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Transport of the Lipophilic Analog Minocycline Differs from That of Tetracycline in Susceptible and Resistant *Escherichia coli* Strains

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Plasmids which specify resistance to tetracycline offer much less resistance to its more lipophilic analog, minocycline. Resistance to minocycline varies for different plasmids. In the case of plasmid R222 (bearing the class B tetracycline resistance determinant on Tn*10*), minocycline resistance is comparatively high (10 μg/ml, or 6% of the tetracycline resistance level). For plasmid pIP7 (bearing the class A determinant), minocycline resistance is only 1% of the tetracycline resistance level. To understand the basis for these differences, we compared the transport of the two tetracyclines by susceptible cells and by resistant cells. Uptake of minocycline by susceptible cells was 10 to 20 times more rapid than uptake of tetracycline and occurred largely via an energy-dependent route. This host-mediated energy-dependent uptake of both analogs was still present in tetracycline-resistant cells. In resistant cells, the same plasmid-mediated active efflux system previously described for tetracycline also exported minocycline. The 15-fold greater susceptibility of tetracycline-resistant R222-bearing cells to minocycline as compared with tetracycline could be explained at least in part by the more rapid influx of minocycline, which more easily overcame the efflux system. The particularly low minocycline resistance offered by pIP7 was due to a weak efflux for minocycline, 10-fold less effective than that mediated by R222. The rate-limiting step for uptake of both analogs appeared to be the outer membrane. That the lipophilic minocycline should cross this membrane more rapidly than tetracycline stands in contrast with other studies which show the outer membrane to be a barrier for entry of lipophilic substances.

Lipophilic substances are generally excluded from gram-negative bacteria because of the outer membrane lipopolysaccharide barrier (28). However, the lipophilic analogs are generally more, not less, active against tetracycline-resistant gram-negative bacteria (24). These findings suggested that the outer membrane might not be a barrier to the entry of lipophilic tetracyclines and that the resistance mechanism might be less effective against more lipophilic analogs.

Minocycline is a semisynthetic analog of tetracycline that is much more lipophilic (3). It has been extensively studied microbiologically and clinically (3, 4, 8, 13, 15). Among the four previously described different classes of tetracycline resistance determinants (A, B, C, and D) (22), the level of minocycline resistance is generally 5 to 10% that of tetracycline; it is considerably less for some of the class A determinants (6a, 22; unpublished results).

Tetracycline is accumulated in susceptible cells by both energy-independent and energy-dependent uptake systems (20). Whether a carrier is involved is unclear (19). Resistance in the *Enterobacteriaceae* is usually plasmid mediated and inducible to high levels by subinhibitory amounts of tetracycline (12). In *Escherichia coli*, resistance specified by each of the four determinants is effected at least in part by an active, carrier-mediated efflux of tetracycline (21). Whether minocycline resistance also involves an efflux system has been unclear, since an accompanying decrease in its uptake in resistant *E. coli* strains is reported by some workers (15) but not by others (31).

We report here that tetracycline and minocycline are exported by the same active efflux system in resistant cells. The host active uptake system for both analogs is still functioning in resistant cells, and minocycline enters largely via this system some 10 to 20 times more rapidly than does tetracycline. Net efflux of minocycline is consequently less, which at least partly explains why tetracycline-resistant cells are more susceptible to minocycline. The efflux system mediated by pIP7 functions minimally for mino-

cycline, accounting for the negligible resistance to minocycline of cells bearing this plasmid. The rate-limiting step for uptake of both analogs in susceptible cells appears to be passage across the outer membrane.

MATERIALS AND METHODS

Bacterial strains and plasmids. The prototroph non-K-12 *E. coli* strain ML308-225 (*lacI lacZ*) (34) was obtained from P. D. Bragg, University of British Columbia, Vancouver, British Columbia, Canada. Wild-type strain SLV41A was isolated from the surface of an apple. *E. coli* K-12 strain DO-1 (CSH2 F^-) and plasmid R222 were obtained from T. Watanabe, Keio University School of Medicine, Tokyo, Japan. *E. coli* B was obtained from E. B. Goldberg and K-12 strain JF50 from J. Felton, both of Tufts University School of Medicine, Boston, Mass. Plasmid pIP7 was received from Y. A. Chabbert, Pasteur Institute, Paris, France. A temperature-sensitive mutant in the tetracycline resistance determinant on R222 was made by mutagenizing R222-containing DO-1 cells with 1.5% ethyl methane sulfonate for 1 h in medium A containing 0.2 M Tris-hydrochloride (pH 7.4) following established procedures (23). Survival was 1.5%. Penicillin selection against induced tetracycline-resistant cells was done in L broth containing tetracycline (30 μg/ml) at 42°C after mutagenesis. The mutant plasmid pLY2 from one clone purified by replica plate selection to be temperature sensitive for tetracycline resistance was transferred by conjugation into ML308-225. Strains JF568, JF701, JF703, and JF694 (see Table 4) from J. Foulds were obtained through B. Bachmann, E. coli Genetic Stock Center, Yale University, New Haven, Conn.

