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Susceptible *Escherichia coli* Cells Can Actively Excrete Tetracyclines

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Escherichia coli shows severalfold less susceptibility to tetracyclines when grown in enriched medium than in minimal medium. Transport studies with cells harvested from these media showed different handling of the drugs. Whereas an energy-dependent uptake of tetracycline and minocycline was observed in susceptible K-12 and wild-type *E. coli* strains grown in minimal medium, an active efflux of minocycline and, to a lesser extent, tetracycline was seen in cells grown in L broth and other enriched media. This efflux was replaced by an active uptake system after treatment of cells grown in L broth with EDTA. When assayed at a lower temperature (27°C), even cells grown in minimal medium showed an efflux of minocycline. Everted membrane vesicles prepared from susceptible cells grown in minimal medium or L broth showed an energy-dependent accumulation of minocycline and tetracycline when supplied with certain divalent cations. These results suggest that an active efflux of tetracyclines occurs in susceptible *E. coli* but is not detected in cells grown in minimal medium because greater permeability of the outer membrane allows a more rapid active uptake. This efflux system is distinct from that specified by tetracycline resistance determinants. Since the active efflux of minocycline in cells grown in L broth disappeared at external antibiotic concentrations of >100 μM, it may be saturable and so mediated by a membrane carrier.

More is known about how a bacterium takes up needed molecules or ions from its environment than how it excretes them. Among the latter processes are energy-dependent efflux mechanisms for sodium (30), calcium (6, 28) and protons (15, 27). Moreover, cells containing the appropriate resistance plasmids are able to excrete tetracyclines (3, 23), cadmium (35), and arsenate (25, 32). Recently, we have detected active efflux of tetracyclines in chromosomal mutants of *Escherichia coli* amplified for resistance (13). Presumably, there are other active excretory pathways which have evolved to remove foreign substances which enter the bacterial cells in their natural environment or are produced internally as waste products.

We have described elsewhere a biphasic uptake of tetracycline in susceptible *E. coli* grown and assayed in L broth (21). Only the second, slower uptake was energy dependent. When grown and assayed in minimal medium, the energy-dependent and -independent components showed similar kinetics but were separable by energy inhibitors. Recently, upon examining minocycline transport in susceptible *E. coli* cells grown in L broth and assayed in phosphate buffer, we unexpectedly found an apparent active efflux of the drug. We present here studies which show that *E. coli* cells grown in enriched medium accumulate less tetracycline and have less drug susceptibility than do cells grown in minimal medium. This decreased uptake is associated with a detectable active efflux of minocycline and, to a lesser extent, tetracycline. The findings demonstrate a previously undetected efflux of tetracyclines which may act via a system used to export other, yet unknown substrates.

MATERIALS AND METHODS

Bacterial strains. The sources of the three non-K-12 *E. coli* strains ML308-225, SLV41A, and Bsu⁻ and of K-12 strains JF50, JF568, and JF703 were described previously (22), as were sources of strains χ984 (11), HB101 (5), DW1021, AG8103, and AG100 (13). Other K-12 strains were obtained from B. Bachmann, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn., or from our laboratory collection.

Media and chemicals. Minimal medium A (23) was supplemented with 0.5% glycerol, and L broth (21) was supplemented with 0.1% glucose. Penassay broth was purchased from Difco Laboratories, Detroit, Mich. B broth was described previously (24). Minocycline hydrochloride and 7-*N*-dimethyl-[^{14}C]minocycline hydrochloride (specific activity, 12.2 mCi/mmol)

were gifts of Lederle Laboratories, Pearl River, N.Y. [7-^{3}H]tetracycline (0.7 Ci/mmol) and a ^{3}H-labeled L-amino acid mixture (1 mCi/ml) were obtained from New England Nuclear Corp., Boston, Mass. Fresh aqueous solutions of the radiolabeled drugs at ≤0.4 mM were prepared weekly and stored at −15°C. Solutions of the unlabeled drugs were prepared on the day of use. Carbonyl cyanide-*p*-trifluoro-methoxyphenylhydrazone (FCCP) and carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) were obtained from Sigma Chemical Co., St. Louis, Mo. Other reagents were prepared as described previously (22).

Susceptibility of cells to tetracyclines. (i) Determination of MIC. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic which prevented visible turbidity after 38 generations of growth in L broth (13 h for strain AG100 and 17 h for strain ML308-225) or medium A (38 h for strain AG100 and 48 h for strain ML308-225) starting from an initial inoculum of 10^4 cells per ml. Concentrations of antibiotic were chosen as described previously (22).

