which the AutoRead sequencing kit (Pharmacia) was used.

Computer analyses. DNA sequences were analyzed by using the software package from the Genetics Computer Group (Madison, Wis.). Putative terminator sequences were recognized by the Terminator program (4), and RNA folding structure predictions and calculations of the free energy were obtained with the program FoldRNA (16, 43). Protein hydrophobicity was predicted according to the method described by Kyte and Doolittle (25) and achieved by the Protean software from DNASTAR (Madison, Wis.). The TopPred II version 1.2 software (10) was used to predict protein topology.

Primer extension. Total RNA (20 μg) from cells harboring pGLambdaE was mixed with 30 U of RNAguard (Pharmacia) and 20 pmol of oligonucleotide primer P62, an 18-mer complementary to the 5′ end of *cmr* and starting 109 bp downstream of the predicted translational start stie. The mixture was heated at 72°C for 1 min and at 30°C for 5 min and then placed on ice. To a final volume of 30 μl, the following was added: deoxynucleotides (1 mM dTTP, dGTP, and dATP and 0.1 mM dCTP), 20 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear), 8 U of avian myeloblastosis virus reverse transcriptase (Promega), and the supplied reaction buffer. The primer extension reaction was performed at 42°C for 30 min before termination with 10 μl of sequencing stop solution.

Antibiotic susceptibility. Initial testing of resistance was performed by monitoring growth in liquid cultures supplemented with antibiotics or by standard agar disk diffusion tests. Extended MIC tests were performed at the Centers for Disease Control and Prevention (Atlanta, Ga.) by using the broth microdilution screening method (37).

Chloramphenicol accumulation studies. Cellular accumulation of chloramphenicol was studied essentially according to method 1 described by McMurry et al. (28). Briefly, cells from log-phase cultures were collected by centrifugation and washed twice in 50 mM potassium phosphate buffer (pH 6.5) containing 0.2% glucose and were then resuspended in the same buffer at cell densities corresponding to 10 optical density at 590 nm (OD_{590}) units. The accumulation of [dichloroacetyl-1,2-^{14}C]chloramphenicol (58 mCi/mmol; New England Nuclear) was determined by collecting 0.5 OD_{590} unit of cells incubated at 37°C in 10 ml of 100 mM LiCl–50 mM potassium phosphate onto 0.45-μm-pore-size HA cellulose filters (Millipore) and then by scintillation counting of the air-dried filters.

To analyze the energy dependence of the accumulation process, aliquots of cells incubated with chloramphenicol were transferred to a new tube containing carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma) at a final concentration of 100 μM and were processed as described above.

Nucleotide sequence accession number. The nucleotide sequence for the *cmr* gene has been deposited in the GenBank database under accession number U44900.

RESULTS

Cloning, mapping, and sequencing of the *cmr* gene. In an unrelated project, we isolated a 1.8-kb *Cla*I-*Bam*HI fragment in a pGEM7Z vector, making up a clone designated pCBD

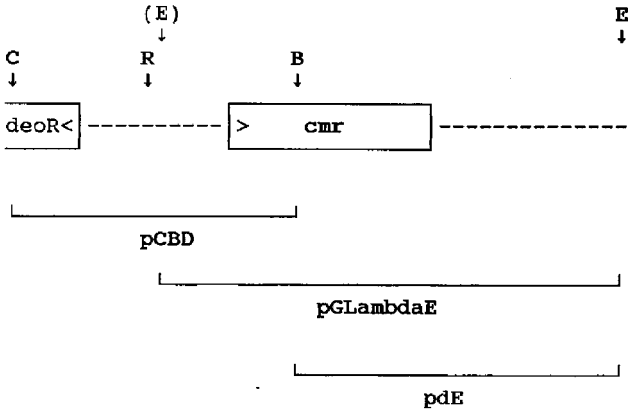

FIG. 1. A schematic presentation of the cloning strategy used to isolate the *cmr* gene. The initial clone pCBD containing a *Cla*I-*Bam*HI insert in pGEM7Zf(+) was cut with *Rsa*I and *Bam*HI, and the released 0.9-kb fragment was used as probe to isolate a 2.8-kb *Eco*RI fragment from an *E. coli* genomic lambda library. This *Eco*RI fragment was inserted into pGEM7Z to produce the pGLambdaE clone. The clone pdE was obtained by cutting pGLambdaE with *Bam*HI and religating the plasmid, thereby removing the 5′ end of *cmr* beyond the (E) site. Open boxes, ORFs representing part of the *deoR* gene (39) and the entire *cmr* gene; arrowheads within the boxes, opposite orientations of the two genes; B, C, E, and R, locations of recognition sites for *Bam*HI, *Cla*I, *Eco*RI, and *Rsa*I, respectively; (E), an *Eco*RI restriction site in the lambda vector used to subclone the 2.8-kb *Eco*RI fragment.

