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Structural and Functional Study of the Phenicol-Specific Efflux Pump FloR Belonging to the Major Facilitator Superfamily

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The florfenicol-chloramphenicol resistance gene *floR* from *Salmonella enterica* was previously identified and postulated to belong to the major facilitator (MF) superfamily of drug exporters. Here, we confirmed a computer-predicted transmembrane topological model of FloR, using the *phoA* gene fusion method, and classified this protein in the DHA12 family (containing 12 transmembrane domains) of MF efflux transporters. We also showed that FloR is a transporter specific for structurally associated phenicol drugs (chloramphenicol, florfenicol, thiamphenicol) which utilizes the proton motive force to energize an active efflux mechanism. By site-directed mutagenesis of specific charged residues belonging to putative transmembrane segments (TMS), two residues essential for active efflux function, D23 in TMS1 and R109 in TMS4, were identified. Of these, the acidic residue D23 seems to participate directly in the affinity pocket involved in phenicol derivative recognition. A third residue, E283 in TMS9, seems to be necessary for correct membrane folding of the transporter.

Multidrug and drug-specific efflux transporters are one of the major causes of failure of drug-based treatments of cancers and infectious diseases. On the basis of bioenergetic and structural criteria, drug efflux transporters have been divided into five superfamilies of transporters (28). One of these, the ATP binding cassette (ABC) superfamily, includes members that are energized by ATP hydrolysis. The other four, the major facilitator (MF) superfamily, the resistance/nodulation/division (RND) superfamily, the drug/metabolite transporter (DMT) superfamily, and the multidrug and toxic compound extrusion (MATE) family, include members that are secondary transporters using the proton motive force to drive the transport.

The MF superfamily comprises membrane transport proteins that are found in all classes of living organisms and are involved in the symport, antiport, or uniport of various substrates such as essential ions, nutrients, and drugs. Drug efflux proteins belonging to this superfamily can be divided into two families, drug:H^+ antiporter (12-spanner) (DHA12) and drug:H^+ antiporter (14-spanner) (DHA14), with either 12 or 14 transmembrane segments (TMS) (21, 32).

Chloramphenicol and florfenicol are two antibiotics of the same structural family. For a long time, chloramphenicol was considered the drug of choice to treat infectious diseases in animals. In August 1994, chloramphenicol was prohibited in the European Union for use in food animals. Florfenicol, a fluor derivative of chloramphenicol, has been introduced to treat respiratory infectious diseases in cattle and pigs and is expected in the near future to have a larger use in animal production.

Multidrug-resistant *Salmonella enterica* serovar Typhimurium, mainly of definitive phage type 104 (DT104), has emerged during the last decade as a world health problem (9). This bacterium, which causes diseases in humans and animals, is resistant to ampicillin, chloramphenicol, florfenicol, spectinomycin, streptomycin, sulfonamides, and tetracyclines. Recently, a chromosomal locus of about 12.5 kb carrying all the above resistance genes, included in a 43-kb genomic island called *Salmonella* genomic island 1 (SGI1), was identified, with the chloramphenicol-florfenicol cross-resistance gene *floR* among them (5–7). Interestingly, *floR* was also found recently in other *S. enterica* serovars such as Agona, Albany, Newport, and Paratyphi B, as well as in other gram-negative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Vibrio cholerae* (33).

From the sequence similarities, the *floR* gene product was predicted to belong to the MF superfamily of drug transporters. This protein shares 48.5% amino acid sequence identity with CmlA1 of *Pseudomonas aeruginosa*, a chloramphenicol efflux protein driven by the proton motive force (12), and 88.8% amino acid sequence identity with Pp-flo of *Photobacterium damselae* subsp. *piscicida*, a chloramphenicol and florfenicol resistance determinant also predicted to belong to the MF superfamily (18).

In the present study, we determined the transmembrane topology of FloR and demonstrated its capacity to pump specific drugs outside the cell by a proton motive force-driven mechanism. By site-directed mutagenesis of transmembrane charged residues, we characterized residues involved in drug selective transport.

MATERIALS AND METHODS

Construction of *floR-phoA* fusions. Fusions of the truncated *floR* gene to the *E. coli* signal sequence-less *phoA* gene were carried out in the pQuantagen(xk) vector (Qbiogen, Illkirch, France). For that purpose, 12 different fragments of *floR* were amplified by PCR from the chromosomal DNA of *Salmonella enterica* serovar Typhimurium DT104 with primer O1 as the forward primer and either

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TABLE 1. Primers used in this study

