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Active Efflux of Bile Salts by *Escherichia coli*

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Enteric bacteria such as *Escherichia coli* must tolerate high levels of bile salts, powerful detergents that disrupt biological membranes. The outer membrane barrier of gram-negative bacteria plays an important role in this resistance, but ultimately it can only retard the influx of bile salts. We therefore examined whether *E. coli* possessed an energy-dependent efflux mechanism for these compounds. Intact cells of *E. coli* K-12 appeared to pump out chenodeoxycholate, since its intracellular accumulation increased more than twofold upon deenergization of the cytoplasmic membrane by a proton conductor. Growth inhibition by bile salts and accumulation levels of chenodeoxycholate increased when mutations inactivating the *acrAB* and *emrAB* gene clusters were introduced. The AcrAB system especially appeared to play a significant role in bile acid efflux. However, another efflux system(s) also plays an important role, since the accumulation level of chenodeoxycholate increased strongly upon deenergization of *acrA emrB* double mutant cells. Everted membrane vesicles accumulated taurocholate in an energy-dependent manner, apparently consuming ΔpH without affecting ΔΨ. The efflux thus appears to be catalyzed by a proton antiporter. Accumulation by the everted membrane vesicles was not decreased by mutations in *acr* and *emrB* genes and presumably reflects activity of the unknown system seen in intact cells. It followed saturation kinetics with V_{max} and K_m values in the neighborhood of 0.3 nmol min^{-1} mg of $protein^{-1}$ and 50 μM, respectively.

Bile salts are detergents made by the liver and secreted into the bile to help the dispersion and enzymatic digestion of fats in the small intestine. Initially, they are made as primary bile acids, which in humans consist of cholate and chenodeoxycholate (CDC) (8). The majority of them are secreted as conjugated bile acids, in a form connected to either glycine or taurine via amide bonds. Intestinal microflora carry out the deconjugation of bile acids as well as their transformation into secondary bile acids, such as the 7α-dehydroxylation reaction that converts cholate and CDC into deoxycholate and lithocholate, respectively (8).

Detergents, including bile salts, are obviously toxic for all cells, since they disaggregate the lipid bilayer structure of the cellular membranes. Since *Escherichia coli* organisms must live in the intestinal tract, where the bile salt concentration can be as high as 20 mM in the duodenum (2) and possibly even higher in other parts of the small intestine, they must defend themselves against the toxic action of bile acids. Indeed, *E. coli* and its relatives show a significant degree of resistance to bile salts, and this property is utilized in their selective enrichment on, for example, MacConkey agar (containing 0.15% bile salts) or deoxycholate agar (containing 0.1% sodium deoxycholate). The mechanism of bile salt resistance of *E. coli*, however, is not understood. Frequently, it is thought that the outer membrane acts as a barrier against bile salts. However, bile salts do penetrate at least the larger channel of the OmpF porin, as indicated by the higher bile salt resistance of *ompF* mutants (see Results). Furthermore, at least the unconjugated bile acids should be able to traverse the lipid bilayer domains of the outer membrane (23), because their pK_a values of 5 to 6.5 (3) mean that a significant portion of these compounds exists in lipophilic, uncharged forms. These considerations suggest the existence of another major mechanism responsible for the bile salt resistance of *E. coli*. In this study, we show that *E. coli* is capable of actively exporting bile salts.

(A preliminary account of this study was presented at the 95th General Meeting of the American Society for Microbiology, Washington, D.C. [27].)

MATERIALS AND METHODS

Bacterial strains and their growth. All strains used were derivatives of *E. coli* K-12 (Table 1). LB broth contained 10 g of Bacto Tryptone, 10 g of Bacto Yeast Extract, and 5 g of NaCl per liter. M63 (12) with 0.2% glucose was the minimal medium. Cells were grown at 37°C, and liquid cultures were aerated by shaking. P1 transduction was by standard methods (12).

Chemicals. [Carboxyl-^{14}C]CDC (50 mCi/mmol) and [24-^{14}C]taurocholate (TOC); (46 mCi/mmol) were obtained from New England Nuclear Corp. Bile salts, nigericin, valinomycin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and acridine orange were from Sigma Chemical Co., and oxonol V was obtained from Molecular Probes, Inc.

Effect of porin composition on the susceptibility to bile salts. Overnight cultures of JF701 and JF703 in LB broth were diluted 50-fold in fresh LB broth containing various concentrations of Na deoxycholate, cultures were incubated with shaking at 37°C, and growth was monitored by measuring turbidity.

Accumulation of CDC by whole cells. Exponential-phase cells grown in M63-glucose were collected by centrifugation, washed once in 25 mM K phosphate (pH 7.0) containing 0.2% glucose and 1 mM $MgSO_4$, and resuspended in the same buffer to 2 mg (dry weight)/ml. [^{14}C]CDC, diluted with nonradioactive CDC to a specific radioactivity of 16 mCi/mmol, was added to a concentration of 0.2 mM, and the suspension was incubated at 37°C. Portions were removed and centrifuged through a mixture of silicone oils, and the cell-associated radioactivity was determined as described earlier (28).

MIC of bile salts. MICs were determined by serial twofold dilutions in LB broth. The MIC values were read after an overnight incubation, at 37°C, of tubes, each inoculated with 10^3 cells.

