ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1975, p. 801-806

Vol. 7, No. 6
Printed in U.S.A.

Comparison of Tetracycline and Minocycline Transport in *Escherichia Coli*

VICTOR E. DEL BENE* AND MARY ROGERS

Infectious Diseases and Immunology Division, Department of Medicine, Medical University of South Carolina, Charleston, South Carolina 29401

Received for publication 20 February 1975

Differences between minocycline and tetracycline transport were demonstrated in an *Escherichia coli* strain with and without an R factor (R46) which confers moderate Tc resistance. Minocycline uptake was similar in R^+ and R^- organisms, whereas tetracycline uptake was decreased in the R^+ as compared to the R^- organism. Sodium azide had little effect on tetracycline uptake by either strain but minocycline uptake was completely inhibited by azide. *p*-Chloromercuribenzoate greatly decreased tetracycline uptake in both strains while minocycline uptake was markedly augmented. Both minocycline and tetracycline were effective inducers of decreased tetracycline transport, but only tetracycline had an effect on minocycline uptake. Mutual inhibition of uptake of one antibiotic by the other could not be demonstrated. These studies indicate different mechanisms of transport of minocycline and tetracycline in *E. coli*.

The transport of tetracycline into *Escherichia coli* is an active process. Although saturation kinetics are poorly defined for this transport system there is evidence for a specific membrane-bound carrier molecule (7). Resistance to tetracycline in R factor bearing *E. coli* has been shown to be due to decreased transport of tetracycline (4, 9), and R^+ cells can be induced to take up even less tetracycline by exposure of the bacteria to small amounts of the drug (5).

E. coli which contain R factors mediating tetracycline resistance are susceptible to minocycline (11), and accumulate more minocycline than tetracycline (6). This difference can be demonstrated in dynamic growth-challenge tests as inhibition of growth of R^+ bacteria by minocycline but not by tetracycline (11). A further distinction between these two analogues is that minocycline is less effective than tetracycline in inducing increased resistance in R^+ organisms to either antibiotic (11).

This study was undertaken to further investigate the differences in the transport of minocycline and tetracycline in *E. coli* with and without an R factor (R46) mediating tetracycline resistance.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 W3110 used in this study was made tetracycline resistant by infection with R factor R46 (Tc^R, Amp^R, S^R, Su^R) which has an fi^- phenotype and belongs to compatibility group N (3).

Media. Trypticase soy broth (BBL) was utilized in studies requiring active bacterial growth. Uptake and loss of radioactive antibiotics from bacterial cells was measured in C medium: ammonium chloride (NH_4Cl), 2 g/liter; sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$), 10.6 g/liter; potassium phosphate (KH_2PO_4), 3 g/liter; sodium chloride (NaCl), 3 g/liter; magnesium chloride ($MgCl_2 \cdot 6H_2O$), 0.02 g/liter; sodium sulfate (Na_2SO_4), 0.026 g/liter; and glucose, 10 g/liter (10). Preliminary experiments indicated a broad Mg^{2+} optimum range of 10^{-3} to 10^{-4} M for maximum tetracycline uptake and a narrow optimum of 10^{-4} M magnesium for maximum minocycline uptake when the incubation concentration of the antibiotic was 10 μg/ml.

Antibiotic transport studies. [^{3}H]tetracycline hydrochloride with specific activity of 1.5 to 2.5 mCi/mg and 8.8 μCi/mg was procured from New England Nuclear and Lederle Laboratories, respectively. [^{14}C]minocycline with a specific activity of 2.4 μCi/mg was provided by Lederle Laboratories.

Prior to measurement of antibiotic transport, bacteria were grown overnight in Trypticase soy broth in a 37 C water bath with shaking. The overnight culture which was in stationary phase was harvested by centrifugation at 2,100 × *g* for 20 min at 23 C and then the bacterial cells were resuspended in C media at approximately 10^7 bacteria/ml, estimated by optical density at 625 nm.

