# R-FACTOR-MEDIATED RESISTANCE TO TETRACYCLINE IN *ESCHERICHIA COLI* K12: AN R-FACTOR WITH A MUTATION TO TEMPERATURE-SENSITIVE TETRACYCLINE RESISTANCE.

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Summary: R-factor-mediated resistance to tetracycline was studied in *Escherichia coli* K12 carrying an *fi*$^+$ F-like R-factor which has a mutation resulting in temperature-sensitive resistance to the drug. The rate of uptake of tetracycline in this mutant is sensitive to temperature, but the rate of efflux is not. These results are consistent with the view that resistance to tetracycline is expressed at the level of the influx rather than of the efflux of tetracycline.

## INTRODUCTION

*Escherichia coli* carrying an R-factor conferring resistance to tetracycline shows reduced permeability to the drug when compared to sensitive *$R^-$ cells which actively accumulate it (1-4). Resistant *$R^+$ bacteria may be induced to higher levels of resistance by preincubation with sub-inhibitory concentrations of tetracycline (3,5). This induction process requires the synthesis of protein and RNA but not DNA (3,6). The isolation of R-factor mutants constitutive for tetracycline resistance (7,8) indicates that an R-factor regulatory gene, apparently coding for a repressor of the resistance gene(s), controls the inducibility of high-level resistance.

Levy and McMurry (9) demonstrated the presence of a unique protein when *E. coli* minicells containing tetracycline-resistant R-factors were incubated in the presence of the drug. This protein was located predominantly in the minicell membrane.

The available evidence indicates that in $R^+$ cells, tetracycline resistance results from the production of one or more proteins which act at

*$R^-$ : lacking an R-factor, $R^+$: containing an R-factor.

the level of the cell membrane by antagonising the active, energy-dependent, accumulation of the drug (2-5), either by reducing its influx or accelerating its efflux.

Tetracycline-sensitive deletion and point mutations in R-factor genes have been described (10). In this communication we report the results of some studies of a mutant which is temperature-sensitive for tetracycline resistance; no such mutant has previously been described. Its importance is that it permits the examination of influx and efflux of tetracycline in an $R^+$ cell under conditions where either sensitivity or resistance to the drug can be expressed.

## MATERIALS AND METHODS

Bacteria. The sensitive strain used was E.coli K12 712 pro trp his lacY str, called 712 $R^-$ in this manuscript.

The resistant strains were: (i) E.coli K12 712 pro trp his lacY str carrying an R-factor determining resistance to the tetracyclines, first described by Franklin and Godfrey (2) and named FR1 (11). We call this strain 712 FR1.

(ii) As (i),but FR1 has a mutation to temperature-sensitive tetracycline resistance (sensitive 42°, resistant 30°). T. J. Franklin supplied the mutant R-factor in a strain of Proteus mirabilis; it was transferred by conjugation to E.coli 712 R-. We call this R-factor FR1tet-ts1, and the strain 712 FR1tet-ts1.

Other strains used were: (i) E. coli RV $lac^{\Delta}$ (from J. A. Cole), (ii) E. coli K12 JM 233 pro thi lac $F'lac^+$ (from B. Ward), (iii) E. coli K12 712 R- $F'lac^+$, (iv) E. coli K12 712 FR1 $F'lac^+$ .

Bacteriophages. Transducing phage P1kc and F-specific phage MS2 were used.

Media. Oxoid Nutrient broth No. 1 and Nutrient agar were used. Minimal salts agar was the medium described by Davis and Mingioli (12) and minimal salts the medium A described by Franklin (3).

Chemicals. Tetracycline hydrochloride was from Lederle Laboratories. [7-$^3$H]-tetracycline hydrochloride was from NEN Chemicals GmbH, Germany, specific activity 700 mCi/mmole. L-amino acids were obtained from BDH, Poole, Dorset.

