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Unusual Expression of New Low-Level-Trimethoprim-Resistance Plasmids

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In a survey, 42% of trimethoprim-resistant clinical members of the family *Enterobacteriaceae* were able to transfer trimethoprim resistance to standard *Escherichia coli* strains when selection was made on complex bacteriological media. When transfer experiments were performed with minimal medium, another 16% of the clinical strains were shown to have transferred trimethoprim resistance. Twelve transconjugants produced negligible trimethoprim resistance in complex media but were resistant in minimal medium. The methionine, glycine, and purine components of complex media appeared to be responsible for the reduced expression of trimethoprim resistance in these strains.

Bacterial resistance to trimethoprim, defined as the ability of organisms to grow in trimethoprim concentrations of 10 mg/liter or more, has increased gradually since the introduction of this drug, in combination with sulfamethoxazole, in 1969 (see reference 13 for a review). Resistance plasmids (R plasmids) conferring resistance to trimethoprim (Tp^r) were first described by Fleming et al. (9). These plasmids, isolated from strains of *Escherichia coli* and *Klebsiella* spp., conferred a characteristically high level of Tp^r on their hosts (MIC >1,000 mg/liter). In subsequent years there have been many reports of transferable Tp^r (1, 2, 12, 15, 19). In addition, two recent reports of R plasmids conferring only moderate levels of Tp^r (MIC = 64 to 256 mg/liter) have appeared (10, 21). In almost all cases, the identification of trimethoprim R plasmids has depended on the ability to detect Tp^r transconjugant strains on suitable complex media after the incubation of donors with suitable recipients. In addition, similar media have usually been used to determine the level of resistance conferred by the plasmids (7, 10, 20, 21).

In a recent survey in South India, we found the incidence of trimethoprim resistance among gram-negative urinary pathogens was 64% (22). In the present paper we describe the methods which detected trimethoprim R plasmids from these resistant bacteria and which allowed the identification of a new type of trimethoprim R plasmid.

MATERIALS AND METHODS

Bacteria and plasmids. Clinical strains of members of the family *Enterobacteriaceae* were isolated in a recent study of urinary pathogens from patients at the Christian Medical College Hospital in Vellore, South India. *E. coli* K-12 J62 *pro*$^-$ *his*$^-$ *try*$^-$, resistant to either rifampin (J62-2) or nalidixic acid (J62-1), was used as the recipient in transfer experiments (5). The transfer factor X^+ (16) was used for the mobilization of non-self-transmissible R plasmids. The standard trimethoprim R plasmids were R483, R67 (14), and pAZ1 (11).

Media. Davis-Mingioli (DM) minimal medium (8) was made up as described by Smith (17) and supplemented with proline, histidine, and tryptophan to allow the growth of J62 strains; the carbon source was 0.28% glucose. Further supplementation was as indicated below. The complex media used were nutrient broth no. 2 (CM67; Oxoid Ltd., London, United Kingdom), Iso-Sensitest broth (CM473; Oxoid), Diagnostic Sensitivity Test Agar (DSTA) (CM216; Oxoid), and Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.). The complex broth and minimal medium were solidified with 1.5% (wt/vol) bacteriological agar no. 1 (L11; Oxoid).

Susceptibility testing. The MIC of trimethoprim was measured by diluting an overnight nutrient broth culture 10,000-fold in DM medium and placing approximately 40 to 100 organisms onto the surface of appropriate agar plates containing increasing concentrations of trimethoprim. The lowest concentration allowing no visible growth after 18 h of incubation at 37°C (or 66 h of incubation on DM medium) was taken as the MIC. Susceptibility testing against other antibacterial drugs was performed by placing a similar dilution onto Oxoid DSTA plates containing 10 mg of drug per liter, except for sulfamethoxazole and spectinomycin, of which 100 mg each per liter was used.

Transfer of R plasmids. Overnight cultures of donor and recipient strains grown at 37°C in nutrient broth were mixed in a ratio of 1 to 10 in 4.5 ml of nutrient broth. The mating mixtures were incubated statically at 37°C for 18 h, after which 0.01 ml was streaked onto selection plates as described in Results. When selection was made on DM minimal medium, the mating mixtures were washed and resuspended in DM medium before they were plated out.