Radiolabeled antibiotic was added to the C medium containing 10^7 bacteria/ml. Samples of 1 ml were obtained immediately and at subsequent intervals of time. These samples were filtered through a membrane filter (Millipore Corp.) (GSW, 0.22 μm pore size) and the bacteria impinged on the membranes were washed once with 2 ml of C medium. The filter

membranes and the bacteria were dried at 37 C for 1 h and suspended in scintillation fluid (5 g of butyl PBD, Beckman Instruments, Inc. in 1 liter of toluene). This fluid renders the filter membranes translucent and results in a counting efficiency of 55 to 60% for [^{3}H]tetracycline and 75% for [^{14}C]minocycline when counted in a Packard Tri-Carb liquid scintillation spectrometer (model 2420).

Studies of the exit of antibiotics from the bacterial cells were performed by incubating the bacteria in C medium with various concentrations of radioactive antibiotic for 20 min. These cells were then removed from the antibiotic by centrifugation at 48,000 × *g* at 23 C and quickly resuspended in antibiotic-free C medium. Aliquots of 1 ml were removed immediately and at subsequent time intervals and filtered onto membrane filters. The radioactivity on these filters was determined as described above.

Inhibition of the uptake and exit of radioactive antibiotics by *p*-chloromercuribenzoate (PCMB) and sodium azide was studied. The effect of these inhibitors on uptake of antibiotic was examined by preincubation of the cells with the inhibitor at final concentration of 10^{-4} M in C medium. Inhibitor effects on the exit of labeled antibiotic from bacteria were evaluated by addition of the inhibitor to the bacterial culture after cells were preloaded with radioactive antibiotics. Measurement of radioactive antibiotic which remained cell associated in these studies was made prior to addition of the inhibitor and at various times after its introduction. In addition, competitive inhibition of uptake by the two analogues was studied by adding the unlabeled analogue in equimolar concentration to the incubation mixture. A control without addition of the nonlabeled analogue was performed simultaneously using the same cell culture.

Induction of decreased antibiotic transport was examined by incubating the bacteria in Trypticase soy broth containing 0.01 of the minimal inhibitory concentration of either tetracycline or minocycline for that organism. After an induction period of 1 h, the cells were recovered by centrifugation and resuspended in C medium containing the appropriate concentration of radioactive tetracycline or minocycline. Uptake of radioactive antibiotic was then measured as described above.

Results are expressed as micromoles of antibiotic per milliliter of bacterial intracellular water (10). Initial readings (time zero) were subtracted from subsequent determinations in each experiment to correct for adsorption to cell surface and membrane filter.

RESULTS

Antimicrobial resistance characteristics. The introduction of the transferable resistance factor, R46, into *E. coli* W3110 resulted in a 10-fold rise in the minimal inhibitory concentration of tetracycline (3.12 to 32 μg/ml). This R factor had no effect on the minimal inhibitory concentration of minocycline for W3110, which remained at 2 μg/ml.

Uptake of antibiotics by R$^+$ and R$^-$ E. coli. Figure 1 shows the effect of the transferable resistance factor, R46, upon tetracycline uptake. The R$^+$ *E. coli* strain accumulated a maximum of 0.4 μmol/ml of tetracycline during a 20-min period. This accumulation was irregular and did not show a linear uptake pattern. When the R$^-$ strain was incubated with 10 μg of tetracycline per ml it accumulated this antibiotic in a linear fashion for the first 10 min and achieved an equilibrium concentration of approximately 2 μmol/ml of intracellular water at 20 min.

Figure 2 shows the influx of minocycline into *E. coli* strains with and without the R factor, R46. At a concentration of minocycline of 10 μg/ml, both R$^+$ and R$^-$ strains accumulated minocycline at about the same rate although after 15 min the rate of accumulation by the R$^+$ bacteria was slower than that in the R$^-$ strain, resulting in less total minocycline accumulation. At 45 min the total accumulation of minocycline was 6.6 μmol/ml by the R$^-$ strain and 3.8 μmol/ml by the R$^+$ strain.

