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Evidence for Chloramphenicol/H$^+$ Antiport in Cmr (MdfA) System of *Escherichia coli* and Properties of the Antiporter[1]

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We detected chloramphenicol/H$^+$ antiport activity in membrane vesicles of *Escherichia coli* and cloned a gene for the antiporter from chromosomal DNA of *E. coli*. Introduction of the gene into *E. coli* cells conferred resistance to chloramphenicol and ethidium. A slight increase in resistance to acridine orange was also observed. Elevated chloramphenicol efflux and ethidium efflux were observed in cells harboring a plasmid carrying the gene. Addition of chloramphenicol to the assay mixture reduced the efflux of ethidium. Elevated chloramphenicol/H$^+$ antiport activity was observed in membrane vesicles prepared from cells harboring the plasmid. The pH optimum for the activity was 6.5. We sequenced the gene and deduced the amino acid sequence of its product. A sequence homology search revealed that it was same as that of Cmr (or MdfA). Thus, it became clear that Cmr (MdfA) is the chloramphenicol(and ethidium)/H$^+$ antiporter.

Key words: chloramphenicol/H$^+$ antiport, *E. coli*, multidrug resistance, sequence.

Microorganisms have several mechanisms by which they can overcome the toxicity of drugs such as antibiotics. Extrusion of toxic drugs from microbial cells is one of these mechanisms. So far, four major groups of drug extrusion systems are known (*1*), the MF (major facilitator) family, the SMR (small multidrug resistance) family, the RND (resistance nodulation cell division) family, and the ABC (ATP binding cassette) family. Membrane transporters of the MF family possess 12 to 14 transmembrane domains. Bcr (*Escherichia coli*) (*2*), EmrB (*E. coli*) (*3*), EmrD (*E. coli*) (*4*), NorA (*Staphylococcus aureus*) (*5*), QacA (*S. aureus*) (*6*), and Bmr (*Bacillus subtilis*) (*7*) are members of this family, and these systems mediate efflux of drugs with different specificities. Transporters of the Smr family are smaller, possessing 4 transmembrane domains. Smr (*S. aureus*) (*8*), QacC (*S. aureus*) (*9, 10*), QacE (*Klebsiella aerogenes*) (*11*), and EmrE (*E. coli*) (*12*) belong to this family. An electrochemical potential of H$^+$ across cell membranes seems to be the driving force for drug efflux by the MF family transporters and the SMR family transporters. Transporters of the RND family consist of several subunits (usually three), and one or more outer membrane proteins are involved in drug transport. AcrAB (*E. coli*) (*13, 14*), AcrEF (*E. coli*) (*15*), MexAB (*Pseudomonas aeruginosa*) (*16, 17*), and MexCD (*P. aeruginosa*) (*17*) are members of this family. Energy coupling in this family is rather complicated, and it seems that both ATP and an electrochemical potential of H$^+$ across cell membranes are necessary to drive the drug efflux. Transporters of the ABC family seem to utilize ATP as an energy source. Members of this family include LmrA (*Lactococcus lactis*) (*18*) and MsrA (*S. aureus*) (*19*).

Efflux systems for chloramphenicol are known in several micoorganisms, and genes encoding these systems have been cloned and sequenced. These systems include the CmlA system of *P. aeruginosa* (*20-22*), the CmlP system of *Rhodococcus fascians* (*23*), and the CmrG system of *Streptomyces lividans* (*24*). In *E. coli*, the *cmlA* gene (*25*), *strA* gene (*26*), *ompF* gene (*27, 28*), and *cmlA1* gene (*27, 28*) have been reported to be involved in chloramphenicol resistance. However, *strA* (ribosomal protein gene), *cmlA* (chloramphenicol acetyltransferase gene), and *ompF* (outer membrane protein gene) are not involved in the efflux of chloramphenicol. Recently, a gene named *cmr* has been cloned and sequenced (*29*). It has been reported that Cmr is involved in a chloramphenicol efflux system and is driven by an electrochemical potential of H$^+$ (*29*). However, no direct evidence for chloramphenicol/H$^+$ antiport *via* this system has been shown. It seemed that the *cmr* gene is the same as *cmlA1*, judging from its map position on the *E. coli* chromosome. Very recently, the *mdfA* gene has been cloned and sequenced (*30*), and shown to be identical with *cmr*.

