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Genetic Analysis of Chromosomal Resistance to Trimethoprim Derived from Clinical Isolates of *Escherichia coli*

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Chromosomal genes conferring resistance to trimethoprim were transferred from three independently isolated *thy*$^+$ clinical strains of *Escherichia coli* to *Escherichia coli* K12 by using P1 transduction. Trimethoprim-resistant transductants were obtained less frequently than transductants of other chromosomal markers, suggesting that there were problems related to the expression of the trimethoprim resistance genes in *E. coli* K12. Mapping studies revealed that one of the resistance determinants was located at a similar position on the chromosome (1 min) to the *fol*-type mutations previously described in *E. coli* K12. The two remaining resistance determinants mapped at separate positions between 2·5 and 3 min on the chromosome. The presence of one of these determinants reduced the efficiency with which either donor or recipient cells carrying it could participate in conjugation mediated by the sex factor F and also resulted in phenotypic interaction with the *azi* gene. The mechanisms of trimethoprim resistance in the three clinical *E. coli* isolates studied were more complex and diverse than was expected from previous studies of *E. coli* K12 mutants.

INTRODUCTION

Since the introduction of trimethoprim for clinical use there have been reports of a number of different forms of resistance in isolates of *Escherichia coli*. Resistance to trimethoprim may result from (i) the acquisition of a plasmid coding for trimethoprim resistance (Datta & Hedges, 1972), such resistance sometimes being transposable (Barth *et al.*, 1976), (ii) the presence of a chromosomally located trimethoprim resistance transposon in the absence of a plasmid (Towner, 1981), (iii) a mutation to thymine auxotrophy (Maskell *et al.*, 1977) or (iv) mutations in other chromosomal genes (Grey *et al.*, 1979).

Trimethoprim resistance in *thy*$^+$ *E. coli* K12 strains which have been either trained to resistance by sequential subculture in increasing concentrations of the drug (Breeze *et al.*, 1975) or isolated following mutagenesis as resistant mutants on trimethoprim-containing medium (Sheldon & Brenner, 1976), has been shown to be determined by mutations in the *fol* gene, coding for production of the enzyme dihydrofolate reductase, located at about 1 min on the *E. coli* K12 linkage map (Bachmann & Low, 1980). Resistance to trimethoprim in *thy*$^+$ clinical isolates of *E. coli* which do not carry either a resistance plasmid or a chromosomally located transposon has been assumed to be determined in the same way, although this has not been investigated by genetic techniques.

We attempted to transfer chromosomal trimethoprim resistance from *thy*$^+$ clinical isolates of *E. coli* to the well-characterized genetic background of *E. coli* K12 using, where possible, P1-mediated generalized transduction. It was hoped that subsequent genetic analysis would not only show whether findings concerning artificially induced trimethoprim resistance in *E. coli*

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K12 were relevant to resistance in strains isolated from human infections, but also throw light upon the factors that may influence the genetic variation and interchange of chromosomal genes in wild strains of *E. coli*.

METHODS

Bacteria, plasmids and bacteriophages. Clinical strains of *Escherichia coli* (339 *thy*$^+$ strains) isolated mainly from samples of infected urine received at the Public Health Laboratory, Nottingham, were investigated in this study. Of these 53 were trimethoprim-resistant (MIC $\geqslant$ 8 mg l^{-1}; tested as below), but had previously been shown not to contain transferable trimethoprim R plasmids or trimethoprim resistance transposons when tested as described by Towner *et al.* (1982). Strains of *E. coli* K12 and the clinical *E. coli* isolates used in gene transfer experiments are listed in Table 1. Also used in conjugation experiments was the P incompatibility group plasmid RP4 (Datta *et al.*, 1971). Bacteriophages used were P1$_{vir}$ (Miller, 1972), P1 *clr100* KM (Goldberg *et al.*, 1974), MS2 (Davis *et al.*, 1961) and R17 (Miller, 1972).