Primer	Nucleotide sequence[a]
O1	5′-CGGGATCCTTAATCGAGGGTTGATTCGTC-3′
O2	5′-CTGGTACCGAACGACAGGGAGATAAATA-3′
O3	5′-TCGGTACCTTCTGAGAGCGGACCAAA-3′
O4	5′-AAGGTACCGTGCAGTTGAAGACCAAGCTG-3′
O6	5′-ACGGTACCGACGGTTGGCATAAACGTCG-3′
O7	5′-ACGGTACCACTCGCCGATCAATGCTCCG-3′
O8	5′-CAGGTACCATCGGCGGCGTCTTGACTTGAT-3′
O9	5′-GGGGTACCCTTGGCCTATGAGCACACGGGGA-3′
O10B	5′-GAGGTACCATCTGGCGACAAAGGACTTCGCGAAA-3′
O11	5′-GTGGTACCGCGAGCCGTAAAGTTCGCCGATCC-3′
O12	5′-TCGGTACCTGTCGTCGAACTCTGCCAAAG-3′
O13	5′-CCGGTACCTATCGCCGTTTAACAGCGTCA-3′
O14	5′-GAGGTACCACTTCTCTCGGTGGCAGCATCACGG-3′
O15	5′-TTGGTACCATTATTGCGCCGCATCGGAGC-3′
D23E/fw	5′-ATGGCTCCTTTCgagATCCTCGCTTCACT-3′
D23K/fw	5′-ATGGCTCCTTTCaagATCCTCGCTTCACT-3′
D23N/fw	5′-ATGGCTCCTTTCaatATCCTCGCTTCACT-3′
D23/rv	5′-CAGCAGCAGTGCTGCCGGCAGCGTATAG-3′
R109E/fw	5′-CCTTTGTCGCTTTCgaaCTACTTCAAGCA-3′
R109K/fw	5′-CCTTTGTCGCTTTCaaaCTACTTCAAGCA-3′
R109N/fw	5′-CCTTTGTCGCTTTCaatCTACTTCAAGCA-3′
R109/rv	5′-CCGGTGCAGTTGAAGACCAAGCTGCTCCC-3′
R283E/fw	5′-CAGGATGCGTGGCGgaaGGGATGGCGTTG-3′
R283K/fw	5′-CAGGATGCGTGGCGaaaGGGATGGCGTTG-3′
R283N/fw	5′-CAGGATGCGTGGCGaatGGGATGGCGTTG-3′
R283/rv	5′-CGATGCCCCATCTGGCGACAAAGGACTTC-3′

[a] Lowercase letters represent new codons introduced by site-directed mutagenesis.

O2, O3, O4, O6, O7, O8, O9, O10B, O11, O12, O13, or O14 as the reverse primer (Table 1). Primer O1 contained a BamHI restriction site at its 5′ end, while primers O2, O3, O4, O6, O7, O8, O9, 010B, O11, O12, O13, and O14 contained a KpnI restriction site at their 5′ ends. Amplifications were carried out with the above primers (1 μM final concentration) and *Pfu* DNA polymerase (Promega, Charbonnières, France) according to the manufacturer's instructions. Amplifications were performed in a thermocycler (Perkin-Elmer, Courtaboeuf, France) with the following cycling conditions: one cycle of 2 min at 95°C; then 35 cycles of 1 min at 95°C, 30 s at 55°C, and 2 min, 30 s, at 72°C; and finally one cycle of 5 min at 72°C. After purification, the amplified DNA fragments were restricted with BamHI and KpnI and inserted between the BamHI and KpnI sites of pQuantagen(xk) (Qbiogen, Illkirch, France) in place of a DNA fragment carrying the first 27 codons of the *phoA* gene.

Alkaline phosphatase assays. An *E. coli* Δ*phoA* strain (Δstar; Qbiogen, Illkirch, France) was transformed by electroporation with the different *floR-phoA* fusion constructs. After overnight incubation at 37°C, the alkaline phosphatase activity of transformed cells was detected on LB plates supplemented with ampicillin (100 μg/ml), 5-bromo-4-chloro-3-indolylphosphate (XP) (40 μg/ml), and isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.5 mM final concentration), inducing the expression of fusion proteins from the *tac* promoter of pQuantagen(xk).

Expression and detection of fusion proteins. *E. coli* Δ*phoA* cells transformed with *floR-phoA* fusion constructs, grown overnight in LB broth supplemented with ampicillin (100 μg/ml), were diluted 1:100 into 20 ml of fresh medium and grown at 37°C until optical densities of the cultures at 600 nm reached 0.6. The expression of fusion proteins was then induced by the addition of IPTG (0.5 mM final concentration), and cultures were incubated further for 3 h at 37°C. Bacteria were then washed with phosphate-buffered saline (PBS), resuspended in 1 ml of PBS containing 0.5 mM phenylmethylsulfonyl fluoride, and finally lysed by sonication with ice bath cooling. Whole-cell extracts containing 50 μg proteins were then fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (4 and 8% polyacrylamide for stacking and separating gels, respectively) under denaturing conditions, but without prior heating samples in SDS buffer. Electrophoresed components were then transferred from the polyacrylamide gel to nitrocellulose membranes using a Transblot unit (217 Multiphor 2; LKB, Bromma, Sweden). The transfer buffer contained 20% (vol/vol) methanol, 0.039 M glycine, and 0.048 M Tris (pH 8.8), and runs were made at 0.8 mA/cm^2 of membrane for 2 h. Transblotted nitrocellulose membranes were incubated first for 30 min with PBS containing 2% (wt/vol) bovine serum albumin (room temperature) and then overnight with a rabbit anti-alkaline phosphatase (Biovalley, Marne-La-Vallée, France) immunoglobulin G preparation (1:5,000 dilution in PBS containing 0.05% [vol/vol] Tween 20 [PBST]). After repeated washes with PBST, membranes were incubated for 4 h with peroxidase-labeled protein A (1:1,000 dilution in PBST). After successive washes with PBST and PBS, a color reaction was developed by addition of α-chloronaphthol to a final concentration of 2.8 mM in the presence of 0.015% (vol/vol) hydrogen peroxide.