Non-self-transmissible plasmids were mobilized with the transfer factor X^+. Overnight nutrient broth cultures of *E. coli* K-12(X^+) and the clinical strains were mixed in a ratio of 1 to 10 in 4.5 ml of nutrient broth. After 18 h of incubation at 37°C, 1.0 ml of this mating mixture was mixed with 1.0 ml of an overnight nutrient broth culture of the appropriate J62 recipient strain in 4.5 ml of nutrient broth. After another 18 h of incubation at 37°C, transconjugants were selected as before.

DNA isolation. Plasmid DNA was isolated by the method of Birnboim and Doly (6). Plasmid sizes were determined in 0.5% agarose 15 Electran (BDH, Poole, United Kingdom) by horizontal gel electrophoresis.

Restriction endonuclease analysis. Plasmid DNA for restriction endonuclease analysis was prepared by the method

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TABLE 1. Resistance profiles and sizes of unusual trimethoprim R plasmids in strain J62-2

Plasmid	Resistance pattern[a]	Size (kilobases)
pUK1123	Tp Su Sm	78
pUK1107	Tp Su Sm	78
pUK1108	Tp Su Sm	78
pUK1110	Tp Su Sm	78
pUK1116	Tp Su Sm Km	81
pUK1117	Tp Su Sm Km	81
pUK1120	Tp Su Sm Km	81
pUK1119	Tp Su Sm Km Ap	79
pUK1115	Tp Su Sm Ap Tc Cm	89
pUK1109	Tp Su Sm Ap Tc Cm	99
pUK1118	Tp Su Sm Ap Tc Cm Km	100
pUK1114	Tp Su Sm Ap Tc Cm Km Gm	122

[a] Ap, Ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Su, sulfamethoxazole; Tc, tetracycline. All plasmids were weakly Tp^r.

of Takahashi and Nagano (18). DNA was restricted with 10 U of *Eco*RI (NBL Ltd., Middlesex, United Kingdom) for 3 h according to the instructions of the manufacturer and then was analyzed by electrophoresis in 0.7% agarose (18).

RESULTS

Transfer of Tp^r. A total of 182 strains, resistant to 10 mg of trimethoprim per liter on Oxoid DSTA, were conjugated for 18 h at 37°C with *E. coli* K-12 recipient strains. The selection for Tp^r transconjugants was initially made on Oxoid DSTA containing trimethoprim (10 mg/liter) and either rifampin (25 mg/liter) or nalidixic acid (10 mg/liter). After incubation, 0.01 ml of the mating mixture was streaked for single colonies onto appropriate selection plates. The plates were incubated overnight, the transconjugant colonies were then purified, and their integrity was checked by testing for specific growth requirements of J62 strains on suitably supplemented DM minimal agar. A total of 74 trimethoprim R plasmids were detected by this method. Another three R plasmids were detected by mobilization with the X^+ transfer factor. No further R plasmids were identified when all transfer experiments were repeated at 25°C.

In a further attempt to detect R plasmids in strains in which no transfer had been demonstrated, the conjugation experiments were repeated. However, this time the selection of the J62 transconjugants was made on DM minimal medium containing proline, histidine, and tryptophan (each at 50 mg/liter), trimethoprim (10 mg/liter), and rifampin or nalidixic acid when appropriate. The selection plates were incubated for 66 h, the transconjugants were subsequently purified, and their integrity was checked. This selection procedure allowed the detection of another 29 trimethoprim R plasmids.

Because additional R plasmids could be detected on suitably supplemented DM medium, the 77 plasmids which had originally been selected on Oxoid DSTA were retested to determine whether they could also be detected when selection was made on DM medium. All 77 plasmids transferred when the selection was made on suitably supplemented DM minimal medium.