In the R$^+$ strain (Table 1), incubated with a concentration of 10 μg of tetracycline per ml, the antibiotic is accumulated to 10 times incubating concentration, whereas the R$^-$ strain accumulates up to 100 times the incubating concentration after 20 min. Both strains accu-

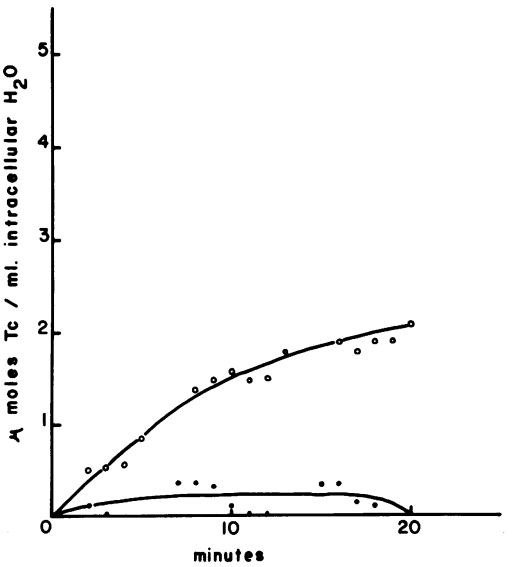

FIG. 1. *Tetracycline uptake by E. coli R$^+$ (●) and R$^-$ (○). The initial concentration of tetracycline in the medium was 10 μg/ml, and the specific activity of [^{3}H]tetracycline was 4.5 μCi/μmol. Activity of sample labeled time zero which was taken within 5 s of addition of antibiotic, subtracted from subsequent determinations.*

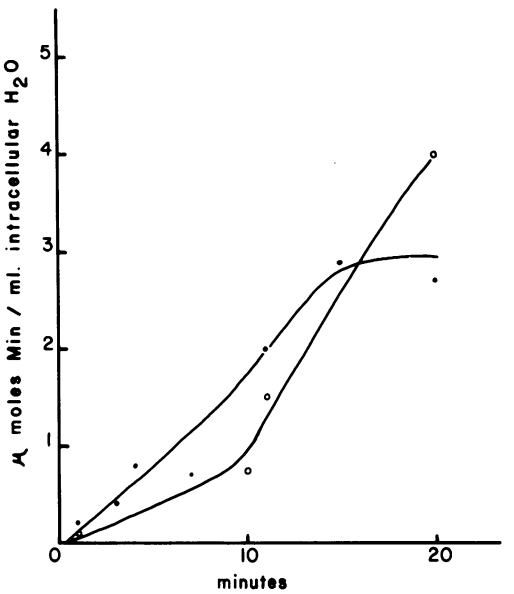

FIG. 2. *Minocycline uptake by E. coli R⁺ (●) and R⁻ (○). The initial concentration of minocycline in the medium was 10 μg/ml, and the specific activity of [¹⁴C]minocycline was 4.4 μCi/μmol. Activity of initial sample which was taken within 5 s of addition of antibiotic was subtracted from subsequent determinations.*

TABLE 1. *Total accumulation[a] and ratio of intracellular/initial (I/E) extracellular antibiotic concentration in R⁺ and R⁻ E. coli*

Antibiotic	R⁺ strain		R⁻ strain	
	μmol/ml[b]	I/E ratio	μmol/ml	I/E ratio
Tc (10)[c]	0.2	10	2.0	100
Min (10)	3.0	150	4.0	200
Tc (100)	20.0	100	29.0	135

[a] After 20 min of incubation.

[b] Micromoles per milliliter of bacterial intracellular water.

