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# Detection of novel trimethoprim resistance determinants in the United Kingdom using biotin-labelled DNA probes

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SUMMARY

Two collections of trimethoprim R plasmids, isolated from strains of *Escherichia coli* during 1978–83 and 1987–8 respectively, were retrospectively screened with specific biotinylated DNA probes for the presence of genes encoding particular DHFR enzymes. The results confirmed that the type I DHFR gene was the predominant plasmid-encoded gene conferring trimethoprim resistance in strains of *E. coli* from the Nottingham area of the UK, but indicated that genes encoding the more recently recognized types of DHFR enzymes had appeared in the bacterial gene pool and could be recognized with increased frequency in the latter plasmid collection. This was particularly true of the type IIIa and type VII enzymes which together accounted for 27% of the trimethoprim R plasmids examined in 1987–8.

## INTRODUCTION

The introduction of trimethoprim-containing products for medical and veterinary use in the United Kingdom, during 1968 and 1969 respectively, provided an unusual opportunity to study the full evolution of bacterial resistance to a novel synthetic antibiotic without the pre-existing influence of resistance genes selected by other related compounds. Several different mechanisms of trimethoprim resistance have subsequently been identified in bacteria, as reviewed by Huovinen [1], but among Enterobacteriaceae isolated from various regions of the UK, high-level plasmid-encoded resistance seems to be of greatest clinical significance [2–5]. Similar findings have also been reported from many other parts of the world [6, 7].

Plasmid-encoded trimethoprim resistance was first reported by Fleming and co-workers [8] and, to date, has always been associated with the plasmid-mediated production of an additional trimethoprim-resistant dihydrofolate reductase (DHFR) that allows the trimethoprim-susceptible DHFR of the host strain to be by-passed [9, 10]. Seven major groups of trimethoprim-resistant DHFRs have now been identified in Gram-negative bacteria and are distinguishable from each other, and from the host chromosomal DHFR, on the basis of a variety of

biochemical and biophysical properties [11]. However, the time required to extract and purify each individual enzyme means that such identification is impracticable to perform for large-scale epidemiological studies.

An alternative method of characterization involves the use of DNA probes specific for particular plasmid-encoded DHFR enzymes. Such probes are available for six of the seven major DHFR groups [11] and can be used in conjunction with a non-radioactive biotin label to screen rapidly large numbers of trimethoprim R plasmids for the presence of a specific DHFR gene [12]. The use of a biotin label not only circumvents the problems associated with the use and disposal of radioactive labels such as $^{32}P$, but also allows probe preparations to be re-used over a period of many months.

Since 1978 we have studied the incidence of trimethoprim resistance found in *Escherichia coli* of both human and animal origin from the Nottingham area of the UK. Resistance levels have risen steadily over this period and it has been possible to demonstrate that much of this increase can be accounted for by the presence of transmissible R plasmids [2, 13]. In a previous study it was demonstrated that 66 of 81 (81%) trimethoprim R plasmids isolated during 1978–83 from strains of *E. coli* encoded the type I DHFR [12]. This predominance almost certainly reflected the widespread distribution of transposon Tn7, the first and most common of the transposons conferring trimethoprim resistance so far described [6, 14].

The objectives of this study were to collate the results of a detailed retrospective analysis of the unresolved plasmids from the 1978–83 collection, carried out with a combination of recently available DNA probes for DHFR genes and biochemical analysis, and to compare the results with those from a more recent collection of trimethoprim R plasmids isolated during the period 1987–8.

## METHODS

### *Bacteria, plasmids and DNA probes*

All genetic manipulations were performed using strains of *E. coli* K12. Trimethoprim R plasmids were initially transferred to strain J53.2 ($F^-$ *pro met rpoB*) from randomly selected trimethoprim-resistant isolates of *E. coli*, obtained from medical or veterinary sources in the Nottingham area, as described previously [2]. The P incompatibility group plasmid RP4 was used to mobilize transposons encoding trimethoprim resistance from certain strains as described by Towner and colleagues [15]. Plasmid DNA was isolated from *E. coli* K12 transconjugants, separated on agarose gels and transferred to nitrocellulose filters using a Semidry Electroblotter (Sartorius) as described previously [12, 16].