Identification of unusual trimethoprim R plasmids. All the R plasmids were characterized by resistance profiles following the transfer to J62 strains. Altogether, 12 J62 transconjugant strains, harboring R plasmids which had originated from 12 different clinical strains, were found to grow either weakly or not at all on Oxoid DSTA plates containing 10 mg of trimethoprim per liter. The resistance profiles of the transconjugants are shown in Table 1. All 12 transconjugants were resistant to sulfamethoxazole and streptomycin, and several of them were also resistant to a variety of other drugs. Analysis of the DNA from these strains revealed the presence of only one plasmid in each transconjugant (Table 1). Restriction endonuclease analysis showed that, with only one exception, plasmids with the same resistance pattern gave identical restriction digest patterns (Fig. 1). Thus, a total of seven different plasmid types were identified.

Determination of the resistance level conferred by unusual trimethoprim R plasmids. Because the transconjugants had been selected for Tp^r and they now appeared to be only weakly Tp^r on Oxoid DSTA, the MIC of trimethoprim for these strains was determined on a variety of complex media and on suitably supplemented DM minimal agar. The results (Table 2) show that, with only one exception [J62-2(pUK1114)], the MIC of trimethoprim for the J62-2 strains harboring these plasmids was much lower in complex media than any MIC previously described for R-plasmid-mediated Tp^r. The level of resistance varied slightly from medium to medium and was not greatly affected by the presence of lysed horse blood. On the other hand, the MIC of trimethoprim for each strain, when tested on suitably supplemented DM minimal agar, was considerably higher (8- to 30-fold) and confirmed that the Tp^r determinant had transferred.

The bacterial response to trimethoprim in minimal medium differs, depending on whether methionine, glycine, and a purine derivative are present in the medium (3). Therefore, the MIC of trimethoprim for each organism was redetermined on suitably supplemented DM agar plates to which methionine, glycine, and adenine had been added, each at a concentration of 50 mg/liter. The results (Table 2) show that the presence of these supplements dramatically reduced the MIC of trimethoprim for each strain to a level similar to that obtained in complex media. The additional presence of uridine, which prevents the uptake of thymine by strains rendered phenotypically thymineless by the presence of trimethoprim (4), had no effect on the MIC of trimethoprim (results not shown).

No significant difference in the MIC of trimethoprim was found for J62-2 strains harboring trimethoprim R plasmids encoding the type I (R483), II (R67), or III (pAZ1) dihydrofolate reductases when the strains were tested on the various media (Table 2).

DISCUSSION

An important observation in this study was that conventional methods for detecting trimethoprim R plasmids may fail with certain plasmids. When Tp^r transconjugant strains were selected on complex media (Oxoid DSTA), 42.3% of the resistant isolates tested were shown to possess trimethoprim R plasmids. However, the repetition of transfer experiments and subsequent selection of Tp^r transconjugants on suitably supplemented DM minimal medium allowed the detection of another 29 trimethoprim R plasmids. Therefore, if the selection of trimethoprim R plasmids had been made exclusively on complex media, 27% of the transferable trimethoprim R plasmids would have remained undetected, even though low-temperature transfer and mobilization experiments had also been performed. We therefore recommend that minimal medium with defined amino acid supplements be used routinely for plasmid transfer experiments.

Characterization of the R plasmids revealed the existence of 12 plasmids, isolated from *E. coli* and *Klebsiella* strains,