Preparation of everted membrane vesicles. Inside-out membrane vesicles were prepared essentially as described by McMurry et al (11). Up to 6 liters of cultures were grown in M63-glucose or LB broth to the late exponential phase. Cells were harvested, washed with precooled 0.1 M K phosphate (pH 6.6)–10 mM disodium EDTA, and resuspended in the same buffer (5 ml per 1 liter of original culture). Phenylmethylsulfonyl fluoride was added to 0.1 mM, and the cells were disrupted by one passage through a French pressure cell at 5,000 lb/in^2. Pancreatic DNase (Sigma) was added to 0.1 mg ml^{-1}, and the mixture was kept on ice for 1 h or until the viscosity decreased significantly. After low-speed centrifugation (27,000 × *g*, 10 min) to remove unbroken cells and much of the outer membrane and cell wall, the vesicles were sedimented by centrifuging at 150,000 × *g* for 1 h. The vesicles were gently resuspended in the same buffer and collected again by

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TABLE 1. *E. coli* strains used

Strain	Relevant genotype	Other genotype	Source or reference
JF701	*ompC264*	F$^-$ *aroA357 ilv-277 metB65 his-53 purE41 cyc-1 xyl-14 lacY29 rpsL77 tsx-6*	4
JF703	*ompF254*	Same as that for JF701	4
CS1253	Δ*ompC178*	F$^-$ *zei-298*::Tn*10*	25
HN849	*emrB*::*kan*	F$^-$	O. Lomovskaya
W4573	*acrA*$^+$ *emrB*$^+$	F$^-$ *lac ara mal xyl mtl gal rpsL*	9
N43	*acrA1*[a]	Same as that for W4573	9
HN790	*emrB*::*kan*	Same as that for W4573	P1 transduction from HN849
HN791	*acrA1* *emrB*::*kan*	Same as that for W4573	P1 transduction from HN849

[a] The *acrA1* mutation involves insertion of IS1 close to the 5′ end of the gene (9).

centrifuging under the same conditions. Vesicles were resuspended in 50 mM K phosphate (pH 7.5) to a protein concentration of about 20 mg ml^{-1}, and aliquots of the vesicle suspension were stored at −70°C after quick freezing in a dry ice-ethanol bath.

Accumulation of bile salts in everted membrane vesicles. Everted vesicles were thawed on ice and diluted to 0.8 to 1.5 mg (protein) ml^{-1} in 50 mM K phosphate (pH 7.5) containing $MgSO_4$ and KCl at indicated concentrations. Vesicles were preincubated for 5 min in a 30°C shaking water bath, and radiolabeled bile acid was added. Vesicles were energized by adding 10 mM NADH or 20 mM Li D-lactate and deenergized with 50 μM CCCP. At various times, samples were removed and assayed by either of the following methods. (i) In the filtration method, the samples (each 50 μl) were immediately diluted into 1 ml of ice-cold solution containing 0.1 M LiCl and 0.1 M K phosphate buffer (pH 7.5), filtered, and washed, and the radioactivity remaining on the filter was determined as described above for intact cells. (ii) In the method involving centrifugation through silicone oil, each sample (0.1 ml) was layered on top of 1.9 ml of a silicone oil mixture (50% Dow Corning fluid no. 550 and 50% Dow Corning no. 510 [50cP], vol/vol) in Quick-Seal centrifuge tubes (11 by 32 mm; Beckman Instruments) kept chilled on ice. The sealed tubes were centrifuged in a TLA100.2 rotor with a Beckman-TL-100 ultracentrifuge at 65,000 rpm for 1.5 h at 4°C. The tubes were then frozen in a dry ice-ethanol bath, and the bottom of the tube containing the pelleted vesicles was sliced off with a razor blade and placed in a scintillation vial. The radioactivity was determined as described above for whole cells.

In an initial series of experiments, CDC was used; no evidence of accumulation was obtained, presumably because CDC diffuses too rapidly through the bilayer domains of the membrane, especially in everted vesicles, which have a much higher surface-to-volume ratio than intact cells. Attempts to measure CDC uptake by flow dialysis (5) were also unsuccessful, because changes in ΔpH, caused by the addition of D-lactate or NADH, produced altered passive distribution of CDC across the membrane since its pKa was close to neutrality.

Because of these reasons, TOC, which has a low pKa and is unlikely to diffuse across the lipid bilayer, was used in subsequent experiments. Both filtration and silicone oil centrifugation methods gave evidence of active concentration of TOC within vesicles (see Results).

For determination of kinetics of TOC transport, a quick-quench filtration method, modified from that of Parker et al. (20), was used. Assay buffer (50 mM K phosphate [pH 7.5] containing 1 mM $MgSO_4$); (45-μl total volume) containing thawed everted vesicles (50 μg of protein), with or without an energy source (20 mM Li D-lactate), was incubated at 30°C. A separate tube containing [^{14}C]TOC in 5 μl of assay buffer was also incubated at 30°C. At time zero, the contents of the former tube was added to the latter tube. At the indicated time, the 50-μl mixture was diluted into 4.5 ml of ice-cold wash buffer (0.1 M K phosphate [pH 7.5] containing 0.5 mM nonradioactive TOC), and the suspension was immediately filtered through a 0.45-μm-pore-size GN-6 Metricel filter (Gelman Sciences). The filter was rapidly washed twice with 5-ml portions of ice-cold wash buffer and dried, and uptake was counted as described above. Energy-dependent uptake was the difference between the tube containing D-lactate and the tube not containing any energy source.