[c] Figures in parenthesis are concentrations of antibiotic in incubation mixture in μg/ml. Specific activity for tetracycline (Tc) (10) was 4.5 μCi/μmol, for Tc (100) 4.4 μCi/μmol, and for minocycline (Min) (10) 4.4 μCi/μmol.

mulate drug to over 100 times the incubating concentration after 20 min when the concentration of minocycline is 10 μg/ml or tetracycline is 100 μg/ml. However, in both cases, the R⁺ strain accumulates less of each drug than the R⁻ strain.

PCMB, a sulfhydryl-binding compound, inhibited the influx of tetracycline into both R⁺ and R⁻ strains (Table 2). A concentration of PCMB of 10^{-4} M inhibited influx by 75% in R⁻ strains incubated for 30 min in tetracycline at 10 and 100 μg/ml. The accumulation of tetracycline by the R⁺ strain was completely inhibited. The effect of PCMB on minocycline uptake was entirely different in both R⁺ and R⁻ strains. The influx of minocycline was increased almost ninefold from 1.4 to 12.3 μmol/ml in the R⁻ strain and by a similar increment in the R⁺ strain from 2.3 to 21.0 μmol/ml.

Sodium azide (10^{-4} M), was used to determine the effect of a metabolic inhibitor on the transport of minocycline and tetracycline (Table 2). The uptake of minocycline was completely inhibited in both R⁺ and R⁻ cells when the incubation concentration of minocycline was 10 μg/ml. In contrast, the influx of tetracycline was inhibited by 30% (2.3 to 1.6 μmol/ml in R⁻ cells) but was not inhibited in R⁺ cells.

Loss of antibiotics from R⁺ and R⁻ bacterial cells. Loss of labeled antibiotics from preloaded R⁺ and R⁻ strains was followed in antibiotic-free medium. Similar rates of exit of both tetracycline and minocycline were seen after prior incubation in medium containing 10 μg/ml (Fig. 3). During the first 5 min the loss of these drugs was linear and thereafter appeared to have a changing slope (probably reflecting recapture of the substrate). Both strains retained a greater amount of minocycline than tetracycline after 2 h.

The addition of azide (10^{-4} M) after a 20-min period of loading had no effect on the efflux of tetracycline or minocycline from R⁺ or R⁻ cells (Table 3). After 30 min in antibiotic-free medium the portion of tetracycline remaining was similar whether or not the inhibitor was present. Even after preloading these cells for 20 min in

TABLE 2. *Effect of PCMB and sodium azide upon minocycline (Min) and tetracycline (Tc) levels in R⁺ and R⁻ E. coli*

Strain (inhibitor)	Antibiotic accumulation (μmol/ml)[a]		
	Tc (10)[b]	Min (10)	Tc (100)
R⁻ (none)	2.3	1.4	39.0
R⁻ (azide)[c]	1.6	0.0	34.0
R⁻ (PCMB)[d]	0.5	12.3	10.0
R⁺ (none)	0.5	2.3	20.0
R⁺ (azide)	0.5	0.0	24.0
R⁺ (PCMB)	0.0	21.0	0.0

[a] Micromoles per milliliter of bacterial intracellular water after 30 min of incubation.

[b] Figures in parenthesis are concentrations of antibiotics in incubation mixture in μg/ml. Specific activity of antibiotics as in Table 1.

[c] Sodium azide, 10^{-4} M.

[d] PCMB, 10^{-4} M.