Media and chemicals. Minimal medium A has been described previously (21); glycerol (0.5%) was the carbon source used. Minocycline hydrochloride and 7-*N*-dimethyl-[^{14}C]minocycline hydrochloride (12.2 mCi/mmol) were gifts of Lederle Laboratories, Pearl River, N.Y. Tetracycline hydrochloride was obtained from Sigma Chemical Co., St. Louis, Mo. [7-^{3}H]tetracycline (0.7 Ci/mmol) was obtained from New England Nuclear Corp., Boston, Mass. Fresh aqueous solutions of the radiolabeled drugs at 0.4 mM or less were prepared weekly and stored at −15°C. Solutions of the unlabeled drugs were prepared on the day of use. A 2 μM concentration of the tetracyclines is approximately equivalent to 1 μg/ml. Other reagents were as described previously (19).

Determination of MIC. Two methods were employed to measure the susceptibility of strains to the tetracyclines. (i) The lowest concentration of antibiotic which prevented visible turbidity after 38 generations of growth in L broth (20) (28 h at 30°C; 17 h at 37°C; 14 h at 42°C) starting from an initial inoculum at absorbance at 530 nm (A_{530}) = 10^{-5} was designated the minimal inhibitory concentration (MIC). The antibiotic concentrations chosen increased in increments of 15% of the magnitude of an initial approximate MIC. Increments began at about 50% of the MIC and stopped at about 200% of the MIC.

(ii) A gradient of antibiotic concentration was formed in a square petri dish by layering drug-containing Penassay agar on top of drug-free agar prehardened with a slanted surface (17, 32). Freshly grown cultures at A_{530} = 0.2 were swabbed onto these gradient plates 0.5 h after the top layer had hardened. The MIC was that concentration of drug at which confluent growth ceased after 40 h of incubation at 37°C.

Uptake of [^{14}C]minocycline and [^{3}H]tetracycline. Unless otherwise stated, cells used in uptake experiments were grown from A_{530} = 0.1 to A_{530} = 0.8 at 37°C in medium A with 0.5% glycerol as previously described (19). Plasmid-bearing cells were induced with 2 μM tetracycline during growth. After washing the cells twice at 37°C in 50 mM potassium phosphate (pH 6.0)–1 mM $MgSO_4$, uptake was measured in the same medium supplemented with 20 mM lithium lactate and 80 μg of chloramphenicol (160 μg for R222-containing cells) per ml; the filtration method previously described (19) was used, and uptake was stopped in 0.1 M LiCl–0.1 M potassium phosphate (pH 6.0). Background incorporation by filters in the absence of cells was subtracted. 2,4-Dinitrophenol (DNP) was added to a final concentration of 1 mM when used. For experiments involving EDTA-treated cells, spheroplasts, and control cells, 20% sucrose was added to the assay and stop buffers.

EDTA treatment and spheroplast formation. A method similar to that described previously (14) was used. Cells grown as described above to A_{530} = 0.8 in medium A were centrifuged at 4°C, washed twice in one-third the volume of 10 mM Tris-hydrochloride (pH 8) at 4°C, and resuspended to A_{530} = 5 in 30 mM Tris-hydrochloride (pH 8) containing 20% sucrose at room temperature. Potassium EDTA was added to a portion of the resuspension to a final concentration of 10 mM. Osmotically sensitive spheroplasts could be formed by immediate subsequent addition of egg white lysozyme to 0.3 mg/ml. After 30 min with stirring, cells or spheroplasts were collected from several milliliters at room temperature by centrifugation and resuspended in 50 μl of assay buffer (containing 20% sucrose) and 2 μg of DNase I. After the suspension had become homogeneous, the A_{530} was adjusted to 3 by the addition of more assay buffer containing 20% sucrose. In some experiments, EDTA or EDTA-lysozyme treatment was for 15 min instead of 30 min; then 15 mM $MgSO_4$ was added (5 mM to cells without EDTA), and the cells, EDTA-treated cells, and spheroplasts were collected as described above.