(Fig. 1). Sequencing of this fragment revealed part of the *E. coli deoR* gene, which has previously been mapped to 18.7 min on the *E. coli* chromosome (39), and a downstream sequence not reported previously. This sequence included a truncated open reading frame (ORF) encoding a sequence of 150 amino acids with significant homology to several transmembrane resistance proteins. Screening of a grid array panel of lambda clones encompassing the entire *E. coli* genome with a 0.9-kb *Rsa*I-*Bam*HI fragment from pCBD (Fig. 1) as a probe yielded two positive clones, 3H12 and 5F4. Clones 3H12 and 5F4 have overlapping inserts originating from 18.7 to 19.1 min of the *E. coli* genome (23). A 2.8-kb *Eco*RI fragment from 5F4, which hybridized to the *Rsa*I-*Bam*HI fragment, was subcloned into pGEM7Z, giving pGLambdaE.

The sequences of the 1,233-nucleotide (nt) *cmr* ORF, the 5′ and 3′ flanking regions, and the deduced 411-amino-acid Cmr sequence are shown in Fig. 2A. Two overlapping hexameric sequences at −7 (TAAACT) or −10 (TATTAA), both with four of six matches to the consensus 5′-TATAAT-3′, may constitute a functional −10 promoter region. No obvious −35 region is apparent. Two putative ribosome-binding sites, a GGCG at positions −14 to −11 and a GAAG at positions −11 to −8 relative to the start of translation, are not optimal Shine-Dalgarno sequences (36). A dyad symmetry within 33 nt downstream of the stop codon was recognized by computer analysis as a putative rho-independent terminator sequence. This 29-nt sequence (Fig. 2A) is predicted to form a stem-loop structure (not shown) with a minimum free energy of −14.5 kcal (ca. −60.7 kJ)/mol. The ORF encoding Cmr contains a rare AGG codon for arginine at position 10 as well as rare glycine GGA codon at position 12.

Hydrophobicity profile and protein sequence homologies. The protein sequence of Cmr was found to display significant similarity to those of many drug resistance transmembrane proteins and also some similarity to those of several sugar transporters. Computer-assisted sequence homology analyses showed 24% identity and 52% similarity to Tn*1696* CmlA (3), and corresponding 25% identity and 52% similarity and 20%