Media. Oxoid nutrient broth (NB), nutrient agar (NA) and sensitivity test agar (DST) were used. Lysed horse blood (4%, v/v) was added when trimethoprim was incorporated into DST. The *E. coli* minimal medium (MM) used was that of Vogel & Bonner (1956), supplemented, where necessary, by amino acids (50 mg l^{-1}) and vitamins (1 mg l^{-1}). Unless otherwise stated, glucose (0·5%, w/v) was added as the carbon source. R plates were made from NA containing 2 × 10^{-3} M-$CaCl_2$. R-top agar was NB with 2 × 10^{-3} M-$CaCl_2$ plus 0·8% Davis agar, dispensed in 2·5 ml amounts. MC medium consisted of 0·1 M-$MgSO_4$ and 0·005 M-$CaCl_2$. Resistance to azide was determined on NA containing 0·005 M-sodium azide.

Trimethoprim MIC determinations. NB cultures (10 ml) of the test organisms, incubated overnight at 37 °C, were diluted 10^{-4} in 1/4 Ringer's solution. MIC values were determined using multipoint inoculation (with a final inoculum of about 100 c.f.u.) on to DST agar containing 4% (v/v) lysed horse blood and doubling dilutions of trimethoprim lactate (Burroughs Wellcome). Plates were examined following overnight incubation at 37 °C and the MIC taken to be the lowest concentration of the drug which prevented growth of the organism.

Determination of bacteriophage sensitivities. The surface of an R plate was inoculated with a single streak of an overnight NB culture of the strain to be tested. Six to eight strains could be tested per plate. A drop of bacteriophage lysate was spotted on to each streak and the plates examined after overnight incubation at 37 °C for inhibition of growth in the area of the spot. In doubtful cases, sensitivity was additionally tested by spotting a drop of bacteriophage lysate on to a lawn of bacterial cells prepared by inoculating R-top agar with 0·1 ml of an overnight NB culture and pouring this on to the surface of an R plate. Bacteriophage sensitivity was indicated by confluent lysis or the formation of plaques following overnight incubation at 37 °C.

Direct selection for P1-sensitive mutants. P1-sensitive mutants of trimethoprim-resistant clinical isolates of *E. coli* which were normally insensitive were selected using P1 *clr100* KM as described by Goldberg *et al.* (1974).

Preparation and use of P1 transducing lysates. These experiments were carried out using standard procedures for the use of P1 as described by Miller (1972). Selection for the transduction of amino acid or vitamin markers into an auxotrophic recipient was made on glucose MM containing all the necessary growth requirements except the one under test. Transduction of the *ara*$^+$ marker into an *ara* recipient was selected by substituting arabinose (0·5%,

Table 1. *Bacterial strains*

		Properties*	Reference/Source
(i)	*E. coli* K12 strains		
	J53.2	F$^-$ *pro met rpoB*	Coetzee *et al.* (1972)
	2K^{--}	F$^-$ *thi ser lac azi rpsL hsdR hsdM*	S. W. Glover
	D13	F$^-$ Su$^-$ *fol*	Breeze *et al.* (1975)
	D5009	HfrC *leu met thr ara lacY mtl xyl thi azi rpsL tonA tsx*	R. E. Glass
	CSH23	Δ(*lac pro*) *supE spc thi* F′ *lac*$^+$ *proA*$^+$,*B*$^+$)	Miller (1972)
	CSH57b	F$^-$ *ara leu lacY purE gal trp his argG malA rpsL xyl mtl ilv met thi*	Miller (1972)
	CSH61	HfrC *trpR thi*	Miller (1972)
(ii)	Clinical isolates of *E. coli*		
	GH9	Tp^r (MIC 32 mg l^{-1}) Su^r Tc^r P1^s	Towner *et al.* (1980)
	GH11	Tp^r (MIC 128 mg l^{-1}) Su^r Tc^r P1^r	Towner *et al.* (1980)
	GH64	P1^s mutant of GH11	This paper
	GH101	Tp^r (MIC 128 mg l^{-1}) Su^r Sm^r Nal^r P1^r	Towner *et al.* (1980)
	GH118	P1^s mutant of GH101	This paper

* Tp, trimethoprim; Su, sulphamethoxazole; Tc, tetracycline; Sm, streptomycin; Nal, nalidixic acid.

w/v) for glucose as the carbon source; a similar method was used for other sugar markers. Transductants were purified by re-streaking on to fresh plates of the same medium. Single colonies were then applied as a grid to the same medium (50 per plate), and replica plating was used to test for unselected marker inheritance.