Construction of site-directed mutants. A plasmid (pQuantagen-FloR) expressing wild-type FloR (unfused to PhoA) from the *tac* promoter of pQuantagen(xk) was constructed. For that purpose, a 1,296-bp DNA fragment encompassing the *floR* gene was amplified by PCR from the chromosomal DNA of *Salmonella* serovar Typhimurium DT104 with primers O1 and O15 (Table 1). Primer O15 contains a KpnI restriction site at its 5′ end. Amplification and cloning were performed as described above for the construction of *floR-phoA* fusions. Mutations were then introduced into the *floR* gene of the pQuantagen-FloR plasmid. For that purpose, a template plasmid (pUC19-*floR*) was created by subcloning the 1,296-bp DNA fragment (BamHI-KpnI) of pQuantagen-FloR between the BamHI and KpnI sites of pUC19. DNA fragments containing the desired mutations and covering pUC19-*floR* in its entirety were amplified with *Pfu* Turbo DNA polymerase (Stratagene, Amsterdam, The Netherlands) according to the manufacturer's instructions, using two nonoverlapping synthetic primers. For each mutation, the forward primer contained the mutation. The primers used are listed in Table 1. Oligonucleotides used to generate D23E, D23K, and D23N were, respectively, D23E/fw, D23K/fw, and D23N/fw as forward primers and D23/rv as the unique reverse primer. To generate R109E, R109K, and R109N, the forward primers were, respectively, R109E/fw, R109K/fw, and R109N/fw, and the reverse primer was R109/rv. To generate R283E, R283K, and R283N, oligonucleotides were, respectively, R283E/fw, R283K/fw, and R283N/fw as forward primers and R283/rv as the unique reverse primer. Amplifications were performed in a thermocycler (Perkin-Elmer, Courtaboeuf, France) with the following cycling conditions: one cycle of 2 min at 98°C; then 30 cycles of 30 s at 98°C, 30 s at 65°C, and 4 min at 72°C; and finally one cycle of 10 min at 72°C. Following amplification, DNA products were treated with the DpnI endonuclease to digest the parental DNA template. DNA products were then purified and ligated to themselves by using T4 DNA ligase (Promega, Charbonnières, France). The resulting plasmids were transformed into *E. coli* DH5α cells by electroporation. The 1,296-bp DNA fragments (BamHI-KpnI) encompassing mutated *floR* genes were then cloned back into pQuantagen(xk) and introduced into *E. coli* AG100A (25) by electroporation.

Expression and detection of mutant proteins. *E. coli* AG100A cells transformed with pQuantagen-*floR* and pQuantagen mutated *floR* constructs were grown as described above for the expression of fusion proteins. Membrane protein fractions were prepared as described previously with some modifications (3). Briefly, after cell lysis by sonication, cell fragments were removed by centrifugation for 5 min at 16,000 × *g*. Membranes were collected by ultracentrifugation (1 h at 50,000 × *g*) and resuspended in 100 μl PBS containing 0.5 mM phenylmethylsulfonyl fluoride. Fifty micrograms of membrane proteins was then

