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PLASMID-MEDIATED TETRACYCLINE RESISTANCE IN ESCHERICHIA COLI INVOLVES INCREASED EFFLUX OF THE ANTIBIOTIC

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SUMMARY

The possibility that decreased accumulation of tetracycline by plasmid-carrying strains of Escherichia coli involves increased efflux as well as reduced influx was investigated. Both decreased uptake and increased efflux of tetracycline occurred in resistant strains under conditions where bacteria were still metabolically functional.

INTRODUCTION

Plasmid-mediated tetracycline resistance in E. coli is at least partially due to decreased accumulation of the antibiotic [1]. This has been ascribed to reduced influx rather than increased efflux of the antibiotic because the rates of loss of tetracycline from sensitive and resistant bacteria pre-loaded with the drug were similar [2]. However, these data are equivocal because the authors did not demonstrate whether bacteria were still metabolically active during loss of tetracycline. Data presented here demonstrate that, under conditions where bacteria are still metabolically functional, increased efflux of the antibiotic does occur from resistant bacteria.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains are listed in Table 1.

Chemicals

All reagents were obtained from Sigma London Ltd. except [^{14}C]labelled amino acid mixture which was obtained from the Radiochemical Centre, Amersham, Bucks.

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Tetracycline uptake

Tetracycline uptake was measured using spectrofluorimetry [3]. Bacteria (5×10^8 cells/ml) grown in nutrient broth [4] were centrifuged (10,000 x g, 5 minutes, 30°C) washed once with 100mM Tris/HCl buffer pH 8.0 and resuspended (5×10^9 cells/ml) in 10mM Tris/HCl buffer pH 8.0. Glucose (0.4% w/v) was added and bacteria circulated through a flow cell attachment (1cm light path) fitted to a Perkin-Elmer spectrofluorimeter (excitation wavelength 400nm, emission wavelength 520nm , excitation and emission monochromator slit widths 5nm). At time zero, tetracycline (100µg/ml) was added and fluorescence (520nm) recorded. The relationship between fluorescence and amount of tetracycline accumulated (Figure 1) was determined as follows: samples (1ml) taken at intervals (5 minutes) during tetracycline uptake were centrifuged (1 minute) using an Eppendorf bench centrifuge. Pelleted bacteria were solubilized by boiling (10 minutes) in HCl (5M). This also converts tetracycline to anhydrotetracycline [5]. The absorbance (440nm) of anhydrotetracycline contained in boiled samples was measured using a Pye Unicam SP1800 spectrophotometer (slit width 1nm). The amount of anhydrotetracycline contained in these samples was determined using a standard curve (0-100µg tetracycline/ml).

Tetracycline efflux

Bacteria were loaded (10 minutes) with tetracycline (100µg/ml) using the method just described for uptake. Bacteria were then centrifuged (4 minutes) using an MSE bench centrifuge and resuspended in 10mM Tris/HCl buffer pH 8.0 containing glucose (0.4% w/v). Tetracycline efflux was measured as the loss of fluorescence at 520nm.

Protein synthesis

Rates of protein synthesis during efflux of tetracycline from loaded bacteria were determined from semi-logarithmic plots of the amount of [^{14}C] labelled amino acids incorporated into hot trichloroacetic acid-precipitable material versus time [6].

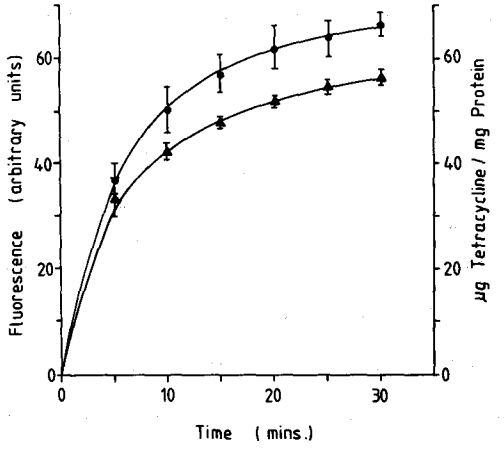


Figure 1 Relationship between fluorescence (▲) and tetracycline accumulated (●). Bacteria were grown, harvested, washed and resuspended as described in Materials and Methods. At time zero, tetracycline (100µg/ml) was added and accumulation monitored using spectrofluorimetry. Samples were also taken to determine total accumulation of tetracycline (see Materials and Methods).