It proved impossible to select directly for *azi*^{r} transfer in transduction experiments owing to heavy background growth when large numbers of cells were plated. The presence or absence of *azi*^{r} as an unselected marker in transductants was tested by inoculating single colonies to plates of NA containing 0·005 M-sodium azide by using a single streak (four tests per plate). When tested in this manner, azide-sensitive transductants failed to show any growth after overnight incubation at 37 °C, while transductants carrying the *azi*^{r} marker produced growth over the entire length of the streak.

Conjugation techniques. Fresh overnight NB cultures of the donor and recipient strains were subcultured (0·1 ml culture : 10 ml NB) and grown at 37 °C to late-exponential phase (4–5 h). Samples (3 ml) of each were then mixed and incubation continued at 37 °C for the time indicated. For crosses other than those involving an Hfr donor, extra aeration was not found to be necessary. For matings between Hfr and F$^-$ cells, cultures were incubated in 100 ml conical flasks in a shaking water bath to provide gentle aeration. Controls of each parent alone were included in all crosses. After incubation, the mating mixtures were centrifuged (5000 *g* for 20 min), washed once and resuspended in 1/4 Ringer's solution. Portions (0·1 ml) of appropriate dilutions were then spread over selection plates and incubated at 37 °C. Transconjugants were tested for the inheritance of unselected markers as described for P1 transductants.

RESULTS

Sensitivity of clinical E. coli isolates to P1

In order to determine whether P1 transduction was a feasible means of transferring genes from clinical isolates of *E. coli* and, in addition, to determine the overall proportion of P1-sensitive isolates, 339 *thy*$^+$ clinical strains of *E. coli* were tested with $P1_{vir}$ propagated on J53.2. Ninety-four (28%) of the 339 strains tested were sensitive to P1, but only one (GH9) of the 53 trimethoprim-resistant strains was P1-sensitive, indicating that the proportion of clinical isolates which were resistant to trimethoprim and also sensitive to P1 was low compared to the overall population of clinical *E. coli* strains.

To examine the basis of trimethoprim resistance in a range of isolates exhibiting various MIC values, an attempt was made to select for P1-sensitive mutants within populations of trimethoprim-resistant organisms which were normally resistant to P1. Two further P1-sensitive *E. coli* strains that were resistant to trimethoprim were isolated (GH64 and GH118) by the technique of Goldberg *et al.* (1974).

Transduction of trimethoprim resistance from clinical isolates of E. coli into E. coli K12 and its subsequent expression

Lysates of P1 were prepared on cultures of donor bacteria (GH9, GH64 and GH118) and transductions performed using strain J53.2 as recipient. Selection for transfer of trimethoprim resistance was initially made on appropriately supplemented MM using a range of drug concentrations up to the MIC of the donor strain. However, it was found that no trimethoprim-resistant transductants were obtained, whatever the MIC of the donor, when more than 8 mg trimethoprim l^{-1} was incorporated into the medium. This concentration was therefore routinely used in all subsequent transduction experiments.

Transduction of trimethoprim resistance from the clinical isolates of *E. coli* to *E. coli* K12 appeared to be a relatively rare event, possibly because the clinical isolates yielded only low titre lysates of P1 (Table 2). In a sequence of experiments using GH9, GH64 and GH118 as donors, trimethoprim-resistant transductants were obtained at a frequency varying between 5×10^{-6} and 1×10^{-8} transductants per p.f.u., whereas transduction of auxotrophic markers from the clinical isolates to J53.2 seemed to occur more readily (Table 2). Although the numbers of trimethoprim-resistant transductants obtained were small, control experiments showed that no colonies were obtained when J53.2 alone was plated on medium containing 8 mg trimethoprim l^{-1} and a separate experiment determined that the frequency of spontaneous mutation of J53.2 to resistance to this concentration of the drug was approximately 2×10^{-11}. As the number of cells routinely plated in each transduction experiment was approximately 10^8, there was

Table 2. *Transduction of markers from clinical strains of E. coli to J53.2 using P1*

Transduction frequencies are expressed in terms of number of transductants per p.f.u. Those shown were obtained in a typical experiment using the same recipient culture of J53.2.