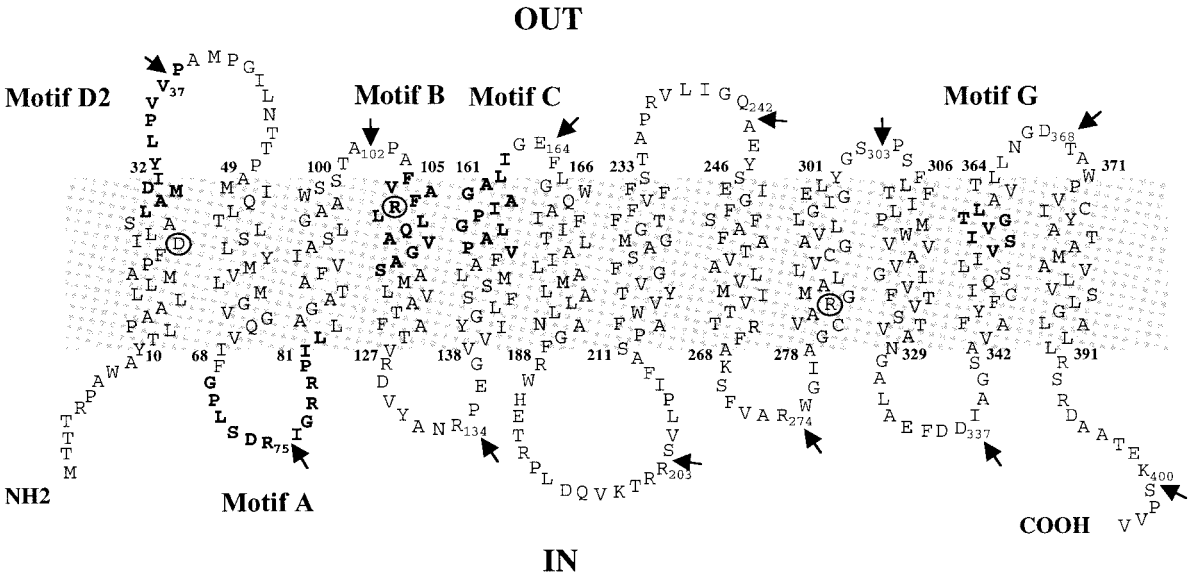

FIG. 1. Membrane topology of FloR. The model is a consensus derived from predictions by the TMHMM, Tmpred, TopredII, PhdTopology, and SOSUI programs. Motifs A, B, C, D2, and G, conserved in the 12-TMS cluster of MF superfamily multidrug transporters, are boldfaced. Arrows indicate the positions of fusion sites with PhoA. The three charged amino acids, found in TMS1, TMS4, and TMS9, respectively, which were mutagenized are circled.

separated by SDS-polyacrylamide gel electrophoresis (4 and 12% polyacrylamide for stacking and separating gels, respectively) and transferred to nitrocellulose membranes as described above. Membranes were saturated as described and incubated overnight with purified polyclonal rabbit anti-FloR peptide (NH_2-CH ETRPLDQVKTRRSV-$CONH_2$ and NH_2-CLLRSRDAATEKSPVV-COOH) antibodies (Eurogentec, Ougrée, Belgium) (2.5 μg/ml in PBST). After repeated washes with PBST, membranes were incubated for 4 h with an alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (1:5,000 dilution in PBST) (Promega, Charbonnières, France). After successive washes with PBST, a color reaction was developed by addition of XP in conjunction with nitroblue tetrazolium according to the manufacturer's instructions (Promega, Charbonnières, France).

Antimicrobial susceptibility assays. For testing susceptibility to antimicrobial agents, *E. coli* cells grown overnight at 37°C in LB broth supplemented with ampicillin (100 μg/ml) were diluted 1:10,000 into 200 μl of fresh LB broth (in 96-well microtiter plates [Nunc, Roskilde, Denmark]) supplemented with IPTG (0.5 mM) and serial twofold dilutions of the desired antimicrobial agent (see Results). Growth was quantified by measuring optical densities of the cultures at 550 nm after 18 h at 37°C with gentle agitation.

Chloramphenicol accumulation. The procedure for determining the uptake of [^{14}C]chloramphenicol by intact cells was adapted from previous studies (11, 23). Exponential-phase bacteria in LB broth were removed by centrifugation. Pellets were washed once and suspended to a density of 10^{10} CFU/ml in 50 mM sodium phosphate buffer, pH 7, containing 5 mM magnesium chloride. [^{14}C]chloramphenicol was a generous gift from Aventis Hoechst Marrion Roussel (Romainville, France). [^{14}C]chloramphenicol (50 μl) (specific radioactivity, 59.46 mCi/mmol) was added to 500 μl of cell suspension at 37°C in a shaking water bath, yielding a final chloramphenicol concentration of 5 μM. At various intervals, 100 μl of the suspension was removed and immediately filtered through GF/C filters (Whatman Ltd., Maidstone, Kent, United Kingdom). After three washes with 4 ml of cold phosphate-LiCl (0.1 M) buffer, filters were dried and radioactivity was measured in a Packard scintillation counter. To de-energize the bacteria, 50 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added 10 min before the radiolabeled antibacterial agent.

Computer analyses. The putative membrane-spanning domains of the FloR protein were predicted using the TMHMM, Tmpred, TopredII, PhdTopology, and SOSUI programs (8, 16, 19, 31).

RESULTS

Determination of the membrane topology of FloR. A prediction of transmembrane domains of the FloR protein by the TMHMM, Tmpred, TopredII, PhdTopology, and SOSUI programs suggested the presence of 12 TMS (8, 16, 19, 31). A consensus predicted topology is presented in Fig. 1. A more detailed analysis of the FloR amino acid sequence revealed the presence of all the previously described conserved motifs found in the sequences of the DHA12 cluster of multidrug transporters of the MF superfamily (Table 2) (26, 28). The locations of these motifs on the topological model are similar to those reported for the other transporters of the family: motif A in the loop between TMS2 and TMS3, motif B in TMS4, motif C at the end of TMS5, motif D2 at the end of TMS1, motif G at the end of TMS11 (Fig. 1). The presence of all these structurally or functionally important motifs, as well as the topological prediction of FloR, strongly suggests that FloR is a