(ii) Inhibition of protein synthesis. Drug susceptibility was also determined by inhibition of protein synthesis. Cells were grown at 37°C in medium A or in L broth, washed twice at 37°C in cell assay buffer, and resuspended in polypropylene tubes at an absorbancy at 530 nm (A_{530}) of 3 in the same buffer containing 0.01% Casamino Acids (Difco) and 20 mM lactate. Fifteen minutes after drug addition, ^{3}H-labeled amino acids were added (final concentration, 4 μCi/ml). After 20 s and 4.0 min, 20-μl samples were precipitated with hot trichloroacetic acid on Whatman 3M paper disks, washed, and counted in Betafluor (National Diagnostics, Somerville, N.J.) in a liquid scintillation counter. The difference in incorporation at the two times was used to determine the rate of protein synthesis (which was shown in prior experiments to be linear for 4 min). The 50% inhibitory concentration was defined as that concentration of drug which inhibited protein synthesis by 50%. In medium A, the 20-s point was also used for comparisons.

EDTA treatment of cells. Cells were washed in 10 mM Tris-hydrochloride (pH 8) at 4°C and resuspended to an A_{530} of 5.0 for treatment with 10 mM potassium EDTA in 30 mM Tris-hydrochloride (pH 8) at 22°C for 30 min (strain ML308-225) or 15 min (strain JF703), as described previously (16, 22), except sucrose was omitted. EDTA treatment was terminated with 15 mM $MgSO_4$. Pelleted EDTA-treated cells were slowly resuspended in cell assay buffer to an A_{530} of 3.0 as described previously (22).

Preparations of everted vesicles. Everted vesicles were prepared from cells by lysis in a French pressure cell (23). The crude lysate was vortexed vigorously for 1 min before being centrifuged at a low speed; this mixing resulted in a clear demarcation between the unlysed cell pellet and the supernatant. Vesicles were sedimented for 1 h at 135,000 × *g* from the supernatant and resuspended in 10 mM Tris-hydrochloride (pH 8) with a thin glass rod (23), divided into 50-μl samples, quickly frozen, and stored at −70°C. An individual sample was used only once. For some vesicle assays involving divalent cations, residual EDTA was removed by washing the vesicles once in 10 mM Tris-hydrochloride (pH 8) before freezing.

Uptake of [^{14}C]minocycline and [^{3}H]tetracycline. (i) Cells. Uptake by cells at 37°C was measured with radiolabeled tetracycline and minocycline in 50 mM potassium phosphate–1 mM $MgSO_4$, (pH 6.1; cell assay buffer) by a filtration method as previously described (22), but without chloramphenicol. The internal concentration of labeled drug was calculated by assuming an internal cell volume of 1.1 μl/U of A_{530} (23).

(ii) Vesicles. Vesicles were thawed and diluted to 0.3 mg of protein per ml at 4°C in one of the following three assay buffers: buffer A, 50 mM potassium phosphate (pH 7.5); buffer B, 10 mM potassium phosphate (pH 7.5); or buffer C, 10 mM Tris-hydrochloride–150 mM KCl (pH 8.0). Cations, as the chloride salts, were added to these buffers as specified in individual experiments. Assays were performed in polypropylene tubes or in glass tubes which had been soaked overnight in sulfuric acid-dichromate solution (Chromerge; Manostat Corp., New York, N.Y.) and then rinsed thoroughly with deionized water. This treatment removed an activator(s) which variably contaminated the glass tubes. Assays were done at 30°C by filtration as described previously (23). Washing buffer was 100 mM LiCl containing 100 mM potassium phosphate, pH 7.5 (for assay buffers A and B), or 50 mM Tris-hydrochloride, pH 8 (for assay buffer C). Generally, radiolabeled drug was first added in the absence of the energy substrate and its incorporation was determined; then an energy substrate was added (lithium D-lactate to 20 mM; sodium ATP to 5 mM, sodium NADH to 5 mM, or 0.1 mM phenazine methosulfate plus 20 mM sodium ascorbate) and additional samplings were made. In experiments in which rates of uptake were measured, the energy substrate was added 1 min before the radiolabeled drug. The internal concentration of antibiotic was calculated with a vesicle volume of 8.4 μl/mg of vesicle protein (23).

Uptake of calcium by vesicles. Accumulation of $^{45}Ca^{2+}$ was performed as described previously (23).

Thin-layer chromatography. Silica gel G plates (Redi Plates; Fisher Scientific Co., Pittsburgh, Pa.) were pretreated with 0.1 M sodium EDTA and dried. Chromatography was performed with butanol–methanol–10% citric acid (1:1:2) in water. After chromatography, the plates were dried, exposed to ammonia vapor to allow visualization of minocycline under UV light, and divided into strips which were scraped off, and the radioactivity of the scrapings was determined in Betafluor.