Here we report evidence for chloramphenicol/H$^+$ antiport in the Cmr (MdfA) system of *E. coli* and properties of the antiporter.

MATERIALS AND METHODS

Bacterium and Growth—*E. coli* W3133-2 (*31*), AS-1 (drug hypersensitive strain) (*32*), EIR104 (a revertant of AS-1 obtained in N. Kamo's laboratory, Hokkaido Univer-

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sity), and WZM120 (*ΔacrAB*) (*14*) were used in this study. The strain W3133-2 was used as a wild type with respect to chloramphenicol sensitivity, strains AS-1 and WZM120 were used as host strains for gene cloning and subcloning, and strain EIR104 was used as a source of chromosomal DNA.

L medium (*33*) was used for cell growth. Where indicated, 20 mM potassium lactate was added to the culture medium. Cells were grown at 37°C under aerobic conditions. Cell growth was monitored turbidimetrically at 650 nm.

Gene Cloning and Sequencing—Chromosomal DNA was prepared from cells of *E. coli* EIR104 by the method of Berns and Thomas (*34*). The DNA was partially digested with *Sau*3A1, and fragments of 4 to 10 kbp were separated by sucrose density gradient centrifugation. The DNA fragments were ligated into pBR322 (which had been digested with *Bam*HI and dephosphorylated with bacterial alkaline phosphatase) by using T4 DNA ligase. Competent cells of *E. coli* AS-1 were transformed with the ligated hybrid plasmids and spread on agar plates consisting of L medium, 6.5 μg/ml chloramphenicol, 100 μg/ml ampicillin, and 1.5% agar. The plates were incubated at 37°C for 1 day and the clones formed were picked up. Plasmids were prepared from the transformants, and competent cells of *E. coli* AS-1 were retransformed and spread on the plates again. The plates were incubated at 37°C for 1 day. Many colonies appeared on the plates. Plasmids contained in the retransformants were prepared. Thus, we obtained two candidate plasmids, pECP5 and pECP9. The two plasmids carried common DNA inserts.

The rough location of the gene responsible for the chloramphenicol resistance was estimated by subcloning. Deletion plasmids for sequencing were constructed using exonuclease III and mung bean nuclease from pECP93. The nucleotide sequence was determined by the dideoxy chain termination method (*35*) using a DNA sequencer (ALF Express, Pharmacia). Sequencing of both sense and antisense strands was completed. The DNA sequence data reported in this paper have been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number AB000968.

Sequence data was analyzed with the GENETYX sequence analysis software (Software Development). The GenBank and the SwissProt databases were searched for sequence similarities.

Fluorescence Quenching Assay—Cells were grown in the L medium (*33*) supplemented with 20 mM potassium lactate. Everted membrane vesicles were prepared by passing cells through a French press as described previously (*36*). Chloramphenicol/H^+ antiport activity was measured by the quinacrine fluorescence quenching method with everted membrane vesicles. The method is basically the same as that reported previously for the measurement of Na^+/H^+ antiport activity (*36*). The standard assay mixture consists of 10 mM Mops-KOH, pH 6.5, 140 mM KCl, 1 μM quinacrine, and membrane vesicles (0.2 mg/ml of protein). The fluorescence was measured at excitation and emission wavelengths of 420 and 500 nm, respectively, when lactate was added as an energy source, or at 440 and 500 nm, respectively, when NADH was added as an energy source. An H^+ conductor, TCS (tetrachlorosalicylanilide), was added to the assay mixture where indicated.