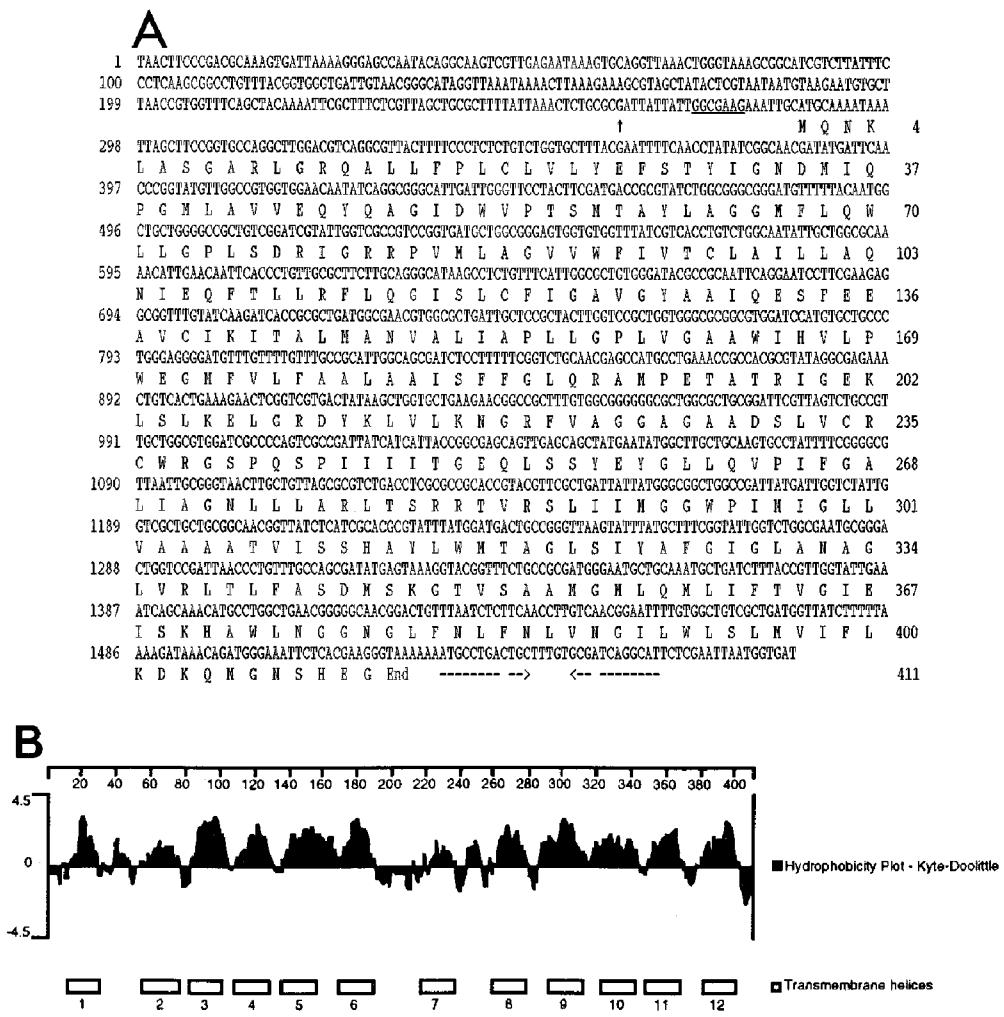

FIG. 2. (A) Nucleotide sequence and predicted amino acid sequence of the *cmr* gene. Vertical arrow, the transcription start G; underlined sequence, two overlapping potential ribosome binding sites; arrows at bottom, region of dyad symmetry 3′ of the *cmr* coding sequence, believed to represent a rho-independent terminator sequence. (B) Hydrophobicity profile and topology predictions of the Cmr protein indicating 12 transmembrane segments.

identity and 50% similarity to *E. coli* EmrD (30) and Bcr (2), respectively. However, the highest level of homology (39% identity and 64% similarity) was found to the hypothetical *E. coli* protein F410 (5), which is encoded by a gene at about 98.3 min.

The predicted hydrophobicity profile of the *cmr* translation product (Fig. 2B) indicates 12 hydrophobic regions of a minimum of 21 amino acids. The TopPred II software used (10) predicts 10 certain and 2 putative (segments 2 and 8) transmembrane segments. A search in the Prosite database (1) revealed residues 76 to 88 (**SDRIGRR**PVMLA**G**), representing the intracellular loop between the transmembrane segments 2 and 3 and part of segment 3, to be a common motif for sugar transporters.

RNA analysis. From Northern blot analyses (Fig. 3A), the size of the transcripts of both the plasmid-contained (lane 2) and the chromosomal *cmr* (lanes 3 to 5) gene was estimated to be 1.3 kb. The weaker hybridization signal at about 8 kb (lane 2) is due to the presence of plasmid DNA (pGLambdaE) that is not efficiently removed during RNA isolation. This signal is seen only in RNA from pGLambdaE cells. An additional smaller transcript of about 0.6 kb is variably recognized by the double-stranded DNA probe. The origin of this smaller transcript is currently unknown. A minor decrease in the level of *cmr* transcription was observed at increasing cell densities (Fig. 3A, lanes 3 to 5). Primer extension analysis of RNA from DH5α cells carrying pGLambdaE (Fig. 3B) revealed a distinct transcription start site at the G in position −24 relative to the expected start of translation. This shows that *cmr* transcription in pGLambdaE is promoted by the *cmr* gene promoter and not by any other elements in the vector. Furthermore, the transcript size of 1.3 kb determined by Northern analysis is completely consistent with a predicted transcript size of 1,293 nt based on the experimentally determined transcription start site and the location of the putative rho-independent terminator downstream of the stop codon.

