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Tetracyclines of Various Hydrophobicities as a Probe for Permeability of *Escherichia coli* Outer Membranes

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The outer membrane of gram-negative cells excludes hydrophobic molecules and is responsible for the resistance of these cells to a number of dyes, detergents, and antibiotics. We describe a test for hydrophobic permeability in which a series of tetracyclines with various hydrophobicities are used. Normal *Escherichia coli* cells became more resistant as hydrophobicity was increased in this series, but mutants altered in outer membrane permeability remained susceptible. A mutant lacking all polysaccharide except 2-keto-3-deoxyoctonic acid in its lipopolysaccharide is virtually as susceptible to the hydrophobic drug 13-phenylmercapto-α-6-deoxytetracycline as to oxytetracycline (MIC 100 times lower than that of the wild type), and a mutant with another, as yet undefined outer membrane defect, *acrA*, also shows increased, although somewhat lesser, susceptibility (MIC 20 times lower than that of the wild type). Increased susceptibility to this tetracycline derivative is associated with greater fluorescence of the derivative when added to the cells, which we interpret as increased interaction of the derivative with hydrophobic domains, such as membranes, in the mutants. This series of tetracyclines may provide an assay for measuring the permeability of gram-negative organisms and their mutants to hydrophobic molecules.

The outer membrane of gram-negative cells is unusual in its permeability properties. Small hydrophilic molecules (in enteric organisms, those ca. ≤600 daltons) enter via pores, but hydrophobic molecules which penetrate other membranes by partitioning into and through the lipid bilayer are excluded (19, 20); the molecular mechanism of this exclusion is still in doubt. Although lipopolysaccharide (LPS), which forms all or most of the lipid of the exterior leaflet of this membrane, is involved (12), it has not been possible to define its role unequivocally by analysis of mutants altered in LPS. Thus, although mutants in which the heptose and more distal polysaccharide is missing from this molecule are more permeable than the wild type to hydrophobic molecules (17, 23), pleiotrophic changes have been reported to occur in such mutants; for instance, they are reduced in several major outer membrane proteins (1, 11). These multiple changes make it difficult to determine whether lack of protein or abbreviation of LPS is the cause of increased permeability. Nikaido (19, 20) has postulated that such mutants are permeable because increased phospholipid fills the extra space in the outer leaflet, permitting the partition of hydrophobic molecules into this membrane, but recent evidence (9, 24) suggests that previous measurements of phospholipid and the ratios of outer membrane components (25) may have been in error and that phospholipid may not be increased relative to LPS. Clearly, further careful studies will be necessary to define the mechanism of hydrophobic impermeability in gram-negative cells.

Throughout these studies, it was difficult to compare the degree of hydrophobic impermeability in different sets of mutants isolated at different times, because no simple test for increase in hydrophobic permeability exists. Workers demonstrating increased permeation to hydrophobic compounds as the result of an experimental variation or mutational event have, until now, relied on the relative susceptibility of the altered bacteria and their controls to a wide variety of unrelated antibiotics, detergents, and dyes (see, e.g., references 6 and 18).

We describe herein a test for altered permeability to hydrophobic compounds based on susceptibility to a series of tetracyclines with various hydrophobicities. All of these tetracyclines are approximately equally effective against gram-positive organisms (3) and apparently are antibacterial via the same mechanism of action. *Escherichia coli* and other gram-negative organisms, as expected, show increasing resistance to the more hydrophobic of this series of tetracyclines (3). In this publication, we show that mutants increased in hydrophobic permeability as shown by other criteria also show increased relative susceptibility to the hydrophobic tetracyclines.

It is reasonable to postulate that this increased susceptibility is based on increased access of the hydrophobic tetracyclines to and through the lipid bilayer. This postulate predicts increased interaction of the hydrophobic tetracyclines with hydrophobic domains in such mutant bacteria. In accord with this prediction, we show herein that the very hydrophobic tetracycline, 13-phenylmercapto-α-6-deoxytetracycline (PTT), which fluoresces in hydrophobic solvents but is quenched in buffers, fluoresces more when added to mutants showing increased hydrophobic permeability than when added to the parent strain.