RESULTS

Tetracycline susceptibility of *E. coli* cells grown in minimal medium or L broth. *E. coli* cells grown in minimal medium were three- to sevenfold more susceptible to tetracyclines than were cells grown in enriched medium (Table 1). Similarly, the drug concentration at which 50% of protein synthesis was inhibited was lower in cells grown in minimal medium than in those grown in L broth (Table 1). There was little difference in the MICs of minocycline and tetracycline for cells grown in minimal medium; a threefold difference was seen for strain AG100 grown in L broth.

TABLE 1. Susceptibility of *E. coli* K-12 and ML308-225 to tetracycline and minocycline

Strain and medium	Mean ± SD inhibitory concn (μM) of following drug[a]:			
	Minocycline		Tetracycline	
	IC_{50}	MIC	IC_{50}	MIC
AG100				
Medium A	3.2 ± 0.8[b]	1.1 ± 0.5	NT[c]	0.8 ± 0.3
L broth	4.8 ± 1.1	6.4 ± 1.9	NT	2.1 ± 0.1
ML308-225				
Medium A	0.6 ± 0.3[d]	0.2 ± 0.07	NT	0.33 ± 0.04
L broth	2.0 ± 0.3	1.5 ± 0.5[e]	NT	1.3 ± 0.3[e]

[a] IC_{50}, Concentration that inhibited protein synthesis by 50%; 2 μM ≈ 1 μg/ml. Values are the averages of two to three determinations.

[b] This value was 1.1 ± 0.1 μM when the 20-s point alone was compared with control. In L broth, use of the 20-s point alone did not give a 50% inhibitory concentration different from that observed with the rate of protein synthesis (20 s to 4 min).

[c] NT, Not tested.

[d] This value was 0.4 ± 0.13 when the 20-s point alone was compared with control.

[e] From reference 18.

Transport of tetracyclines in susceptible cells grown in enriched and minimal media. An energy-dependent accumulation of the tetracyclines occurred in susceptible *E. coli* ML308-225 grown in medium A and assayed in phosphate buffer; subsequent blocking of energy production by the uncoupler 2,4-dinitrophenol (DNP) caused the drug to flow out of the cells (e.g., see minocycline, Fig. 1A) (21–23). However, when the same strain was grown in L broth before being assayed in buffer, minocycline and tetracycline accumulation was much smaller, and the addition of DNP either decreased the uptake very little or, particularly in the case of minocycline, actually increased uptake (Fig. 1A). This result suggested the presence of an energy-dependent efflux of tetracyclines in susceptible cells.

Since this effect was more prominent for minocycline than for tetracycline, further studies of this possible efflux system were done with minocycline. Eleven genetically different *E. coli* K-12 strains showed an apparent efflux when grown in L broth (e.g., see strain JF50, Fig. 1B). The average (± standard deviation) minocycline accumulation for these K-12 strains examined at the same external minocycline concentration (5 μM) was 73 ± 6 μM before the addition of DNP (22 min after addition of label) and 145 ± 46 μM at 9 min after the addition of DNP (at 22.5 min after addition of label). The effect of DNP could be seen during the first minute of uptake (Fig. 1C) and after final steady-state concentrations were achieved. Four of these strains were sampled for uptake after being grown in medium A, and all showed an active uptake (data not shown). In some instances, cells grown in L broth showed no change or a small decrease in accumulation when tested with DNP; this was particularly true of strains ML308-225, Bsu$^-$, and SLV41A, all non-K-12 strains (see Fig. 2B1). In all cases, however, we observed much less active uptake in these strains than in cells

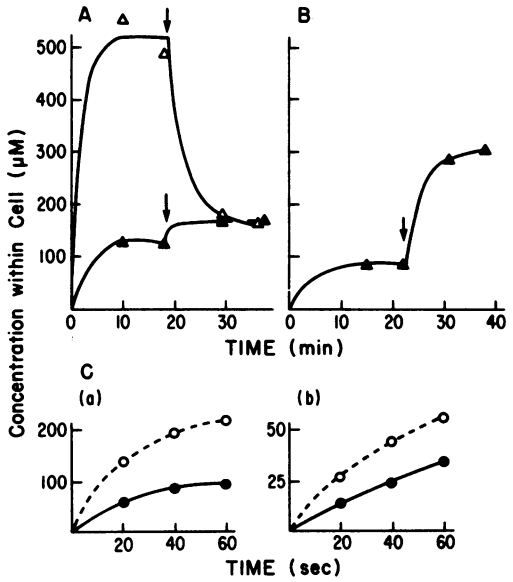

FIG. 1. Uptake of minocycline and tetracycline (5 μM each) by susceptible cells grown in medium A or L broth. (A) Uptake of minocycline by strain ML308-225. DNP (1 mM) was added at the times indicated by the arrows. △, Medium A; ▲, L broth. (B) Uptake of minocycline by strain JF50 grown in L broth. (C) Strain JF703 grown in medium A–glycerol–3% L broth supplemented with vitamin B1 (1 μg/ml) and proline, histidine, methionine, isoleucine, valine, adenine, and pyridoxine (100 μg/ml). (a), Minocycline; (b), tetracycline; ●, control cells; ○, 1 mM DNP added 10 min before antibiotic.