Monitoring of ΔpH and ΔΨ with fluorescent dyes. The two components of the proton motive force across the membrane of everted vesicles were determined by use of fluorescent dyes. Thawed vesicles were diluted to 0.1 mg of protein ml^{-1} in 50 mM K phosphate (pH 7.5) containing 1 mM $MgSO_4$ and the indicated concentration of KCl. Acridine orange, a weak base, was added to 2 μM for the measurement of ΔpH (21). Fluorescence was measured at room temperature with a Perkin-Elmer MPF-44B spectrofluorimeter, with an excitation wavelength of 490 nm and an emission wavelength of 530 nm. For the measurement of the ΔΨ component, oxonol V was added instead to 2 μM (30), and an excitation wavelength of 580 nm and an emission wavelength of 635 or 650 nm were used.

Other methods. Protein was determined by use of bicinchoninic acid reagent (Pierce), with bovine serum albumin as the standard (26).

RESULTS

Bile acid susceptibility and the porin composition. Since bile acids have dimensions that may barely allow them to penetrate through the porin channels (7), the growth of *E. coli* strains expressing different porin species was tested in the presence of deoxycholate. JF701 and JF703 are an isogenic pair expressing only the OmpF porin or OmpC porin, respectively, in normal (phosphate-rich) media (Table 1). Figure 1 shows that strain JF701, expressing the OmpF porin which produces the wider-diameter channel (7, 15), was more susceptible to the growth-inhibitory effect of deoxycholate than strain JF703, which expresses the porin with the smaller-diameter channel, OmpC (15). In view of these results, we used in some experiments CS1253 (Table 1), which expresses only the OmpF porin.

Accumulation of bile acids in whole cells. Cells of W4573, a K-12 strain that is wild type in terms of its efflux genes (Table 1), were incubated in the presence of radiolabeled CDC, and its accumulation in the cells was initially measured by filtration and washing (see Materials and Methods). These experiments showed only low levels of uptake in comparison with the extracellular levels. We suspected that this result was due to leakage of intracellular CDC during the washing process, since CDC has a pK_a of 6 to 6.5 (3) and a small, but significant

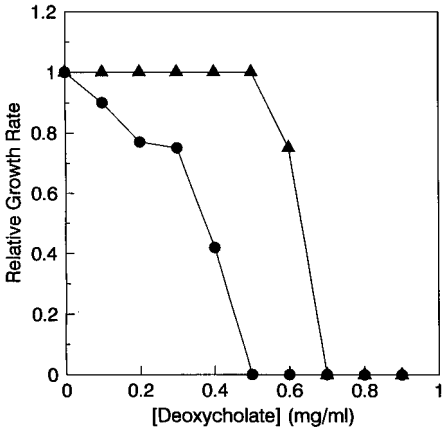

FIG. 1. Effect of deoxycholate on the growth of isogenic K-12 strains producing only OmpF or OmpC porin. Relative exponential growth rates of strains JF701 (OmpF$^+$ OmpC$^-$) (circles) and JF703 (OmpF$^-$ OmpC$^+$) (triangles) were measured in LB broth at 37°C in the presence of various concentrations of sodium deoxycholate. The rates observed were normalized to the growth rates of the respective strains in the absence of deoxycholate.

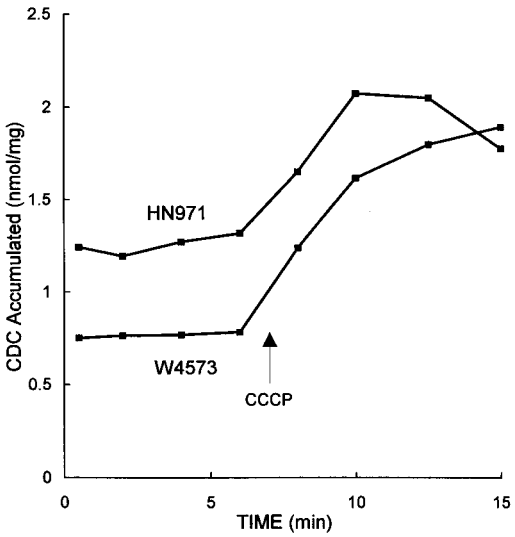

FIG. 2. Accumulation of [^{14}C]CDC by W4573 (wild-type) and HN971 (*acrA emrB*) cells. Cells were incubated in phosphate buffer containing glucose, and CDC was added to a final concentration of 200 μM. The accumulation was measured by centrifugation of cells through a layer of silicone oil (see Materials and Methods). At the time indicated by the arrow, CCCP was added to a final concentration of 100 μM. The values shown are averages of the data from two experiments, carried out with the same set of bacterial suspensions.

fraction will exist in the uncharged, protonated form that can easily diffuse across the cytoplasmic membrane.

When CDC accumulation was assayed by centrifugation of cells through silicone oil (see Materials and Methods), a method that does not require any washing, the amount of CDC taken up by W4573 cells was shown to reach a steady state rapidly (Fig. 2). Assuming that 1 mg (dry weight) of *E. coli* has a volume of 3 μl (13), at the steady state, the estimated intracellular concentration corresponds to about 230 μM. This was somewhat higher than the external concentration of CDC (200 μM), and thus the steady-state level alone does not necessarily suggest the existence of active efflux. However, when the cytoplasmic membrane was deenergized by the addition of the proton conductor CCCP (0.1 mM), the accumulation level increased rapidly more than twofold. This result strongly suggested that *E. coli* K-12 strains actively pump out bile acids, probably by using the proton motive force as an energy source, and that the steady-state accumulation level was increased once the activity of the pump was abolished due to the deenergization of the membrane. The fact that the apparent intracellular concentration under the deenergized condition was so much higher than the external one was probably due to the partitioning and adsorption of CDC into apolar structures in the bacterium.