MATERIALS AND METHODS

Chemicals and media. The tetracyclines and their sources are described in Table 1. Tetracycline itself was purchased from Sigma Chemical Co., St. Louis, Mo., and minocycline was obtained from Lederle Laboratories, Pearl River, N.Y., but all other tetracycline derivatives were gifts. Experimental tetracyclines were gifts from Pfizer Laboratories, Groton, Conn., and methacycline was a gift from Wallace Laboratories, Cranbury, N.J. Luria broth (14) was used for growth

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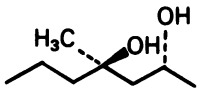
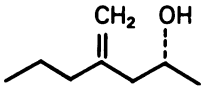
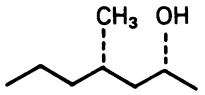
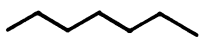
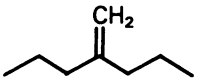
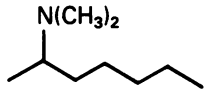
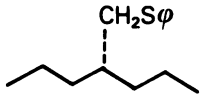
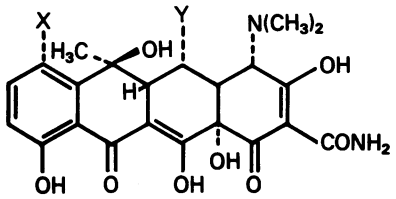
TABLE 1. Name, structure, and partition of tetracyclines[a]

Code no.	Chemical name	Generic name	Source[b]	Partial structure[c]	Log K_D
1	5-Hydroxytetracycline	Oxytetracycline	Pfizer		1.91
2	Tetracycline	Tetracycline	Sigma		1.32
3	6-Methylene tetracycline	Methacycline	Wallace		0.93
4	α-6-Deoxytetracycline	Doxycycline	Pfizer		0.20
5	6-Demethyl-6-deoxytetracycline		Pfizer (GS 2147)		−0.89
6	6-Methylene tetracycline		Pfizer (GS 2830)		−1.22
7	7-Dimethylamino-6-demethyl-6-deoxytetracycline	Minocycline	Lederle		−1.60
8	13-Phenylmercapto-α-6-deoxy-tetracycline	PTT	Pfizer (GS 3265)		<−2.0

[a] Data in this table are summarized from reference 3.
[b] Pfizer identification numbers are given in parentheses for antibiotics available only for research purposes.
[c] The basic tetracycline structure is as follows:



Carbons 5, 6, and 7 and their substituents are pictured in the partial structures.
[d] Log of the distribution constant at pH 7.0; buffer/$CHCl_3$.

of bacteria, and phosphate-buffered saline (0.01 M sodium phosphate–0.15 M NaCl, pH 7.2) was used as the buffer, unless otherwise indicated.

Bacterial strains. *E. coli* CL93 (*thi lacY malA mtlI gal-3 supE44 rpsL117*) and *E. coli* CL94, isogenic with CL93 except also *acrA*, were derived by transduction of *E. coli* PCO135 (from the *E. coli* Genetic Stock Center, isogenic with CL93, except also *purE55*). P1 phage grown on CL2 (original *acrA* strain; see reference 6) was used. Pur^+ transductants were selected and then scored for novobiocin susceptibility (6) to identify a strain with (CL94) and without (CL93) the *acrA* locus. *E. coli* PC1349 (*thr leu delgpt-proA his thi argE lacY galK xyl mtl recC22 sbc6 supE*) and *E. coli* PC2040 (isogenic with PC1349 except also *del thrA-proB-cad* [10]) were the gift of B. Lugtenberg, Utrecht, The Netherlands.

Determination of MICs. Cultures were grown to mid-logarithmic phase (ca. 4×10^8 to 5×10^8/ml) and stored at 4°C for up to 10 days. They were diluted to a final concentration of 10^5/ml in 3 ml of medium in wide test tubes (20 mm). Appropriate concentrations of antibiotics were added, and the cultures were incubated at 37°C for 18 h. Multiple (three to five), separate determinations were done to determine the MIC of a single antibiotic. After initial determinations of the appropriate range, we tested concentrations used in a narrower range until each tube differed by ca. 10% of the final MIC. Clear positive or negative growth results were determined visually, but intermediate values were recorded spectrophotometrically and considered negative when the optical density was <3% that of a full-grown culture. For final concentrations of PTT of ≥50 μg/ml, which became slightly cloudy owing to drug insolubility, the optimal density of a blank containing drug but no bacteria was subtracted. The final titer of the control culture was always $>10^9$/ml.

