Tetracycline Resistance in *Escherichia coli* K12 is not Associated with a Decrease in Cyclopropane Fatty Acid Content

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INTRODUCTION

In *Escherichia coli* plasmid-mediated resistance to tetracyclines is associated with the synthesis of membrane proteins (resistance proteins) (Levy & McMurry, 1974; Yang, Zubay & Levy, 1976; Wojdani, Avtalion & Sompolinsky, 1976; Chopra, unpublished). Since resistance results from decreased accumulation of the antibiotic (Franklin, 1973; Young & Hubball, 1976) the resistance proteins probably have a role in the exclusion of tetracycline from the cell. Both Dunnick & O'Leary (1970) and Ringrose & Higgins (1974) found that *E. coli* harbouring plasmids which specify resistance to tetracycline contained decreased quantities of cyclopropane fatty acids compared with plasmid-less bacteria. These authors suggested that the reduction in cyclopropane fatty acid content might be related to the formation of the permeability barrier in tetracycline-resistant cells. It is impossible, however, to attribute the reported cyclopropane fatty acid changes (Dunnick & O'Leary, 1970; Ringrose & Higgins, 1974) to expression of tetracycline resistance since plasmid products other than those specified by the tetracycline resistance regions could mediate the changes. We have therefore re-examined the possibility of an association between cyclopropane fatty acid content and tetracycline resistance in *E. coli* by (i) comparing the sensitivity of wild-type and cyclopropane fatty acid deficient mutants to tetracycline and (ii) analysing the fatty acid composition of strains which are isogenic apart from the tetracycline resistance region.

METHODS

Bacteria. Bacterial strains are listed in Table 1.

Culture media. These have been described previously (Chopra, Lacey & Connolly, 1974).

Other materials. Tetracycline and straight-chain fatty acid methyl ester standards were purchased from Sigma. Cyclopropane fatty acid methyl ester standards (*cis*-9,10-methylene hexadecanoic acid and *cis*-9,10-methylene octadecanoic acid) were purchased from Applied Science Laboratories, State College, Pennsylvania, U.S.A.

Isolation and methylation of fatty acids. Bacteria in the mid-exponential phase of growth (5×10^8 bacteria ml^{-1}) were harvested by centrifugation (15000 g, 15 min, 4 °C) and washed twice at 4 °C in distilled water. Lipids were extracted by the method of Bligh & Dyer (1959) and purified by re-extraction in benzene (Radin, 1969). Dimethoxypropane-induced transmethylation of fatty acids was performed as described by Mason & Waller (1964) using 3 M anhydrous methanolic HCl.

Gas chromatography of fatty acid methyl esters. Fatty acid methyl esters were analysed in a Pye series 104 gas chromatograph. Acid-washed diatomite C (100 to 120 mesh) was coated either with polyethylene glycol adipate (10 %, w/w) or Apiezon L grease (10 %, w/w) and packed into columns (1.5 m $\times$ 4 mm i.d.). Columns were run isothermally at 180 °C for polyethylene glycol adipate and 197 °C for Apiezon L. The flow of carrier gas (nitrogen) was 45 ml min^{-1} for each type of column and the flame ionization detectors were lit by a mixture of hydrogen (45 ml min^{-1}) and air (700 ml min^{-1}). Under these conditions, the efficiency of the Apiezon L column for methyl palmitate was 2400 theoretical plates and that of the polyethylene glycol adipate column was 560 theoretical plates.

Table 1. *Escherichia coli* K12 strains

Strain no.	Strain description	Reference and source
JC3272	<i>his trp ma lgal lys lacΔX74 su⁻ λ^R T6^R T1⁸ Str^R Spc⁸ rec⁺ F⁻</i>	Achtman, Willetts & Clark (1971)
DUI203	Derived by translocation of the tetracycline resistance region (Tn10; Cohen, 1976) of pDU301* to the chromosome of JC3272	Foster, Howe & Richmond (1975)
FT1	<i>proC32 purE42 metE70 metB1 lysA23 thi-1 trpE38 str-109 F⁻</i>	Taylor & Cronan (1976)
FT16	Cyclopropane fatty acid deficient (CFA ⁻) derivative of FT1	Taylor & Cronan (1976)
FT17	CFA ⁻ derivative of FT1	Taylor & Cronan (1976)
UB1593	FT1(pDU301)	This paper
UB1594	FT16(pDU301)	This paper
UB1595	FT17(pDU301)	This paper

* pDU301 is a mutant of R100-1 which expresses tetracycline resistance constitutively.

Identification of the E. coli fatty acid methyl esters. Mixtures of standard saturated, monoenoic and cyclopropane fatty acid methyl esters were chromatographed on the polar (polyethylene glycol adipate) and non-polar (Apiezon L) columns. The logarithms of the retention times were plotted against the number of carbon atoms in the ester, and the points were connected by straight lines as established by James (1959). Chromatographic analysis of the *E. coli* fatty acid methyl esters was performed under the same conditions. All but one of the fatty acid esters were identified by their positions on these graphs. One unknown peak which was resolved between methyl myristate (C_{14:0}) and the C₁₆ fatty acid esters was tentatively identified as the methyl ester of β-hydroxymyristic acid (see Fig. 2).