An alternative explanation of these results is that deenergization of the cells caused a passive redistribution of weak acids (including CDC) across the membrane due to the collapse of ΔpH. The passive equilibration of weak acids occurs so that the concentrations of the membrane-permeant, protonated species stay equal on both sides of the membrane. In respiring energized cells, the cytoplasmic pH is higher than the extracellular pH. Thus, weak acids are passively accumulated inside energized cells, and the abolition of ΔpH should decrease, rather than increase, the cytoplasmic accumulation of acids such as CDC. This alternative hypothesis therefore cannot explain the observed changes in CDC accumulation.

E. coli is known to express at least two multidrug efflux pumps, AcrAB and EmrAB (reviewed in reference 14). AcrAB pumps out dyes, detergents (including bile salts [9]), and a wide variety of lipophilic and amphiphilic antimicrobial agents, such as β-lactams, chloramphenicol, tetracycline, novobiocin, and erythromycin. EmrAB pumps out lipophilic compounds such as CCCP and thiolactomycin. Indeed, the *acrA* mutant was confirmed to be hypersentive to bile salts, and the sensitivity to some of them was even higher in the *acrA emrB* double mutant (Table 2). Accumulation kinetics of CDC in the *acrA emrB* double mutant is also shown in Fig. 2. Two features are evident. (i) The accumulation level of CDC is much higher in the *acrA emrB* double mutant, a result suggesting that the AcrAB (and possibly also EmrAB) pump plays a significant role in the efflux of bile salts. The comparison of W4573 and HN971 was carried out in five independent experiments, and the accumulation level in the wild-type parent was always significantly lower (59.6% ± 7.9% [average ± standard deviation]) than that in the *acrA emrB* mutant. The *acrA* single mutant N43 also accumulated CDC to a level similar to that seen with the *acrA emrB* double mutant (data not shown). (ii) However, deenergization with CCCP still produces an almost twofold increase in the accumulation level in the double mutant, a result indicating that another important part of the efflux occurs through systems other than AcrAB and EmrAB.

TABLE 2. MICs of bile salts in isogenic strains of *E. coli* K-12

Strain	MIC (mg/ml) of:			
	Na cholate	Na DOC[a]	Na CDC	Na TOC
W4753 (*acr*$^+$ *emr*$^+$)	>64	>16	>64	>64
N43 (*acrA emr*$^+$)	8	1	16	4
HN970 (*acrA*$^+$ *emrB*::*kan*)	>64	>16	>64	>64
HN971 (*acrA emrB*::*kan*)	2	0.25	16	4

[a] DOC, deoxycholate.

Accumulation of TOC by everted membrane vesicles. To study the active efflux further, we utilized everted membrane vesicles. If bile acids are actively pumped out by intact cells, then the same process should produce an active accumulation against the concentration gradient in everted (inside-out) membrane vesicles. However, CDC could not be used in this system without the outer membrane barrier, presumably because it went through the lipid bilayer too rapidly (see Materials and Methods). We therefore turned to the use of TOC, which is unlikely to pass through lipid bilayers because its pK_a is very low (<1.5) and it is thus completely ionized at physiological pH values. When vesicles were incubated with radiolabeled TOC and its accumulation was measured by filtration of vesicles, a clear evidence for its energy-dependent uptake was obtained (Fig. 3). However, the values obtained at some time points were often erratic. We believe that this was due to the binding of TOC to the membrane filter.

Because of this difficulty, we turned to the centrifugation of vesicles through silicone oil. In this assay, everted membrane vesicles were incubated in the presence of 10 μM [^{14}C]TOC without an energy source for 10 min, an energy source (usually D-lactate) was then added, and the vesicles were incubated for an additional 10 min (see Materials and Methods). Energized and unenergized vesicles were finally centrifuged through a layer of silicone oil in a Beckman ultracentrifuge, and the radioactivity in the sedimented vesicles was determined. This method clearly showed that there was an energy-dependent accumulation of TOC (Table 3). The estimated intravesicular space in everted membrane vesicles varies from 0.51 to 8.4 μl per mg of protein (11, 22, 24, 30). Even when we use a fairly

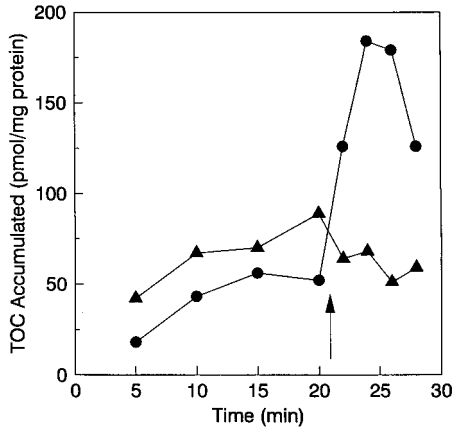

FIG. 3. Accumulation of TOC by everted membrane vesicles of CS1253. Accumulation was measured by filtration through a membrane filter. [^{14}C]TOC was added to 10 μM. Vesicles were incubated in the absence (circles) or presence (triangles) of 50 μM CCCP, and 10 mM NADH was added to both samples at 21 min (arrow). In separate experiments, similar results were obtained by the addition of 20 mM Li D-lactate, instead of NADH, as the energy source.