R-factor transfer. (a) Conjugation. Nutrient broth cultures of exponentially growing donors and recipients were mixed in a ratio of 1:2, incubated statically at 37° for 60 min before samples were streaked onto media selective for transconjugants.
(b) Transduction. The method used for transduction with phage P1kc was that described by Lennox (13), and Watanabe and Fusakawa (14).

Tetracycline Uptake. To estimate the net uptake (influx less efflux) of tetracycline, 712 $R^-$ and 712 FR1*tet-ts1* were grown to logarithmic phase at 37° and 30° respectively in supplemented minimal salts medium, which for 712 FR1*tet-ts1* contained 1 µg/ml tetracycline to induce maximum resistance. 6 ml samples of cells were equilibrated at 0°, 30°, 33°, 36°, 39° and 42°, and tetracycline and [7-$^3$H] - tetracycline were added to final concentrations of 150 µg/ml and 0.1 µCi/ml respectively. After 3 and 6 min incubation, 3 ml samples were withdrawn, centrifuged, the pellets washed once in 3 ml 0.9% NaCl at room temperature and resuspended in 3 ml distilled water. The drug was extracted by heating at 100° for 20 min (3). After removal of denatured cells duplicate 1 ml samples were mixed with 10 ml of Triton X-100: Toluene (1:2) containing scintillants 2,5 diphenyloxazole (2.67 g/l), and 1,4 - di [2-(5-phenyloxazolyl)] benzene (0.08 g/l). The radioactivity was determined using a Phillips liquid scintillation spectrometer, with an efficiency of counting of about 30% for $^3$H. After correcting for the amount of drug non-specifically bound at 0° and slight differences (10% or less) in culture absorbances at 500 nm, the rate of uptake was calculated as µg tetracycline absorbed/min/ml culture.

Tetracycline Efflux. 712 $R^-$ and 712 FR1*tet-ts1* were grown as described above. 30 ml of cells were equilibrated at 42° and tetracycline to a final concentration of 150 µg/ml and [7-$^3$H] -tetracycline to 0.1 µCi/ml were added. The bacteria were then preloaded with drug by incubation at 42° for 30 min when two 15 ml samples of the culture were filtered through prewarmed membrane filters (25 mm 0.22 µm). The filters were then transferred to 15 ml drug-free supplemented minimal salts at 30° and 42°. 3 ml samples were taken immediately and at 10 min intervals for 30 min, and treated as described above. The efflux of tetracycline in drug-free medium was determined as the amount of drug bound to cells measured as a percentage of the drug initially bound.

## RESULTS AND DISCUSSION

The R-factor, FR1, with which the mechanism of tetracycline resistance has been most thoroughly studied by Franklin (2, 3, 7, 11, 15-17), has not previously been characterised genetically. The data of Tables 1 and 2 show that FR1 represses the fertility of the sex-factor F, and produces sensitivity to F-specific phage in the host. Accordingly, FR1 is *fi*$^+$ and F-like (18). The molecular weight of this transmissible plasmid is approximately 42 megadaltons (unpublished work).

Transduction and conjugation data (Table 3) show that FR1*tet-ts1* transductants and transconjugants (selected on medium containing tetracycline at 25°) grow on nutrient agar but not on nutrient agar plus tetracycline at 42°. Since the mutant R-factor, FR1*tet-ts1* is not temperature-sensitive for plasmid replication (data not shown),

TABLE 1. Fertility inhibition property of FR1.

| Donor | Frequency of plasmid transfer by conjugation | |
|---|---|---|
| | F'*lac*$^+$ | FR1 |
| 712 FR1 | - | $4.4 \times 10^{-4}$ (347) |
| 712 F'*lac*$^+$ | $8.3 \times 10^{-1}$ (1775) | - |
| 712 F'*lac*$^+$ FR1 | $8.7 \times 10^{-4}$ (2215 | $7.0 \times 10^{-4}$ (1188) |

Donors were grown to logarithmic phase in nutrient broth supplemented with lactose (1%), tetracycline (10 µg/ml), or both, mixed with recipient *E. coli* RV *lac*$^\Delta$ in a ratio of 1:10, and incubated for 60 min at 37°. Samples were plated onto media selective for RV FR1 or RV F'*lac*$^+$ transconjugants. The frequency of transfer was estimated as the number of transconjugants/donor. Figures in brackets refer to number of colonies counted. Cotransfer of resistance to tetracycline and ability to use lactose from 712 F'*lac*$^+$ FR1 occurred at a frequency of $7.0 \times 10^{-6}$ (154 colonies).