RESULTS AND DISCUSSION

Accumulation of tetracycline is a reversible process [7,8] and can be represented as follows:

$$[Tc_{out}] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [Tc_{in}] \qquad \text{(i)}$$

where $[Tc_{out}]$ = concentration of tetracycline outside the cell suspension,

$[Tc_{in}]$ = concentration of tetracycline inside the cell suspension,

k_1 = rate constant for tetracycline influx

$$= \frac{\text{initial velocity of uptake}}{\text{initial } [Tc_{out}]}$$

k_{-1} = rate constant for tetracycline efflux

$$= \frac{\text{initial velocity of efflux}}{\text{initial } [Tc_{in}]}$$

and at equilibrium (i.e., when uptake has reached a plateau):

$$\frac{k_1}{k_{-1}} = \frac{[\overline{Tc}_{in}]}{[\overline{Tc}_{out}]} \qquad \text{(ii)}$$

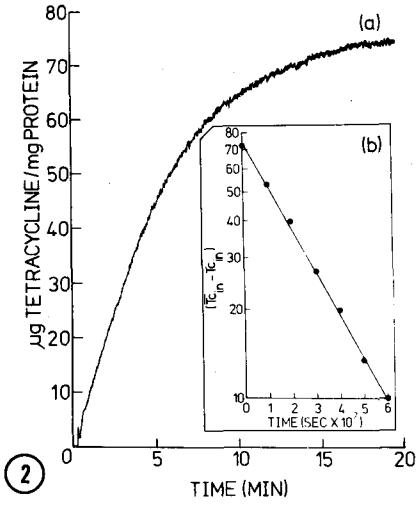
where $[\overline{Tc}_{out}]$ and $[\overline{Tc}_{in}]$ are respectively the concentrations of tetracycline outside and inside the cell suspension at equilibrium. If tetracycline accumulation approximates to a first-order reaction, as implied by equations (i) and (ii), then the rate of approach to equilibrium is defined as:

$$\frac{d[Tc_{in}]}{dt} = (k_{-1} + k_1)([\overline{Tc}_{in}]-[Tc_{in}]) \qquad \text{(iii)}$$

and plots of $\ln([\overline{Tc}_{in}]-[Tc_{in}])$ against time should be linear with the gradient equal to $k_1 + k_{-1}$. Linear plots were indeed obtained for tetracycline uptake in JC3272 (see Figures 2a and 2b).

Confirmation that efflux itself approximates to a first-order reaction in JC3272 was obtained by measuring efflux of tetracycline from pre-loaded bacteria (Figure 3a). Plots of $\ln([Tc_{in}]-[\overline{Tc}_{in}]$ versus time were linear (Figure 3b) implying that efflux is indeed first-order.

Examination of the kinetics of tetracycline uptake using several tetracycline-resistant strains and their parental tetracycline-sensitive wild-



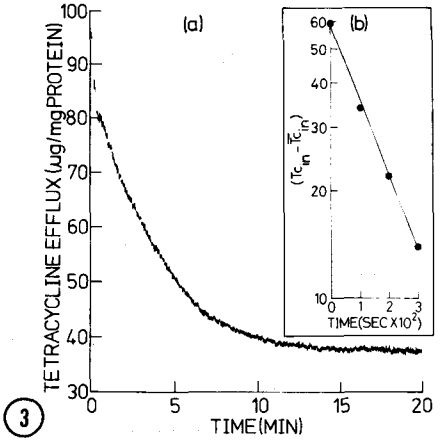


Figure 2 (a) Accumulation of tetracycline by JC3272 and (b) a plot of $\ln([\overline{Tc}_{in}]-[Tc_{in}])$ constructed using curve (a). Accumulation of tetracycline was measured using spectrofluorimetry (see Materials and Methods) and $[\overline{Tc}_{in}]$ and $[Tc_{in}]$ were calculated as described in the text.