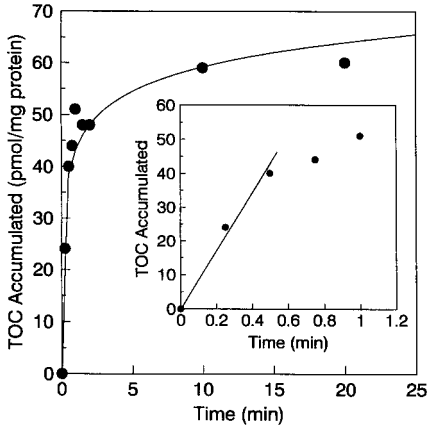

FIG. 4. Time course of TOC accumulation by everted membrane vesicles of CS1253, determined by a quick-quench filtration method. The accumulation was determined as described in Materials and Methods, in the presence of 10 μM [^{14}C]TOC and with or without 20 mM Li D-lactate. Energy-dependent accumulation was the difference between the lactate-containing system and the system without lactate. The inset shows the accumulation during early time points.

large estimate of 5 μl per mg of protein, the energy-dependent accumulation of 120 pmol of TOC per mg of protein by the W4573 vesicles (Table 3) translates to an intravesicular concentration of 24 μM, a concentration higher than the extravesicular concentration of 10 μM. It should be mentioned, however, that vesicles incubated without the addition of an energy source also showed substantial accumulation, an average of 413 pmol of TOC per mg of protein. Presumably, this reflects the (partial) partitioning of this amphiphilic compound into the membranes, a phenomenon similar to the observation of high values of a nominal intracellular concentration of CDC in intact cells of W4573, where an active efflux should be occurring (Fig. 2).

An alternative interpretation of the high levels of accumulation in nonenergized vesicles is that the vesicles were energized somewhat even without the addition of D-lactate. This appears to be true to a certain extent, because the addition of 50 μM CCCP often decreased the nonenergized uptake level by as much as 50%. (This means that the energy-dependent uptake values in Table 3 may be underestimated.) However, this mechanism does not appear to be the whole explanation, because even in CCCP-preincubated vesicles, the nominal accumulation levels were still higher than the concentration of TOC in the extravesicular fluid.

The silicone oil centrifugation technique also allowed us to estimate the accumulation by various mutant strains of *E. coli*.

TABLE 3. Energy-dependent accumulation of TOC by everted membrane vesicles[a]

Strain	Amt accumulated (pmol mg of protein^{-1})		
	Without energy source	With energy source	Energy dependent
W4573	413 ± 39	533 ± 7	120 ± 39
N43 (*acrA*)	470 ± 15	541 ± 21	121 ± 38
HN970 (*emrB*)	427 ± 31	542 ± 20	115 ± 33
HN971 (*acrA emrB*)	429 ± 16	565 ± 5	136 ± 15

[a] All experiments were repeated four times, and the means ± standard deviations are shown. Vesicles were incubated in the presence of 10 μM [^{14}C]TOC and energized by the addition of 20 mM D-lactate. Accumulation in a 10-min period was measured by the silicone oil centrifugation method.

Although an *acrA emrB* strain of *E. coli* was more susceptible to bile salts (Table 2) (9), vesicles from *acrA* and *acrA emrB* mutants accumulated amounts of TOC similar to that accumulated by vesicles from the isogenic parent (Table 3). (Possibly, the *acrA* mutant used was not sufficiently polar, so that it expressed enough AcrB transporter molecules. However, in a separate series of experiments, we saw no difference in TOC accumulation between the CS1253 vesicles and those from a strain with the deletion of most of the *acrAB* operon [data not shown].) The vesicle experiments thus suggest that a significant part of efflux of bile salts is carried out by a system(s) other than AcrAB and EmrAB, a conclusion in agreement with the accumulation data in intact cells (Fig. 2). We have attempted to inactivate this other gene(s) responsible for bile acid efflux by Tn*phoA* and Tn*phoA′* mutagenesis (29), but to date we have only obtained mutations that affect the process in an indirect manner (see Discussion). In another experiment, the accumulation of TOC was tested with W4573 vesicles without added Mg^{2+}. The absence of Mg^{2+} did not have any significant effect on the accumulation (data not shown).