TABLE 2. Alignment of consensus sequences of conserved motifs in transporters of the MF superfamily with similar sequences found in FloR[a]

Sequence	Motif A	Motif B	Motif C	Motif D2	Motif G
Consensus	GxLaDrxGrkxxxl	lxxxRxxqGxgaa	gxxxGPxxGGxl	lgxxxxxPvxP	GxxxGPL
FloR	GPLSDRIGRRPILL	FVAFRLLQAVGAS	VPALGPIAGALI	LAMDIYLPVVP	VSIVGTL

[a] The consensus sequences of the motifs are displayed as follows: x, any amino acid; capital letters, amino acids occurring in >70% of the MF sequences analyzed by Putman et al.; lowercase letters, amino acids occurring in >40% of these sequences (28).

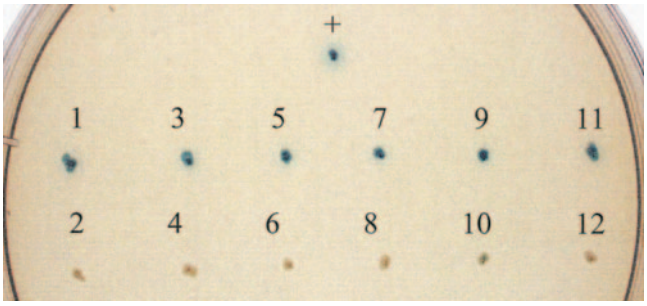

FIG. 2. Alkaline phosphatase activities of *E. coli* Δ*phoA* cells expressing FloR-PhoA fusion proteins. *E. coli* cells transformed with *floR-phoA* constructs (fusion site after the *floR* codon encoding V37 in position 1, R75 in position 2, A102 in position 3, R134 in position 4, E164 in position 5, R203 in position 6, Q242 in position 7, R274 in position 8, S303 in position 9, D337 in position 10, D368 in position 11, and K400 in position 12) were grown on LB agar plates supplemented with 40 μg/ml XP, 100 μg/ml ampicillin, and 0.5 mM IPTG. *E. coli* cells harboring a pQuant type of plasmid containing an uninterrupted *phoA* gene (Qbiogen) were grown similarly and used as a positive control (+).

member of the DHA12 family of efflux transporters of the MF superfamily.

The topology of various bacterial membrane proteins has been studied using the *phoA* gene fusion method (35). The use of alkaline phosphatase as a reporter molecule, enzymatically active only after translocation and complete assembly in the periplasm, is advantageous because a positive result requires the active export of the mature reporter enzyme in the periplasm. Fusion points were chosen from the predicted model in the middle portion of each putative loop (Fig. 1). However, to prevent the disruption of known topological determinants, such as charged residues, fusion points were placed after most of the charged residues of each loop (except for charged residues which are located in the C-terminal portion of the loop, close to a predicted TMS).

The alkaline phosphatase activities of fusion proteins were evaluated by blue-white screening on XP plates, in an *E. coli* strain lacking the *phoA* gene so as to decrease the background. Cells expressing fusion proteins fused at position V37, A102, E164, Q242, S303, or D368 of FloR grew as blue colonies, reflecting a periplasmic location of these residues, while those expressing fusions fused at R75, R134, R203, R274, D337, or K400 appeared white, consistent with the predicted cytoplasmic location of these amino acids (Fig. 2). Fusion proteins were all detected by Western blotting with anti-alkaline phosphatase antibodies, indicating that the absence of phosphatase activity was not caused by low protein expression (results not shown). These results confirmed the 12-TMS structure model of FloR.

Drug specificity of FloR. Some transporters of the MF superfamily mediate the extrusion of a given drug or class of drugs (30, 34). In contrast to these specific transporters, the so-called multidrug transporters can handle a wide variety of structurally unrelated compounds (20, 24, 27, 36). Because FloR shows similarity with proteins belonging to the MF superfamily and was confirmed to contain 12 TMS, we tested the substrate specificity of FloR. For this purpose, we produced this protein in the drug-hypersusceptible *E. coli* strain AG100A, which was deleted of the major multidrug efflux system AcrAB (25). The susceptibilities of AG100A strains carrying pQuantagen and pQuantagen-FloR were tested with various structurally unrelated molecules (tetracycline, sulfamethoxazole, nalidixic acid, erythromycin, berberine, tetraphenylphosphonium bromide, ciprofloxacin, ethidium bromide, florfenicol, chloramphenicol, and thiamphenicol). As shown in Table 3, FloR confers on the susceptible strain AG100A increasing levels of resistance to chloramphenicol, florfenicol, and thiamphenicol, while no effect was noted with the other agents tested. These results suggest that FloR exhibits a specific pump activity for phenicols.

