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Trimethoprim-resistant mutants of *E. coli* K12: preliminary genetic mapping

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SUMMARY

Trimethoprim-resistant mutants of *E. coli* K12 have been isolated by serial subculture in progressively higher concentrations of trimethoprim. High-level resistance depends on the accumulation of several mutational changes. Transduction with bacteriophage P1 has shown that all the mutations involved in resistance are associated with a locus, to be called *tmr*, between *pyrA* and *pdxA* and closely linked to *pdxA*. Resistance is accompanied by, and presumably due to, an increased activity of the target enzyme, dihydrofolate reductase. The *tmr* locus may include the structural gene for dihydrofolate reductase but the only mutations that have so far been observed are concerned with regulation.

1. INTRODUCTION

Trimethoprim,§ an inhibitor of the enzyme dihydrofolate reductase, is much used clinically because it enters many bacteria relatively easily and is active against the bacterial enzyme at a concentration one hundred thousandth of that necessary to inhibit the mammalian enzyme (Burchall & Hitchings, 1965). It is also useful for the selection of thymine-requiring mutants of *E. coli* (Stacey & Simson, 1965) because the mutants are very much more resistant to the drug when the principal drain on the tetrahydrofolate pool, the synthesis of thymidylate, no longer operates. Although much less frequently, mutants can be isolated that are resistant to high concentrations of trimethoprim in the absence of thymine, and we hoped these would be useful to study the regulation of the cellular concentration of dihydrofolate reductase. Amethopterin (MTX), a drug with the same target enzyme, has been extensively used to study the same problem. Many MTX-resistant mutants have been isolated from those bacteria which, unlike *E. coli*, take up this drug easily, and they usually show two changes: they have a higher concentration of dihydrofolate reductase and the enzyme has altered properties (Albrecht, Palmer & Hutchinson, 1966; Sirotnak, Donati & Hutchinson, 1964). In many such mutants, resistance appears to be in direct proportion to the intracellular level of dihydrofolate reductase (Sirotnak, 1970). We anticipated isolating similar mutants but have found the situation more complex: the evolution

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§ Trimethoprim: 2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine.

of resistant strains occurred in several steps, each presumably by mutation, but in the more resistant strains the increase in enzymic activity (30-fold) was much less than the increase in resistance (1000-fold), and we have not been able to show any difference between the enzymes prepared from wild-type and resistant strains. Increased levels of dihydrofolate reductase in an MTX resistant strain of *E. coli* B (Poe *et al.* 1972) and in a trimethoprim resistant strain of *E. coli* K12 (Burchall, 1970) have been reported. Similar mutants have been isolated in *Salmonella typhimurium* using trimethoprim (Berberich & Levinthal, 1969; Kemper, 1974).

We report here a genetic analysis that shows that the mutations involved in trimethoprim resistance in our mutant strains are located in one cluster, very closely linked to *pdxA* on the *E. coli* chromosome.

2. MATERIALS AND METHODS

(i) *Strains*

All the strains listed in Table 1 are mutants of *E. coli* K12:

Table 1. *Bacterial strains*

Strain	Markers	Origin
A	$Su^- \lambda^- F^-$	Stock laboratory strain
D series mutants	Isolated as trimethoprim resistant from strain A	This work (see Table 1)
AB1157	*thr leu thi his arg pro str* F^-	P. Howard-Flanders
AT739	*thr pyrA thi* F^-	A. L. Taylor
AT3201	*leu lac his pdxA str met thi* F^-	A. L. Taylor
D13 *ara*$^-$	Spontaneous *ara*$^-$ mutant obtained by penicillin selection from D13	This work
AT3293	*pdxA thi* (HfrH)	A. L. Taylor
UR1	*thr pyr pdx his met thi lac str*. Isolated by sequential transduction of AT3201 using AT739 and AT3293 as donors	This work

(ii) *Media*

M9 (Adams, 1959), supplemented where necessary with the appropriate amino acids, and nutrient broth Oxoid No. 2 were normally employed. Solid media were made by the addition of 15 g/l and 12·5 g/l of Davis agar respectively. Casamino acids medium contained 10 g Difco Bacto casamino acids, 8 g sodium chloride and 2 g glucose per litre. Trimethoprim was supplied as the lactate (Burroughs Wellcome Ltd).