Tetracyclines were dissolved immediately before use at concentrations determined by their water solubility. At low concentration (≤20 μg/ml), all except 6-methylene tetracycline and PTT could be dissolved in water. These latter two were usually dissolved in dimethyl sulfoxide and diluted to a final concentration of <0.5% dimethyl sulfoxide in medium

for use; this concentration of dimethyl sulfoxide, when used alone, did not affect bacterial growth. In several experiments, these antibiotics were dissolved initially in either water or dimethyl sulfoxide for measurement of the MIC. The MICs obtained were one to two times higher when water was used than when dimethyl sulfoxide was used, presumably because the drugs were incompletely dissolved in water, but the relative susceptibilities (i.e., susceptibility of the mutant relative to that of the wild type) were unchanged by solvent.

Fluorescence measurements. Cells were grown to 5×10^8/ml, harvested at room temperature, and washed twice in phosphate-buffered saline in room temperature. Cells at the indicated concentration were suspended in phosphate-buffered saline, prewarmed to 37°C, and placed in the cuvette. PTT was added to the indicated final concentration, and the contents were rapidly mixed. The fluorescence was determined by scanning the entire emission spectrum in cuvettes maintained at 37°C in a Perkin-Elmer MPF 44B fluorescence spectrophotometer with excitation at 402 nm unless otherwise indicated. The first measurement was within 30 s of the addition of antibiotic, and measurements were continued with time as indicated. It was found that cells washed at 4°C did not show reproducible spectra until variable times after rewarming; for this reason, cells were washed at room temperature.

RESULTS

Susceptibilities of wild-type and mutant strains to tetracyclines with various hydrophobicities. The structures of the tetracyclines used and their relative partition into buffer-chloroform is shown in Table 1 (adapted from reference 3). All have approximately equivalent charges, except for minocycline, which has one more positive charge. All of these drugs are active against the gram-positive organisms *Staphylococcus aureus* and *Streptococcus pyogenes* (MIC, <1.0 μg/ml); in contrast, *E. coli*, *Aerobacter aerogenes*, and *Salmonella typhosa* show more susceptibility to the first four tetracyclines than to the latter four (see reference 3 for a review).

To determine whether a mutant previously known to be susceptible to hydrophobic compounds was altered in susceptibility to the hydrophobic tetracyclines, we compared the susceptibility of the LPS mutant PC2040 with that of its parent, PC1349. The deletion in PC2040 yields an organism with LPS lacking heptose and all distal sugars; we shall refer to this strain by its phenotype, Hep$^-$. In addition, as with other Hep$^-$ strains, the change in LPS is associated with reduction in several outer membrane proteins (10). This mutant showed increased susceptibility to tetracyclines of increasing hydrophobicity relative to the parent (Table 2, line 3). The difference was most striking for PTT (compound 8; see Table 1), with a 100-fold difference in susceptibility between mutant and wild type. The increase in susceptibility to tetracyclines of increasing hydrophobicity is in accord with the previously observed increase in susceptibility of these and other Hep$^-$ strains to hydrophobic drugs, dyes, and detergents (18, 23). The mutant showed a small but reproducible decrease in susceptibility to the two most water-soluble tetracyclines (compounds 1 and 2). Since prior evidence suggests that these hydrophilic drugs enter via the pores, this decreased susceptibility may reflect the lowered levels of porins that occur secondarily to the primary Hep$^-$ lesion in such strains.

TABLE 2. MIC of tetracyclines as a function of hydrophobicity

Strain	Relevant gene or phenotype[a]	MIC (μg/ml) of following tetracycline derivative[b]:							
		1	2	3	4	5	6	7	8
PC1349	WT	0.7	0.7	1.0	1.5	2.0	11	4	150
PC2040	Hep$^-$	1.0	1.0	0.8	0.4	0.2	2	0.3	1.5
	WT/Hep$^-$ ratio[c]	0.7	0.7	1.2	3.8	10.0	5.5	13	100
CL93	WT	0.8	0.7	0.7	0.6	1.2	12	ND[d]	200
CL94	*acrA*	1.0	0.6	0.7	0.3	0.5	4	ND	10
	WT/*acrA* ratio	0.8	1.1	1.0	2.0	2.4	3	ND	20

[a] WT, Wild type.
[b] Code numbers of tetracycline derivatives are from Table 1.
[c] Ratios are rounded to one-place accuracy to reflect imprecision of measuring a ratio of two MICs.
[d] ND, Not determined.