TABLE 2. Sensitivity to phage MS2.

| Strain | Number of phage present in infected culture (plaque-forming units/ml) | |
|---|---|---|
| | 0.5h | 3h |
| 712 R$^-$ | $2.6 \times 10^3$ (518) | $1.7 \times 10^3$ (671) |
| 712 FR1 | $2.2 \times 10^3$ (528) | $1.1 \times 10^5$ (210) |

2 ml of exponentially growing bacteria ($2 \times 10^8$/ml) were infected with phage MS2 at a multiplicity of 10. After 10 min at 37° the mixtures were washed 3 times by centrifugation and resuspension in 10 ml 0.9% NaCl, 2mM $CaCl_2$, and the final pellet suspended in 2 ml nutrient broth. 1 ml was removed and immediately assayed for phage on *E.coli* JM233 F'*lac*$^+$, and the remainder incubated for a further 2.5 hours before assaying. Figures in brackets refer to number of plaques counted.

TABLE 3. Transfer of temperature-sensitive tetracycline resistance by conjugation and by transduction with phage P1*kc*.

| Nature of transconjugants and transductants | Number tested | Number growing on nutrient agar + tetracycline | | Number growing on nutrient agar at 42° |
|---|---|---|---|---|
| | | 25° | 42° | |
| FR1*tet-ts1* transconjugants | 30 | 30 | 0 | 30 |
| FR1*tet-ts1* transductants | 180 | 180 | 0 | 180 |
| FR1 transconjugants | 10 | 10 | 10 | 10 |
| FR1 transductants | 30 | 30 | 30 | 30 |

Donors were mixed with suitably marked recipients as described in Materials and Methods. Transconjugants and transductants were selected on minimal agar containing 20 µg/ml tetracycline and incubated for 48 hours at 25°. Resistant colonies were tested for temperature-sensitivity by replica-plating to plates of nutrient agar, and nutrient agar + tetracycline (20 µg/ml) at 25° and 42°. (FR1 transductants were fertile in subsequent conjugation experiments indicating that P1 transduces the entire R-factor).

it is likely that the mutation is located in the R-factor gene(s) determining resistance to tetracycline. The existence of such a mutation provides further evidence that a protein is directly involved in R-factor-mediated resistance to tetracycline.

At 42° the mutant R-factor does not render its host resistant to tetracycline (Table 4). At 30°, however, resistance is expressed although at a consistently lower level than strains carrying FR1. 712 FR1*tet-ts1* bacteria can be induced to high-level resistance at 30°, but after shifting to 42° these induced cells are sensitive to 1 µg/ml drug. These results indicate that the same protein is

TABLE 4. Minimal inhibitory concentration (MIC) of tetracycline (μg/ml) required to inhibit E.coli strains uninduced and induced to high level resistance at different temperatures.

| Strain | Uninduced | | | Induced | | |
|---|---|---|---|---|---|---|
| | 30° | 37° | 42° | 30° | 37° | 42° |
| 712 FR1 | 45 | 50 | 25 | 120 | 150 | 50 |
| 712 FR1tet-tsl | 25 | 5.0 | 0.5 | 90 | 6.0 | 1.0 |
| 712 $R^-$ | 0.4 | 0.5 | 0.5 | - | - | - |

5 ml of nutrient broth containing different concentrations of tetracycline were inoculated with 2 x $10^6$ cells and incubated for 20 hrs at the specified temperature. 712 FR1 and 712 FR1tet-tsl cells were induced to high level resistance by overnight growth at 37° and 30° respectively in nutrient broth containing 1 μg/ml tetracycline. The MIC was recorded as the lowest concentration of tetracycline required to prevent visible turbidity after incubation.

involved in both low-level (uninduced) and high-level (induced) resistance to tetracycline.