Determination of rate constant *k*. Every 15 to 20 s after the addition of radiolabeled tetracycline or minocycline to cells in assay medium, samples were removed to determine uptake as described above. Three or four such samplings were done in sequence. If DNP was used, it was added 10 min before the addition of the labeled drug. The initial rate of tetracycline uptake was determined by the slope of the first three or four determinations excluding time zero. Uptake was linear during this interval. The energy-dependent rate for tetracycline was defined as the difference between the slopes calculated for cells with and without DNP. For minocycline, since the uptake rate was faster and began to slow down even after 15 s, only the time zero point and the first point at 15 or 20 s were used to determine slopes and rates. The uptake rate was converted to nanomoles per A_{530} unit per minute. Previously, we had determined that 1 A_{530} unit represented 0.3 mg of cell protein (20). Assuming that 15% of this was membrane protein (14), then 1 A_{530} unit represented 0.045 mg of membrane protein. The uptake rate was then converted to nanomoles per milli-

gram of membrane protein per minute by dividing the original rate (in A_{530} units) by 0.045 mg/A_{530} unit. Finally, the absolute rate constant k was derived by dividing this rate by the external concentration of drug expressed in nanomoles per liter. The units of k are liters per milligram per minute. In the graphs, uptakes are presented as concentration (micromolar) of drug within cells. These values are calculated from values as nanomoles per A_{530} unit by dividing by 0.0011 ml, the volume of 1 A_{530} unit of cells (21); the volume in 20% sucrose is probably somewhat less, but the same value was used. To convert micromolar concentration to nanomoles per milligram of membrane protein, one multiplies the micromolar concentration by 0.0244.

Standard deviation. Values are reported as ±1 standard deviation.

RESULTS

Susceptibility to tetracycline and minocycline. The susceptability to tetracycline and minocycline of ML308-225 cells bearing plasmids pIP7 (class A determinant) or R222 (class B determinant) was compared (Table 1). Cells without either plasmid were equally susceptible to both analogs. Plasmid-bearing cells induced for resistance were much less resistant to minocycline than to tetracycline. We calculated the net increase in resistance due to the presence of the plasmid. On this basis, R222 offered 6% as much resistance to minocycline as to tetracycline, whereas pIP7 offered only 1% as much resistance to minocycline.

TABLE 1. Tetracycline and minocycline MICs[a]

Antibiotic	MIC (μM) ± SD		
	Susceptible cells	pIP7	R222
Tetracycline	1.3 ± 0.3	105 ± 10	330 ± 30
Minocycline	1.5 ± 0.5	2.5 ± 0.7	21 ± 2.5
Minocycline ÷ Tetracycline[b]		0.01	0.06

[a] The host strain was ML308-225. Tetracycline (2 μM) was present during MIC determinations (by method i) on resistant cells to maintain induction. Two to four determinations were made.

[b] MIC of susceptible cells was subtracted before calculation of ratio.

Uptake of minocycline and tetracycline by susceptible cells. Differences in the susceptibilities of resistant cells to tetracycline and minocycline probably involved transport differences. Before examining resistant cells, it was necessary to compare the transport of these two drugs in susceptible cells. As before (19, 21), DNP was used as an energy inhibitor to distinguish energy-dependent from energy-independent uptake. The total initial rate of uptake of minocycline in strain ML308-225 was about 20 times that for tetracycline at external concentrations of 4 μM (Fig. 1 and Table 2). Minocycline uptake also proved to be faster than that of tetracycline in the three other strains of *E. coli* tested (Table 2). Except in the case of SLV41A, the initial rate for minocycline appeared to reflect mostly energy-dependent uptake; for tetracycline, the proportion attributable to active uptake was a little lower. In deenergized cells, an extremely rapid initial uptake or binding took place in less than 15 s, followed by a slower equilibration (Fig. 1).

Accumulation had reached a plateau by 30 min. We measured this "steady-state" accumulation as a function of external drug concentration in the absence and presence of DNP (Fig. 2). DNP was added either before the labeled drug or after the 30 min of uptake with similar results. Data are presented as the ratio of internal to external concentrations versus external concentration. At low levels of drugs, net actively accumulated minocycline was about 60 times the external concentration; for tetracycline, the value was 7 to 8 times the external concentration. The energy-independent accumulation appeared to be 30 to 40-fold above the external concentration for minocycline and 6 to 10-fold for tetracycline. Presumably, most of this incorporation was bound rather than free (see Discussion). However, above external concentrations of 10 μM (minocycline) or 40 μM (tetracycline), an abrupt increase in the ratio of internal to external concentration occurred in the energized cells (Fig. 2). This unusual step-up was not unique to the tetracyclines; when proline (at 0.4 μM) was the labeled transported molecule, a step-up was also seen when external unlabeled minocycline was 40 μM or tetracycline was 80 μM (data not shown). Although yet unexplained, this phenomenon did not interfere with interpretation of subsequent experiments.

