THE INTER-RELATIONSHIP OF TETRACYCLINE RESISTANCE, DECYNOYL-N-ACETYL CYSTEAMINE AND MEMBRANE FATTY ACID COMPOSITION IN *ESCHERICHIA COLI*

P. S. RINGROSE and J. E. HIGGINS

Department of Biochemistry, Roche Research Laboratories,
Welwyn Garden City, England

(Received for publication July 17, 1974)

Fatty acid compositions of strains of *Escherichia coli* were compared before and after transference of tetracycline resistance factors. Tetracycline-resistant strains contained significantly less C 16:1 and C 17-cyclopropane but more C 16:0 fatty acids than sensitive strains. Doxycycline (10 μg/ml) enhanced this trend in resistant strains. 3-Decynoyl-N-acetylcysteamine (DNAC) reversed the effect of doxycycline on resistant strains to a slight degree with respect to C 16:1 and C 17 cyclopropane fatty acids, but produced a significant increase in C 16:0 and decrease in C 18:1 fatty acids. DNAC however did not increase sensitivity to doxycycline in sensitive or resistant strains of *E. coli* and in certain cases antagonism was observed.

Tetracycline-resistance is due almost entirely to a decrease in the active accumulation of the drug.[1,2] This effect is inducible in resistant strains[3] and is associated with changes in membrane lipid composition.[4] DUNNICK and O'LEARY have observed increases in the ratio of unsaturated (C 16:1 and 18:1) to cyclopropane (C 17 cy and 19 cy) fatty acids in tetracycline-resistant organisms.[5] A method of interfering with this induced change in lipid composition could be to use a known inhibitor of bacterial unsaturated fatty acid synthesis, *i.e.* 3-decynoyl-N-acetylcysteamine.[6]

Materials and Methods

Materials

Bacto-Dextrose and Bacto Vitamin-free Casamino acids were obtained from Difco. Individual L-amino acids and streptomycin sulphate were from Sigma and tetracycline hydrochloride from Calbiochem. Doxycycline hydrochloride (6-deoxy-5-hydroxytetracycline) was a kind gift from Pfizer (Sandwich, Kent). 3-Decynoyl-N-acetylcysteamine was a kind gift from Dr. K. BLOCH (Harvard University, Mass.)

Abbreviations

Ap —ampicillin	His —histidine	Su —sulphonamides
Cm —chloramphenicol	Met—methionine	Tc —tetracycline
DMSO —dimethyl sulphoxide	Pro —proline	Trp—tryptophan
DNAC—decynoyl-N-acetylcysteamine	Sm —streptomycin	

Bacteria

All strains of *Escherichia coli* described were provided by Prof. G. G. MEYELL, Department of Microbiology, University of Kent. Strains J 53 (R^+ donors) are pro^-, his^-. J 53—R 46 is resistant to Tc, Ap, Sm and Su; J 53—R 64 is resistant to Tc, Sm and Su (I-like) and J 53—R 192 is resistant to Tc, Cm, Sm and Su (F-like). Tc sensitive strains were 711, 712 and CL 142.

711 and 712 are pro$^-$, his$^-$, trp$^-$, lac$^-$. 711 is nalidixic acid resistant and Sm^S. 712 and CL 142 are Sm^R.

Media and Growth Conditions

Growth medium M9 (50 mM Na_2HPO_4, 20 mM NH_4Cl, 20 mM KH_2PO_4, 0.1 mM $MgSO_4$, 1.0 μM $FeCl_3$, 20 mM glucose) was supplemented with either 3 g casamino acids per litre (plus tryptophan), or 50 μg individual essential amino acids per ml. Unless otherwise stated tetracycline or doxycycline was routinely used at 10 μg/ml and streptomycin (100 μg/ml) was added to all media used for Sm^R organisms. Duplicate cultures were harvested for lipid analysis after diluting an overnight culture to a cell density of 1×10^8 bacteria/ml and incubating for 2 hours at 37°C with shaking.