DNA probes specific for known DHFR genes comprised the following: type I 499 bp *Hpa*I fragment of pFE872 [17]; type II, 275 bp *Sau*3A/*Eco*RI fragment of pWZ820 [18]: type IIIa, 700 bp *Pst* I/*Eco*RI fragment of pUN972 [19] or 855 bp *Eco*RI/*Hin*dIII fragment of pFE1242 [11]; type IV, 1·7 kb *Cla* I fragment of pUK1148 [16]; type V, 500 bp *Hin*cII fragment of pLK09 [16]; type VII, 300 bp *Eco*RV fragment of pUN1042 [20]. There is no currently available probe for the type VI DHFR. Probes were isolated as described previously [16] and labelled with biotin-14-dATP using a BioNick Translation Kit and the conditions recommended by the manufacturer (Gibco/BRL).

*Hybridization experiments*

Labelled DNA probes were hybridized with plasmid DNA immobilized on nitrocellulose filters and the conditions described previously [12]. Detection of a positive hybridization result was by means of a BlueGENE Kit (Gibco/BRL) using the high-stringency post-hybridization washes and procedures recommended by the manufacturer.

*Confirmatory biochemical tests*

Where necessary, DHFR activity was extracted and characterized using the procedures and parameters described by Amyes and co-workers [21].

## RESULTS

*Trimethoprim R plasmids from 1978–83*

Of 81 trimethoprim R plasmids isolated during this period from strains of *E. coli*, 66 (81%) had already been shown to carry a gene encoding a type I DHFR and have been described in detail elsewhere [12]. The remaining 15 plasmids (Table 1) were re-examined using a combination of newly available DNA probes for DHFR types I-VII and known biochemical parameters for DHFR enzymes. In the majority of cases a single large plasmid (> 30 kb) was detected on agarose gels, but in four instances more than one plasmid was identified in transconjugants and this is indicated in Table 1 where applicable.

(i) *pUN108, pUN189, pUN510 and pUN545.* An initial re-examination using the type I DHFR probe generated a positive hybridization result with pUN510 DNA and this plasmid has now been reclassified as a type I.

*E. coli* K12 transconjugants carrying pUN108, pUN189, or pUN545 yielded plasmid DNA that failed to hybridize with any of the available DHFR gene probes. Nevertheless, detailed biochemical examination of purified DHFR extracts determined that the properties of the trimethoprim-resistant DHFRs produced by these transconjugants were similar of those of a type I-like enzyme [11]. Experiments were performed to determine whether the trimethoprim resistance genes in these strains had transposed to the host cell chromosome and been lost from the original plasmid. Mobilization experiments using the P incompatibility group plasmid RP4 as mobilizing vector, combined with screening by agarose gel electrophoresis, identified secondary trimethoprim-resistant transconjugants in which mobilization of trimethoprim resistance was associated with an approximately 13·5 kb increase in the size of RP4. This increase in size is characteristic of Tn7 and subsequent hybridization experiments generated a positive hybridization result between the type I DHFR gene probe and the RP4 derivatives encoding trimethoprim resistance. The transconjugants carrying pUN108, pUN189, pUN510 and pUN545 were therefore also reclassified as owing their trimethoprim resistance to the production of a type I DHFR.

(ii) *pUN589.* The trimethoprim resistance gene from this plasmid has now been cloned [19] and has enabled the resistance gene to be classified as a type IIIa DHFR.

Table 1. *Plasmids isolated during 1978–83 that were originally identified as not encoding a type I DHFR*

| Trimethoprim R plasmid designation | Resistance* phenotype of transconjugant | DHFR† type |
|---|---|---|
| pUN108 | Tp | I |
| pUN166 | Tp Tc Km Cm Su | V |
| pUN189 | Tp Sm | I |
| pUN241 | Tp Tc Km Cm | V |
| PUN283 | Tp Sm Su‡ | V |
| pUN365 | Tp Su | UT |
| pUN394 | Tp Su‡ | V |
| pUN433 | Tp Sm | V |
| pUN445 | Tp Su‡ | V |
| pUN494 | Tp Tc Km Cm Su | V |
| pUN510 | Tp Cm Su Ap | I |
| pUN545 | Tp Ap | I |
| pUN589 | Tp Tc Su Sm | IIIa |
| pUN663 | Tp Tc Su Sm Ap‡ | V |
| pUN835 | Tp Km Tc Cm Su Sm Ap | VII |

UT, untyped.

* Tp, trimethoprim; Tc, tetracycline; Km, kanamycin; Cm, chloramphenicol; Su, sulphonamides; Sm, streptomycin; Ap, ampicillin.

† Trimethoprim-resistant DHFRs encoded by plasmids were determined primarily using specific DNA probes, but with confirmatory biochemical tests where necessary as described in the text.

‡ Indicates the presence of more than one plasmid in a transconjugant. Plasmid designations refer to the plasmid band (~ 5 kb) which generated a positive hybridization result with the specific DNA probe indicated.