Chloramphenicol resistance and accumulation studies. As described above, protein sequence analyses revealed homology between Cmr and efflux pumps. Consequently, cells were tested for growth in the presence of various antibiotics. A significant tolerance to chloramphenicol was found in pGLambdaE cells harboring the entire *cmr* transcription unit. As controls, cells carrying pCBD, which contained only the coding region for the N-terminal 150 amino acids of Cmr, or pdE, which harbored only 260 amino acids of the Cmr C

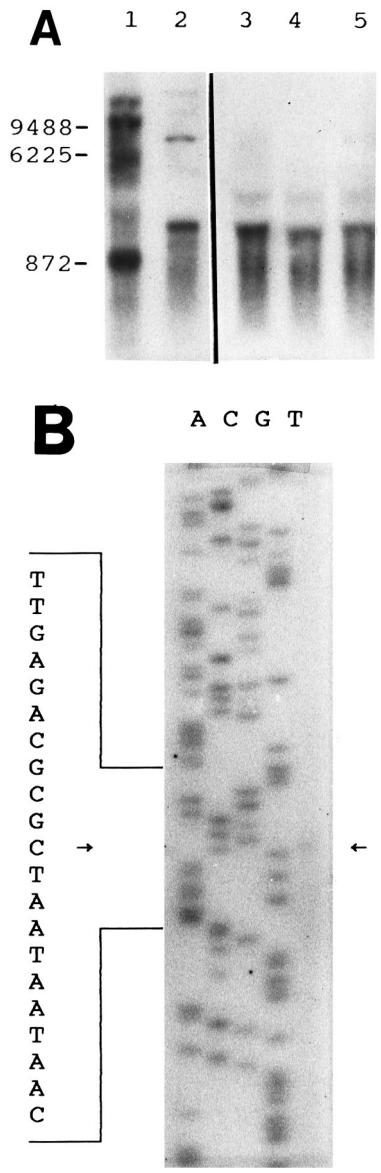

FIG. 3. *cmr* transcription analyses. (A) Northern blot of chromosomally transcribed RNA from DH5α cells (lanes 3 to 5) and RNA from cells carrying the pGLambdaE plasmid (lane 2), showing the *cmr* transcription size to be close to 1.3 kb (major signal lanes 2 to 5). The RNA in lane 2 is 50-fold diluted. The RNA samples in lanes 3, 4, and 5 were isolated from cultures grown to densities at 0.35, 0.55, and 0.75 OD_{590} units, respectively. The RNA MW marker used (lane 1) included fragments of 9,488, 6,225, 3,911, 2,800, 1,898, 872, 562, and 362 nt. It is unknown why the three denoted fragments in lane 1 hybridize to the *cmr* probe. The hybridizing fragment of approximately 8 kb in lane 2 is pGLambdaE plasmid DNA not efficiently removed during RNA isolation. (B) Primer extension mapping of the *cmr* transcription start site with RNA isolated from cells containing pGlambdaE (same as in lane 2 of panel A) to determine the 5′ end of the *cmr* transcript as shown in the lane to the right of lane T in the DNA control sequence (lanes A, C, G, and T). The extension reaction and the DNA sequencing were obtained with the same primer, and the resulting complementary DNA sequence is shown.

terminus (Fig. 1), were tested and were found not to mediate chloramphenicol resistance (not shown).

Extended antibiotic susceptibility tests show that the *cmr* gene increases the chloramphenicol MIC by a factor of 8 when it is present in a high-copy-number vector (Table 1). Furthermore, no significant effect on the resistance to other antibiotics tested was observed.

TABLE 1. *cmr*-mediated antibiotic resistance

Antimicrobial agent[a]	MIC (μg/ml)	
	DH5α	pGLambdaE(+)
Amikacin	<2	<2
Aztreonam	<1	<1
Cefazolin	<4	<4
Cefotetan	>64	>64
Ceftazidime	<2	<2
Ceftriaxone	<2	<2
Cefuroxime	<4	<4
Chloramphenicol	4	32
Ciprofloxacin	<0.1	<0.1
Gentamicin	<0.5	<0.5
Imipenem	<2	<2
Tetracycline	<1	2
Tobramycin	<0.5	<0.5
Trimethoprim/sulfa	<0.5/9.5	<0.5/9.5

[a] The *ampR* gene of the vector confers resistance to several agents not shown here.