Kinetics of TOC uptake by everted membrane vesicles. The silicone oil centrifugation method was convenient for measuring near-steady-state levels of uptake without complications such as loss of the bile acid during washing or adsorption of the bile acid to the membrane filter. However, it did not allow us to measure the initial rates of uptake. For this purpose, we developed a modified filtration method, based on the rapid quench filtration method described for the measurement of facilitated glucose transport in *Zymomonas mobilis* (20). We found that the use of ice-cold dilution-and-wash buffer, containing an excess of unlabeled TOC, was sufficient to give us reproducible results. The presence of unlabeled TOC was apparently crucial in lowering the binding of TOC to the filters, and the use of a wash solution cooled to subzero temperature (20) was not necessary in our system. The time course of accumulation of TOC by everted membrane vesicles of CS1253, measured by this method, showed that the accumulation process was nearly linear with time during the first 30 s and essentially reached the steady state in about 1 min (Fig. 4). We also emphasize that the data obtained by this method were highly reproducible; for example, in one experiment in which

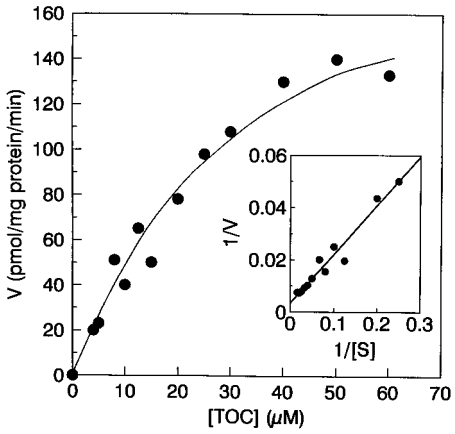

FIG. 5. Accumulation rates of TOC at various concentrations of the substrate. Accumulation during the first 30 s was determined at TOC concentrations of 4 to 60 μM. The nonenergized uptake values, used for the calculation of energy-dependent uptake in this experiment, were derived from an average of four different experiments at different concentrations of TOC. This is a composite figure in which results from four independent experiments have been combined. The inset shows the plot in the double reciprocal form.

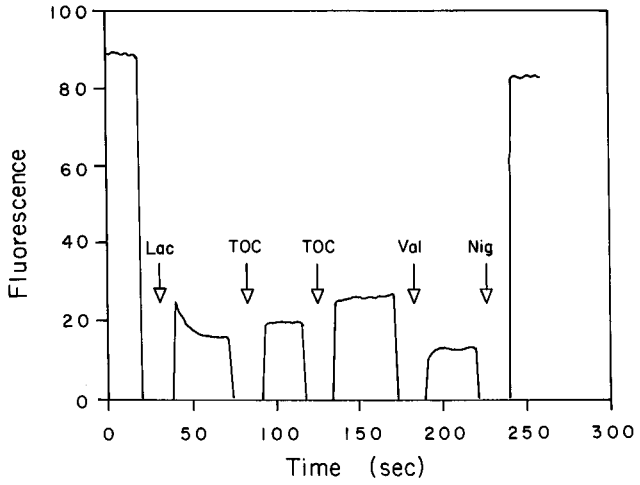

FIG. 6. Changes in ΔpH upon the addition of TOC. Everted vesicles from CS1253 were incubated in 50 mM K phosphate (pH 7.5) containing 1 mM $MgSO_4$, 30 μM KCl, and 2 μM acridine orange. The fluorescence emission was measured at 530 nm. Addition of 20 mM Li D-lactate (Lac) generated ΔpH as indicated by the strong quenching. Subsequent additions of 10 and 50 μM TOC caused immediate decreases in ΔpH as shown by decreased quenching. Valinomycin (1 μM; Val) produced an increase in ΔpH, whereas nigericin (1 μM; Nig) totally abolished it. The intensity of fluorescence emission is shown in arbitrary units.

duplicate samples were taken at eight time points, the difference between duplicate samples was on average only 5%.

The initial rates of TOC accumulation were determined at various external concentrations of the substrate. As shown in Fig. 5, the process follows saturation kinetics, with the reciprocal plot showing a good fit with a line indicating a K_m of about 50 μM and a V_{max} of about 0.3 nmol min^{-1} mg of $protein^{-1}$. These data were again highly reproducible. Similar experiments were repeated five times, and the K_m values obtained were all in the range of 30 to 65 μM and the V_{max} values were in the range of 0.15 to 0.4 nmol min^{-1} mg of $protein^{-1}$. The saturable nature of the accumulation process was also confirmed by utilizing the silicone oil centrifugation assay, where the steady-state accumulation levels plotted against the concentration of TOC showed signs of asymptotic tailing off at high concentrations (20 to 100 μM) (data not shown).

Nature of the energy source. Susceptibility of the TOC transport process to proton conductors such as CCCP suggested that the efflux is energized by a component(s) of the proton motive force. To determine whether efflux is driven by the proton gradient, ΔpH, or the electrical potential across the membrane, ΔΨ, we examined the effect of TOC transport on ΔpH and ΔΨ in respiring, everted, membrane vesicles. The ΔpH and ΔΨ components were measured by standard methods, i.e., ΔpH by the accumulation in the intravesicular space of the weakly basic, lipophilic, fluorescent dye acridine orange, and the consequent quenching of its fluorescence (21) and ΔΨ by the intravesicular accumulation and subsequent quenching of the always negatively charged fluorescent dye oxonol V (6).