(iii) *pUN166, pUN241, pUN283, pUN394, pUN433, pUN445, pUN494 and pUN663*. Each of these plasmids generated a positive hybridization result with the type V gene probe. All of these plasmids encoded DHFR enzymes with biochemical properties that were compatible with those encoded by the prototype type V gene from Sri Lanka [22].

(iv) *pUN835*. This plasmid originally generated negative hybridization results with the then available DNA probes. Subsequent biochemical studies [21] demonstrated that this plasmid encoded a new enzyme, the type VII DHFR. The gene encoding this enzyme has been cloned and a specific DNA probe identified [20] which was subsequently used in the remainder of this study.

(v) *pUN365*. This plasmid generated negative hybridization results with all DNA probes, including the newly available type VII probe. Attempts to extract and characterize the resistant DHFR encoded by this plasmid have shown that the enzyme is produced at an unusual low specific activity that has so far made it impossible to identify.

*Trimethoprim R plasmids from 1987–8*

A total of 69 trimethoprim R plasmids isolated from randomly-selected strains of *E. coli* during this period were initially examined using the DNA probe for the type I DHFR. A positive hybridization with this probe was obtained for 43 plasmids (62%), which were therefore classified as encoding type I DHFRs. The

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Table 2. *Plasmids isolated during 1987–8 that did not encode a type I DHFR*

| Trimethoprim R plasmid designation | Resistance* phenotype of transconjugant | DHFR† type |
|---|---|---|
| pUN963 | Tp Su | IIIa |
| pUN964 | Tp Su Sm Ap | V |
| pUN965 | Tp | V |
| pUN969 | Tp Su | IIIa |
| pUN970 | Tp Su | VII |
| pUN971 | Tp Su‡ | UT |
| pUN978 | Tp Su‡ | IIIa |
| pUN981 | Tp Su | V |
| pUN984 | Tp Su‡ | IIIa |
| pUN988 | Tp Su Sm Ap | V |
| pUN993 | Tp Sm | UT |
| pUN994 | Tp‡ | VII |
| pUN995 | Tp Tc Su‡ | IIIa |
| pUN996 | Tp Su‡ | IIIa |
| pUN1011 | Tp Km Su | II |
| pUN1012 | Tp Su Sm Ap | IIIa |
| pUN1015 | Tp Tc Su Sm‡ | IIIa |
| pUN1016 | Tp Tc Su‡ | IIIa |
| pUN1018 | Tp Su Sm Ap‡ | IIIa |
| pUN1019 | Tp Su Sm Ap‡ | IIIa |
| pUN1024 | Tp Tc Km Su Sm Ap‡ | VII |
| pUN1025 | Tp Tc Su Sm Ap‡ | IIIa |
| pUN1026 | Tp Km Su Sm Ap‡ | IIIa |
| pUN1029 | Tp Tc Cm Ap‡ | VII |
| pUN1030 | Tp Tc Cm Ap‡ | VII |
| pUN1032 | Tp Tc Su Sm Ap‡ | IIIa |

UT, untyped

* Tp, trimethoprim; Tc, tetracycline; Km, kanamycin; Cm, chloramphenicol; Su, sulphonamides; Sm, streptomycin; Ap, ampicillin.

† Trimethoprim-resistant DHFRs encoded by plasmids were determined primarily using specific DNA probes, but with confirmatory biochemical tests where necessary as described in the text.

‡ Indicates the presence of more than one plasmid in a transconjugant. Plasmid designations refer to the plasmid band which generated a positive hybridization result with the specific DNA probe indicated.

remaining 26 plasmids (Table 2) were re-examined using the available probes for the other known DHFR types. The presence of more than one plasmid in a transconjugant is indicated in Table 2 where applicable.

(i) *pUN1011*. This plasmid generated a positive hybridization result only with the type II DHFR gene probe.

(ii) *pUN963, pUN969, pUN978, pUN984, pUN995, pUN996, pUN1012, pUN1015, pUN1016, pUN1018, pUN1019, pUN1025, pUN1026 and pUN1032*. These 14 plasmids generated positive hybridization results only with the type IIIa DHFR gene probe. In many instances more than one plasmid was present in transconjugants (Table 2) and a positive result with the type IIIa gene probe was associated with both small ($< 5$ kb) and large ($> 30$ kb) plasmids.