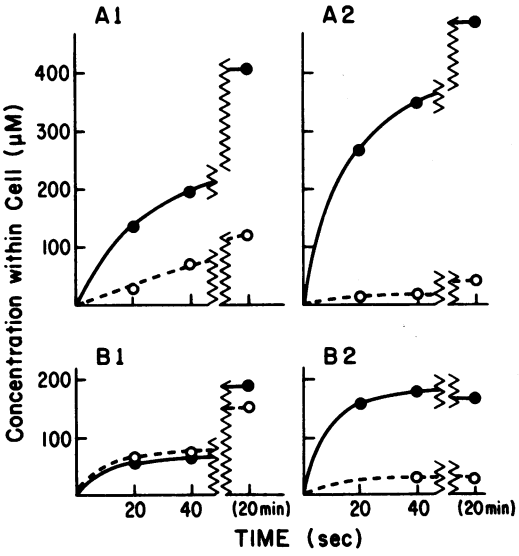

FIG. 2. Uptake of minocycline (4 μM) by control and EDTA-treated cells of strain ML308-225 grown in medium A (A) or L broth (B). EDTA treatment was carried out as described in the text. A1 and B1, control cells; A2 and B2, EDTA-treated cells; ●, cells without CCCP; ○, 100 μM CCCP added 10 min before [^{14}C]minocycline.

grown in minimal medium (22; Fig. 1A). Addition of 3% L broth to medium A was sufficient to produce an apparent efflux for strains grown in this medium (e.g., see strain JF703, Fig. 1C). Energy-dependent efflux was also seen for some cells grown in other enriched media, namely, Penassay broth and B broth (data not shown). Exchanging glucose for glycerol in medium A did not remove the active uptake system.

DNP also revealed an apparent active efflux of tetracycline in four strains grown in L broth (χ984, JF568, JF703, and HB101 [Fig. 1C]), but was not seen for AG100, in which little or no active uptake was noted.

The purity of [^{14}C]minocycline was checked by thin-layer chromatography, and ≥90% of the radioactivity was found to comigrate with unlabeled drug in an area 1 to 2 cm wide. Since it was not clear that this was a single component, and to eliminate the possibility that a radiolabeled contaminant in [^{14}C]minocycline was the molecule being effluxed, we measured uptake at cell densities from an A_{530} of 1 to 16 and found that the magnitude of the energy-dependent decrease in accumulation increased with cell concentration, reaching 40% of the total added radioactivity at an A_{530} of 16. We concluded that minocycline was the molecule being actively kept out of these cells.

To check whether, in our assay, cells grown in L broth could excrete substances which should as that used for minocycline. No active efflux occurred; rather, there was a large active uptake, about half that of cells grown in medium A. Thus, active efflux of minocycline occurred in cells actively accumulating proline.

Decreased outer membrane permeability of cells grown in L broth. EDTA is known to remove some lipopolysaccharide from the outer membranes of cells (17), making them more permeable to lipophilic substances (16, 17), including minocycline (22). EDTA treatment of strains ML308-225 and JF703 grown in L broth replaced the efflux with an active uptake (Fig. 2B1 and B2). Production of the active efflux itself was not exclusively the result of growth in L broth; susceptible ML308-225 and AG100 cells grown in medium A at 37°C but assayed at 27°C (pH 7.0) manifested an active efflux (data not shown). These findings suggested that growth in L broth decreased outer membrane permeability to the drug and so revealed a preexisting efflux system.

Transport of tetracyclines in everted membrane vesicles. Everted energized cytoplasmic membrane vesicles will concentrate substances which are actively excreted in intact cells as long as all necessary components for this transport are associated with the cytoplasmic membrane (10, 28). Everted vesicles prepared from strain ML308-225 showed a lactate-dependent accumulation of minocycline (Fig. 3A and B). Since NADH and ATP also served as energy sources (Fig. 3A), this uptake could not be due to contaminating right-side-out vesicles (1). Divalent cations were required for this accumulation. With lactate as the energy source, Ca^{2+}, Mn^{2+}, Zn^{2+}, and Mg^{2+} (in order of decreasing effectiveness) stimulated uptake with approximate optima of 0.1 to 0.5 mM (data not shown). These cations also stimulated NADH-dependent uptake. With NADH, calcium and zinc ions stimulated uptake even when vesicles were washed once in 10 mM Tris-hydrochloride (pH 7.5) to remove any EDTA remaining from the lysis buffer. ATP-dependent uptake required both Mg^{2+} and Ca^{2+} (0.5 mM each) or high levels (10 mM) of Mg^{2+} alone. This appeared to reflect a requirement of the membrane ATPase.