(iii) *Isolation of TMR mutants*

Resistant mutants of strain A were isolated without mutagenesis by repeated subculture in minimal media containing progressively greater amounts of trimethoprim. In this and other attempts at selection using other strains of *E. coli* K12 (A. S. Breeze, 1972) it was clear that resistance was acquired in steps, although occasionally mutants isolated in one medium were resistant to the concentration used at the next stage (Table 2).

Table 2. *Isolation of resistant strains of* E. coli *K12*

Mutant ...	A	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13
Source*	.	A	A	1	3	4	5	6	6	6	6	6	11	11
Trimethoprim†	.	2	4	10	15	20	90	150	250	300	440	600	1000	1400
M.I.C.‡	1	16	16	16	16	256	256	256	1024	1024	1024	1024	2048	2048
Relative specific activity§	1·0	1·0	0·9	1·0	2·2	3·0	2·9	3·7	25	26	27	29	25	26

These mutants are derived by sequential isolation from one clone of a F⁻Su⁻ derivative from a stock laboratory strain of *E. coli* K12 – strain A.

* Strain inoculated into isolation tube.

† Concentration of trimethoprim in isolation tube in μg/ml.

‡ Minimum inhibitory concentration of trimethoprim in μg/ml in liquid minimal medium.

§ Enzymic activity, measured as μmoles of dihydrofolate reduced per min per mg protein, compared with the wild-type, strain A.

Table 3. *P1 transductions using D13* ara⁻ *as donor and AT739 and AT3201 as recipients*

Strain	Selected marker	Colonies scored	% shown to have unselected markers in these combinations			
AT739	*pyrA*	300	*thr*⁻ *tmr*^{S}	*thr*⁻ *tmr*^{R}*	*thr*⁺ *tmr*^{S}	*thr*⁺ *tmr*^{R}
			27·9	12·6	53	6·3
	thr	300	*pyr*⁻ *tmr*^{S}	*pyr*⁻ *tmr*^{R}	*pyr*⁺ *tmr*^{S}	*pyr*⁺ *tmr*^{R}
			59·3	0	37	3·3
AT3201	*pdxA*	250	*leu*⁺ *ara*⁺ *tmr*^{S}	*leu*⁺ *ara*⁺ *tmr*^{R}†	*leu*⁻ *ara*⁺ *tmr*^{S}	*leu*⁻ *ara*⁺ *tmr*^{R}
			2	6	5·2	34
			leu⁻ *ara*⁻ *tmr*^{S}	*leu*⁻ *ara*⁻ *tmr*^{R}	*leu*⁺ *ara*⁻ *tmr*^{S}	*leu*⁺ *ara*⁻ *tmr*^{R}
			2·1	14	2·4	34·4
	leu	300	*pdx*⁺ *ara*⁺ *tmr*^{S}	*pdx*⁺ *ara*⁺ *tmr*^{R}	*pdx*⁻ *ara*⁺ *tmr*^{S}	*pdx*⁻ *ara*⁺ *tmr*^{R}
			1	0·7	59	0·3
			pdx⁻ *ara*⁻ *tmr*^{S}	*pdx*⁻ *ara*⁻ *tmr*^{R}	*pdx*⁺ *ara*⁻ *tmr*^{S}	*pdx*⁺ *ara*⁻ *tmr*^{R}
			32·6	0·3	1	5

* The maximum level of trimethoprim resistance observed in these recombinants was an M.I.C. of 512 μg/ml which corresponded to that of D13 under the same conditions.

† The level of resistance scored in these transductants was only 256 μg/ml but this probably corresponds to that of D13; AT3201 is more sensitive than strain A.

(iv) *Conjugation and P1 transduction*

The map location of trimethoprim resistance was obtained by standard methods. The linkage to *ara* was determined by conjugation with HfrH and, in the main, the detailed mapping was done by P1 transduction. P1 grown upon the resistant strains was used to transfer *thr*$^+$, *pdxA*$^+$, *pyrA*$^+$, *leu*$^+$, as appropriate, to AB1157, AT739, AT3201 and UR1. After purifying by restreaking on selective media the inheritance of the unselected markers and the level of trimethoprim resistance was determined by replica plating. Resistance was scored on solid minimal media using 2-fold steps in trimethoprim concentration. The degree of resistance depended on the growth medium used to provide the test culture. Minimal grown cells were 4 times as resistant as those grown in nutrient broth. The highest concentration showing distinct growth was taken as half the minimal inhibitory concentration. The scoring of recombinants that require threonine was complicated by the sparing effect of threonine on pyridoxine starvation (Dempsey & Sims, 1973). The only way to obtain unambiguous results was to prevent 'carry-over' in the sequence of replica plating.