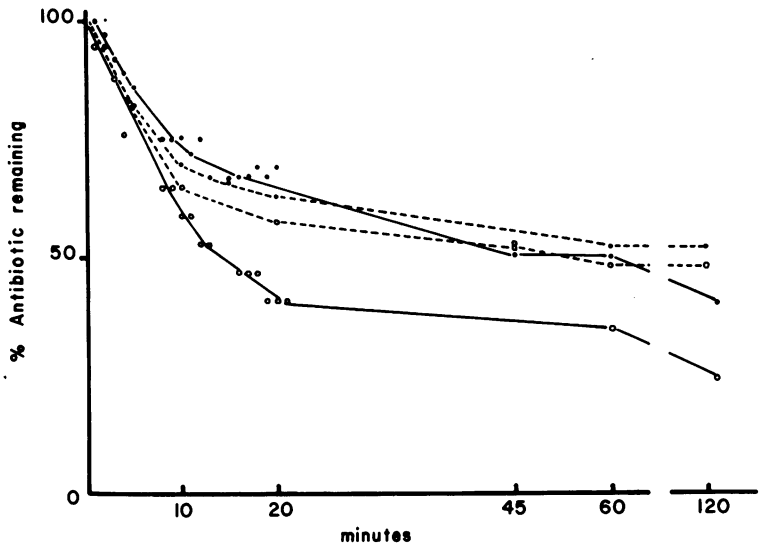

FIG. 3. *Loss of minocycline and tetracycline from R⁺ and R⁻ E. coli strains. Cells were preincubated in 10 μg/ml of antibiotic for 20 min, then resuspended in antibiotic-free medium. Samples were taken immediately and at timed intervals following resuspension. Symbols: R⁺ (●), R⁻ (○), minocycline (-----), tetracycline (——). Specific activity of minocycline, 4.4 μCi/μmol; of tetracycline, 4.5 μCi/μmol.*

TABLE 3. *Effect of PCMB and sodium azide upon minocycline (Min) and tetracycline (Tc) loss in R⁺ and R⁻ E. coli*

Strain (inhibitor)	Intracellular antibiotic (% remaining)[a]	
	Tc (100)[b]	Min (10)[b]
R⁻ (none)	63	55
R⁻ (azide)[c]	62	53
R⁻ (PCMB)[d]	16	69
R⁺ (none)	48	62
R⁺ (azide)	49	63
R⁺ (PCMB)	17	70

[a] Percentage of antibiotic remaining after 30 min in antibiotic free medium. Actual 100% values were 51.0 and 53.6 μmol of Tc/ml for R⁺ and R⁻ strains and 2.7 and 4.0 μmol Min/ml for R⁺ and R⁻ strains, respectively.

[b] Figures in parenthesis are concentration of antibiotic in medium during 20-min incubation period prior to addition of inhibitor. Specific activities same as in Table 1.

[c] Sodium azide, 10^{-4} M.

[d] PCMB, 10^{-4} M.

100 μg of tetracycline per ml, which increased the accumulated amount of tetracycline to 10 times that obtained using an incubation concentration of 10 μg of tetracycline per ml, the levels of the drug remaining after 30 min were not affected by sodium azide.

When the effect of PCMB (10^{-4} M) on minocycline efflux was studied (Table 3), both strains retained slightly more minocycline than did the control after 30 min; 69 versus 55% in the R⁻ strain and 70 versus 62% in the R⁺ strain. An entirely different result was seen in R⁺ and R⁻ strains after loading in 100 μg of tetracycline per ml. Treatment with PCMB increases the loss of tetracycline from R⁺ and R⁻ strains to the same degree (to about 15% of control after 30 min).

Effect of preincubation with subinhibitory levels of antibiotics on uptake. Minocycline uptake levels in the R⁺ strain were decreased to about 30% of the control after preincubating for 1 h in 2.5 μg of tetracycline per ml, whereas 0.2 μg of minocycline per ml failed to produce this effect (Table 4). Both minocycline and tetracycline significantly decreased the uptake of tetracycline in R⁺ strains. After 7 min the R⁺ strain incubated in 100 μg of tetracycline per ml accumulated only 22% of control when previously exposed to subinhibitory levels of tetracycline and 40% of control when pre-exposed to minocycline. Minocycline pre-exposure produces a similar reduction in tetracycline uptake (40% of control) when the incubating concentration of tetracycline is 10 μg/ml. When R⁻ strains were studied such marked results are not obtained after preincubation with either analogue.

When chloramphenicol (0.2 μg/ml) was added at the beginning of the 1-h inducing period the effect of subinhibitory amounts of minocycline and tetracycline upon uptake of either analogue was blocked.