At 0.6 mg of vesicle protein per ml, about 25% of the radioactive [^{14}C]minocycline was actively accumulated, an amount too large to represent an impurity of the radiolabeled drug. Everted vesicles prepared from three other susceptible *E. coli* strains (HB101, AG8103, and DW1021) also manifested active uptake of minocycline with lactate, ATP, and NADH.

Everted vesicles also concentrated tetracycline with NADH, but only to two to five times the external concentration and only when Ca^{2+} (0.25 to 0.5 mM) was present. In our earlier

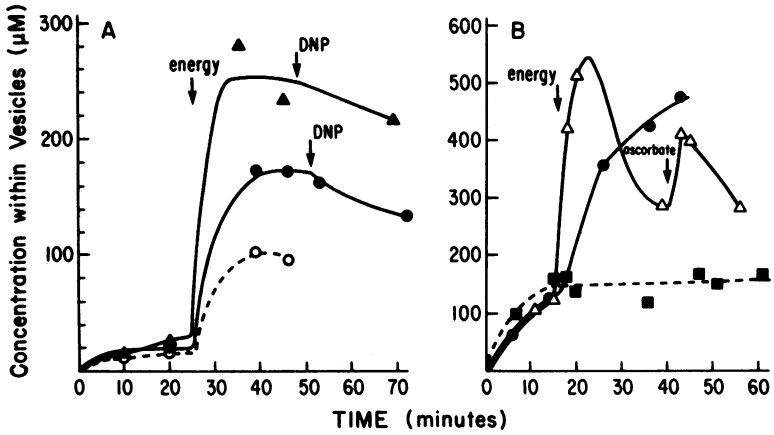

FIG. 3. Uptake of minocycline by everted vesicles of cells of strain ML308-225. (A) Everted vesicles prepared from cells grown in medium A were assayed in vesicle assay buffer B at 4 μM external minocycline. $CaCl_2$ concentration was 0.5 mM for NADH (▲) and lactate (●) and 0.3 mM for ATP (○); the assay with ATP also contained 1 mM $MgSO_4$. Energy substrates were added at 25 min. DNP (1 mM) was added at the times indicated by the arrows. (B) Everted vesicles prepared from cells grown in medium A plus 3% L broth were assayed in vesicle assay buffer C at 8 μM external minocycline and 0.25 mM $CaCl_2$. More ascorbate (20 mM) was added at the second arrow to the phenazine methosulfate-ascorbate-energized vesicles. ●, Lactate; △, phenazine methosulfate-ascorbate; ■, no energy.

work, in which we compared tetracycline uptake in everted vesicles prepared from susceptible and resistant cells (23), no calcium had been added. For this reason, tetracycline accumulation in vesicles from susceptible cells, as described here, had not been seen.

The pH of the assay buffer also affected minocycline accumulation. With lactate as energy source and 50 mM potassium phosphate as buffer, there was no active uptake of minocycline at pH 6.0 with 0.25 mM Ca^{2+}, whereas under the same conditions at pH 7.5, the lactate-dependent uptake concentrated the drug about 40-fold. With NADH, 0.25 mM Ca^{2+}, and 10 mM Tris-maleate buffer, a broad pH optimum for steady-state active uptake was seen between pH 8 and 9 (data not shown).

It seemed likely that this active accumulation was dependent on protonmotive force. When added before minocycline, 1 to 2 mM DNP, which destroys protonmotive force (15), inhibited NADH-dependent minocycline uptake by about 45 to 55%; DNP inhibited calcium uptake in these vesicles by 55 to 85%. Another dissipator of protonmotive force, FCCP, inhibited NADH-dependent minocycline uptake almost completely at 20 μM. For an unexplained reason, a third such inhibitor, CCCP, inhibited active minocycline uptake by only 15% even at 100 μM, whereas calcium transport was nearly eliminated in the same vesicle preparation. This lack of effect by CCCP was not due to an inhibition of its activity by minocycline, since CCCP inhibited Ca^{2+} accumulation in vesicles in the absence and presence of 100 μM minocycline to an equal extent (data not shown).

The minocycline which accumulated in energized vesicles appeared to be free in some cases but not in others. When phenazine methosulfate-ascorbate was the energy source, minocycline accumulation increased for 5 min but subsequently dropped, apparently because of ascorbate exhaustion. Addition of more ascorbate resulted in a repeat of this pattern (Fig. 3B), suggesting that accumulated minocycline was mostly free within the vesicle. However, with lactate or NADH as energy substrates, addition of DNP or FCCP after antibiotic accumulation often caused only a 10 to 15% loss of antibiotic from the vesicles (see Fig. 3A). This finding implied that the antibiotic was irreversibly bound or precipitated in the vesicle.