The cellular accumulation of chloramphenicol was examined in order to test the mechanism by which Cmr might act. An instant entry of chloramphenicol was observed in DH5α cells before a plateau level was reached after 4 min (Fig. 4). Cells containing multiple copies of *cmr* accumulated less than 50% of the chloramphenicol found in cells carrying the vector alone or in cells with no plasmid. The concentration of chloramphenicol used (2.1 μM) corresponds to 0.7 μg/ml, a concentration which slightly inhibits the growth of DH5α cells without pGLambdaE (not shown). It should be noted that time zero probably reflects the situation 10 to 15 s after addition of chloramphenicol because of the delay in handling these samples during the assay.

Addition of CCCP, an uncoupler of oxidative phosphoryla-

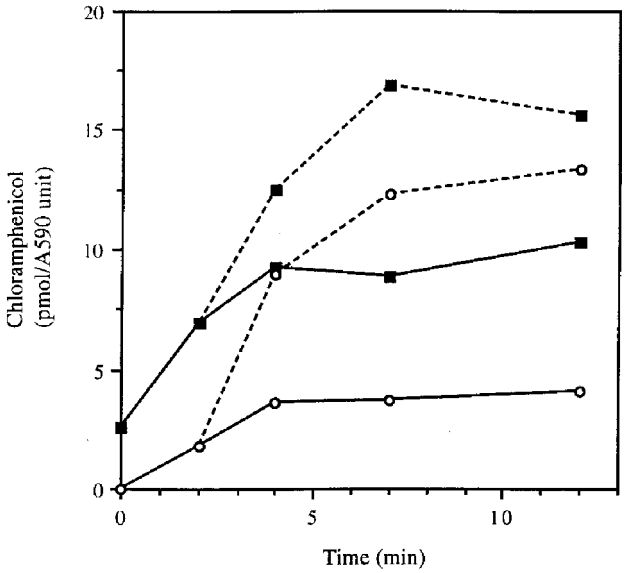

FIG. 4. Accumulation of chloramphenicol by DH5α cells without (closed symbols) or with (open symbols) the *cmr*-containing plasmid pGlambdaE. At time zero, chloramphenicol was added to a final concentration of 2.1 μM (solid lines). After 2 min, the incubation mixtures were split in two and CCCP was added to a final concentration of 100 μM (dashed lines).

tion (35), resulted in a rapid increase in the cellular levels of chloramphenicol (Fig. 4). The relative CCCP-induced increase was higher in cells harboring pGLambdaE (150% increase) than in cells lacking this plasmid (35% increase). The levels of accumulated chloramphenicol in pGLambdaE-containing DH5α cells after addition of CCCP did not reach the corresponding levels in cells carrying an insert-less vector. Most likely, this reflects the lower level of chloramphenicol bound to ribosomes or acetylated by the CAT enzyme prior to the addition of CCCP in pGLambdaE cells compared with DH5α cells. These results show that pGLambdaE mediates an energy-dependent exclusion of chloramphenicol.

DISCUSSION

Until recently, chloramphenicol resistance in *E. coli* was considered to be associated with a reduced passive entrance of the drug as mediated by the *mar* locus (12) or by the enzymatic inactivation of the drug by an acetyltransferase (29). The finding of an active chloramphenicol efflux (28) expanded the known mechanistical repertoire of *E. coli* chloramphenicol resistance.

The novel *cmr* gene described in this study shows the phenotypical characteristics of active chloramphenicol efflux as reported by McMurry et al. (28), based on the mediated resistance and the hydropathic profile of Cmr and the energy-dependent decrease in chloramphenicol accumulation by pGLambdaE. Since pCBD and pdE gave no insert-mediated resistance, we conclude that the chloramphenicol resistance produced by pGLambdaE is due to the *cmr* gene.