Serial dilutions of DNAC and doxycycline were incubated with *E. coli* at an initial cell density of 2×10^7 bacteria/ml and minimum growth inhibitory concentrations determined after 4~5 hours. Isobolograms and fractional inhibitory concentrations of inhibitor combinations were determined according to the method of ELION *et al.*[7]

Extraction and Trans-methylation of Fatty Acids

After harvesting and washing, the cell pellet was extracted with chloroform-methanol (1:1) for 4 hours at 20°C, followed by overnight extraction with chloroform-methanol (2:1).[8] The extraction solvents were combined, adjusted to 2:1 chloroform-methanol, and shaken with one fifth their volume of 0.29% (w/v) saline.[9] After centrifugation the top layer was removed. The interface was washed gently with 3×2 ml of "top phase" solvents (3:48:47 chloroform-methanol-0.29% (w/v) NaCl). Methanol was added to give a homogeneous mixture which was evaporated to dryness under vacuum at 70°C. The residual lipid fraction was transmethylated by refluxing with methanol-benzene-conc. Sulphuric acid (20:10:1) for 90 minutes over steam. The methylated fatty acids were extracted with 1:1 diethylether-petroleum ether (60~80°) and washed five times with water to remove acid. The extraction solvents were dehydrated with anhydrous sodium sulphate and evaporated to dryness under vacuum at 30°C. Evaporation was repeated after addition of a small amount of *n*-hexane. The esters were finally dissolved in 1 ml *n*-hexane and stored at −20°C.

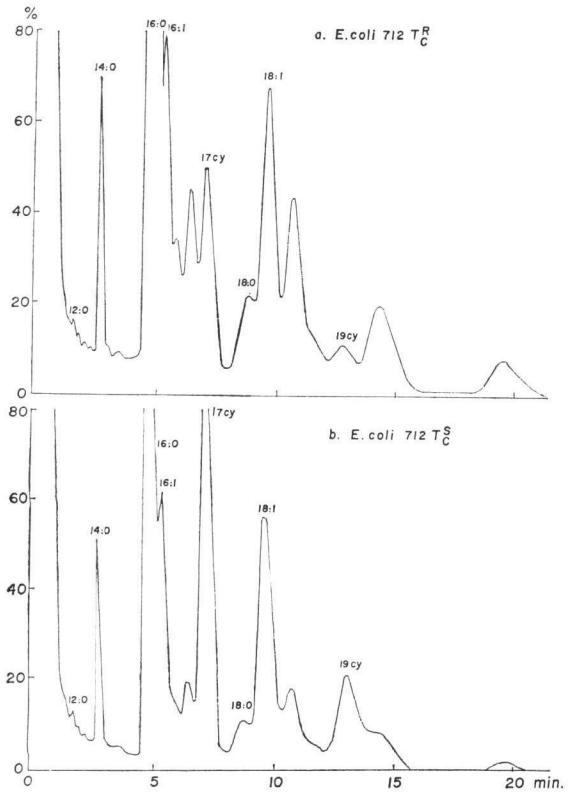
Identification of Fatty Acids by Gas Liquid Chromatography

Gas-liquid chromatography analyses were carried out using a Pye-Unicam Series 104 Chromatograph with a flame ionisation detector. The liquid phase was 10% polyethylene glycol adipate on Universal support, 80~100 mesh (Phase Separations Ltd., Deeside Industrial Estate) in a five foot siliconised glass column, diameter 1/16". The carrier gas used was oxygen-free nitrogen, at a flow rate of 50 ml/minute. Temperature was maintained isothermally at 197°C. Sample size was routinely 2 μl. Retention times were measured and expressed relative to methyl palmitate. Peak attenuation was varied from 20 to 5,000 to give 40~100% full scale deflection. A standard mixture was prepared from 13 methyl fatty acid standards (0.4 mg/ml) obtained from Applied Science Labs. Inc. (Field Instruments Company, Richmond, Surrey). Esters of even-numbered saturated fatty acids from 6:0 to 22:0 inclusive were used, plus the mono-unsaturates; myristoleate (14:1ω5), palmitoleate (16:1ω7), oleate (18:1ω9) and eicosanoate (20:1ω9). The standard was run before and after each set of analyses and the average relative retention times used to determine the identity of the methylated fatty acids in the samples (Fig. 1). Isomers of 18:1 were not distinguished. Cyclopropane fatty acids were identified by comparison with their reported retention times.[10,11]