TABLE 3. Susceptibilities to various compounds of *Escherichia coli* cells expressing or not expressing the FloR protein of *Salmonella enterica* serovar Typhimurium DT104

Compound	MIC (μg/ml) for:	
	AG100A/ pQuantagen	AG100A/ pQuantagen-FloR
Chloramphenicol	1	64
Florfenicol	1	16
Thiamphenicol	8	1,024
Tetracycline	0.25	0.25
Sulfamethoxazole	16	16
Nalidixic acid	0.8	0.8
Berberine	16	16
Tetraphenylphosphonium bromide	4	4
Erythromycin	8	8
Ciprofloxacin	0.1	0.1
Ethidium bromide	2	2

Drug resistance is mediated by an energy-dependent mechanism. To decipher the FloR resistance mechanism, we measured chloramphenicol accumulation in *E. coli* AG100A cells harboring the pQuantagen-FloR construct. Experiments were carried out in the absence and in the presence of CCCP, a proton motive force uncoupler collapsing the efflux pump energy (11, 22). In bacteria expressing FloR, the addition of CCCP induced a strong increase in the intracellular accumulation of chloramphenicol, which reached about 250% of the level obtained in the resistant strain in the absence of the uncoupler (Fig. 3). These results clearly indicated that FloR is involved in an active efflux of chloramphenicol driven by the proton motive force.

Mutagenesis of membrane-embedded charged residues. The postulated transmembrane domains of FloR contain seven charged residues: D23 and D31 in TMS1, R109 in TMS4, E247 and R266 in TMS8, and R283 and E301 in TMS9. Among these, only three residues, D23, R109, and R283, are undoubtedly membrane embedded. D31, E247, and E301 are located close to regions which are mapped as periplasmically accessible from phosphatase analyses, while R266 is located close to cytoplasmic regions. Since charged residues in putative TMS were observed to play an important role in the proton translocation or substrate recognition of many transporters (3), including drug transporters, we investigated the importance of D23, R109, and R283 for the activity of FloR. By site-directed mutagenesis of pQuantagen-FloR, we replaced the aspartate residue (D23) of FloR with either another negatively charged residue (D23E), a positively charged residue (D23K), or a neutral residue (D23N). Similarly, the two arginine residues

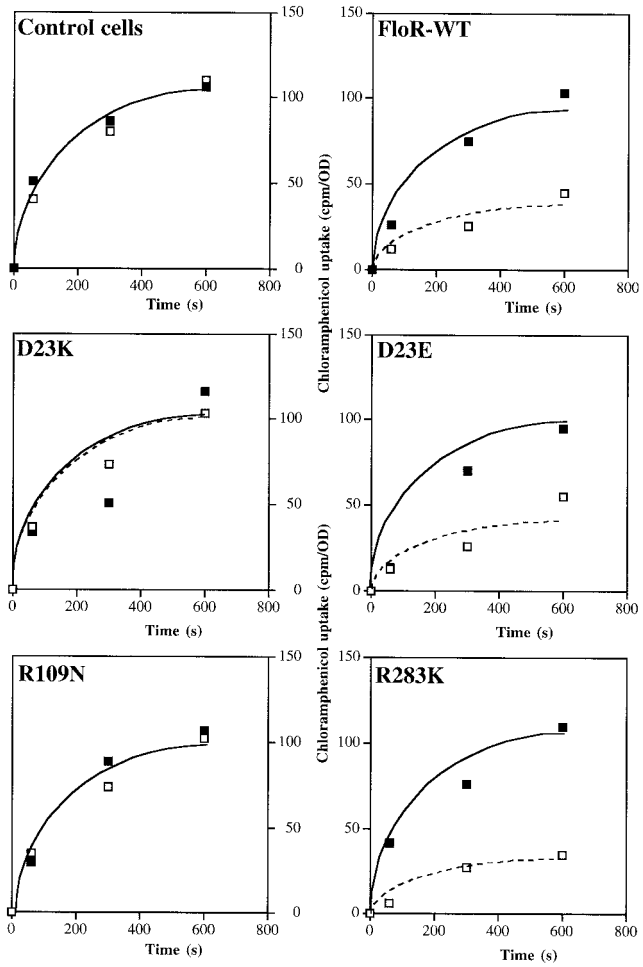

FIG. 3. Uptake of chloramphenicol by AG100A *E. coli* cells expressing the various FloR mutations. The intracellular accumulation of [^{14}C]chloramphenicol in various strains was determined in the absence (white symbols, dashed lines) and in the presence (black symbols, continuous lines) of 50 μM carbonyl cyanide *m*-chlorophenylhydrazone. Each point represents the mean from three independent assays.

(R109 and R283) were systematically replaced by a negatively charged residue (R109E and R283E), another positively charged residue (R109K and R283K), or a neutral residue (R109N and R283N). The expression of the mutant proteins in *E. coli* AG100A cells was checked by Western blotting with anti-FloR antibodies after electrophoresis of membrane protein fractions on an SDS-polyacrylamide gel (results not shown). The mutant proteins were all expressed equally except for R283E and, to a lesser extent, R283N, which presented altered expression.