Cellular Accumulation of Chloramphenicol—Chloramphenicol accumulation in cells and efflux from cells were measured as follows. Cells grown in the medium supplemented with 20 mM potassium lactate were harvested, washed with a minimal salt medium (*37*) (Na^+ salts were replaced with K^+ salts), and suspended in the same medium. Potassium lactate (final 20 mM) was added to the cell suspension (0.5 mg protein/ml), and the cell suspension was kept at 25°C with gentle shaking. The assay was initiated by addition of [^{14}C]chloramphenicol (final 1 μM) to the cell suspension. An H^+ conductor, CCCP (carbonylcyanide *m*-chlorophenylhydrazone), was added (final 100 μM) when necessary. Samples were taken at intervals and filtered through a membrane filter (0.45 μm pore size). The filter was washed with the minimal salt medium (*37*), dried, and the radioactivity was counted.

Cellular Accumulation of Ethidium—Cells were prepared as described above for the chloramphenicol efflux assay. Potassium lactate (final 20 mM) was added to the cell suspension (0.5 mg protein/ml), and the cell suspension was kept at 25°C for 5 min with gentle stirring. Ethidium bromide was added at 10 μM to the cell suspension to initiate the assay. CCCP was added at 100 μM where indicated. When necessary, the indicated concentration of chloramphenicol was added to the assay mixture 5 min prior to the addition of ethidium bromide. The fluorescence of the assay mixture was measured at excitation and emission wavelengths of 500 and 580 nm (*38*), respectively, using a Hitachi F2000 fluorescence spectrophotometer.

Other Methods and Chemicals—The minimum inhibitory concentrations of drugs were determined in Mueller Hinton broth (Difco). Cells were incubated at 37°C for 24 h, then growth was estimated. Protein contents were determined by the method of Lowry *et al.* with bovine serum albumin as a standard (*39*). [^{14}C]Chloramphenicol was from DuPont NEN. Reagents for DNA manipulation and sequencing, bacteriological media, and other chemicals were obtained from the usual commercial sources.

RESULTS

Chloramphenicol/H^+ Antiport Activity in Membrane Vesicles—The quinacrine fluorescence quenching assay is a convenient method for detection and measurement of solute/H^+ antiport. During the course of our studies on drug/H^+ antiporters, we observed chloramphenicol/H^+ antiport activity by using the fluorescence quenching technique in membrane vesicles of several microorganisms (unpublished results). Figure 1 shows that chloramphenicol/H^+ antiport activity is present in everted membrane vesicles prepared from wild type *E. coli* cells, as measured by the fluorescence quenching method. The AcrAB system is the principal drug efflux system in *E. coli* (*13, 14*), and chloramphenicol is a substrate for this system. We tested whether the chloramphenicol/H^+ antiport activity is observed in membrane vesicles prepared from mutant cells lacking the AcrAB system. No significant difference in the chloramphenicol/H^+ antiport activity between the wild type and the mutant lacking the AcrAB was detected (data not shown). Therefore, the chloramphenicol/H^+ antiport activity was not due to the AcrAB system.

Cloning of the Gene Responsible for Chloramphenicol Resistance—It seems that the chloramphenicol/H^+ anti-

