Tetracycline Resistance Determinants from Groups A to D Vary in Their Ability to Confer Decreased Accumulation of Tetracycline Derivatives by *Escherichia coli*

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The ability of four genetically distinct plasmid-located tetracycline resistance determinants (TetA, B, C and D) to confer decreased accumulation of tetracycline and some of its analogues by *Escherichia coli* K12 was examined. Accumulation of oxytetracycline, tetracycline, demethylchlorotetracycline, 6-demethyl-6-deoxy-5-hydroxy-6-methylene-tetracycline, chlorotetracycline, doxycycline and 6-demethyl-6-deoxytetracycline was examined by fluorescence spectroscopy. The determinants varied in their ability to promote decreased accumulation of tetracyclines, defined as an R^+/R^- fluorescence ratio of <0.85 . Plasmid pIP7 (TetA) caused reduced accumulation of only oxytetracycline, tetracycline and chlorotetracycline, but plasmid pDU301 (TetB) promoted reduced accumulation of all the compounds tested except 6-demethyl-6-deoxytetracycline. The TetC determinant of pBR322 caused decreased uptake of five derivatives, but not doxycycline or 6-demethyl-6-deoxytetracycline. Plasmid RA1 (TetD) encoded reduced accumulation of oxytetracycline, tetracycline, 6-demethyl-6-deoxy-5-hydroxy-6-methylenetetracycline and chlorotetracycline. In general, the resistance determinants were more efficient in promoting decreased accumulation of hydrophilic tetracyclines. These accumulation studies provide a satisfactory method for the phenotypic identification of Tet resistance determinants.

INTRODUCTION

In many bacterial species the genes that specify resistance to tetracycline are plasmid-located (for a recent review, see Chopra *et al.*, 1981*a*). The use of DNA–DNA hybridization techniques on plasmids found in Gram-negative bacteria has revealed the presence of at least four classes (TetA to TetD) of tetracycline resistance determinants (Mendez *et al.*, 1980). However, each determinant specifies a resistance mechanism which involves decreased accumulation of tetracycline (Ball *et al.*, 1980; McMurry *et al.*, 1980; Shales *et al.*, 1980). The lack of nucleotide sequence homology between the resistance determinants implies that the products conferring decreased tetracycline accumulation are not identical. In view of this, it seems plausible that the resistance determinants may code for products which promote decreased accumulation of tetracycline, but which vary in their ability to confer decreased accumulation of other tetracyclines. We have now examined this question.

METHODS

Bacterial strains and plasmids. The plasmid-free host strain used throughout this work was *Escherichia coli* K12 strain JC3272 (Achtman *et al.*, 1971). Plasmids pIP7 (TetA) (Shales *et al.*, 1980; Mendez *et al.*, 1980), pDU301 (TetB) (Shales *et al.*, 1980) and RA1 (TetD) (Mendez *et al.*, 1980) were introduced into JC3272 by conjugation. Plasmid pBR322 (derived from TetC) (Sutcliffe, 1978; Mendez *et al.*, 1980) was obtained from Dr P. M. Bennett and introduced into JC3272 by transformation.

Tetracyclines. Tetracycline, oxytetracycline and chlorotetracycline were purchased from Sigma. Demethylchlorotetracycline, 6-demethyl-6-deoxy-5-hydroxy-6-methylenetetracycline, 6-demethyl-6-deoxytetracycline and minocycline were gifts from Lederle Laboratories. Doxycycline was kindly donated by Pfizer Ltd.

General methods. Concentrations of antibiotic causing a 50% reduction in growth rate (IC_{50}) were determined as described by Shales *et al.* (1980). Resistance was induced by growth of cultures in the presence of tetracycline ($0.5 \mu\text{g ml}^{-1}$). Accumulation of tetracyclines was assayed by spectrofluorimetry (Samra *et al.*, 1978; Ball *et al.*, 1980; Shales *et al.*, 1980).