TABLE 4. *Effect of prior exposure to 0.1 minimal inhibitory concentration of minocycline (Min) and tetracycline (Tc) upon the uptake of both antibiotics by R^+ and R^- E. coli*

Strain	Inducer	Accumulation of antibiotic[a]		
		Min (10)[b]	Tc (100)[b]	Tc (10)[b]
R^+	Min	95 (3.3)	40 (6.6)	44 (0.02)
R^+	Tc	32	22	ND[c]
R^-	Min	100 (0.09)	ND	100 (1.3)
R^-	Tc	100	ND	72

[a] Antibiotic accumulated after 7 min following incubation in 10 μg of Min or Tc per ml or 100 μg of Tc per ml expressed as percentage of uptake of noninduced controls. Control values in micromoles of antibiotic/milliliter of intracellular water in parenthesis.

[b] Figures in parenthesis are concentration of antibiotic in medium during 7-min uptake period. Specific activities same as in Table 1.

[c] ND, Not done.

Analogue inhibition studies. Inhibition of uptake of radiolabeled tetracycline or minocycline in R^+ and R^- strains by equimolar concentrations of the other nonlabeled analogue was attempted. No inhibition was demonstrated.

DISCUSSION

Our studies indicate that the mechanisms of transport of tetracycline and minocycline in *E. coli* are different. This conclusion is based on the following findings. (i) The introduction into *E. coli* of an R factor (R46) conferred moderate resistance to tetracycline but no change in minocycline resistance. This implies a basic difference between the tetracycline and minocycline uptake mechanism since a tetracycline resistance determinant was not effective in protecting cells exposed to minocycline. A previous study showed similar results with the same R factor (R46) (11). (ii) Minocycline was accumulated by the R^+ strain, whereas tetracycline was not. In addition, more minocycline than tetracycline was accumulated by the tetracycline-susceptible (R^-) strain. The data demonstrate a correlation between the susceptibility of *E. coli* to tetracycline and minocycline and the differential uptake of each compound. Similar findings were reported by Kuck and Forbes (6), but the differences in uptake which they observed between R^+ and R^- bacteria were not as large as we have found, probably because of differences in methodology and differences in R factor resistance. The R factors used by Kuck and Forbes caused an increase of 30-fold in the minocycline resistance of the recipient as well as greatly increasing tetracycline resistance. Our transport studies also confirm the suggestions of Robertson and Reeve (11) that tetracycline resistance conferred by R46 was related to decreased tetracycline uptake and that R46 would not affect minocycline uptake. (iii) Sodium azide completely inhibited uptake of minocycline in R^+ and R^- strains, whereas it had relatively little effect on tetracycline uptake. This finding indicates that minocycline transport is energy dependent, although whether a specific permease is inhibited or a more general effect on membrane integrity is involved remains to be determined. The failure of sodium azide to inhibit the tetracycline permease system when measurements are made with low to moderate concentrations of tetracycline in the medium was also reported by Shipley and Olsen (13). (iv) PCMB inhibited tetracycline uptake while it enhanced the uptake of minocycline in both R^+ and R^- strains. PCMB also inhibited the efflux of minocycline and facilitated efflux of tetracycline. It has been previously shown that covering sulfhydryl groups causes failure of disulfide bonding which disrupts cell wall structure (12) and effects the membrane permeability of sodium (1). Such effects could be responsible for nonspecific permeation of many compounds. Minocycline might gain entry into intact cells by a mechanism which is impeded by intact disulfide groups, whereas tetracycline is not. It is not improbable that after PCMB treatment highly lipophillic compounds, such as minocycline (2), might be preferentially bound and absorbed when compared to a less lipophillic compound like tetracycline. For minocycline uptake in *E. coli*, mechanisms such as this might be quantitatively more significant than an active permease mechanism. (v) Tetracycline effectively induced decreased uptake by R^+ *E. coli* of both minocycline and tetracycline but minocycline induced decreased tetracycline uptake only and not minocycline uptake. We also have found that tetracycline was more effective as an inducer of decreased uptake than minocycline. These findings are compatible with the observations made by Robertson and Reeve (11) of increased resistance of *E. coli* to antibiotic inhibition induced by pre-exposure of the bacteria to each drug. If two mechanisms are postulated for minocycline uptake, namely a specific permease and a nonspecific absorption, our induction studies would indicate that the latter is quantitatively more important in minocycline accumulation than the former. Thus, maneuvers which change the tetracycline permease regulatory mechanism would result in little or no reduction of uptake of minocycline since it is primarily accumulated by another mechanism.