Host-mediated active uptake system retained in resistant cells; weak minocycline efflux specified by pIP7. Both pIP7 and R222 offer less resistance to minocycline than to tetracycline. In addition, cells bearing pIP7 are more sensitive to minocycline than are those bearing R222. If the endogenous uptake system remained functioning in resistant cells, one possible explanation for lowered minocycline resistance would be that net efflux was less because of the higher minocycline uptake rate noted above. Alternatively, the efflux system might be less able to export minocycline than tetracycline. Moreover, pIP7 might not mediate minocycline efflux at all.

To see whether the host active uptake remained in resistant cells and to measure the strength of the efflux in resistant cells, we used the following rationale. We had shown, using everted membrane vesicles, that the active efflux carrier was saturable by tetracycline (21). Therefore, if enough drug could be added externally to bring the internal levels sufficiently above the K_m for efflux, this carrier should become saturated in whole cells. Such satura-

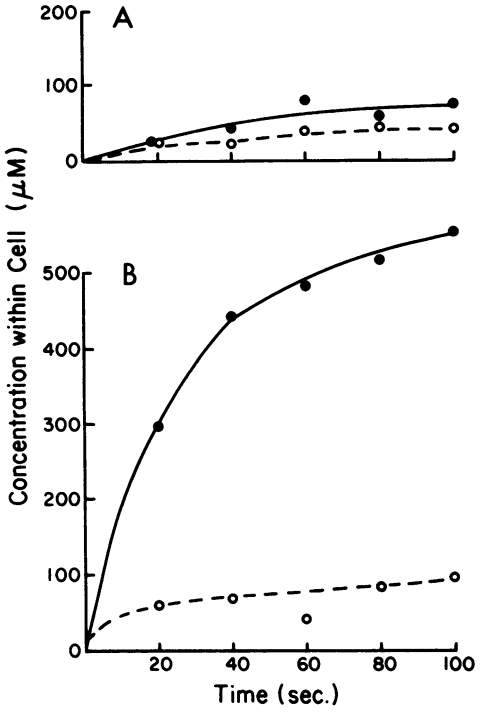

FIG. 1. Initial uptake of 4 μM tetracycline (A) and 4 μM minocycline (B) by susceptible cells. Uptake was measured during the first 100 s after the addition of radiolabeled antibiotic to ML308-225 cells. The internal concentrations were calculated as described in the text; the external concentration of each drug was 4 μM. DNP (1 mM) was added 10 min before the addition of labeled drug. ○, DNP; ●, control.

tion could be detected by measuring steady-state accumulation of drug by cells in the absence and presence of DNP. Normally, addition of energy inhibitors such as DNP or cyanide to resistant cells causes an increase in steady-state tetracycline levels (18), since active efflux is inhibited in these deenergized cells (21). However, if this efflux were saturated, accumulation in energized cells would no longer be lower. In fact, if resistant cells retained the active uptake system of the host cell (which we found to be unsaturable), this active uptake system might become detectable at high external drug levels when the efflux system had been saturated.

We therefore measured steady-state accumulation (30 min after addition of label) in resistant cells as a function of external drug concentration in the presence and absence of DNP. These experiments were identical to those described above for susceptible cells. Net active efflux was declared if steady-state uptake in the presence of the energy inhibitor DNP was greater than that in its absence.

We first used cells bearing plasmid R222 which were induced for resistance. Net efflux of tetracycline was unimpaired, even at external tetracycline levels of 1,000 μM (Fig. 2A). The findings were different with minocycline. Whereas an efflux of minocycline was seen at concentrations less than 6 to 7 μM (Fig. 2B), an active uptake of minocycline was clearly revealed above this level (Fig. 2B): the crossover point was called *Cx*. Above 20 μM, the active uptake was 100 times the external concentration, nearly equal to the 200-fold factor seen for susceptible cells (Fig. 2B).

We next examined cells harboring plasmid pIP7. These cells (Table 1) had a 3-fold lower resistance to tetracycline than did the R222-containing cells and a 20-fold lower net resistance to minocycline. Unlike the R222-bearing cells, net active tetracycline efflux in cells with pIP7 disappeared at a *Cx* of about 5 μM (Fig. 2A). As the external level increased, active uptake appeared. However, the amount of tetracycline within the cells remained below that of susceptible cells at the same external concentration (indicating that the tetracycline efflux was not yet saturated) until the abrupt step-up which

TABLE 2. Rate constant *k* for initial uptake[a]

Susceptible *E. coli* strain	Uptake	*k* (liters/mg of membrane protein per min) × 10^3		Mc/Tc
		Mc	Tc	
ML308-225[b]	Total	5.6 ± 0.2	0.26 ± 0.05	22
	Energy dependent	4.8 ± 0.5	0.16 ± 0.05	30
B[c]	Total	8.4	0.88	9.6
	Energy dependent	6.8	0.58	11.7
JF50[c]	Total	7.8	0.67	11.6
	Energy dependent	5.7	0.37	15.4
SLV41A[b]	Total	3.6 ± 0.6	0.45 ± 0.05	8.0
	Energy dependent	1.7 ± 0.2	0.28 ± 0.006	6.1

[a] Mc, Minocycline; Tc, tetracycline.
[b] Two experiments; mean ± standard deviation.
[c] One experiment.