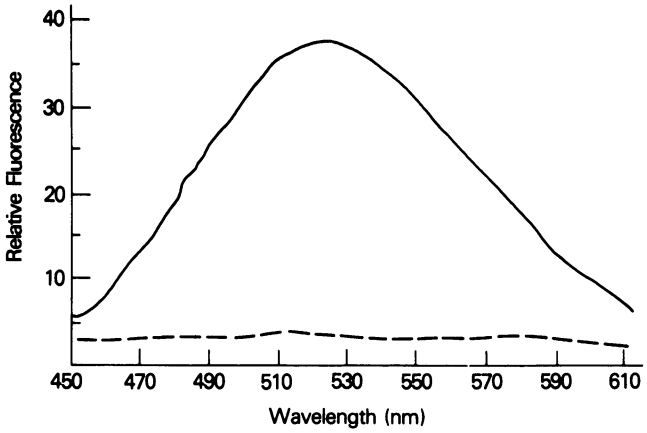


FIG. 1. Fluorescence spectrum of PTT. Ten micrograms of PTT in 110 μl of ethanol-water (9:1, vol/vol) (——) and in PBS (– – –).

This assay will be most useful if it permits detection and characterization of strains that show increased hydrophobic permeability because of different outer membrane treatments or mutations. We therefore tested an *acrA* mutant and its isogenic parent with this series of compounds. The *acrA* mutation increases hydrophobic permeability but with somewhat different specificity of entry than does the Hep$^-$ mutation (6). *acrA* mutants have normal levels and types of outer membrane proteins, as assessed by sodium dodecyl sulfate gels, and normal amounts and types of outer membrane lipids (6; Leive and Rick, unpublished observations). In previous work, we reported that the LPS is lowered in amount of phosphate; we found more recently, however, that this was not the case and that mutant and parent had the same phosphate content in LPS. We are currently attempting to further define the defect. We therefore chose this organism, since the mutant showed increased hydrophobic permeability due to an undefined defect, to further assess the utility of this assay.

Table 2 shows the susceptibility of the *acrA* mutant and a strain otherwise isogenic but normal for this locus. As with PC2040, the *acrA* mutation conferred increased susceptibility to the hydrophobic tetracyclines, although less than for the Hep$^-$ lesion. A decrease in susceptibility to oxytetracycline (compound 1; Table 2, line 5) was less reproducible for the *acrA* than for the Hep$^-$ strain.

Increased fluorescence of a hydrophobic tetracycline in outer membrane mutants. Fluorescence of tetracycline has been used as a measure of its entry (5, 7, 8); however,

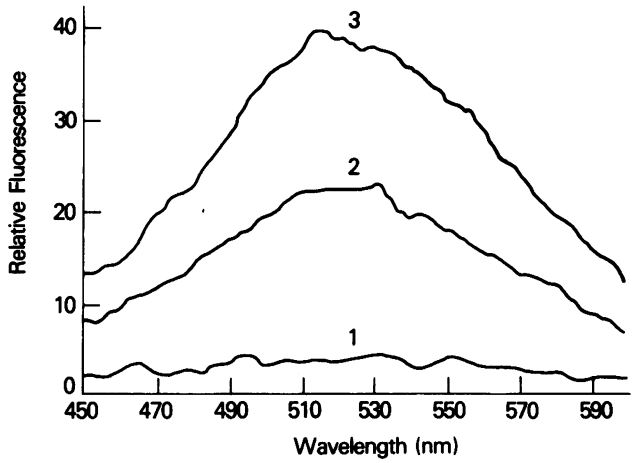


FIG. 2. Relative fluorescence of PTT in the presence of cells of *E. coli* Hep$^-$ and *E. coli* PC1349 (wild type) at a final concentration of 4 × 10^8/ml in phosphate-buffered saline and PTT at 20 μg/ml. Fluorescence was measured 20 min after the addition of drug. In Fig. 2 through 4, the scale of relative fluorescence is three times that shown in Fig. 1; i.e., the curves would be one-third as high if drawn on the scale of Fig. 1. 1, Buffer alone; 2, wild type; 3, Hep$^-$