porter extrudes chloramphenicol from cells, conferring resistance to chloramphenicol. Thus, we tried to clone the gene(s) encoding the chloramphenicol/H⁺ antiporter based on the resistance to chloramphenicol by shotgun cloning. The *E. coli* mutant AS-1, which is hypersensitive to many drugs, was used as the cloning host. The *E. coli* mutant EIR104, which is more resistant to many drugs than AS-1, was used as a source of chromosomal DNA. As a result of the shotgun cloning, we obtained two candidate plasmids, pECP5 and pECP9. Analysis of their restriction fragments revealed that these two plasmids carried several identical fragments derived from chromosomal DNA and some different fragments (data not shown). Thus, we concluded that pECP5 and pECP9 carried a common region of chromosomal DNA. Since cells harboring pECP9 showed a higher resistance to chloramphenicol than cells harboring pECP5, we used pECP9 for further analyses. Figure 2 shows the growth of AS-1 and AS-1/pECP9 in the absence or presence of chloramphenicol. The host cells AS-1 did not grow in the presence of 6.5 μg/ml (20 μM) chloramphenicol or higher concentrations. On the other hand, cells of AS-1/pECP9 were able to grow in the presence of 6.5 μg/ml of chloramphenicol. Even in the presence of 13 μg/ml (40 μM) chloramphenicol, the AS-1/pECP9 cells grew, although the growth rate was reduced. Thereafter, we determined minimum inhibitory concentrations (MICs) of many drugs for AS-1 and AS-1/pECP9 (Table I). We observed an 8-fold increase in the MIC for chloramphenicol with AS-1/pECP9 compared with AS-1. We also observed an 8-fold increase in MIC for ethidium bromide with AS-1/pECP9. A 2-fold increase in MIC for acridine orange was observed with AS-1/pECP9. No significant change in MIC was detected with the other drugs tested (Table I). Thus, it seems that ethidium bromide is a common substrate for the system which confers resistance to chloramphenicol. Acridine orange may be a poor substrate for the system.

Four plasmids (pECP92 to pECP95) carrying different portions of the DNA insert were constructed from pECP9 (Fig. 3). Cells harboring either pECP92 or pECP93 showed chloramphenicol resistance. pECP93 carried a shorter DNA insert than pECP92. Thus, we further characterized the chloramphenicol/H⁺ antiport system using cells harboring plasmid pECP93.

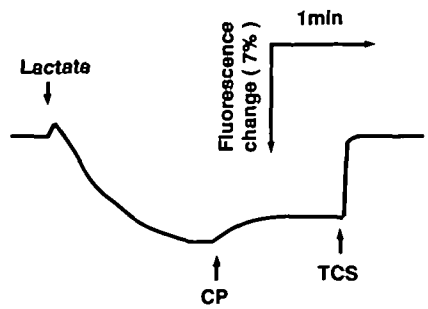

Fig. 1. **Chloramphenicol/H⁺ antiport activity in membrane vesicles of *E. coli*.** Everted membrane vesicles were prepared from cells of W3133-2. The chloramphenicol/H⁺ antiport activity was measured by the quinacrine fluorescence quenching method. Lactate was added to the assay mixture to initiate respiration at 1 mM. Chloramphenicol (CP) was added to the assay mixture at 1 mM. TCS, an H⁺ conductor, was added to a final concentration of 0.25 μM to collapse the H⁺ gradient.

Transport Activity Due to the Cloned Gene—Since the gene product(s) seemed to confer resistance to several drugs, it is likely that the gene or genes code for a multidrug efflux system whose best substrates are chloramphenicol and ethidium bromide. We first tested whether it is really an efflux system or not, using chloramphenicol as a substrate. The accumulation of chloramphenicol in cells was measured, and the effect of the H⁺ conductor CCCP on the accumulation was tested. For this experiment, we used *E. coli* WZM120, which is a deletion mutant of the principal drug efflux system AcrAB (*13, 14*), as the host. This strain is valuable for analysis of drug extrusion systems other than AcrAB, because we can neglect the effect of the principal system. A certain level of accumulation of chloramphenicol was observed with cells of WZM120, and the accumulation level increased after the addition of CCCP (after deenergization of the membranes), and reached a plateau level, indicating the presence of an H⁺-coupled efflux system for chloramphenicol in WZM120 cells (Fig. 4). A much lower accumulation of chloramphenicol was observed with cells of WZM120/pECP93 than was observed with cells of WZM120, and the accumulation level increased greatly after the addition of CCCP, then reached