Possible saturability of active efflux assayed in cells and vesicles. By increasing the external concentration of minocycline, we could detect saturation of plasmid-mediated tetracycline efflux systems in intact cells, whereupon an active uptake of drug was revealed (22). Using this approach, we measured steady-state uptake levels of [^{14}C]minocycline in susceptible cells of strain AG100 grown in L broth at various external minocycline concentrations before and after the addition of 100 μM CCCP or 1 mM DNP. The active efflux seen at 5 μM external minocycline disappeared at about 100 μM minocycline, and an active uptake appeared at higher external drug concentrations (Fig. 4). This result could be attributed to saturation of an active carrier-

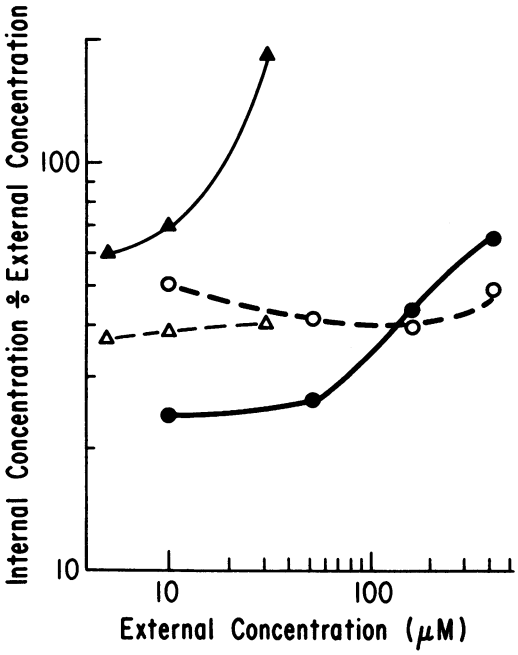

FIG. 4. Uptake of minocycline by cells of strain AG100 grown in medium A or L broth as a function of external minocycline concentration. Steady-state accumulation was measured 20 min after addition of labeled drug; 100 μM CCCP was then added, and the new steady-state level was determined 20 min later. ▲, Medium A, control; △, medium A, CCCP; ●, L broth, control; ○, L broth, CCCP. A $\log_{10}$ scale was used for both the abscissa and ordinate. Casamino Acids (0.01%) were included in the assay buffer to reproduce the conditions used in determination of the 50% inhibitory concentration.

mediated efflux, a specific enhancement of active minocycline uptake, or a nonspecific enhancement of active transport in general, as seen previously in cells grown in minimal medium (22). This last possibility was eliminated by examining the effect of high external minocycline concentrations on proline uptake in AG100 cells grown in L broth. No change in proline accumulation was noted in the presence of up to 200 μM minocycline.

To look for saturation in everted membrane vesicles, we added [^{14}C]minocycline (3 to 200 μM) 1 min after energization with NADH at Ca^{2+} concentrations of 0.25, 0.5 or 2 mM and measured the energy-dependent uptake rate with samples taken at 0.5 and 4.0 min. Vesicles from ML308-225 cells grown in minimal medium were used. Saturation was seen in some but not all experiments; the K_m in experiments which showed saturation was well above 100 μM. Unlabeled tetracycline and chlortetracycline were also used in competition experiments with [^{14}C]minocycline with equally equivocal results. The reason for this variability could not be determined, but it occurred even at 2 mM Ca^{2+}, at which concentration Ca^{2+} limitation due to chelation by high tetracycline concentrations (200 μM) should not be a problem.

Relation of efflux to chromosomal multiple antibiotic resistance (*marA*) gene. The *marA* gene (12) in *E. coli* K-12 is associated with amplifiable chromosomal resistance to tetracycline and minocycline and an efflux of these drugs (13). To determine whether the *marA* gene was involved in the efflux described here, we tested AG1005 *marA*, a mutant of AG100 in which the *marA*$^+$ locus was inactivated by Tn*5* insertion (12). This mutant was no more susceptible to tetracycline than the parent, as measured on gradient plates (12). When the mutant was grown in L broth and accumulation of minocycline by cells was measured at different external concentrations of drug before and after addition of CCCP, an energy-dependent efflux pattern identical to that of the parent (Fig. 4) was observed (data not shown). Therefore, the *marA* locus was not essential for this efflux in susceptible cells.

DISCUSSION

Susceptible *E. coli* cells and everted membrane vesicles of both K-12 and ML origin can use energy to efflux minocycline and tetracycline. The active efflux is detectable in intact cells only when they are grown in enriched media. The decreased accumulation of antibiotic correlated qualitatively, at least, with decreased inhibition of protein synthesis (Table 1). Growth in different media is known to cause changes in the protein species in the outer membrane (14). Changes in lipopolysaccharide have been associated with different growth temperatures (20), although correlations with growth rate or medium have not been reported. Our results suggest that growth in L broth has made the outer membrane less permeable to incoming tetracyclines. EDTA treatment eliminated at least part of this difference in permeability, whereupon net efflux was replaced by net active uptake. One study of susceptible *E. coli* suggested the existence of an active efflux of tetracycline, since the rate of loss of drug from cells treated with DNP or cyanide decreased by 60% (3). Still, there was net active accumulation of drug in these cells.