According to the physical map of the *E. coli* genome (23), the *cmr* gene in the overlapping lambda clones 5F4 and 3H12 is located at approximately 18.8 min in the genome. We find the distance between *cmr* and *deoR* to be 850 nt. The *deoR* gene was reported to map to 18.7 min (39), but this is contradicted by the compiled sequence update from 1993 (24), locating *deoR* to 18.61 min, and by the public-accessible *E. coli* database (40), in which the gene is mapped to 18.929 min. In consideration of the discussed discrepancies, we approximate the *cmr* location to 18.8 min, and we specify the physical distance from *deoR* to be 850 nt.

The presence of a putative −10 promoter region and the absence of a −35 region could indicate that an activator is needed for the RNA polymerase to bind to and subsequently transcribe *cmr*, which is analogous to the class II CAP-dependent promoters in which the −35 determinant for binding RNA polymerase is replaced by a CAP-binding motif (42).

The proposed nonconsensus ribosome-binding site could indicate weak binding to ribosomes and consequently a low level of translation of the gene. Additional translational limitations may be caused by the codon usage for this gene, i.e., the AGG codon of arginine in position 10 and the GGA codon of glycine in position 12. The occurrence of such minor codon usage in the N terminus of a protein has been suggested as a feature of essential gene products repressed at high densities (7, 8). Although a slight decrease in *cmr* transcription at higher cell densities was observed, we have no evidence to claim that *cmr* is an essential gene in *E. coli.*

An element of dyad symmetry that allows the formation of stem-loop structures in the RNA transcripts is the basic component of rho-independent terminators (4). Additionally, described features of such terminators are a stretch of uracil residues 3′ of a G+C-rich stem-loop (4, 6) and a G-C base pair closing the stem (6). The putative rho-independent terminator of *cmr* was predicted to be closed by two A-T base pairings instead and lacks a 3′ poly(U) tail in the *cmr* transcript. However, the absence of a 3′ poly(U) tail does not necessarily weaken the efficiency of termination (20).

The amount of Cmr in cells containing pGLambdaE is not known. However, a 50-fold increase in pGLambdaE-directed *cmr* transcription (Fig. 3A) probably does not reflect a similar increase in translation, since this is expected to have a drastic effect on the membrane composition. The sequence homology of Cmr to EmrD, CmlA, and Bcr suggests that the Cmr protein is close to the alignment ratio cluster of these drug extrusion pumps as previously established (18, 26). Interestingly, these proteins show specificity toward substrates of a common structure. Chloramphenicol (CmlA and Cmr), CCCP and phenylmercury acetate (EmrD), and bicyclomycin (Bcr) have a single benzene ring as the major common component. Thus, their protein sequence homology could reflect a structural homology of the substrates that these proteins actively exclude. The *bcr* gene has been claimed (26) to be identical to the *sur* gene, which, after amplification, contributes to sulfonamide resistance (32), a drug that has a more complex structure than the discussed benzen derivatives.

Attempts to make *cmr* disruption mutants have thus far not been successful. Thus, the relative contribution of *cmr* to the intrinsic resistance to chloramphenicol is unknown. The constitutive expression of *cmr* could indicate that the Cmr protein may provide beneficial functions to the host cell. The energy-dependent decrease in cellular levels of chloramphenicol (reference 28 and the present study) shows an active process by which the cells could achieve part of their intrinsic resistance to the drug. As demonstrated in the present study, the presence of multicopy *cmr* leads to an efficient removal of chloramphenicol from its intracellular target. The accumulation of chloramphenicol in pGLambdaE cells is lowered by 60%, and the MIC is increased by eightfold compared with MICs of cells lacking the plasmid. These results suggest that relatively small changes in the cellular drug level produce large changes in drug resistance. Consequently, the observed energy-dependent 35% decrease in chloramphenicol accumulation in DH5α host cells may be a significant contributor to intrinsic resistance. The documented active efflux of chloramphenicol is probably not mediated by *cmr* alone. Multiresistant homologs of Bmr (31) could be alternative candidates contributing to this process.

The increased chloramphenicol efflux in the resistant Mar mutants (28) indicates a role for MarA in the transcriptional regulation of the chloramphenicol efflux gene(s) in *E. coli*. A low-efficiency efflux in a sensitive *mar* deletion mutant was concluded to demonstrate alternative chloramphenicol efflux systems in which one is *mar* dependent (28). A possible regulation of *cmr* transcription by MarA is currently under investigation.

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