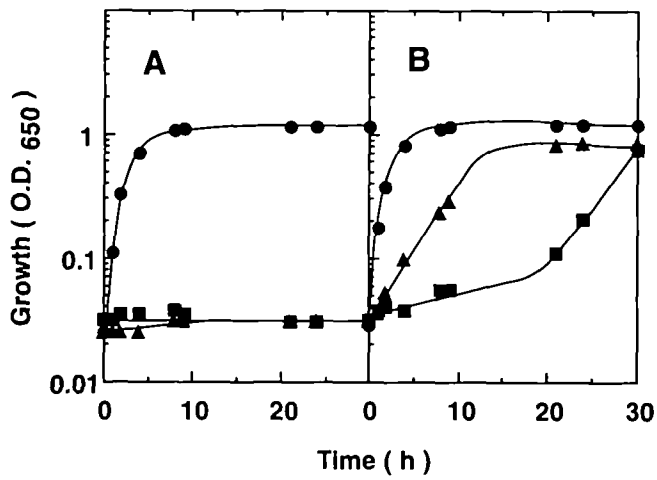

Fig. 2. **Effect of chloramphenicol on growth of AS-1 and AS-1/pECP9.** Cells of *E. coli* AS-1 (A) or AS-1/pECP9 (B) were grown in L medium in the absence (●) or presence of 6.5 μg/ml (▲) or 13 μg/ml (■) of chloramphenicol at 37°C under aerobic conditions. Cell growth was monitored turbidimetrically at 650 nm.

TABLE I. **MICs of several drugs for AS-1 and AS-1/pECP9.**

Drugs	MIC (μg/ml)	
	AS-1	AS-1/pECP9
Chloramphenicol	1	8
Ethidium bromide	4	32
Acridine orange	4	8
Chlorpromazine	32	32
Erythromycin	1	1
Ofloxacin	0.015	0.015
Tetracycline	1	1
Triphenyltetrazolium chloride	256	256
Benzalkonium ion (300 μM)	S*	S
Methylene blue (100 μM)	S	S
Rhodamine 6G (10 μM)	S	S

*S, sensitive.

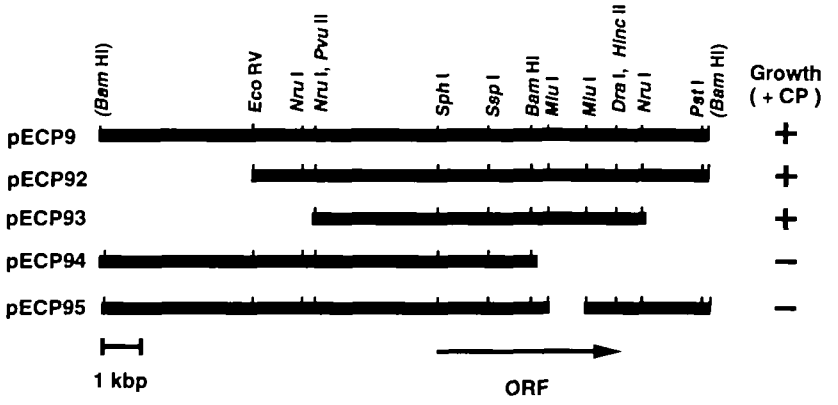

Fig. 3. **Plasmids and restriction maps of cloned *E. coli* DNA containing the *cmrA* gene.** Physical maps of DNA inserts derived from the *E. coli* chromosome in pECP9 and its derivatives are shown. Restriction sites determined in pECP9 are shown. The growth capabilities of *E. coli* AS-1 cells harboring each plasmid in L medium containing 6.5 μg/ml of chloramphenicol (+CP) are shown on the right. "+" means that cells grew and "−" means that cells did not grow. The position and direction of an open reading frame (ORF) revealed by sequencing are shown at the bottom.

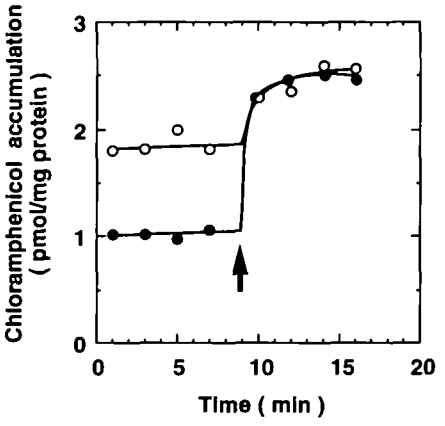

Fig. 4. **Chloramphenicol efflux assay in *E. coli* WZM120 and WZM120/pECP9.** Cells of *E. coli* WZM120 (○) or WZM120/pECP9 (●) were incubated with [^{14}C]chloramphenicol (final concentration 1 μM). Samples were taken at intervals, and intracellular chloramphenicol was measured. An H^+ conductor, CCCP (final concentration 100 μM), was added to the assay mixture at the point indicated by an arrow.