Statistical analysis of cyclopropane fatty acid methyl ester contents. A regression analysis (A. J. Hedges, unpublished data) was used to determine the statistical significance of differences in cyclopropane fatty acid content between strains JC3272 and DUI203.

RESULTS AND DISCUSSION

Addition of 0.8 µg tetracycline ml⁻¹ resulted in a reduction of about 50 % in the growth rate of strain FT1 (Fig. 1*a*). The cyclopropane fatty acid deficient (CFA⁻) mutants (FT16 and FT17) were both slightly more sensitive to tetracycline than the wild-type parental strain (FT1) (Fig. 1*a*). Thus, 0.4 µg tetracycline ml⁻¹ caused a reduction of about 50 % in the rate of growth of FT16 and about 60 % for FT17 (Fig. 1*a*).

Although these data suggest that a reduction in cyclopropane fatty acids results in a decrease of the basal level of resistance to tetracycline, expression of plasmid-mediated resistance could require reduction in the cyclopropane fatty acid content. For instance this could reflect the requirements for correct insertion into the membrane of the plasmid-specified resistance proteins. To assess this possibility, the plasmid pDU301 was transferred to strains FT1, 16 and 17, producing strains UB1593, 1594 and 1595 respectively, and the resistances of these strains to tetracycline were compared. Although UB1594 was slightly less resistant to tetracycline than UB1593 (Fig. 1*b*), the inhibition curves for UB1593 and UB1595 were virtually identical (Fig. 1*b*). We therefore conclude that reduction in the cyclopropane fatty acid content is not a requirement for the integration or function of the membrane resistance proteins determined by pDU301.

Since the synthesis or insertion into the membrane of the tetracycline resistance proteins could indirectly affect the cyclopropane fatty acid content of the cell, we determined the cyclopropane fatty acid content of bacteria known to differ only in the possession of the tetracycline resistance region. Representative chromatograms of the methyl esters of fatty acids from JC3272 (tetracycline sensitive) and DUI203 (constitutively resistant to tetracycline) are shown in Fig. 2. The C₁₇ cyclopropane fatty acid content of JC3272 was 4.83 % (three determinations) and that of DUI203, 4.63 % (three determinations) ($P > 10\%$).

Fig. 1. The effects of tetracycline on the growth rate of (a) strains FT1 (●), FT16 (○) and FT17 (×) and (b) strains UBI593 (●), UBI594 (○) and UBI595 (×). Cultures were grown at 37 °C with aeration in the presence of the indicated concentrations of tetracycline. Growth rates were determined from semi-logarithmic plots of culture absorbance at 675 nm versus time. The growth rate of each strain in the absence of antibiotic has been normalized to 1.0. Other rates are expressed as a proportion of the 'drug-free' rates.

Fig. 2. Gas chromatograph recorder tracings of fatty acid methyl esters prepared from (a) JC3272 and (b) DUI203. Fatty acids were extracted, esterified and analysed on a polyethylene glycol adipate column as described in Methods. The peaks were identified as described in Methods (cyc = cyclopropane).

The C₁₉ cyclopropane fatty acid content of JC3272 was 1.07 % (three determinations) and that of DUI203, 1.36 % (three determinations) ($P > 10\%$).

The data presented here therefore suggest that there is no correlation between expression of tetracycline resistance and cyclopropane fatty acid content. The data reported by Dunnick & O'Leary (1970) and Ringrose & Higgins (1974) which showed decreased cyclopropane fatty acid contents in tetracycline-resistant strains of *E. coli* may be explained in one of two ways. Either cyclopropane fatty acid synthesis was affected by products of the plasmids other than those of the tetracycline resistance regions, or it was affected by the presence of tetracycline which was used to induce resistance. The difficulty of attempting to correlate fatty acid content with tetracycline resistance in non-isogenic strains is illustrated by comparison of Dunnick & O'Leary's (1970) data with those of Ringrose & Higgins (1974). Dunnick & O'Leary (1970) report that the ratio of saturated to monoenoic plus cyclopropane fatty acids is the same in sensitive and resistant strains, yet Ringrose & Higgins (1974) show that this ratio is increased in resistant bacteria.

Sub-inhibitory concentrations of tetracycline reduce the synthesis of envelope proteins to a greater extent than that of cytoplasmic proteins in *E. coli* (Hirashima, Childs & Inouye, 1973). Reduction in cyclopropane fatty acid content could therefore reflect partial inhibition of the synthesis of cyclopropane fatty acid synthetase by tetracycline. The experiments described in this paper avoid such difficulties since pDU301 confers constitutive expression of tetracycline resistance.

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