Figure 3 (a) Efflux of tetracycline from JC3272 and (b) a plot of $\ln([Tc_{in}]-[\overline{Tc}_{in}])$ constructed using curve (a). Efflux of tetracycline from pre-loaded bacteria was measured using spectrofluorimetry (see Materials and Methods) and $[Tc_{in}]$ and $[\overline{Tc}_{in}]$ were calculated as described in the text.

type strain (Table 1) showed that:

a) $k_1 > k_{-1}$ in the tetracycline-sensitive parental strain JC3272 (Table 2);

b) $k_1 + k_{-1}$ is similar in both the tetracycline-sensitive strain and its tetracycline-resistant derivatives (Table 2);

c) $k_1 < k_{-1}$ in those strains expressing tetracycline resistance constitutively (Table 2);

d) k_{-1} is increased on induction of tetracycline resistance in UB1528 (Table 2).

Thus the decreased accumulation of tetracycline observed in the resistant strains is due both to increased efflux and to reduced influx.

To confirm that increased efflux occurs when resistance is expressed, efflux from JC6310 and UB5783 (see Table 1) was followed. UB5783 carries the plasmid pUB905 (see Table 1) which determines temperature-conditional expression of tetracycline resistance (resistant 30°C, sensitive 42°C).

Table 1. Bacterial strains

Strain	Strain description	Source/Reference Bacteria	Plasmids
JC3272	*his*⁻ *trp*⁻ *lys*⁻ *strA*	[9]	
UB1553	JC3272 pDU301 (Tc Sm/Su Fa Cm Hg)	"	[10]
UB6012	JC3272 pUB5554 (Tc Tp Su)	"	J.M. Ward[1]
UB6014	JC3272 pUB5555 (Tc Tp)	"	J.M. Ward[2]
UB1528	JC3272 pIP (Tc Ka)	"	[11]
JC6310	*his*⁻ *trp*⁻ *lys*⁻ *strA recA*	[12]	
UB5783	JC6310 pUB905 (Tc Ap)	"	P.M. Bennett[3]

1. pUB5554 was constructed by transposition of Tn10 from the chromosome of DU1200 [10] to R388 [13]
2. pUB5555 was constructed by ligation of a PstI fragment of pUB5554 containing Tn10 into the PstI site of pUB5502 [13]
3. pUB905 was constructed by transposition of Tn3 into FR1 tet ltsl [2]

Table 2. Values for $k_1 + k_{-1}$ and calculated values for k_1 and k_{-1} for several tetracycline-resistant strains (Table 1) and their plasmidless tetracycline-sensitive parent. Tetracycline accumulation was measured using spectrofluorimetry (see Materials and Methods) and $k_1 + k_{-1}$ and k_1 and k_{-1} calculated as described in the text

Strain	$k_1 + k_{-1}$ [a]	k_1 [a]	k_{-1} [a]
JC3272	3.36 ± 1.54	2.16	1.20
UB1553	2.45 ± 0.13	0.20	2.25
UB6012	2.33 ± 0.59	0.38	1.95
UB6014	2.86 ± 0.74	0.29	2.57
UB1528	2.82 ± 0.93	1.76	1.06
UB1528 + Tc [b]	3.09 ± 0.16	0.44	2.65

a. k_1, k_{-1} and $k_1 + k_{-1}$ are all $\times 10^{-3}$ sec^{-1}

b. UB1528 grown for several generations in the presence of tetracycline (0.5μg/ml)

Resistance is inducible by growth in the presence of low concentrations of tetracycline [2]. At 35°C, UB5783 accumulates tetracycline to a level intermediate between the fully sensitive and resistant states (Table 3). Efflux of tetracycline at 30°C was measured after JC6310 and UB5783 had been loaded with drug at 35°C. The rates of tetracycline efflux were almost identical from JC6310 and UB5783 grown in the absence of tetracycline (Table 4) but the rate from UB5783 was increased approximately 2-fold following induction (Table 4). In each case protein synthesis occurred