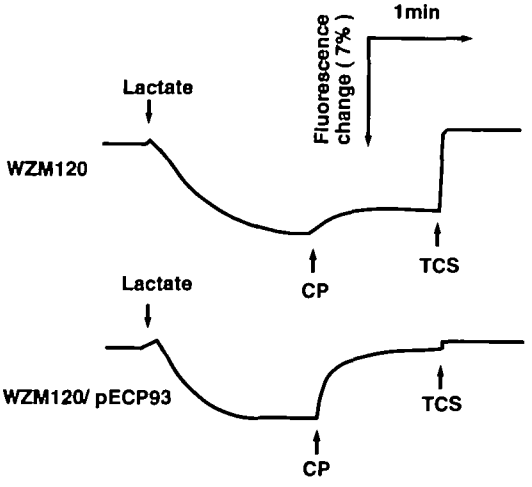

Fig. 5. **Chloramphenicol/H^+ antiport activities in membrane vesicles prepared from the host and transformant.** The chloramphenicol/H^+ antiport was measured by the quinacrine fluorescence quenching assay. At the time points indicated by arrows, potassium lactate (5 mM) was added to initiate respiration. After the fluorescence quenching reached a steady state, chloramphenicol (CP, final concentration 1 mM) was added to the assay mixture. Finally, at the time points indicated by arrows, TCS was added at a concentration of 0.25 μM to collapse the H^+ gradient.

a plateau level. Thus, it is highly likely that the gene product(s) derived from plasmid pECP93 is (are) involved in an H^+-coupled efflux system for chloramphenicol.

If this is correct, membrane vesicles prepared from cells of WZM120/pECP93 may have greater chloramphenicol/H^+ antiport activity than vesicles from WZM120, and this elevated activity may be detectable by the fluorescence quenching method. In fact, the antiport activity was much higher in membrane vesicles of WZM120/pECP93 than in those of WZM120 (Fig. 5). Thus, it is clear that the chloramphenicol/H^+ antiport activity in WZM120/pECP93 was greatly elevated by the gene(s) carried on the plasmid pECP93.

We found that the pH optimum for the chloramphenicol/H^+ antiport activity in membrane vesicles prepared from WZM120/pECP93 cells was 6.5 (Fig. 6). A similar pH profile was obtained with membrane vesicles prepared from WZM120 cells, although the activity was lower. In the pH range from 6.5 to 7.5, we observed severalfold higher activity with vesicles prepared from WZM120/pECP93 than with those prepared from WZM120. However, no significant difference in the activity was observed between the two at pH 8.0 or 8.5 (Fig. 6). This suggests that there is at least one other chloramphenicol/H^+ antiporter in *E. coli*. The activity of such a system may be pH-independent or it may be higher at slightly alkaline pH than at neutral pH.

As shown in Table I, cells harboring pECP9 were more resistant to ethidium than the host cells. We therefore measured efflux of ethidium from cells of WZM120 and WZM120/pECP93. A certain level of ethidium accumulation was observed with WZM120 cells, and the level increased when CCCP was added, then reached a plateau level (Fig. 7A, curve a). This represents H^+-coupled efflux of ethidium from the cells. We observed a much lower accumulation of ethidium in cells of WZM120/pECP93 (Fig. 7A, curve b), and the level increased after addition of CCCP and reached the same plateau level as that observed with WZM120 cells (Fig. 7A). This represents an elevated H^+-coupled efflux of ethidium from cells of WZM120/pECP93 compared with cells of WZM120.