Method of Transferring Tc Resistance

The Tc^R transfer factor from strain J 53—R 46 was used to confer resistance on strain 712. Exponentially growing cultures of donor and receptor strains were mixed in the proportion of 1:4 and incubated without agitation at 37°C for 2 hours in M9 medium supplemented with

Fig. 1. Gas/liquid chromatogram of fatty acid methyl esters prepared from tetracycline resistant and sensitive strains of *E. coli*



Fatty acid methyl esters are prepared from chloroform-methanol extracts of exponential cultures of (a) the Tc^R recombinant of *E. coli* strain 712 and (b) the Tc^S parent strain 712. Esters are subjected to gas-liquid chromatography on 10% PEGA at 197°. Peak traces are at constant attenuation and chart speed.

essential amino acids. A 0.1 ml aliquot was diluted with 9.9 ml 0.85% saline, agitated, and plated onto plain agar containing medium M9 supplemented with Pro, His, Trp, Sm and 10 μg/ml Tc. This medium was selective for Tc^R, Pro^-, His^-, Trp^- recombinants. Clonal isolates of the recombinant 712 Tc^R were subsequently cultured in selective medium for lipid analysis.

Results and Discussion

In Table 1 the mean fatty acid composition of 3 Tc^S strains is compared with that of three Tc resistance-factor carrying strains.

Table 1. Fatty acid compositions of tetracycline-sensitive and resistant strains of *E. coli*

Fatty acid	% Fatty acid composition ±S.E.M.	
	Tc^S [12]	Tc^R [12]
16:0	44.2±1.8	52.7±1.3*
16:1	16.0±1.9	9.1±0.7*
17 cy	10.5±1.2	2.6±0.2*
18:0	1.2±0.2	1.8±0.3
18:1	19.0±1.4	16.0±0.9
19 cy	1.7±0.3	0.3±0.1*

Fatty acid compositions of *E. coli* Tc^S strains (CL 142, 711 and 712) and TC^R strains (J 53—R 46, —R 64 and —R 192) are determined from chloroform/methanol extracts of exponential cultures by gas liquid chromatography (see Fig. 1). Percentages are based on the total fatty acid content including C 12:0, 14:0 and unidentified peaks, and are expressed ±S.E.M. of 12 determinations (4 per strain). Asterisks mark statistically significant values ($p<0.01>0.001$).

Resistant strains have significantly more ($p<0.01$) palmitic (16:0) but less palmitoleic (16:1) and cyclopropane (17 and 19) acids than sensitive strains. The change in stearic (18:0) and vaccenic (18:1) acids is not significant. The decreased proportion of cyclopropane fatty acids in Tc^R strains is consistent with the low occurrence of these fatty acids in membranes of naturally resistant mammalian cells[12] and has been observed by other workers.[5,10] In Table 2 fatty acid compositions of strain 712 are compared before (Tc^S) and after (Tc^R—) transference of a Tc^R factor from J 53—R 46. The same changes as in Table 1 for C 16:0, C 16:1 and C 17 cy fatty acids are observed after resistance factor transference and this trend is enhanced in the recombinant strain after incubation with doxycycline (Tc^R+). In Tables 1 and 2 there is a net decrease in the relative amounts of mono-unsaturated and cyclopropane fatty acids which is countered by an increase in saturated fatty acids. These observations are contrary to those of DUNNICK and O'LEARY[5] who find that the proportion of saturated to mono-unsaturated plus cyclopropane fatty acids remains constant, *i.e.* the decrease in cyclopropane fatty acids is