The chloramphenicol, florfenicol, and thiamphenicol resistance levels conferred by wild-type FloR and the mutants, which are correctly expressed, were compared (Table 4). Concerning the effects of various substitutions on efflux capacity, wild-type FloR and the D23E, D23K, R109K, R109N, and R283K mutants were assayed for chloramphenicol accumulation (Table 5; Fig. 3).

As shown in Table 4, the D23K and D23N substitutions in putative TMS1 resulted in serious decreases in the levels of

TABLE 4. Chloramphenicol, florfenicol, and thiamphenicol resistance levels conferred by FloR and mutant FloR proteins on *Escherichia coli* AG100A

Strain	MIC (μg/ml) of:		
	Chloramphenicol	Florfenicol	Thiamphenicol
AG100A/pQuantagen	1	1	8
AG100A/pQuantagen-FloR	64	16	1,024
AG100A/pQuantagen-FloR-D23E	32	2	512
AG100A/pQuantagen-FloR-D23K	2	1	16
AG100A/pQuantagen-FloR-D23N	4	1–2	64
AG100A/pQuantagen-FloR-R109E	8	1	256
AG100A/pQuantagen-FloR-R109K	16	2–4	512
AG100A/pQuantagen-FloR-R109N	4	1	128
AG100A/pQuantagen-FloR-R283K	32	8	1,024

resistance to chloramphenicol, florfenicol, and thiamphenicol. Accordingly, cells expressing D23K did not preserve the efflux activity, and the intracellular accumulation of chloramphenicol reached the level obtained in the host cells that do not express FloR (Table 5; Fig. 3). In contrast, the D23E substitution rendered cells susceptible to florfenicol but retained chloramphenicol and thiamphenicol resistance levels similar to those observed in cells expressing wild-type FloR. In accordance, cells expressing D23E showed a reduction in the intracellular chloramphenicol level similar to that observed with wild-type FloR (Table 5; Fig. 3).

The substitutions R109N and, to a lesser extent, R109E in postulated TMS4 resulted in cells susceptible to florfenicol, chloramphenicol, and thiamphenicol. Accordingly, cells expressing R109N did not exhibit chloramphenicol efflux activity. In contrast, the R109K substitution, retaining significant chloramphenicol, florfenicol, and thiamphenicol resistance levels, showed a reduction of intracellular accumulation of chloramphenicol (Table 5).

The R283K substitution preserved the levels of resistance to chloramphenicol, thiamphenicol, and florfenicol.

To determine the precise effects of FloR mutations, the kinetics of chloramphenicol accumulation were measured in the absence and in the presence of CCCP (Fig. 3). The uncoupler was able to completely restore the intracellular concentration of chloramphenicol when the mutations preserved the pump activity: drug efflux remained CCCP sensitive in the D23E and R283K mutants at levels similar to that of wild-type FloR. For the other mutation, intracellular chloramphenicol

TABLE 5. Accumulation of chloramphenicol in *Escherichia coli* AG100A expressing FloR or mutated FloR proteins

Strain	Chloramphenicol accumulation (cpm/OD measured at steady state)[a]
AG100A/pQuantagen	110 ± 20
AG100A/pQuantagen-FloR	45 ± 10
AG100A/pQuantagen-FloR-D23E	55 ± 10
AG100A/pQuantagen-FloR-D23K	103 ± 8
AG100A/pQuantagen-FloR-R109K	63 ± 5
AG100A/pQuantagen-FloR-R109N	102 ± 14
AG100A/pQuantagen-FloR-R283K	35 ± 10

[a] OD, optical density. Values are means from three independent experiments.

accumulation exhibited the same level as that obtained in the host cell and the accumulation was not increased by addition of the uncoupler. This result suggests that FloR was the sole active pump, CCCP sensitive, which expelled chloramphenicol.

DISCUSSION

Structure-function relationships of FloR, encoded by the *floR* resistance gene of *S. enterica*, have not yet been provided, and it was interesting to identify protein domains or amino acids that are selectively involved in this drug transport. After analysis of the FloR protein with five topology prediction programs, a consensus model with 12 TMS was proposed. We checked this model experimentally by using the *phoA* gene fusion method and assigned FloR to the DHA12 family of efflux transporters of the MF superfamily.

Knowing that some transporters of the MF superfamily are drug specific (30, 34) while others can handle a wide variety of structurally unrelated compounds, we tested the drug specificity of FloR. For that purpose, we produced this protein in a drug-hypersusceptible *E. coli* strain and evaluated the capacity of the resulting construct to confer drug resistance on the host cell. We showed that FloR is a transporter specific for structurally associated phenicol drugs. To investigate the mechanism by which FloR confers phenicol resistance, studies of chloramphenicol accumulation in this host cell were performed in the presence or absence of CCCP, which collapses membrane energy (11, 22). The observed increase of chloramphenicol accumulation in the presence of CCCP indicated that FloR mediates an active efflux process driven by a proton motive force.

Taking advantage of the structural phenicol derivatives chloramphenicol, thiamphenicol, and florfenicol, we analyzed the effects of specific residues on the respective susceptibilities.