In the initial series of experiments, conditions were optimized so that the vesicle membranes behaved as expected in response to the addition of various ion carriers. This was necessary because some of the components of the bathing buffer, especially the chloride ion, slowly penetrate across lipid bilayers, increasing the ΔpH component and decreasing the ΔΨ component of the proton motive force (21). By changing the concentration of Cl^-, we discovered that in the presence of 30 μM Cl^-, the addition of valinomycin, a K^+-specific ionophore, increased the ΔpH as assayed by acridine orange fluorescence (Fig. 6). This is the expected response because of the following. Valinomycin carries the K^+ ion out of the vesicles following the outside-negative ΔΨ, and this collapses the ΔΨ. Respiring membrane vesicles tend to maintain the sum of ΔpH and ΔΨ more or less constant (24), and because of this homeostasis, the decrease in ΔΨ is followed by an increased pumping of H^+, resulting in a compensating increase in the ΔpH component, as shown (Fig. 6). (In contrast, when a higher concentration, e.g., 0.1 M, of Cl^- was present, the addition of valinomycin paradoxically decreased the ΔpH [results not shown].) The effect of Cl^- concentration was also tested in oxonol V-treated everted vesicles. Upon addition of nigericin, a K^+/H^+ antiporter, accumulated H^+ should flow out of the vesicles, collapsing ΔpH. This electroneutral exchange should have no direct effect on ΔΨ, but ΔΨ will eventually increase in respiring vesicles because of the homeostasis of the total proton motive force, mentioned above. In fact, in vesicle suspensions containing 70 μM Cl^-, the addition of nigericin increased ΔΨ as expected (Fig. 7). (In contrast, ΔΨ paradoxically decreased upon the addition of nigericin if the suspending buffer contained no Cl^- [results not shown].)

Thus, the behavior of ΔpH during TOC accumulation was tested in the presence of 30 μM Cl^-, and the behavior of ΔΨ was tested in the presence of 70 μM Cl^-. Figure 6 clearly shows that the accumulation of TOC was accompanied by an immediate, small, but reproducible lowering of ΔpH. This suggests that the influx of TOC is accompanied by an efflux of H^+, i.e., that the transport process involves an antiport of TOC and H^+. In contrast, the addition of TOC to everted membrane vesicles in the presence of 70 μM Cl^- produced little immediate change in ΔΨ (Fig. 7), suggesting that ΔΨ is not consumed by the influx of TOC. There was also a hint of a slight increase in ΔΨ after the second addition of TOC, possibly caused by the compensation for the decrease in ΔpH. These results then suggest that ΔpH energizes the TOC transport process, most likely in an electroneutral manner.

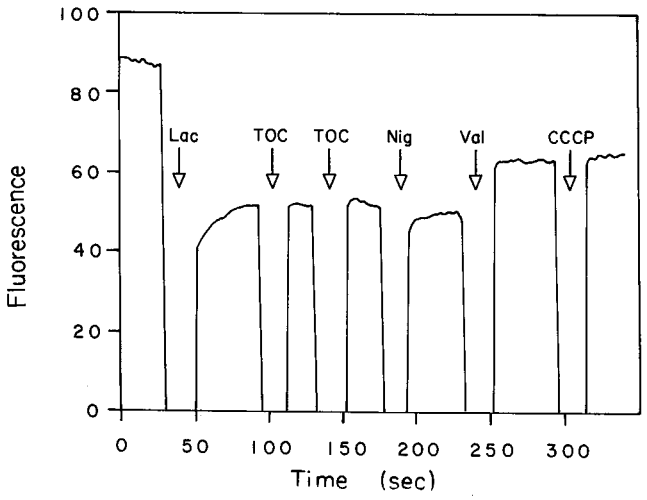

FIG. 7. Changes in $\Delta\Psi$ upon the addition of TOC. Everted vesicles from CS1253 were incubated in 50 mM K phosphate buffer (pH 7.5) containing 1 mM $MgSO_4$, 70 μM KCl, and 2 μM oxonol V. The initial addition of 20 mM Li D-lactate (Lac) generated a strong $\Delta\Psi$ as indicated by the quenching of oxonol fluorescence. Two additions of 10 μM TOC did not cause the decrease of $\Delta\Psi$, whereas nigericin (1 μM; Nig) increased $\Delta\Psi$ and valinomycin (1 μM; Val) apparently totally abolished $\Delta\Psi$, as confirmed by the absence of effect of the subsequent addition of 20 μM CCCP. The intensity of fluorescence emission is shown in arbitrary units.

DISCUSSION

The results of this study, particularly the energy-dependent prevention of CDC accumulation in intact cells (Fig. 2) and the energy-dependent TOC accumulation by everted membrane vesicles (Table 3; Fig. 3), leave no doubt that bile acids are actively pumped out by *E. coli*. Furthermore, the saturation kinetics of the efflux (Fig. 5) indicates that the process is catalyzed by specific transporter protein(s). The presence of this active efflux mechanism(s) is also consistent with the known properties of the membrane barriers of this organism. Thus, bile acids are able to penetrate at least through the OmpF porin channel of the outer membrane (Fig. 1), and unconjugated bile acids should be able to penetrate, albeit slowly, through the lipid bilayer domain of this membrane (see reference 23). Since these unconjugated bile acids exist largely in uncharged protonated forms at the acidic pH of the periplasm, they should cross the lipid bilayer domain of the cytoplasmic membrane easily, and the cloning from *E. coli* of a 7α-hydroxysteroid dehydrogenase gene, whose product appears to have no signal sequence (32), suggests that the substrates of this enzyme, bile acids, do reach the cytoplasm. In the presence of a constant influx of bile acids from the environment, an active efflux mechanism would be needed to avoid the toxic, detergent action of these compounds, in addition to their possible detoxification through metabolism. Interestingly, a *Eubacterium* sp., a gram-positive bacterium that metabolizes bile acids, was recently shown to produce an importer of bile acids that appears to be energized by the proton motive force (10).