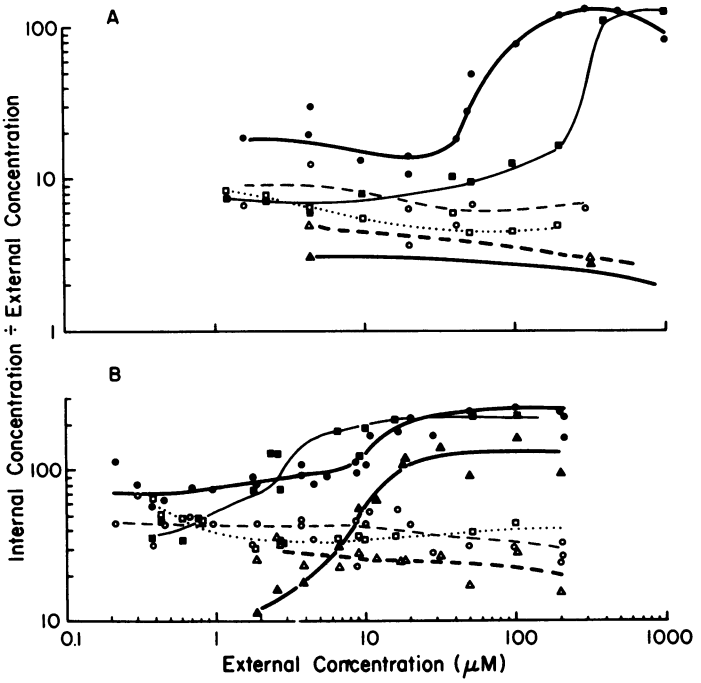

FIG. 2. Ratio of internal steady-state concentration of drug to external concentration of drug as a function of external concentration of tetracycline (A) and minocycline (B) in susceptible and resistant cells. Steady-state levels of uptake achieved after 30 min in drug were measured in the absence (closed symbols) or presence (open symbols) of 1 mM DNP. These levels, calculated as internal micromolar concentration (see text), were divided by the external concentrations (micromolar) to give the ratios plotted on the ordinate. ML308-225 cells containing plasmid R222 or plasmid pIP7 were induced to resistance before the assay. Symbols: ● and ○, no plasmid; ▲ and △, R222; ■ and □, pIP7.

occurred between 250 and 400 μM. Active efflux of minocycline in these cells was seen only below 0.6 μM. Above this level, an active uptake of minocycline was seen (Fig. 2B), reaching an in/out ratio of 200 at about 8 μM, which was somewhat lower than the external concentration required by susceptible cells to achieve the same ratio.

From these results it was evident that the host-mediated active uptake system for the tetracyclines was retained in resistant cells. It also appeared that the particularly low resistance to minocycline in cells harboring pIP7 could be explained by a weaker minocycline efflux system mediated by pIP7, inasmuch as cells bearing pIP7 could maintain net minocycline efflux only up to an external drug concentration of 0.6 μM, $^1/_{10}$ the concentration found for cells bearing R222.

Efflux system export of minocycline and tetracycline. To ascertain whether both tetracyclines were exported by the same carrier, we measured steady-state accumulation of labeled tetracycline in the presence of unlabeled minocycline, and vice versa. Cells bearing R222 were used. First, various concentrations of unlabeled minocycline were added with 3.4 μM [^{3}H]tetracycline. At about 10 μM unlabeled minocycline, the efflux disappeared, and at higher minocycline levels an active tetracycline uptake was seen. At 200 μM unlabeled minocycline, the highest concentration tested, the in/out ratio of energized cells was 50 and that of deenergized cells was 5. Presumably, minocycline was saturating the tetracycline efflux system. We then examined whether unlabeled tetracycline could saturate active efflux of minocycline. Indeed, at about 100 μM unlabeled tetracycline, efflux of [^{14}C]minocycline (at 1.8 μM) disappeared and an active uptake appeared. At 400 μM tetracycline, the highest concentration tested, the in/out ratio of [^{14}C]minocycline in energized versus deenergized cells was 30 and 15, respectively, so the minocycline efflux system had only begun to saturate.