Donor	P1 lysate titre (p.f.u. ml^{-1})	Transduction frequency		
		Tp^r	*pro*$^+$	*met*$^+$
GH9	3×10^7	$3{\cdot}0 \times 10^{-7}$	$3{\cdot}3 \times 10^{-6}$	$2{\cdot}8 \times 10^{-6}$
GH64	2×10^7	$5{\cdot}0 \times 10^{-7}$	$3{\cdot}8 \times 10^{-5}$	$6{\cdot}0 \times 10^{-5}$
GH118	2×10^6	$5{\cdot}0 \times 10^{-6}$	$5{\cdot}0 \times 10^{-6}$	$1{\cdot}0 \times 10^{-5}$

therefore a negligible chance that the putative trimethoprim-resistant transductants observed arose spontaneously.

Resistant transductants were purified and tested to ensure that they retained the characteristics of J53.2. Their trimethoprim MIC values were then determined and compared on the same plate with the MIC of the original donor strain. Although in the initial experiments no transductants were obtained on selection plates containing trimethoprim concentrations in excess of 8 mg l^{-1}, after purification, transductants obtained using any of the three donors were found to have an MIC of 32 mg l^{-1}; however, for GH64 and GH118 this was still below the value exhibited by the original donor strain (Table 1). More than one gene might be involved in conferring a higher level of resistance in GH64 and GH118, but attempts to use the resistant transductants from the initial experiments as recipients in further transductions, with selection on medium containing 64 mg trimethoprim l^{-1}, failed to produce any recombinants with an increased MIC.

It was recognized that the resistance determinants transferred into J53.2 might not be a single gene or might correspond to previously described genes in *E. coli* K12; however, for clarity in presentation, the trimethoprim resistance determinants transferred from GH9, GH64 and GH118 were temporarily designated *tim-1, tim-2* and *tim-3*, respectively, and these designations will be used in the remainder of this paper.

Possible factors influencing the yield of transductants

In order to examine whether the action of the restriction/modification system of *E. coli* K12 was responsible for limiting the yield of transductants, the initial experiments were repeated with GH9, GH64 and GH118 as donors and using J53.2 and the restrictionless strain $2K^{--}$ as recipients. No significant increase in the recovery of trimethoprim-resistant transductants was obtained using $2K^{--}$.

A second possibility, especially in view of the MIC results, was that the determinants encoding resistance to trimethoprim were not immediately expressed in *E. coli* K12. Breeze *et al.* (1975) found that when *fol* mutations were transduced between strains of *E. coli* K12, the yield of transductants was increased by allowing two cycles of division following infection with P1 before plating out. This was tested by carrying out duplicate sets of transductions in which one set of samples was plated out immediately, while extra growth medium (0·1 ml NB) was added to the remaining mixture and incubation continued at 37 °C. At hourly intervals (up to 4 h) further samples were transferred to trimethoprim selection plates. No increase was observed in the numbers of transductants obtained when post-transduction growth was allowed compared with the numbers obtained after immediate plating.

Attempts to produce Hfr strains carrying the trimethoprim resistance determinants

For preliminary mapping of the trimethoprim resistance determinants by conjugation experiments, resistance was transduced from the original J53.2 transductants to the HfrC strain CSH61. No difficulty was experienced in transducing the *tim-2* and *tim-3* determinants to CSH61 and the resulting transductants continued to act as Hfr-type donors and exhibit sensitivity to the F-specific bacteriophages MS2 and R17. In contrast, although it was possible

Table 3. *Co-transduction of markers between strains of E. coli K12*

Transduction experiments were performed as described in the text. Co-transduction frequencies (x) are expressed in terms of the proportion of transductants examined which inherited the unselected marker. Map distances (d; in min) and 95% confidence intervals (CI; in min) were calculated from the formula of Wu (1966).