We have attempted to identify the gene(s) that is responsible for the efflux of bile salts. Growth inhibition and accumulation studies using isogenic *acrA* and *emrB* mutants (Table 2; Fig. 2) showed clearly that these two known efflux systems, especially AcrAB, played a significant role in pumping out various bile salts. These two systems, however, could not entirely explain the efflux of these compounds. Thus, the accumulation level of CDC went up significantly upon deenergization with CCCP, even in the *acrA emrB* double mutant. Furthermore, with some bile salts, such as CDC or TOC, the double mutant still showed significant levels of resistance (Table 2). Finally, the TOC accumulation in everted vesicles was not diminished in the *acrA emrB* mutant (Table 3). The transport activity of the Acr-type pumps, which are composed of three proteins (14), is not easily detected in everted vesicle systems (16), possibly because of the disruption of the three-component structure. It appears, therefore, that our everted vesicle assay measured primarily the activity of the systems other than AcrAB and EmrAB. We have tried to identify the gene(s) responsible for this unknown efflux system by carrying out random insertion mutagenesis with Tn*phoA* and Tn*phoA′* elements (29). However, to date the only mutants obtained that were hypersensitive to bile salts were either deep rough lipopolysaccharide mutants producing a hyperpermeable outer membrane or regulatory mutants such as *hns*. One possible reason for our failure could have been the redundancy of the efflux systems mentioned above.

Efflux systems in bacteria can be classified, on the basis of energy source used, into proton antiporters and ATP-binding cassette (ABC) systems (17). Active accumulation of TOC in everted membrane vesicles without the addition of ATP, as well as the immediate inhibition of the transport process by CCCP (Fig. 2 and 3), strongly suggests that this bile acid excretion system, as well as AcrAB and EmrAB (14), is driven by the proton motive force. Accumulation of TOC in everted membrane vesicles was accompanied by the immediate lowering of ΔpH, but not of $\Delta\Psi$, across the membrane (Fig. 6 and 7). This suggests that this system functions by consuming ΔpH and that it may act as an electroneutral proton antiporter, pumping out bile acids in exchange for a proton that enters into the cytoplasm. Bile acids are anions, but a bile acid combined with a multivalent cation would make a cationic complex which could participate in an electrically neutral exchange with a proton. A good precedent for such a process is the tetracycline efflux protein Tet-catalyzed efflux of tetracycline by bacteria, in which a magnesium salt of tetracycline, with a +1 net charge, is exchanged for H^+ (31). Tetracycline chelates divalent cations (1), and this process enhances efflux. Bile acids appear to bind Mg^{2+} only weakly (3), but perhaps enough Mg^{2+} salts can be formed in the presence of the presumably high concentrations of this cation in the cytoplasm. We could not show this experimentally, however, because the accumulation of TOC occurred at undiminished rates in everted vesicles even without the addition of Mg^{2+}.

Another major division among the efflux systems in gram-negative bacteria depends on whether they transport their substrates across only the cytoplasmic membrane and into the periplasm or across both the cytoplasmic and outer membranes and directly into the medium (14). One distinction between these two classes of exporters is that the outer membrane permeability will not affect the susceptibility of cells if the transport occurs only across the cytoplasmic membrane, because what matters is the balance between the active efflux and the spontaneous influx across the cytoplasmic membrane. In contrast, the outer membrane barrier will have a pronounced effect if the export occurs across both cytoplasmic and outer membranes (14, 28). Indeed, the mutational loss of the OmpF porin has no effect on Tet-mediated resistance to tetracycline, whereas it increases the tetracycline MIC eightfold when the resistance is mediated by a mechanism, presumably AcrAB (18), that pumps out the drug directly into the medium (28). On this basis, at least some mechanisms of bile acid export appear to involve transport across both membranes, because (i) the nature of the porins produced influences the suscepti-

bility of *E. coli* to bile salts (Fig. 1) and (ii) Tn*phoA* transposon mutagenesis followed by screening for bile salt-hypersusceptible mutants led to the isolation of a number of deep rough mutants with more-permeable outer membranes, as mentioned above. In fact, it has been known for many years that deep rough mutants were hypersusceptible to bile salts (for a review, see reference 18). These results are as expected, since a significant part of efflux occurs through the AcrAB and EmrAB systems, known to catalyze transport of molecules directly into the external medium (14).

We can also approach the question of the nature of the transport system in a different way. Mathematical modeling suggests that a system exporting drugs only across the cytoplasmic membrane requires very high V_{max} values with amphiphilic and lipophilic substrates, since it is necessary for the efflux process to compete effectively against the rapid influx of such compounds through the lipid bilayer domains of the membrane (28). In contrast, the system catalyzing efflux across both the cytoplasmic and outer membranes can function with much lower V_{max} values, since the competing influx process occurs only slowly through the outer membrane barrier. Thus, the Tet system, which exports tetracycline only across the cytoplasmic membrane, is estimated to have a V_{max} of 5 to 20 nmol min^{-1}, in contrast to the estimated V_{max} of 0.2 nmol min^{-1} for the endogenous tetracycline efflux system, which exports the drug directly into the medium, presumably catalyzed by the AcrAB system (19). The V_{max} value of 0.3 nmol min^{-1} for TOC, observed in everted vesicles, was indeed in the range expected for systems catalyzing transport across both the inner and outer membranes, although it is not clear whether the V_{max} in vesicles accurately reflects the transport rate in intact cells.

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