It appeared, therefore, that the efflux of both analogs probably occurred via the same saturable carrier, since each analog antagonized the efflux of the other. To verify this finding, we examined uptake in cells bearing a temperature-sensitive tetracycline resistance determinant on R222. At increasing external drug concentrations we measured steady-state levels of minocycline and tetracycline accumulation at 30°C, then shifted the cells to 42°C and repeated the measurements. The external concentration at

which active efflux just disappeared and active uptake appeared (*Cx*) was determined. For both tetracycline and minocycline, *Cx* was lower and more temperature sensitive for cells bearing pLY2 (the mutant R222) as compared with wild-type R222 (Table 3). This result confirmed that both drugs used the same efflux system.

Rate-limiting barrier for tetracycline and minocycline uptake in susceptible cells. Although it was more lipophilic than tetracycline, minocycline entered susceptible cells more rapidly. This finding contrasted with other work which has shown lipophilicity to hinder, not enhance, entry of molecules into gram-negative bacteria; the outer membrane was considered the barrier particularly effective against lipophilic substances (28). We therefore examined whether the outer membrane might not be the rate-limiting step for uptake of minocycline and tetracycline in susceptible cells.

The removal of the outer membrane during formation of spheroplasts by EDTA-lysozyme treatment (see reference 2) increased the rate constant *k* (see Materials and Methods) for active uptake of minocycline more than 3 to 5 times (Table 4 and Fig. 3). The initial rate was too rapid to determine precisely with 15-s samplings (Fig. 3), as noted in Table 4, so the increases are minimal estimates. The active tetracycline uptake rate in spheroplasts was about 1.7 times that in cells (Table 4 and Fig. 3). In this case, uptake during the first minute was linear, so this slope was used to determine the rate constant; the active accumulation at 15 s was too small to use for accurate measurement of rate. The results with the two analogs indicated that the outer membrane was rate limiting for entry of minocycline and tetracycline into cells.

We then asked whether the two analogs crossed the outer membrane via the hydrophilic channels formed by porin proteins (25, 27–29). Earlier work had shown that cells deficient in outer membrane porin protein OmpF were 3 times as resistant to tetracycline but only 1.5 times more resistant to minocycline (6), suggesting that tetracycline, but not minocycline, crossed the outer membrane via OmpF porins. We reexamined the role of porins on susceptibility to the two analogs and confirmed the previous results. Cells lacking the OmpF porin were 2.7 times as resistant to tetracycline but only 1.5 times as resistant to minocycline (Table 5). Absence of OmpC porin had no effect, as reported previously (6). Thus, tetracycline indeed entered primarily via OmpF, whereas minocycline did not. When OmpF (and OmpC) were lacking, the presence of the new membrane porin NmpA restored susceptibility to tetracycline (Table 5). In these experiments, we noted that the parent strain was 2.5 times more resistant to minocycline than to tetracycline, whereas no difference had been seen for ML308-225 (Table 1); several other K-12 strains were also similarly less susceptible to minocycline (data not shown).

Permeability of the outer membrane, particularly to lipophilic substances, can be increased by treating cells with EDTA (16, 17, 30). We found that EDTA treatment of cells increased the rate constant *k* for active minocycline uptake more than 2.5 times but had no effect on tetracycline uptake (Table 4).

DISCUSSION

Plasmids bearing tetracycline resistance determinants mediate considerably less resistance to the lipophilic analog minocycline than to tetracycline. Cells bearing pIP7 are particularly susceptible to minocycline. We showed here

TABLE 3. Efflux mediated by R222 and a mutant R222 temperature sensitive for resistance[a]

Determination and plasmid	μM Tc		μM Mc		Ratio[b] 42°C/30°C	
	30°C	42°C	30°C	42°C	Tc	Mc
Cx[c]						
R222	>600	≥600	12	4.5	<1	0.38
R222 temperature sensitive (pLY2)	150	11	1.3	≤0.08	0.073	≤0.062
MIC[d]						
R222	540 ± 18	180 ± 45	24 ± 1	13 ± 3	0.33	0.54
R222 temperature sensitive (pLY2)	425 ± 35	24 ± 1.4	18 ± 2	1.6 ± 0.1	0.055	0.035
Susceptible cells	0.75 ± 0.07	0.65 ± 0.07	0.75 ± 0.07	1.03 ± 0.04	0.87	1.37

[a] Tc, Tetracycline; Mc, minocycline.

[b] MIC of susceptible cells was subtracted from that of resistant cells before calculation of ratio.