Selected marker	No. scored	Unselected marker	x	d	95%CI
tim-1	496	*leu*	0·014	1·52	0·10
tim-1	402	*ara*	0·014	1·51	0·11
tim-1	353	*azi*	0·632	0·28	0·04
tim-2	399	*leu*	0·260	0·72	0·07
tim-2	399	*ara*	0·360	0·58	0·06
tim-2	98	*azi*	0·061	1·21	0·17
tim-3	180	*leu*	0	>2	
tim-3	180	*ara*	0	>2	
tim-3	260	*azi*	0·227	0·78	0·09
fol	153	*leu*	0·405	0·52	0·09
fol	153	*ara*	0·509	0·40	0·08
fol	342	*azi*	0·158	0·92	0·08

to transduce the *tim-1* determinant to CSH61, the transductants were consistently no longer able to act as donors in conjugation experiments and were resistant to MS2 and R17. When attempts were made to transfer F′*pro*$^+$*lac*$^+$ from CSH23 into J53.2 carrying *tim-1*, transconjugants were obtained at a frequency of 9×10^{-6} per recipient organism. This compared with a frequency of 6×10^{-2} per recipient organism when transfer was to the parental J53.2. In addition, whilst 100% of J53.2 *pro*$^+$ transconjugants were R17-sensitive, only 17% of J53.2 *tim-1 pro*$^+$ transconjugants were R17-sensitive. Thus the presence of *tim-1* had a considerable effect on both the donor and recipient ability of a cell in relation to the fertility factor F. This observation will be further discussed later.

Preliminary mapping of the trimethoprim resistance determinants using conjugation

An R17-sensitive J53.2 derivative carrying *tim-1* and F′*pro*$^+$*lac*$^+$ was used as donor in a mating with CSH57b. The map positions of the markers carried by CSH57b are shown by Bachmann & Low (1980). Trimethoprim-resistant transconjugants were obtained at a frequency of 2×10^{-5} transconjugants per recipient cell, 44% of which had received only *ara*$^+$ and *leu*$^+$ in addition to *tim-1*. When *ilv*$^+$ transconjugants were selected, the co-inheritance of the unselected markers (*ara*$^+$, *leu*$^+$ and *tim-1*), was *ara*$^+$ 59%; *leu*$^+$ 36%; *tim-1* 12%, suggesting that *tim-1* was located on the far side of *leu* from *ara*, at about 2 min on the *E. coli* K12 chromosome. Similar crosses, in which the CSH61 HfrC *tim-2* and *tim-3* derivatives were used as donors, indicated that *tim-2* mapped at about the 1 min region, while *tim-3*, like *tim-1*, mapped at about 2 min.

Mapping using P1 co-transduction frequencies

Having determined the approximate map locations of *tim-1, tim-2* and *tim-3* by conjugation experiments, it was feasible to examine their P1 co-transduction frequencies with markers positioned close to their apparent locations. Many of the genes positioned at or around 2 min on the *E. coli* K12 linkage map are concerned with cell wall functions and cell division and are therefore less easily handled than resistance or nutritional markers. In view of this, co-transduction of *tim-1, tim-2* and *tim-3* with *ara, leu* and *azi* was investigated. The co-transduction frequencies of a known *fol* mutation with the same markers was also determined (Table 3).

The *leu* and *ara* markers are located between 0·2 and 0·3 min apart on the *E. coli* K12 linkage map (Bachmann & Low, 1980). However, the map distance between these two markers, calculated using the data obtained from transductional crosses with a CSH57b recipient and the donors mentioned above, varied from 0·21 to 0·39 min. Variations also occurred depending on which of the two markers was selected, a not unusual finding (Bachmann *et al.*, 1976), which