recently it has been shown that fluorescence does not parallel entry as measured by radioactive drug (26) and that it is more likely that the degree of fluorescence reflects the entry plus the location (whether enhancing or quenching to fluorescence) of the drug (22, 26). Because of its structure, PTT would be expected to fluoresce when in a hydrophobic environment; as for many other such compounds, fluorescence may be quenched in polar solution (21). This prediction was confirmed experimentally: PTT dissolved in ethanol yielded an emission spectrum (Fig. 1); the excitation spectrum maximum was at 402 nm (data not shown). This fluorescence was dependent on a hydrophobic environment, as the addition of water (data not shown) and, even more severely, the addition of phosphate-buffered saline (Fig. 1) quenched the fluorescence. We therefore decided to use PTT as a probe for access to hydrophobic (fluorescence-enhancing) environments within the cell.

When PTT was added to cells of the Hep$^-$ mutant and the corresponding wild type, the mutant yielded more fluorescence than the wild type (Fig. 2). This fluorescence was maximal as soon as it could be measured (by ≤30 s) for the wild type; however, fluorescence for the mutant was always slightly higher initially and increased with time to a plateau at ca. 15 min (Fig. 3). Fluorescence was proportional to the concentration of PTT over a range of 5 to 15 μg/ml (data not shown). Thus, as predicted, PTT showed greater fluorescence when added to the mutant than when added to the wild type, suggesting greater association with hydrophobic regions.

When the *acrA* mutant was compared with its parent (Fig. 3B), fluorescence was also increased in the mutant, but the final difference between the two strains was less than for the Hep$^-$ strain. In addition, fluorescence was maximal for both strains within 30 s. Thus, fluorescence, like MIC, shows less difference from the wild type for *acrA* than for Hep$^-$ strains.

DISCUSSION

Tetracycline has at least two means of entry into gram-negative bacteria. As shown by Levy and his colleagues (13), transport into the cytoplasm is both active and passive at the inner membrane (16), the former using energy derived from proton motive force (16). Accumulation may also be affected by an energy-dependent efflux from the cell, where functional activity is dependent on growth (14a). The rate of uptake appears to be limited by the outer membrane (15).

We show in the current work that there is an increase in resistance to tetracyclines in wild-type *E. coli* as hydrophobicity is increased. Minocycline is especially interesting in that it carries an additional positive charge, which makes it more water soluble despite its hydrophobicity as judged by octanol-water partition, but wild-type bacteria still show resistance relative to a Hep$^-$ mutant. This finding emphasizes the relevance of partition rather than water solubility in determining outer membrane penetration. McMurry et al. (15) recently demonstrated increased permeability (transport) of minocycline in EDTA-treated cells and concluded that the outer membrane was the rate-limiting step in its entry. Our demonstration of increased susceptibility of a Hep$^-$ mutant to tetracyclines of increasing hydrophobicity, as compared with a strain with a normal outer membrane, confirms that the outer membrane is rate limiting for entry of tetracyclines.