In cells grown in L broth, the minocycline efflux disappeared at external concentrations of >100 μM, which could be explained by saturation of a carrier-mediated efflux (22). Alternatively, this loss of efflux could result from drug-induced changes in or damage to the outer membrane (as was seen with EDTA) which enhanced uptake of the drug. Saturation experi-

ments with everted vesicles were inconclusive.

Molecules which are lipophilic cations or bases may be excluded from energized cells without the need for carriers (27, 29); this effect results from equilibration with the electrical potential or the pH gradient component of the protonmotive force (29). The tetracyclines in aqueous solution are complex dipolar ions with both acidic and basic protonated groups (2, 4, 7). They also chelate certain cations (7, 26). Whether they can cross membranes by diffusion as chelates, nonionic molecules, or dipolar ions is a subject of debate (8, 9, 31, 33). Therefore, whether they might equilibrate passively with protonmotive force and so be excluded from cells is not known. Since both active efflux and active uptake occur in susceptible cells, and probably both cannot be explained by passive equilibration, at least one of the two is likely to be carrier mediated.

It is puzzling how cells can show active excretion of drug at external concentrations to which they are susceptible. Several considerations may help explain this apparent paradox. First, a proportion of the accumulated drug is presumably not free, but is bound in the cell. Our estimates of the internal concentration of the drug are based on the assumption that the drug is mostly soluble in cell water. This cannot be the case, since the internal/external concentration ratio in the absence of energy is not 1, but about 40 (Fig. 4). In this situation, only about 2.5% of total drug associated with cells is free. Second, much more drug is associated with each cell than is needed to inhibit the 15,000 ribosomes (one drug molecule per ribosome [34]). At the MIC (6 μM), net minocycline accumulation in the absence of energy in cells grown in L broth is about 3.6×10^5 molecules per cell; with energy, only half of this amount is excreted (Fig. 4). We therefore propose that most of the tetracyclines which accumulate in these cells exist in at least three cellular compartments: the membrane, the ribosome, and the efflux system. Furthermore, since binding is reversible (3, 21, 22), it is likely that membrane-bound drug is in equilibrium with that on the ribosomes. Most of the active net uptake seen in cells grown in minimal media may also represent increased bound drug and not free drug. With energy, the amount of free drug is, therefore, difficult to estimate, but may not be so different for cells grown in L broth or minimal medium at the respective MICs.

It would be of interest to determine the identity of the usual substrate for the efflux system. Even if no carrier is involved, growth in enriched media lowered the capacity for net active uptake of the tetracyclines. This appeared to account for the diminished susceptibility of cells grown in enriched media.