[c] *Cx* was obtained from a graph of log of analog uptake versus log of external concentration of that analog; data were obtained both with and without DNP. The external concentration at which the curves with and without DNP intersected equaled *Cx*, which signaled efflux saturation.

[d] Measured by method i as described in the text.

TABLE 4. Effect of EDTA alone or plus lysozyme upon the rate constant k for initial uptake[a]

Treatment	Uptake	k[b] (liters/mg of membrane protein per min)	
		Minocycline	Tetracycline
Part 1			
Cells	Total	2.8×10^{-3}	0.28×10^{-3}
	Energy dependent	2.1×10^{-3}	0.21×10^{-3}
Spheroplasts	Total	11.3×10^{-3}[c]	0.42×10^{-3}
	Energy dependent	10.8×10^{-3}[c]	0.36×10^{-3}
Part 2			
Cells	Total	3.2×10^{-3}	0.26×10^{-3}
	Energy dependent	2.3×10^{-3}	0.21×10^{-3}
Cells + EDTA	Total	5.9×10^{-3}[c]	0.28×10^{-3}
	Energy dependent	5.5×10^{-3}[c]	0.24×10^{-3}
Spheroplasts	Total	8.0×10^{-3}[c]	ND[d]
	Energy dependent	7.5×10^{-3}[c]	ND

[a] Part 1: cells were treated for 30 min. Part 2: cells were treated for 15 min; the EDTA was then neutralized with $MgSO_4$ as described in the text before centrifugation. Spheroplasts are cells treated with both EDTA and lysozyme.

[b] k Determined as described in the text.

[c] Uptake too rapid for determination of precise values; these are minimal values.

[d] ND, Not determined.

that cells containing pIP7 or R222 manifested an active efflux for minocycline (as we had previously shown for tetracycline), but that the efflux specified by pIP7 was weaker than that specified by R222. Competition experiments with tetracycline and minocycline in cells bearing R222 indicated that the two analogs were using the same efflux carrier; this conclusion was substantiated by the finding that a temperature-sensitive mutation in the resistance determinant on R222 led to temperature-sensitive efflux of both tetracycline and minocycline. Susceptible cells actively accumulated minocycline at a much greater rate than they did tetracycline. By saturating the efflux system, we could demonstrate that this endogenous host active uptake system for minocycline and tetracycline was still functioning in resistant cells. Part of the 30-fold greater rate of uptake of minocycline via this system was reflected in an 8-fold higher steady-state accumulation of minocycline over tetracycline in susceptible cells without any increase in susceptibility. The remaining portion of the greater rate of minocycline uptake presumably contributes to the increased susceptibility to minocycline in resistant cells. It is also possible that the efflux system itself is less able to transport minocycline than tetracycline. If both drugs were exported equally well in cells containing R222, net tetracycline efflux might be expected to disappear at external concentrations some 30 times greater than that at which net minocycline efflux disappeared. In fact, however, net tetracycline efflux had not disappeared by 1,000 μM, whereas net minocycline efflux was gone above 6 to 7 μM, implying that minocycline might be exported less well.

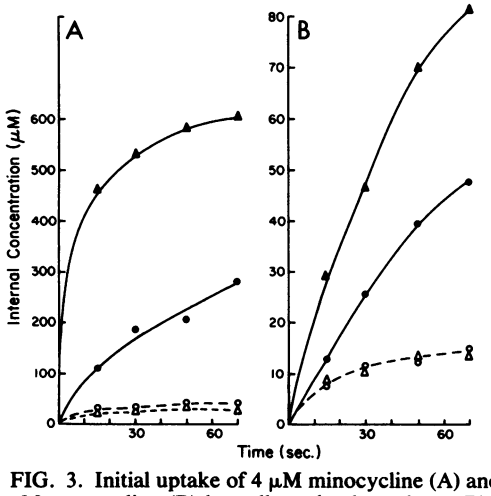

FIG. 3. Initial uptake of 4 μM minocycline (A) and 4 μM tetracycline (B) by cells and spheroplasts. The spheroplasts (▲, △) were made by treating ML308-225 cells (●, ○) with EDTA and lysozyme in sucrose as described in the text. Closed symbols, without DNP; open symbols, with DNP. The assay was performed in 20% sucrose; the external concentration of the antibiotics was 4 μM.