RESULTS

Phenotypic patterns of resistance mediated by determinants of groups A to D

Mendez *et al.* (1980) reported that TetB determinants encode resistance to both tetracycline and minocycline. Previous studies in this laboratory (Shales *et al.*, 1980; Chopra *et al.*, 1981*b*) have confirmed these observations, but in contrast to the data of Mendez *et al.* (1980) we have found that RA1 (TetD) encodes minocycline resistance (Table 1). Since pSC101 only confers resistance to tetracycline (Shales *et al.*, 1980; Mendez *et al.*, 1980) a similar phenotype might also be expected for pBR322, which is a derivative of pSC101 (Sutcliffe, 1978). However, pBR322 does mediate resistance to minocycline (Table 1).

Ability of determinants to confer decreased accumulation of tetracycline and some of its analogues

Various tetracyclines can be used as fluorescent probes of their own accumulation by bacteria (Samra *et al.*, 1978; Ball *et al.*, 1980; Shales *et al.*, 1980). Using a spectrofluorimetric method, the ability of determinants from groups A to D to confer decreased accumulation of tetracycline and some of its analogues was examined. Decreased accumulation was considered to occur when the R^+/R^- fluorescence ratio was <0.85 . On this basis the determinants varied in their ability to promote decreased accumulation of tetracyclines (Table 2). Plasmid pIP7 (TetA) only caused reduced accumulation of oxytetracycline, tetracycline and chlorotetracycline. Plasmid pDU301 (TetB) promoted reduced accumulation of oxytetracycline, tetracycline, demethylchlorotetracycline, 6-demethyl-6-deoxy-5-hydroxy-6-methylenetetracycline, chlorotetracycline and doxycycline. Plasmid pBR322 (TetC) caused reduced accumulation of oxytetracycline, tetracycline, demethylchlorotetracycline, 6-demethyl-6-deoxy-5-hydroxy-6-methylenetetracycline and chlorotetracycline. Plasmid RA1 (TetD) encoded reduced accumulation of oxytetracycline, tetracycline, 6-demethyl-6-deoxy-5-hydroxy-6-methylenetetracycline and chlorotetracycline.

In general, the resistance determinants were more efficient in promoting decreased accumulation of hydrophilic tetracyclines (Table 2). For example, most determinants caused reduced accumulation of analogues with partition coefficients ≤ 0.32 (hydrophilic compounds), but none encoded reduced uptake of 6-demethyl-6-deoxytetracycline and only one (pDU301, TetB) conferred decreased accumulation of doxycycline. Both these antibiotics are hydrophobic (partition coefficients >0.32) (Table 2).

DISCUSSION

In a previous paper (Shales *et al.*, 1980) we reported our inability to detect decreased tetracycline accumulation in cells harbouring pSC101. In contrast, cells containing pBR322 exhibit decreased drug accumulation (Table 2). Although pBR322 contains only part of the tetracycline resistance region of pSC101, the copy number of pBR322 (20–30 copies per chromosome; Boyer *et al.*, 1977) is greater than that of pSC101 (6–8 copies per chromosome; Bukhari *et al.*, 1977). These findings imply that the fluorescence assay might measure reduced drug accumulation only when the quantity of plasmid products reaches a certain level within the cell.

Table 1. *Resistance profiles to tetracycline and minocycline of uninduced and induced cultures of E. coli K12 JC3272 containing different plasmids*

Plasmid	Phenotypic classification of resistance determinant†	Resistance level (IC ₅₀) to tetracycline (µg ml ⁻¹)‡		Resistance level (IC ₅₀) to minocycline (µg ml ⁻¹)‡	
		Uninduced	Induced	Uninduced	Induced
None	None	0.29 ± 0.02	ND	0.42 ± 0.08	ND
pIP7	TetA	3.1 ± 0.1	21.0 ± 2.0	0.37 ± 0.04	0.45 ± 0.01
pDU301*	TetB	61.0 ± 1.0	ND	2.35 ± 0.25	ND
pBR322*	Derived from TetC	32.0 ± 5.5	ND	1.20 ± 0.30	ND
RA1	TetD	15.3 ± 1.1	47.3 ± 6.3	1.1 ± 0	2.7 ± 0.2

ND, Not determined.