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LITERATURE CITED

1. **Adler, L. W., and B. P. Rosen.** 1977. Functional mosaicism of membrane proteins in vesicles of *Escherichia coli*. J. Bacteriol. **129**:959–966.
2. **Asleson, G. L., and C. W. Frank.** 1976. pH dependence of carbon-13 nuclear magnetic resonance shifts of tetracycline. Microscopic dissociation constants. J. Am. Chem. Soc. **98**:4745–4749.
3. **Ball, P. R., S. W. Shales, and I. Chopra.** 1980. Plasmid-mediated tetracycline resistance in *Escherichia coli* involves increased efflux of the antibiotic. Biochem. Biophys. Res. Commun. **93**:74–81.
4. **Barringer, W. C., W. Shultz, G. M. Sieger, and R. A. Nash.** 1974. Minocycline hydrochloride and its relationship to other tetracycline antibiotics. Am. J. Hosp. Pharm. **146**:179–191.
5. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
6. **Brey, R. N., and B. P. Rosen.** 1979. Cation/proton antiport systems in *Escherichia coli*: properties of the calcium/proton antiporter. J. Biol. Chem. **254**:1957–1963.
7. **Brown, J. R., and D. S. Ireland.** 1978. Structural requirements for tetracycline activity. Adv. Pharmacol. Chemother. **15**:161–202.
8. **Caswell, A. H., and J. D. Hutchison.** 1971. Visualization of membrane bound cations by a fluorescent technique. Biochem. Biophys. Res. Commun. **42**:43–49.
9. **Colaizzi, J. L., and P. R. Klink.** 1969. pH-partition behavior of tetracyclines. J. Pharm. Sci. **58**:1184–1189.
10. **Futai, M.** 1978. Experimental systems for the study of active transport in bacteria, p. 7–41. *In* B. P. Rosen (ed.), Bacterial transport. Marcel Dekker, Inc., New York.
11. **Frazer, A. C., and R. Curtiss III.** 1975. Production, properties and utility of bacterial minicells. Curr. Top. Microbiol. Immunol. **69**:1–84.
12. **George, A. M., and S. B. Levy.** 1983. Gene in the major cotransduction gap of the *Escherichia coli* K-12 linkage map required for the expression of chromosomal resistance to tetracycline and other antibiotics. J. Bacteriol. **155**:541–548.
13. **George, A. M., and S. B. Levy.** 1983. Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in *Escherichia coli*: involvement of a non-plasmid-determined efflux of tetracycline. J. Bacteriol. **155**:531–540.
14. **Hall, M. N., and T. J. Silhavy.** 1981. Genetic analysis of the major outer membrane proteins of *Escherichia coli*. Annu. Rev. Genet. **15**:91–142.
15. **Harold, F. M.** 1972. Conservation and transformation of energy by bacterial membranes. Bacteriol. Rev. **36**:172–230.
16. **Leive, L.** 1968. Studies on the permeability change produced in coliform bacteria by ethylenediaminetetraacetate. J. Biol. Chem. **243**:2373–2380.
17. **Leive, L.** 1974. The barrier function of the gram-negative envelope. Ann. N.Y. Acad. Sci. **235**:109–129.
18. **Levy, S. B., and L. McMurry.** 1978. Plasmid-mediated tetracycline resistance involves alternative transport systems for tetracycline. Nature (London) **276**:90–92.
19. **Levy, S. B., L. McMurry, P. Onigman, and R. M. Saunders.** 1977. Plasmid-mediated tetracycline resistance, p. 181–203. *In* J. Drews and G. Hogenauer (ed.), Topics in infectious disease, vol. 2. Springer-Verlag, Vienna, Austria.
20. **McConnell, M., and A. Wright.** 1979. Variation in the structure and bacteriophage-inactivating capacity of *Sal-*

monella anatum lipopolysaccharide as a function of growth temperature. J. Bacteriol. **137**:746–751.

21. **McMurry, L., and S. B. Levy.** 1978. Two transport systems for tetracyclines in sensitive *Escherichia coli*: critical role for an initial rapid uptake system insensitive to energy inhibitors. Antimicrob. Agents Chemother. **14**:201–209.
22. **McMurry, L. M., J. C. Cullinane, and S. B. Levy.** 1982. Transport of the lipophilic analog minocycline differs from that of tetracycline in susceptible and resistant *Escherichia coli* strains. Antimicrob. Agents Chemother. **22**:791–799.
23. **McMurry, L., R. E. Petrucci, Jr., and S. B. Levy.** 1980. Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **77**:3974–3977.
24. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 433. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Mobley, H. L. T., and B. P. Rosen.** 1982. Energetics of plasmid-mediated arsenate resistance in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **79**:6119–6122.
26. **Newman, E. C., and C. W. Frank.** 1976. Circular dichroism spectra of tetracycline complexes with Mg^{++} and Ca^{++}. J. Pharm. Sci. **65**:1728–1732.
27. **Rosen, B. P., and E. R. Kashket.** 1978. Energetics of active transport, p. 559–620. *In* B. P. Rosen (ed.), Bacterial transport. Marcel Dekker, Inc., New York.
28. **Rosen, B. P., and T. Tsuchiya.** 1979. Preparation of everted membrane vesicles from *Escherichia coli* for the measurement of calcium transport. Methods Enzymol. **56**:233–241.
29. **Rottenberg, H.** 1979. The measurement of membrane potential and ΔpH in cells, organelles, and vesicles. Methods Enzymol. **60**:547–569.
30. **Schuldiner, S., and H. Fishkes.** 1978. Sodium-proton antiport in isolated membrane vesicles of *Escherichia coli*. Biochemistry **17**:706–711.
31. **Schumacher, G. E., and E. E. Linn.** 1978. Kinetic and thermodynamic aspects of *in vitro* interphase transfer of tetracyclines. II. Influence of divalent metal salts. J. Pharm. Sci. **67**:1717–1720.
32. **Silver, S., and D. Keach.** 1982. Energy-dependent arsenate efflux: the mechanism of plasmid-mediated resistance. Proc. Natl. Acad. Sci. U.S.A. **79**:6114–6118.
33. **Stezowski, J. J.** 1976. Chemical-structural properties of tetracycline derivatives. 1. Molecular structure and conformation of the free base derivatives. J. Am. Chem. Soc. **98**:6012–6018.
34. **Tritton, T. R.** 1977. Ribosome-tetracycline interactions. Biochemistry **16**:4133–4138.
35. **Tynecka, Z., Z. Gos, and J. Zajac.** 1981. Energy-dependent efflux of cadmium coded by a plasmid resistance determinant in *Staphylococcus aureus*. J. Bacteriol. **147**:313–319.