Whether lipophilicity accounts for the differences seen between minocycline and tetracycline is not certain. However, in general, lipophilic tetracycline analogs are more effective than are hydrophilic analogs against resistant cells (24) and, in certain cases, might be excluded from these cells less well (6a). It is possible that lipophilicity for the tetracyclines increases

TABLE 5. Resistances of porin mutants to tetracycline and minocycline

Strain	Characteristics	MIC (μM)[a]			
		Tetracycline	R	Minocycline	R
JF568	Parent[b]	1.5 ± 0.2	1.0	3.6 ± 0.3	1.0
JF701	OmpC missing[c]	2.1 ± 0.3	1.4	4.2 ± 0.8	1.1
JF703	OmpF missing[d]	4.0 ± 0.8	2.7	5.5 ± 1.2	1.5
JF694	OmpC and OmpF missing; NmpA present[e]	1.4 ± 0.2	0.93	3.4 ± 0.6	0.93

[a] MICs were measured by using method ii as described in the text. Four determinations were made. R is the MIC of each strain divided by the MIC of JF568.
[b] *proC, aroA, his, purE, ilv, met, lacY, xyl, rspL, cycA, cycB?, tsx,* λ^-, F^- (10).
[c] *ompC264* (11).
[d] *ompF254* (5).
[e] *ompC263, ompF254; nmpA1* (10).

the rate of uptake and at the same time decreases the ability of the efflux system to handle the drug. The active site of the efflux carrier or access to it may accommodate hydrophilic molecules better.

The net efflux of tetracycline in R222-containing cells was not saturated, even at an external concentration of 1,000 μM, yet in cells bearing pIP7 (which were one-third as resistant) net active uptake appeared (Fig. 2) at the unexpectedly low external concentration of 5 μM. Although saturation was not complete and active uptake was not maximal until levels above 400 μM, cells containing pIP7 were actively accumulating tetracycline at external levels of drug in which they were presumably resistant. This finding raises questions about whether efflux can be the sole mechanism for resistance.

Our results can account for a puzzling observation that in resistant *E. coli* (bearing R64 with a class B resistance determinant [22]), minocycline was actively accumulated to levels as high as those seen in susceptible cells, whereas tetracycline uptake was lower and was not energy dependent (8). We can now understand that these findings were a result of the use of an external concentration of 20 μM for minocycline, sufficient to saturate the active efflux system (particularly in the uninduced cells used) and thereby reveal the active uptake system. A similar effect presumably explains the equal uptake of minocycline seen in susceptible cells and cells containing a constitutive Tn*10* (class B) mutant (31), since in that case 200 μM external minocycline was used.

Although minocycline achieves a higher energy-dependent steady-state accumulation than does tetracycline, susceptible cells are not more susceptible to this analog. This would occur if minocycline were less effective at the ribosomal level, although it has been reported both that the two analogs had similar activities in a cell-free system (9) and that minocycline was more active (32a). Alternatively, some discrepancy may result from measurement of susceptibility in enriched broth and uptakes in simple buffer. Finally, much of the actively accumulated minocycline could be located in an "irrelevant" compartment, e.g., within the cytoplasmic membrane, where it could not inhibit protein synthesis. If this were the case, the greater rate constant *k* for active minocycline uptake compared with that for tetracycline would be immaterial as far as susceptibility to these two drugs was concerned. In deenergized cells, the greater uptake of minocycline may be caused by a greater solubility of this analog in membranes.

We found that the rate-limiting step for active uptake of both minocycline and tetracycline was the outer membrane. The route used to cross this membrane was not the same for the two drugs. Tetracycline (molecular weight, 444) appeared to use the hydrophilic pores formed by OmpF proteins. The exclusion size for these pores is about 600 (28). Minocycline (molecular weight, 457) either used both OmpF and OmpC pores or, more likely, used neither. Its rate of uptake, like that known for other lipophilic molecules, was enhanced by EDTA treatment. Such treatment removes part of the lipopolysaccharide from the outer membrane (17) and may thereby form phospholipid patches through which lipophilic substances can diffuse (28). Further evidence for a lipophilic pathway for minocycline entry is that some "deep rough" mutants are more susceptible to minocycline than are the parent strains, whereas little difference is seen for tetracycline (1; unpublished experiments). The lipopolysaccharide of the outer membrane of deep rough mutants is defective, and with respect to permeability these mutants behave much like EDTA-treated cells (26, 28).

Even tetracycline is a relatively lipophilic molecule (26). Our results suggested that it was hydrophilic enough to cross the outer membrane via the hydrophilic pores, but that this step was slower than passage across the cytoplasmic membrane and was therefore rate limiting. Minocycline appeared instead to cross the outer membrane by another pathway more character-

istic of lipophilic substances; this pathway was also rate limiting for active transport into the cell. Generally, hydrophilic substances are thought to use hydrophilic pores to cross the outer membrane of gram-negative cells more rapidly than lipophilic substances can cross by some other mechanism not involving these pores (26, 28). Our results showed, however, that the more lipophilic molecule, minocycline, crossed the outer membrane 10 to 20 times faster than tetracycline.

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