If ethidium is transported by the same system as chloramphenicol in WZM120/pECP93, then competition between the two substrates should take place. Thus, we measured ethidium efflux from cells of WZM120/pECP93

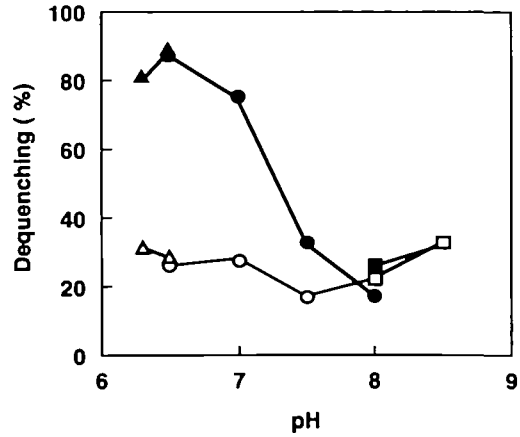

Fig. 6. **pH profiles of the chloramphenicol/H⁺ antiport activity.** The chloramphenicol/H⁺ antiport activity was measured at various pHs with membrane vesicles prepared from cells of WZM120 (open symbols) or WZM120/pECP9 (closed symbols). Three buffer systems, Mes-KOH buffer (▲, △), Mops-KOH buffer (●, ○), and Tricine-KOH buffer (■, □), were used to cover the indicated pH range.

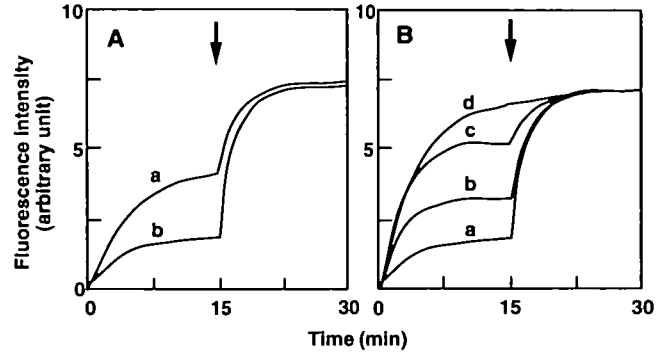

Fig. 7. **Ethidium efflux assay.** Ethidium efflux was measured with cells of WZM120 or WZM120/pECP93. (A) Cells of WZM120 (a) or WZM120/pECP93 (b) were used. Ethidium bromide (final concentration 10 μM) was added to the assay mixture at 0 time, and fluorescence intensity was measured. At the time point indicated by an arrow, CCCP (final concentration 100 μM) was added to the assay mixture. (B) Chloramphenicol was added to the assay mixture (a, 0 μM; b, 100 μM; c, 200 μM; d, 300 μM) 5 min prior to the addition of ethidium bromide, and fluorescence intensity was measured. At the time point indicated by an arrow, CCCP (final concentration 100 μM) was added to the assay mixture.

in the absence or presence of various concentrations of chloramphenicol. As shown in Fig. 7B, the energy dependent efflux of ethidium was inhibited by chloramphenicol in a concentration dependent manner in cells of WZM120/pECP93. Chloramphenicol also inhibited the efflux of ethidium from WZM120 cells in a similar manner (data not shown). Thus, we conclude that ethidium is a substrate for the chloramphenicol/H⁺ antiporter.

Sequencing—We determined the nucleotide sequence of the DNA insert carried on the plasmid pECP93. We found one open reading frame (ORF) in the DNA region, and the corresponding amino acid sequence was deduced from the ORF. The hydropathy pattern calculated by the method of Kyte and Doolittle (*40*) from the deduced amino acid sequence (data not shown) supported the idea that the gene encodes a membrane transport protein with about 12 putative transmembrane domains.