Table 2. Effects of resistance factor transference, tetracycline induction and decynoyl-NAC on *E. coli* strain 712 fatty acid composition

Fatty acid	% Fatty acid composition ±S.E.M.			
	Tc^S [6]	$Tc^R(-)$ [6]	$Tc^R(+)$ [4]	+DNAC [4]
16:0	41.5±1.2	45.5±1.3	55.9±1.4	59.8±0.8
16.1	18.4±1.5	12.1±0.7	9.2±1.3	14.7±0.4
17 cy	13.6±0.6	2.2±0.2	2.8±0.1	5.1±0.9
18:0	1.3±0.2	1.6±0.4	1.3±0.2	1.9±0.4
18:1	20.0±0.7	13.3±0.4	15.6±0.3	8.3±0.5
19 cy	2.4±0.6	5.8±0.4	4.4±0.3	1.6±0.2

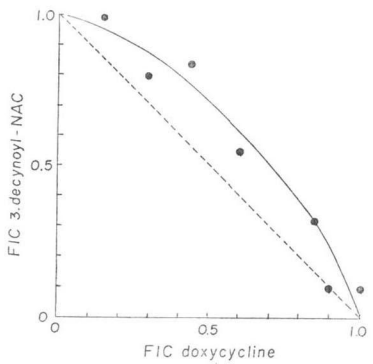
Fatty acid compositions of *E. coli* strain 212 are determined before (Tc^S) and after (Tc^R-) recombination with strains J 53—R 46 (see Methods). Determinations are also made of the recombinant strain (a) in the presence of 10 μg doxycycline/ml (Tc^R+) and (b) in the presence of doxycycline but after pre-treatment with 6 μg DNAC/ml(+DNAC). Percentages are expressed as described in Table 1; the number of determinations are given in parenthesis.

countered by an increase in unsaturated fatty acids.

The effect of pre-incubating the Tc^R recombinant with sub-growth inhibitory concentrations of the β-OH-decanoyl-thioester dehydrase inhibitor, DNAC,[6] before adding doxycycline is also shown in Table 2. DNAC reversed the effect of doxycycline on the recombinant strain to a slight degree with respect to C 16:1 and C 17 cy fatty acids, but produced a significant increase in C 16:0 and decrease in C 18:1 fatty acids consistent with its site of action.[6]

The original premise that induced tetracycline resistance is associated with an increased synthesis of unsaturated fatty acids was not confirmed and therefore interference with the expression of tetracycline resistance by inhibitors such as DNAC, as postulated, was not observed in growth inhibition tests. The minimum growth inhibitory concentration of DNAC using Tc^S strains 711, 712 and CL 142 was 4~6 μg/ml whereas for Tc^R strains J 53—R 46 and the 712 recombinant, it increased to 20~30 μg/ml. Pre-incubation of Tc^S and Tc^R strains with DNAC at sub-growth inhibitory concentrations did not alter their susceptibility to doxycycline and no potentiation was observed. In Fig. 2 antagonism of a low order was observed.

Fig. 2. Isobologram of doxycycline *vs* decynoyl-NAC



E. coli strain CL 142 is incubated with serial dilutions of combinations of doxycycline and DNAC, and the concentrations required to inhibit growth by 50 % after 4~5 hours determined. Fractional inhibitory concentrations are calculated and an isobologram drawn according to the method of ELION *et al.*[7]