(iii) *pUN964, pUN965, pUN981 and pUN988*. Of these four plasmids, pUN964 generated a positive hybridization result only with the type V DHFR gene probe,

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Table 3. *Numbers of plasmids isolated which encoded different DHFR types*

| DHFR type | Period 1978–83 | Period 1987–8 |
|---|---|---|
| I | 70 (86·4%) | 43 (62·3%) |
| II | 0 | 1 (1·5%) |
| IIIa | 1 (1·2%) | 14 (20·3%) |
| IV | 0 | 0 |
| V | 8 (9·9%) | 4 (5·8%) |
| VI | NT | NT |
| VII | 1 (1·2%) | 5 (7·2%) |
| Untyped | 1 (1·2%) | 2 (2·9%) |

NT, Not tested.

while pUN965, pUN981 and pUN988 reacted with both the type V and the type VII gene probes. It is, however, known that some cross-hybridization occurs between the type VII probe and the type V gene, but not between the type V probe and the type VII gene [21]. These four plasmids were therefore assigned to the type V DHFR group.

(iv) *pUN970, pUN994, pUN1024, pUN1029 and pUN1030*. These five plasmids reacted strongly with the probe for the type VII DHFR gene. In addition, pUN1029 and pUN1030 reacted with the type IIIa probe, while pUN994 reacted with the type IV probe. It is known that the type IIIa and type IV probes contain DNA sequences extraneous to the structural gene and that the type VII probe does not hybridize with the type IIIa or type IV structural genes. Confirmatory biochemical tests also suggested that the DHFR enzymes produced did not belong to types IIIa or IV. All five plasmids were therefore classified as type VII.

(v) *pUN971 and pUN993*. Both of these plasmids generated negative hybridization results with each of the available DHFR gene probes and have so far proved untypable using biochemical tests.

*Comparison between 1978–83 and 1987–8*

Table 3 summarizes the types and frequency of identification of plasmid genes encoding trimethoprim-resistant DHFRs in strains of *E. coli* from the Nottingham area. Whereas the gene encoding DHFRs belonging to the type I group remained predominant, the type IIIa and type VII genes were identified with increased frequency from the 1987–8 plasmid collection.

## DISCUSSION

Recent reports of new plasmid-encoded trimethoprim-resistant DHFRs [11] indicate that these enzymes are continuing to emerge and evolve, perhaps in a manner comparable to the different families of plasmid-encoded $\beta$-lactamase enzymes. This increased complexity, coupled with the difficulties of performing biochemical analysis on an epidemiological scale, means that DNA hybridization procedures probably offer the best approach for monitoring the evolution and distribution of these medically important enzymes. The type I DHFR gene probe consists almost entirely of the structural gene [17] and, when used in combination

with high stringency wash conditions, forms an extremely useful and highly specific probe for initial screening purpose [11]. The work described in this paper confirmed that the type I group remains the predominant plasmid-encoded group conferring trimethoprim resistance among strains of *E. coli* from the Nottingham area of the UK. Nevertheless, the results indicated that genes encoding the more recently recognized types of trimethoprim-resistant DHFRs have indeed appeared in *E. coli* from the Nottingham area and can be isolated with increasing frequency.

Of particular interest was the apparent increasing importance of the type IIIa and type VII DHFR enzymes. Prior to its identification in Nottingham, the type IIIa enzyme had previously been reported only in a clinical isolate of *Salmonella typhimurium* from New Zealand [2, 3]. It confers a moderate degree of trimethoprim resistance on its host (with a typical MIC of 64 mg/l) and has a low molecular size (16·9 kDa). When considered in the context of the Nottingham gene pool, the type IIIa gene seems to provide an example of the appearance and spread of a 'new' gene in a bacterial population. In contrast, the type VII enzyme has biochemical properties that are very similar to those of the type I and type V enzymes, although its molecular size and hybridization properties are quite different [21]. Indeed, the type VII may possibly have evolved from the type I or type V genes. If this is the case then the emergence and spread of the type VII gene may be an example of advantageous evolution of an 'existing' gene which was already distributed in a bacterial population.

The emergence of different genes conferring trimethoprim resistance provides an excellent model for the study of bacterial evolution in response to new antibiotics. The use of specific DNA probes allows large numbers of bacterial isolates to be screened rapidly for the presence of particular resistance genes. Little is known about the evolutionary relationships between the different trimethoprim-resistant DHFRs and full biochemical characterization may still be required to resolve occasional ambiguities and identify possible inter-relationships. Subsequent application of nucleotide and automatic amino acid sequencing procedures to related genes should then allow a more complete understanding at the molecular level of the evolutionary processes which occur following the exposure of bacteria to the challenge posed by new antimicrobial agents in general.

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