* Expression of resistance by these plasmids is constitutive.

† According to the scheme of Mendez *et al.* (1980). The tetracycline resistance determinant of pBR322 is derived from that in pSC 101 (Sutcliffe, 1978); the latter contains a TetC determinant (Mendez *et al.*, 1980).

‡ Mean values ± 1 standard deviation.

Table 2. *Partition coefficients and antibiotic accumulation in strains of E. coli containing different plasmids*

Antibiotic	Partition coefficient (octanol/aqueous buffer) at pH 6.6*	Antibiotic accumulation (arbitrary fluorescence units) in R ⁻ host†	Ratio of antibiotic accumulation (R ⁺ /R ⁻) in strains containing different plasmids			
			pIP7 (TetA)	pDU301 (TetB)	pBR322 (TetC)	RA1 (TetD)
Oxytetracycline	0.087	45.8	0.11	0.19	0.43	0.26
Tetracycline	0.052	79.2	0.08	0.13	0.31	0.15
Demethylchlorotetracycline	0.19	58.3	1.62	0.31	0.53	1.39
6-Demethyl-6-deoxy-5-hydroxy-6-methylenetetracycline	NR	65.0	0.95	0.25	0.29	0.47
Chlorotetracycline	0.32	73.8	0.67	0.13	0.31	0.60
Doxycycline	0.92	122.0	1.22	0.58	1.08	0.86
6-Demethyl-6-deoxytetracycline	0.96	85.0	0.92	0.85	0.87	1.04

* Data from Colaizzi & Klink (1969). NR, Not reported.

† Mean of duplicate determinations.

Despite limitations on the use of fluorescence spectroscopy to examine tetracycline accumulation in cells containing pSC101, the method is generally versatile and permits studies on the accumulation of tetracycline derivatives which are not readily available in a radioactive form (Samra *et al.*, 1978). Data presented here show that different classes of tetracycline resistance determinants vary in their ability to promote decreased accumulation of tetracycline derivatives. Thus, the lack of nucleotide sequence homology between these determinants (Mendez *et al.*, 1980) could be due at least partially to differences in the products mediating decreased drug accumulation. Reduced tetracycline accumulation mediated by Tn10 (TetB) results from insertion of a 36 kilodalton protein into the cytoplasmic membrane of *E. coli* (Levy *et al.*, 1977; Chopra *et al.*, 1981c) and proteins of similar molecular weights have been implicated in reduced accumulation of tetracycline mediated by pBR322 (see Chopra *et al.*, 1981a) and pRSD1 (TetA) (R. Schmitt, personal communication). However, attempts to compare these proteins on the basis of mass alone could be misleading because requirements for membrane insertion may necessitate that the various resistance proteins have similar sizes. Isoelectric point determinations on the membrane proteins could answer the question of whether they differ, but to date only the Tn10 (TetB) product has been examined (pI 6.4) (Chopra *et al.*, 1981c).

The ability of the resistance determinants to promote decreased accumulation of hydrophobic derivatives such as doxycycline and 6-demethyl-6-deoxytetracycline is poor (Table 2). Accumulation of minocycline (partition coefficient 1.48 at pH 6.6) was not investigated, but it is presumed to behave in a similar fashion to 6-demethyl-6-deoxytetracycline. Indeed, *E. coli* carrying a TetB determinant accumulates equivalent amounts of minocycline to the plasmid-free host (Shales *et al.*, 1980). Plasmid-determined resistance to such hydrophobic tetracyclines probably involves ribosomal alterations (Shales *et al.*, 1980).

Mendez *et al.* (1980) reported that the levels of resistance to tetracycline, minocycline and β -chelocardin conferred by determinants A to D could be useful in distinguishing between them. Apart from the case of TetA determinants, we have found resistance levels to be a less useful criterion for phenotypic identification of the three classes B to D (Table 1). In contrast, the accumulation studies reported here clearly distinguish the four categories of resistance determinant.

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