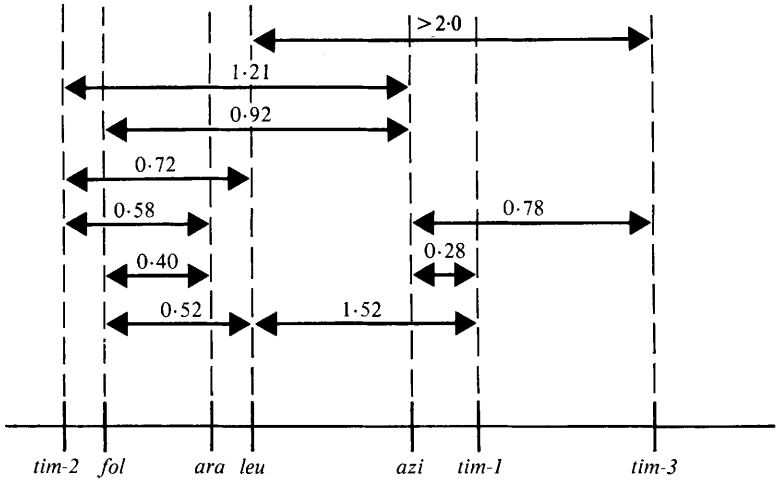

Fig. 1. Map summarizing the co-transduction data in *E. coli* K12. Map distances (min) shown are mean values and are therefore not additive.

represents a potential source of error in the determination of map distances. Bearing in mind this qualification, a comparison of the co-transduction frequencies of *tim-1*, *tim-2*, *tim-3* and *fol* with *ara* and *leu* showed that *tim-2* mapped at a position very close to that of *fol*. In contrast, *tim-1* showed some linkage with *ara* and *leu*, but the low co-transduction frequencies obtained were not consistent with *tim-1* being located close to *fol*. In the case of *tim-3* no linkage with *ara* and *leu* was detected. When co-transduction frequencies with *azi* were determined, results obtained were again consistent with *tim-2* and *fol* mapping closely together near *leu* and *ara* and at about 1 min from *azi*, while *tim-1* and *tim-3* mapped closer to *azi* than *ara* or *leu*. The linkage data obtained are summarized in Fig. 1.

Possible interaction of tim-1 and azi

When *tim-1* transductants were tested as described in Methods for the co-transduction of either *azi*^{s} or *azi*^{r}, a varying proportion of transductants displayed an 'intermediate' degree of growth on sodium azide. In these cases there was growth which covered only the initial area of the streak; this contrasted with the zero growth obtained with the sensitive control, but was considerably less than that obtained with the resistant control. As this phenomenon was not observed in parallel crosses involving the transduction of *tim-2*, *tim-3* or *fol*, the presence of *tim-1* might in some way be connected with the phenotypic appearance of the transductants on sodium azide. This observation will be discussed later.

Evidence that the positions taken by tim-1, tim-2 and tim-3 on the E. coli K12 chromosome were not affected by the mechanism of transfer

It was possible that the positions taken by *tim-1*, *tim-2* and *tim-3* on the *E. coli* K12 chromosome were influenced by the mechanism of gene transfer from the original strain (i.e. P1 transduction) rather than reflecting the positions which were originally occupied on the chromosome of their respective clinical strains. In order to investigate this, the plasmid RP4 was introduced into each original clinical strain. RP4 is known to be capable of mobilizing the chromosome of *E. coli* strains at a low frequency, possibly as a result of an integration event following the transposition of Tn1 from RP4 to the host cell chromosome (Harayama *et al.*, 1980). When the clinical strains containing RP4 were crossed with J53.2, trimethoprim-resistant transconjugants were obtained at frequencies of about 5×10^{-8} per recipient cell. No trimethoprim-resistant colonies were obtained in RP4$^-$ control experiments. The map positions taken by *tim-1*, *tim-2* and *tim-3* on the *E. coli* K12 chromosome in the transconjugants produced

in RP4-mediated matings were the same as those taken when resistance was originally mobilized from the clinical isolates using P1 transduction. Thus it appeared that these map positions reflected genuine regions of DNA homology between the genomes of the naturally occurring *E. coli* strains and *E. coli* K12.