(v) *Enzyme assays*

The level of dihydrofolate reductase activity for each strain was determined in cell free extracts. These were obtained by growing cultures to $2–4 \times 10^8$ cells/ml in casamino acids medium, harvesting, resuspending in 0·01 M phosphate buffer, pH 7·0, containing 1 mM EDTA, and sonicating with a M.S.E. Ultrasonic Disintegrator. This extract, after centrifugation to remove the cellular debris, was treated with one-tenth vol. of 5% streptomycin sulphate solution. After centrifugation, ammonium sulphate was added (0·351 g/ml) to bring it to 55% saturation. After removal of the precipitate enough solid ammonium sulphate was added to bring the solution to 90% saturation. The precipitate formed was removed by centrifugation and redissolved in 0·01 M phosphate buffer (pH 7·0) containing 0·001 M EDTA. This, the 55–90% fraction, was used for the assays of enzymic activity after dialysis against 0·001 M phosphate buffer containing 1×10^{-4} M (EDTA (pH 7·0)). This procedure removed the small amount of NADPH oxidase observed in some crude extracts and concentrated the activity some 2- to 3-fold.

(vi) *Assay of enzymic activity*

The method of assay was that of Burchall & Hitchings (1965). The reaction mixtures contained varying amounts of protein, 90–900 μg, and dihydrofolate, 18–200 nmoles, in 0·1 M phosphate buffer (pH 7·0) with 0·6 mM mercaptoethanol. These were incubated for 5 min before the reaction was started by the addition of NADPH, 60–180 nmoles. The change in absorbance at 340 nm was recorded continuously for 5 min in a Pye–Unicam spectrophotometer (SP 800). Under these conditions a decrease of 0·01 corresponds to a reduction of 2·6 nmoles of FH_2. The preparation of dihydrofolate was slightly modified from that given by Friedkin, Crawford & Misra (1962). Folic acid, 300 mg, was dissolved in 25 ml 1 M mercaptoethanol by adjusting to pH 7·5 with 1 M potassium hydroxide; 2 g of sodium

dithionite were added and the mixture stirred at 4 °C for 1 h. The mixture was left overnight in ice-water and the precipitate removed, washed three times in 1 M mercaptoethanol and after resuspension in the same liquid dispensed into vials which were stored frozen at −20 °C.

3. RESULTS

(i) *Isolation of mutants*

In the series reported here 13 mutants were isolated and their properties given in Table 2.

(ii) *Genetical analysis*

(*a*) *Transduction*

AB1157. *thr*$^+$, *ara*$^+$ and *leu*$^+$ transductants of AB1157 were isolated after transduction with P1 grown on D13. These results confirmed the linkage of trimethoprim resistance to *ara* (~ 30%) and suggested that it might be nearer to *pyrA* and *pdxA* than any other easily scored marker.

AT739. *thr*$^+$ and *pyr*$^+$ transductants of AT739 were obtained using P1 grown on D13*ara*$^-$. The much better growth of clones that were *pyrA*$^+$ might complicate the analysis (see Table 3) because of the powerful selection for the *pyr*$^+$ phenotype. The interaction of threonine and the *pyrA* phenotype led us, at first, to suspect the presence of a second locus for trimethoprim resistance close to *pyrA*; those recombinants that had inherited *pyr*$^+$ were more resistant than the parent strain. We communicated this belief to Dr A. L. Taylor and this is the reason for the two *tmr* loci that appear on the current Taylor map of the *E. coli* chromosome (Taylor & Trotter, 1972). We have now concluded that this low-level increase in resistance is due simply to the better growth of the arginine–uracil independent recombinants; transductants that had inherited *pyr*$^+$ from the trimethoprim-sensitive parent, strain A, showed the same increase in resistance.