Unsaturated fatty acids have been implicated in other active transport systems[13~15] and have been shown to increase cell permeability[16] probably by binding other lipid molecules together as pores.[13] The decreased proportion of unsaturated fatty acids in Tc^R strains of *E. coli* observed in this report is therefore consistent with a change in permeability. The results of FRANKLIN[3] however indicate that tetracycline sensitivity of a microorganism is due to an induced inhibition of drug "outflow"

whilst "inflow" remains unimpeded. A net accumulation of the drug therefore occurs. Resistance is thought to be due to a failure in preventing "outflow" rather than interference with "inflow" permeability.[8] Changes observed in the relative amounts of cyclopropane and mono-unsaturated-fatty acids in resistant strains may therefore reflect this failure of the resistant micro-organism to prevent drug "outflow" although no significant change was observed with Tc^S strains and low doxycycline concentrations. The antagonism of DNAC and doxycycline observed in Fig. 2 could therefore be the result of either DNAC preventing doxycycline induced inhibition of 'outflow' or doxycycline impeding DNAC permeability.

References

1) Izaki, K. & K. Arima: Disappearance of oxytetracycline accumulation in the cells of multiple drug-resistant *E. coli*. Nature 200: 384~385, 1963
2) Franklin, T. J. & B. Higginson: Active accumulation of tetracycline by *E. coli*. Biochem. J. 116: 287~297, 1970
3) Franklin, T. J.: Changes in permeability to tetracyclines in *E. coli* bearing transferable resistance factors. Biochem. J. 105: 371~378, 1967
4) Norrington, F. E. & A. M. James: The cell wall lipids of cells of tetracycline-sensitive and resistant strains of *Streptococcus pyogenes*. Biochim. Biophys. Acta 218: 269~277, 1970
5) Dunnick, J. K. & W. M. O'Leary: Correlation of bacterial lipid composition with antibiotic resistance. J. Bact. 101: 892~900, 1970
6) Endo, K.; G. M. Helmkamp & K. Bloch: Mode of inhibition of β-hydroxydecanoyl thioester dehydrase by 3-decynoyl-N-acetylcysteamine. J. Biol. Chem. 245: 4293~4296, 1970
7) Elion, G. B.; S. Singer & G. H. Hitchings: Antagonists of nuclic acid derivatives. VIII. Synergism in combinations of biochemically related anti-metabolites. J. Biol. Chem. 208: 477~488, 1954
8) Russell, N. J.: Alteration in fatty acid chain length in *Micrococcus cryophilus* grown at different temperatures. Biochim. Biophys. Acta 231: 254~256, 1971
9) Folch, J.; M. Lees & G. H. Stanley: A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226: 467~509, 1957
10) Kaneshiro, T. & A. G. Marr: *Cis*-9, 10-methylene hexadecanoic acid from the phospholipids of *E. coli*. J. Biol. Chem. 236: 2615~2619, 1961
11) Bishop, D. G. & J. L. Still: Fatty acid metabolism in *Serratia marcescens*. III. The constituent fatty acids of the cell. J. Lipid Research 4: 81~86, 1963
12) Law, J. H.: Bacterial lipids in "The Specificity of Cell Surfaces", Davies, B. D. & L. Waren (Eds.) Prentice-Hall Inc., Engelwood Cliffs, New Jersey, 87~105, 1967
13) Gale, E. F. & J. M. Llewellyn: Effect of unsaturated fatty acids on aspartate transport in *S. aureus* and on staphylococcal lipid monolayer. Biochim. Biophys. Acta 233: 237~242, 1971
14) Cronan, J. E. & P. R. Vagelos: Metabolism and function of the membrane phospholipids of *E. coli*. Biochim. Biophys. Acta 265: 25~60, 1970
15) Fox, C. F.: Lipid requirement for induction of lactose transport in *E. coli*. Proc. Natl. Acad. Sci. U.S. 63: 850~855, 1969
16) De Gier, J.; C. Haest, J. Mandersloot & L. Van Deenen: Valinomycin-induced permeation of Rb^+ of liposomes with varying composition through bilayers. Biochim. Biophys. Acta 211: 373~375, 1970