DISCUSSION

The transfer of the chromosomal genes controlling trimethoprim resistance in clinical isolates of *E. coli* into the laboratory strain *E. coli* K12 was found to present a number of difficulties. Fewer naturally occurring isolates were sensitive to P1 than had been anticipated and, of those which were, only one was resistant to trimethoprim. Only 28% of the 339 clinical *E. coli* isolates tested were sensitive to P1 compared with 70% in a survey reported by Robeson *et al.* (1980). This group of workers used P1CM*clr100* to lysogenize sensitive cells and allow for their selection in a largely resistant population. The method used in this paper was likely to detect only a predominantly sensitive population and this may account for the wide disparity in the results obtained.

It was only possible to produce low titre lysates from the P1-sensitive strains of *E. coli* which were trimethoprim-resistant. As only about 0·3% of particles in a P1 lysate are transducing particles (Stent & Calendar, 1978), this considerably reduced the likelihood of the transduction of any particular chromosomal gene compared with the use of a lysate of higher titre. Trimethoprim-resistant transductants were detected less often than other classes of transductants. When coupled with the observation that no trimethoprim-resistant transductants were detected if more than 8 mg trimethoprim l^{-1} was incorporated into the selection plates, this suggested that there were problems related to the expression of the genes conferring resistance in *E. coli* K12. Restriction of the incoming DNA was shown not to be a significant factor and although the possibility of delayed expression was suggested by the work of Breeze *et al.* (1975), our results indicated that incubation prior to plating on trimethoprim-containing medium did not greatly influence the number of resistant transductants obtained. It would therefore seem that there must be some basic differences between 'wild' and 'laboratory' strains of *E. coli* which affect the ease with which different types of chromosomal resistance to trimethoprim can be expressed.

A possible insight into one of these differences was provided by the influence of the *tim-1* determinant on the expression of the fertility factor F. It was not possible to transduce *tim-1* into an Hfr donor and retain the Hfr character. In addition, F′ plasmids could only be transferred at a reduced frequency into J53.2 carrying *tim-1* and a proportion of the transconjugants were insensitive to F-specific bacteriophages. Similar findings have been reported previously: Bushby (1973) could not demonstrate the transfer of resistance by conjugation in *E. coli* strains trained to trimethoprim resistance, while Breeze *et al.* (1975) could not train an *E. coli* K12 Hfr strain to trimethoprim resistance and retain the Hfr character. One of the main structural components of the outer membrane of Gram-negative bacteria is LPS. It has been suggested that LPS has an essential role in the formation and stabilization of mating pairs in *E. coli* K12 (Glowacka & Mieczylawa, 1981) and, in addition, that rough cells with defective LPS acquire plasmids at a higher frequency than smooth strains (Okada & Watanabe, 1968). An alteration in the LPS component of the membrane might lead to a change in the conjugational ability of the organism and at the same time contribute to the acquisition of trimethoprim resistance.

The possibility that the *tim-1* determinant might mediate resistance to trimethoprim by causing an alteration in the cell envelope was supported by its mapped location at about 2 min on the K12 linkage map, among a cluster of genes coding for cell envelope functions, and also by its phenotypic interaction with the *azi* gene, which is itself thought to be involved in the formation, activity and maintenance of the membrane structure (Yura & Wada, 1968). Further detailed biochemical study will obviously be required to elucidate the precise nature of the changes brought about by *tim-1*, *tim-2* and *tim-3* in *E. coli* K12.

One of the original purposes of this work was to investigate genetically whether findings concerning artificially-induced trimethoprim resistance in *E. coli* K12 were relevant to

chromosomal resistance in naturally occurring clinical strains. Mapping studies indicated that *tim-2* was probably a *fol*-type mutation, although the observation that only a reduced MIC was expressed in the K12 transductants suggested that other factors contributed to the total resistance in the original clinical strain. In contrast, *tim-1* and *tim-3* mapped at completely different loci compared with *fol*. It would therefore appear that mechanisms of chromosomal resistance to trimethoprim in *E. coli* isolates from human infections are considerably more complex and diverse than was indicated by previous studies of *E. coli* K12 mutants.

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