Table 3. Accumulation of tetracycline by JC6310 and UB5783. Bacteria were grown at 30°C and tetracycline accumulation measured at the temperature indicated using spectrofluorimetry (see Materials and Methods)

Strain	Tetracycline accumulated after 20min (μg/mg protein) 30°C	35°C	42°C
JC6310	67.1 ± 1.1	66.0 ± 2.6	66.0 ± 5.2
UB5783	55.2 ± 2.1	59.7 ± 3.6	70.5 ± 2.6
UB5783 + Tc[1]	5.2 ± 0.9	38.7 ± 2.4	63.6 ± 5.4

1. UB5783 grown for several generations in the presence of tetracycline (0.5μg/ml)

Table 4. Values for tetracycline efflux (expressed as k_{-1}) from JC6310 and UB5783. Bacteria were grown at 30°C, loaded with tetracycline at 35°C and efflux (30°C) measured using spectrofluorimetry (see text)

Strain	$k_{-1} \times 10^3$ sec^{-1}
JC6310	0.97 ± 0.19
UB5783	0.86 ± 0.11
UB5783 + Tc[1]	2.17 ± 0.30

1. UB5783 grown for several generations in the presence of tetracycline (0.5μg/ml)

during efflux (Table 5). However, the rate of protein synthesis during efflux of tetracycline from induced cultures of UB5783 was about 2-fold higher than in uninduced cultures or the plasmidless host (Table 5). Since tetracycline primarily inhibits protein synthesis, the increased rate of protein synthesis during tetracycline efflux from induced cultures of UB5783 suggests that the accelerated loss observed (Table 4) confers some protection. Efflux of tetracycline from JC6310 was inhibited by 2,4-dinitrophenol and by potassium cyanide (Table 6). These results contradict other reports that tetracycline efflux is energy-independent [8] and the apparent energy dependence is currently being examined in more detail.

Taken together, the data strongly suggest that the increased efflux of tetracycline from resistant bacteria represents a physiological process acting to protect them from inhibition by the antibiotic. The precise

Table 5. Rates of protein synthesis in JC6310 and UB5783 during tetracycline efflux. Bacteria were grown, harvested, washed and resuspended as described in Materials and Methods. Resuspended bacteria were divided into two aliquots, one of which was then loaded with tetracycline (see Materials and Methods). The second aliquot served as a control. Bacteria from each aliquot were resuspended in drug-free medium and [^{14}C]-labelled amino acid mixture (0.5μCi/ml) added immediately. Rates of protein synthesis in both bacterial suspensions were calculated (see Materials and Methods). The rates quoted for pre-loaded cells are expressed as a percentage of the control rates in bacteria not loaded with tetracycline.

Strain	Rate of protein synthesis during tetracycline efflux (% of rate in bacteria not loaded with tetracycline)
JC6310	19.2 ± 4.7
UB5783	20.8 ± 3.8
UB5783 + Tc[1]	50.0 ± 8.0

1. UB5783 grown for several generations in the presence of tetracycline (0.5μg/ml)

Table 6. Effect of 2,4-dinitrophenol (DNP) and potassium cyanide (KCN) on efflux of tetracycline from JC6310. Bacteria were loaded with tetracycline (see Materials and Methods) and DNP or KCN added immediately after resuspension of bacteria in drug-free medium. Tetracycline efflux from bacteria treated with either DNP or KCN is expressed as a percentage of efflux from untreated bacteria.

Concentration of inhibitor		Tetracycline efflux after 20min (% of control)
DNP (μM)	10	63.7 ± 18.7
	100	41.2 ± 10.1
KCN (mM)	10	67.8 ± 19.8
	100	37.7 ± 4.8

mechanism of efflux of tetracycline from resistant bacteria is unknown. However, since tetracycline uptake is apparently an equilibrium process, accelerated efflux may represent interaction of plasmid products with the transport site in such a way that efflux is stimulated at the expense of influx.

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