(vi) Competitive inhibition of uptake could not be demonstrated for either compound. This finding, however, is not conclusive in view of the poorly defined affinity of tetracycline for its permease (8, 13).

Our studies give evidence that accumulation of minocycline by *E. coli* depends upon at least two mechanisms: (i) the tetracycline permease system and (ii) some other mechanism, possibly nonspecific absorption.

ACKNOWLEDGMENTS

We wish to express our appreciation to our colleagues, Grace M. Thorne and W. Edmund Farrar, Jr., for their helpful advice and criticisms.

This study was supported by South Carolina Allocations for Biomedical Research.

LITERATURE CITED

1. Bernheim, F. 1970. The effect of sulfhydryl compounds and mercurials on the swelling rate of cells of *Pseudomonas aeruginosa* in sodium and potassium buffers. Proc. Soc. Exp. Biol. Med. **133**:841-844.
2. Colaizzi, J. L., and P. R. Klink. 1969. pH Partition behavior of tetracyclines. J. Pharm. Sci. **58**:1184.
3. Datta, N., and R. W. Hedges. 1971. Compatibility groups among *fi*⁻ R factors. Nature (London) **234**:222-223.
4. Franklin, T. J., and A. Godfrey. 1965. Resistance of *Escherichia coli* to tetracyclines. Biochem. J. **94**:54-60.
5. Izaki, K., K. Kiuchi, and K. Arima. 1966. Specificity and mechanisms of tetracycline resistance in a multiple drug resistant strain of *Escherichia coli*. J. Bacteriol. **91**:628-633.
6. Kuck, N. A., and M. Forbes. 1973. Uptake of minocycline and tetracycline by tetracycline-susceptible and -resistant bacteria. Antimicrob. Agents Chemother. **3**:662-664.
7. Levy, S. B., and L. McMurry. 1974. Detection of an inducible membrane protein associated with R-factor mediated tetracycline resistance. Biochem. Biophys. Res. Commun. **56**:1060-1068.
8. Reynard, A. M., and L. F. Nellis. 1972. Uptake of tetracycline by *Escherichia coli*: lack of binding of tetracycline to the uptake system. Biochem. Biophys. Res. Commun. **48**:1129-1132.
9. Reynard, A. M., L. F. Nellis, and M. E. Beck. 1971. Uptake of ^{3}H-tetracycline by resistant and sensitive *Escherichia coli*. Appl. Microbiol. **21**:71-75.
10. Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britten. 1955. Studies of biosynthesis in *Escherichia coli*, p. 4-5. Carnegie Institution of Washington publication 607. Washington, D. C.
11. Robertson, J. M., and E. C. R. Reeve. 1972. Analysis of the resistance mediated by several R-factors to tetracycline and minocycline. Genet. Res. **20**:239-252.
12. Schaechter, M., and K. A. Santomassino. 1962. Lysis of *Escherichia coli* by sulfhydryl-binding reagents. J. Bacteriol. **84**:318-325.
13. Shipley, P. L., and R. H. Olsen. 1974. Characteristics and expression of tetracycline resistance in gram-negative bacteria carrying the pseudomonas R factor RP1. Antimicrob. Agents Chemother. **6**:183-190.