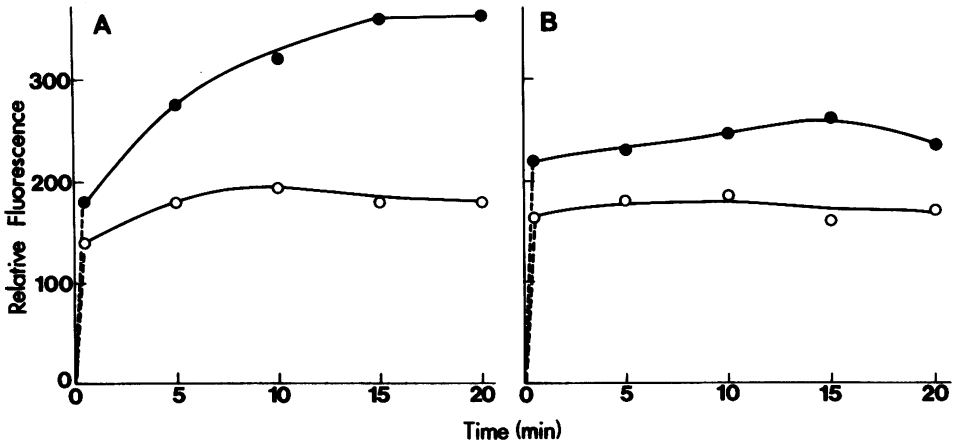


FIG. 3. Fluorescence of PTT as a function of time after addition of *E. coli*. Conditions were exactly as those described in the legend to Fig. 2. Fluorescence was measured at peak (520 nm) at 30 s after the addition of drug and at various times thereafter. (*A*) *E. coli* PC2040 (Hep$^-$) (●) and PC1349 (Hep$^+$) (○); (*B*) *E. coli* CL94 (*acrA*) (●) and CL93 (*acr*$^+$) (○).

These results provide the basis for a new assay for determining hydrophobic susceptibility, in which this series of tetracyclines graded in hydrophobicity is used. This method is preferable to measuring susceptibility to structurally diverse, unrelated molecules, since the tetracyclines have known, analogous structures and known partition coefficients, and those that have been tested have the same mechanism of action (13).

This assay should prove useful in distinguishing mutants with different outer membrane changes and assaying degree and type of hydrophobicity in new mutant strains. Thus, even if one did not know the genetics or biochemistry of the *acrA* strain, it could be predicted to be an outer membrane mutation on the basis of this assay. The *acrA* mutation defines an outer membrane change that we previously thought was due to reduced phosphate in LPS (6). This conclusion is, however, not correct (unpublished data), and the biochemical lesion remains to be defined.

If this assay permits measurement of the degree and type of hydrophobic permeability in new mutant strains, it will be analogous and complementary to a similar assay in which cephalosporins are used to measure access, through the pores of the outer membrane, to periplasmic β-lactamase. Zimmerman and Rossalet (27) have shown that the more hydrophobic the cephalosporin, the more restricted the entry into wild-type *E. coli*, and Bavoil et al. (2) have shown that organisms lacking porins or with modified porins show lowered entry. Our current experiments cannot strictly differentiate the mechanism of entry of the hydrophobic tetracyclines in the mutants. Entry might be through partition directly through the bilayer or it might reflect a mechanism whereby the pores become less able to exclude hydrophobic compounds. However, we believe it is the former mechanism because (i) PTT is far more hydrophobic by octanol-water partition than are any of the cephalosporins used by Nikaido and his colleagues (L. Cullinane and L. Leive, unpublished data) and thus would be unlikely to pass via hydrophilic pores, and (ii) the *acrA* mutant, which also shows increased penetration of PTT, albeit less striking than in the Hep$^-$ strain, has normal amounts of porins as indicated on gels (6) and normally functioning porins as indicated by growth on extremely low levels of required nutrients (L. Leive, unpublished data).

Fluorescence of tetracyclines when added to bacteria is a complicated phenomenon. It was believed initially to accurately reflect transport into the cell (5, 7, 8), but recent work indicates that there are pitfalls in this assumption because the fluorescence will be affected by whether the molecule is in a hydrophobic or hydrophilic environment, internal pH, and degree of association with divalent cations (22, 26). With the highly hydrophobic PTT, we found that fluorescence is completely quenched in aqueous solutions with physiological concentrations of ions, and in preliminary experiments, although we observed some fluorescence enhancement by millimolar concentrations of Mg^{2+} and Ca^{2+}, it is far less than observed in apolar solutions (L. Leive, unpublished observations). We therefore postulate that the fluorescence we observed reflects association with hydrophobic regions, presumably membranes, in the cells, but further work will be necessary to prove this postulate.

The entry of the three most hydrophilic tetracyclines is also interesting. The first two, oxytetracycline and tetracycline are more effective against wild-type cells than against Hep$^-$ cells. If hydrophilic tetracyclines enter via pores, as appears likely (20), this result might reflect the reduced number of pores, owing to decreased porin content found in such Hep$^-$ cells (1, 11). In accord with this postulate, we (unpublished observations) and others (4, 15) have found that mutants lacking the *ompF* or *ompC* porins are less susceptible to oxytetracycline and tetracycline than the wild type.

Tetracycline susceptibility can be affected by many facets of cell metabolism, but the rate-limiting nature of entry through the outer membrane suggests that increased susceptibility to hydrophobic tetracyclines can be used as an assay of outer membrane permeability.

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