Mutations were constructed in D23, R109, and R283, which were selected because they belong to putative membrane regions. Thus, it was assumed that these charged amino acids could be involved in the efficiency of drug transport.

Interestingly, D23 appears to play a strategic role for the recognition of expelled substrate: (i) the negative charge located at this position is probably necessary for chloramphenicol, thiamphenicol, and florfenicol diffusion, and in addition, (ii) the size of the side chain exhibited by D23 is important for florfenicol recognition and not for that of chloramphenicol and thiamphenicol.

These results are quite divergent from those observed for the MF superfamily transporter MdfA of *E. coli*, a multidrug transporter with broad substrate specificity that recognizes both charged substrates and uncharged substrates, such as chloramphenicol (3, 4, 10). This transporter contains a membrane-embedded Asp residue in its first TMS (E26), and replacing E26 with D drastically decreased the resistance levels to chloramphenicol and thiamphenicol. Similar results were obtained with E26H and E26L substitutions, which abolished the efflux resistance, while E26Q, E26V, and E26I variants exhibited a higher resistance level than that conferred by the wild-type pump (4, 10). These observations suggest that MdfA activity tolerates a certain flexibility for charges located at this position. In contrast, both the size and the charge appear as key parameters at position D23 in FloR. An increase in the side chain length or a charge inversion appears to drastically reduce the florfenicol resistance level. This indicates that D23 could be involved, at least partly, in the ligand recognition pocket of the pump. Interestingly, when we compare this FloR sequence region with those of the oxalate transporter, the glycerol-3-phosphate transporter, and the LacY permease, this region, also located in the first TMS, plays an important role during substrate transport (1, 2, 13, 14, 17). In addition, this D23 residue is preserved in *E. coli*, *Enterobacter aerogenes*, *V. cholerae*, *K. pneumoniae*, and *P. aeruginosa* putative chloramphenicol and florfenicol transporters, suggesting its involvement in the specificity for florfenicol transport.

Similar results were obtained for thiamphenicol and chloramphenicol with D23 variants, which ruled out a possible involvement of the protruding methylsulfonyl group that is present in florfenicol and thiamphenicol. The analysis of phenicol derivative structures pinpoints the fluor, which is present only in florfenicol in place of the hydroxyl group in the other two. During translocation through the FloR channel, the fluoromethyl group could be more interactive with the charge exposed by residue D23. The transition D→E, which introduces one additional methylene for E, probably induces a steric hindrance with the fluor group harbored by florfenicol and thus alters florfenicol transport. This FloR jamming results in a significant restoration of florfenicol susceptibility.

Regarding the second position, R109 belonging to TMS4 is a highly conserved arginine residue in motif B of MF superfamily members which is also preserved in described chloramphenicol-florfenicol pumps (33). In addition, helix 4 is involved in the transporter cavity of the 12-TMS transporters and is close to helix 1 (15). The three substitutions tested in this location modified levels of resistance to phenicol derivatives. R109K preserves a significant chloramphenicol efflux, and the resistance level is partially conserved, indicating that not only the positive charge but also the orientation and size of the ammonium group at position 109 are important for chloramphenicol recognition. A similar effect was noted with florfenicol, while in contrast, no effect was observed with thiamphenicol. The other two substitutions, R→E and R→N, appeared to be more drastic for the efflux of the phenicol molecules. This position (R109) may participate in the orientation of the substrate inside the pump central cavity, especially for chloramphenicol and florfenicol. With the model proposed for 12-TMS transporters (13, 14), it is also possible that D23 and R109 work together to organize the orientation of these two molecules inside the channel.

The last residue, R283, seems to be important for membrane assembly and pump stability. Only the R283K substitution preserved a normal synthesis of the mutant compared to wild-type FloR, while R283E and, to a lesser extent, R283N induced serious decreases in levels of mutated protein. Taking into account the model for 12-TMS transporters (15) and the 3-dimensional structure of the oxalate transporter and the LacY permease (1, 2, 14), we propose that R283 acts similarly to R302 in LacY in order to maintain associations between TMS9 and TMS10 in FloR. These associations, which are probably involved in the folding of the FloR pump in the membrane, are important for protein stability. Consequently, substitutions such as R283E and, to a lesser extent, R283N, impairing the helix-helix organization, caused a misfolding which allowed a

degradation of abnormal membrane proteins, as previously reported for inner membrane proteins (29). In addition, the R283K substitution, which maintains the charge and the orientation of the positive group in TMS9, preserved the expression level of FloR and the efflux activity.

For the first time, this study documents the structure-function relationships of FloR. The 12-TMS organization defines a phenicol-specific efflux pump driven by a membrane potential energy-dependent mechanism. In this transporter, R283 seems to be necessary for correct membrane folding, whereas two residues, D23 in TMS1 and R109 in TMS4, are essential for the active efflux function. Of these, the acidic residue D23 seems to directly participate to the affinity pocket involved in phenicol recognition, and the transport of florfenicol appears dependent on the side chain size/orientation of this amino acid.

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