AT3201. *leu*$^+$ and *pdxA*$^+$ transductants were obtained with P1 grown on D13*ara*$^-$. The linkage of the *ara*$^-$ marker to *leu* was lower than that normally obtained but again this might be the result of a bias against the inheritance of *ara*$^-$ and in favour of *pdx*$^+$. AT3201 was appreciably more sensitive to trimethoprim than any other strains studied and we believe that the maximum level of resistance scored in the recombinants (256 μg/ml) corresponds to that of the donor (512 μg/ml).

UR1. Although these crosses establish the degree of linkage they do not establish the order unambiguously, so a new strain carrying *thr*, *pyrA* and *pdxA* markers was derived from AT301. P1 stocks were obtained from strains A, D5, D10 and D13 and used to transduce UR1. Recombinants were selected for each of the three markers and the linkage of the unselected markers determined for a randomly picked 100 of each type of recombinant. The data (Table 4) confirmed the known gene order and put *trm* very close to *pdxA* (90% co-transduction) and suggest, though not conclusively, that the order is *thr–pyrA–trm–pdxA*. In addition they suggest that all of the mutations that confer resistance to trimethoprim map in the same small segment of chromosome.

Transduction with direct selection for trimethoprim resistance yielded very few transductants but in a series of experiments carried out for a quite different purpose we have found that allowing two cycles of division post-transduction does permit the direct selection of trimethoprim resistant transductants. This result

Table 4. *P1 transduction using UR1 as recipient and strains A, D5, D10 and D13 as donors*

Donor strain	Recombinant genotype	% shown to have unselected markers in these combinations								Colonies tested
		Selected phenotype *thr*+								
	pyr	−	+	+	+	+	−	−	−	
	*tmr**	S	S	R	R	S	R	R	S	
	pdx	−	−	−	+	+	−	+	+	
A	.	59	27	.	.	13	.	.	1	100
D5	.	70	23	1	.	.	.	4	.	98
D10	.	81	17	.	.	.	2	.	2	100
D13	.	79	20	.	.	.	1	.	.	100
		Selected phenotype *Pyr*+								
.	*thr*	−	+	+	+	+	−	−	−	.
.	*tmr*	S	S	R	R	S	R	R	S	.
.	*pdx*	−	−	−	+	+	−	+	+	.
A	.	25	34	.	.	20	.	.	20	99
D5	.	47	10	.	8	.	2	29	4	100
D10	.	28	14	1	18	.	2	38	1	80
D13	.	40	29	2	8	.	.	20	1	100
		Selected phenotype *Pdx*+								
.	*thr*	−	+	+	+	+	−	−	−	.
.	*pyr*	−	−	+	+	−	+	−	+	.
.	*tmr*	S	S	S	R	R	R	R	S	.
A	.	60	.	20	.	.	.	.	20	100
D5	.	7	1	.	9	.	39	41	2	99
D10	.	9	.	1	10	.	29	51	.	100
D13	.	11	1	.	11	.	33	44	.	100

A few recombinants expressed an intermediate phenotype and are not listed here.

* R indicates here the full phenotypic resistance of the donor and S the same phenotype as the recipient.

may explain the non-reciprocal nature of the crosses involving trimethoprim resistance observed by Kemper (1974). In these experiments linkage to *ara* was found to be 70%.

(iii) *Enzyme activity of the transductants*

The enzymic activities of 15 partially resistant transductants of AB1157 have been measured and in 11 the enzyme level corresponds with that expected from the M.I.C. of the donor (Table 2).

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4. DISCUSSION

The results in Tables 2 and 4, especially those for AT3201 and UR1, suggest that trimethoprim resistance results from mutation in one region of the chromosome, closely linked to *pdxA*. We suggest that this site be given the symbol *tmr* until the biochemical nature of its function(s) has been determined.

Experiments with partially diploid strains have shown that some of the mutations are in a regulatory function and are consistent with both operator and repressor components. We have no evidence for structural gene mutations. This position for *tmr* corresponds to that mapped for the gene called *fol* in *Salmonella typhimurium* (Kemper, 1974).

The changes in the level of dihydrofolate reductase were not accompanied by any change in the specific activities of thymidylate synthetase and methylene tetrahydrofolate dehydrogenase (Stowell, 1973), and it would appear that the regulation of these enzymes is not directly coupled to that of dihydrofolate reductase.

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