Uptake experiments (Fig. 1a) show that 712 $R^-$ cells accumulate tetracycline at a rate dependent on temperature in the range 30-42°. Cells harbouring FR1tet-tsl, however, take up little or no drug at 30° or 33°, but at 36° and above, it is accumulated at rates comparable to those found with the 712 $R^-$ strain at the same temperatures.

Net uptake of tetracycline must result from an excess of influx over efflux. Franklin and Higginson (4) have shown that in $R^-$ cells increasing intracellular concentrations of tetracycline progressively inhibit efflux of the drug. The results presented in Fig. 1b, show that efflux of tetracycline from 712 FR1tet-tsl occurs at a rate

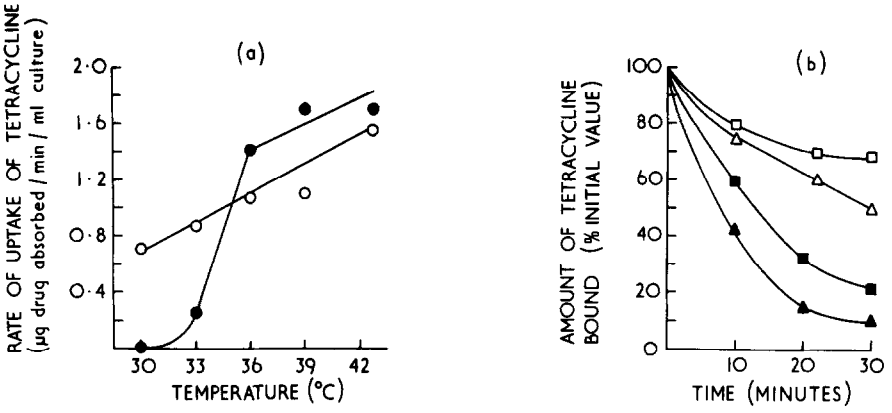


Figure 1. Tetracycline uptake and efflux studies in E.coli 712 $R^-$ and 712 FR1tet-tsl

(a) Uptake. 712 $R^-$ (o) and induced 712 FR1tet-tsl (●) cells were incubated for 3 and 6 min over the temperature range 30-42° in supplemented minimal salts medium containing 150 µg/ml tetracycline and 0.1 µCi/ml [7-$^3$H]-tetracycline. The amount of drug taken up was determined as described in Materials and Methods.
(b) Efflux. 712$R^-$ (△ ▲) and induced 712 FR1tet-tsl (□ ■) cells were preincubated at 42° for 30 min in supplemented minimal salts medium containing 150 µg/ml tetracycline and 0.1 µCi/ml [7-$^3$H]-tetracycline. After membrane filtration the cells were transferred to drug-free medium at 30° (△ □) and 42° (▲ ■) and the loss of drug determined as described in Materials and Methods.

Both uptake and efflux experiments were performed several times with drug concentrations ranging from 50 to 200 µg/ml. Figures 1a and 1b show typical results.

similar to or less than that from 712 $R^-$ cells at both 42° and 30°. This indicates that resistance is not the result of increased efflux, and therefore is probably due to decreased influx of the drug in $R^+$ cells. The less likely possibility that at 42° tetracycline binds to the resistance protein and prevents a conformational change at 30°, is being investigated.

Since tetracycline is accumulated by an energy-dependent process in $R^-$ cells (2,4) growing in either complex or simple media, it is likely that the process occurs by means of a constitutive permease system. We are currently studying chromosomal mutants which produce

higher levels of resistance in $R^+$ and $R^-$ cells, with a view to identifying the transport system by which tetracycline is actively accumulated.

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