A homology search in a protein sequence database (SwissProt) using the BLAST algorithm (*41*) revealed that our deduced amino acid sequence was almost same as that of Cmr (*29*) (16 alterations). Very recently, the sequence of MdfA has been reported (*30*). We found one alteration of amino acid residue in the reported sequence of MdfA (position 59 is Asn in MdfA, and Thr in our sequence and in Cmr). The DNA sequence determined by the *E. coli* genome project (Accession numbers, AE000186) indicates that position 59 is Thr. The MdfA system has been reported to be a multidrug facilitator with an extraordinarily broad spectrum of drug recognition, which is driven by the proton electrochemical potential (*30*).

DISCUSSION

We found chloramphenicol/H⁺ antiport activity in membrane vesicles of *E. coli*, and cloned and sequenced a gene encoding the chloramphenicol/H⁺ antiporter. Ethidium is also a substrate for this antiporter. A homology search of the deduced amino acid sequence revealed that the antiporter was same as the Cmr (MdfA) system, an energy-dependent chloramphenicol (and other drugs) efflux system, reported by Nilsen *et al.* (*29*) and Edger and Bibi (*30*). Thus, we clearly demonstrated that this system is the drug/H⁺ antiporter which extrudes several drugs from cells.

The Cmr (MdfA) drug/H⁺ antiporter seems to be a constitutive system, because we observed the antiport activity in wild-type cells grown in L medium or in membrane vesicles prepared from such cells. The increase in the MIC in cells harboring pECP9 carrying the *cmr* (*mdfA*) gene would be due to an increase in the copy number of the gene, and consequently the increase in the level of the Cmr (MdfA) protein in cell membranes.

The existence of another chloramphenicol/H⁺ antiporter in *E. coli* was suggested by our results. This second system is different from the principal drug efflux system in *E. coli*, the AcrAB system, because the antiport activity was observed in membrane vesicles prepared from cells of WZM120, which is a deletion mutant of the *acrAB* (*14*). The Cmr (MdfA) system was very different from the AcrAB system with respect to its substrate specificity. In particular, the Cmr (MdfA) system appeared not to mediate the efflux of erythromycin, rhodamine 6G, or methylene blue, although the AcrAB system extrudes these drugs (data not shown). The extent of ethidium bromide resistance was different between the Cmr (MdfA) system and the AcrAB system. The AcrAB system conferred a higher level of resistance to ethidium bromide than did the Cmr (MdfA) system (data not shown). Edger and Bibi reported recently that the MdfA confers resistance to many drugs, such as erythromycin and rhodamine 6G (*30*). The discrepancy between our results and theirs may be due to differences in host cells, copy number of the plasmids and/or expression level of the gene.

Several other drug efflux systems have been reported in *E. coli* (*2-4, 13-15*). Compared with such systems, the Cmr (MdfA) system is unique with respect to its substrate specificity. Chloramphenicol and ethidium are good substrates, and acridine orange is a poor substrate. Other drugs tested were not substrates for this system in our experi-

ments. It is likely that a broader spectrum of drug recognition would be observed if we could get higher expression of the gene. Perhaps the Cmr system operates for extrusion of certain substances that are unfavorable for *E. coli* cells. In wild type *E. coli* cells, the activity of the Cmr (MdfA) system is not very high, so the cells are sensitive to chloramphenicol (the MIC is several micrograms/ml).

The sequence similarity between Cmr and members of the major facilitator (MF) family, Bcr (*2*), EmrD (*4*), and CmlA (*9, 21, 22*), has been reported by Nilsen *et al.* (*29*). As reported by Edger and Bibi (*30*), a hypothetical membrane transport protein, YjiO, of *E. coli* (*42*) showed the higher similarity, 39% identity and 80% similarity in 409 amino acid residues, to the MdfA. In addition to Bcr, EmrD, and CmlA, we found that Cmr (MdfA) has sequence similarity with LmrP of *Lactococcus lactis* (*38*) (21% identity and 60% similarity in 347 amino acid residues), ProP of *E. coli* (*43*) (20% identity and 58% similarity in 348 amino acid residues), and Fsr (fosmidomycin resistance conferring protein) of *E. coli* (*44*) (19% identity and 60% similarity in 348 amino acid residues).

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