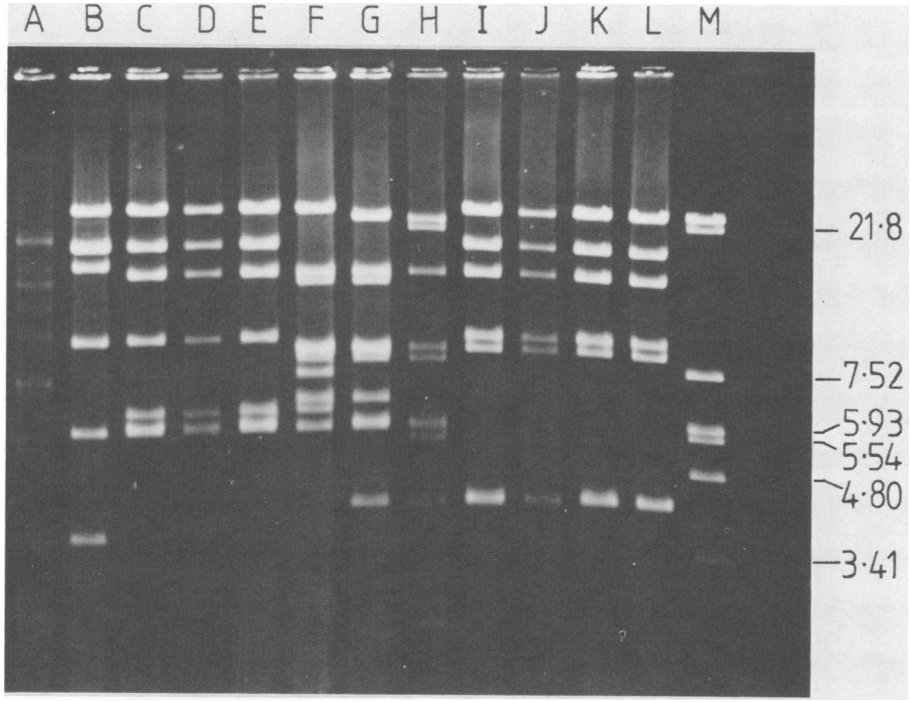

FIG. 1. *Eco*RI restriction endonuclease digests of unusual trimethoprim R plasmids. Lanes: A, pUK1114; B, pUK1119; C, pUK1120; D, pUK1117; E, pUK1116; F, pUK1118; G, pUK1115; H, pUK1109; I, pUK1110; J, pUK1108; K, pUK1107; L, pUK1123; M, *Eco*RI digests of phage λ DNA used as size standards. Numbers indicate sizes (kilobases).

which conferred an unusually low level of Tp^r on their *E. coli* transconjugant hosts. Although the Tp^r determinant of all 12 plasmids could be detected on suitably supplemented DM agar, the determinant could be distinguished for only 4 on Oxoid DSTA. This probably resulted from a poorer expression of the Tp^r gene in complex media, as much lower MICs were obtained for this type of medium than for minimal medium.

Amyes and Smith (3) demonstrated that in minimal medium the bacterial response to trimethoprim is bacteriostatic in the absence of methionine, glycine, and adenine but is bactericidal in their presence. The difference in the MIC of trimethoprim for strains harboring these plasmids suggests that the presence of the metabolites influences the expression of Tp^r. This is quite unlike any plasmid-determined Tp^r previously described. The trimethoprim R plasmids reported by Towner and Pinn (21) and Fling et al. (10) also confer low levels of Tp^r. However, the MICs in these studies were determined in complex media, and these plasmids produced levels of resistance similar to those reported here for DM

TABLE 2. MIC of trimethoprim for *E. coli* J62-2 transconjugants harboring unusual R plasmids

Plasmid in strain J62-2	MIC of trimethoprim on various media[a] (mg/liter)					
	DM	DSTA	DSTA-LB	M-H	IA	DM+Met+Gly+A
pUK1107	160	20	10	5	5	10
pUK1108	160	20	10	5	5	10
pUK1109	160	20	10	5	5	10
pUK1110	160	20	10	5	5	10
pUK1123	160	20	10	5	5	10
pUK1116	160	10	10	5	5	10
pUK1117	160	10	10	5	5	10
pUK1118	160	10	10	5	5	10
pUK1120	160	10	10	5	5	10
pUK1115	80	10	10	5	5	10
pUK1119	160	20	40	10	10	40
pUK1114	320	40	160	40	40	160
R483	2,560	2,560	2,560	2,560	2,560	2,560
R67	2,560	2,560	2,560	2,560	2,560	2,560
pAZ1	160	80	80	80	80	80
No plasmid	0.5	0.5	0.5	0.5	0.5	0.5

[a] DM, Davis-Mingioli minimal medium supplemented with proline, histidine, and tryptophan; DSTA-LB, Oxoid Diagnostic Sensitivity Test Agar containing 5% lysed horse blood; M-H, Difco Mueller-Hinton agar; IA, Oxoid Iso-Sensitest agar; Met, methionine; Gly, glycine; A, adenine.

minimal medium. It seems, therefore, that the resistance mechanism mediated by the plasmids in these previous studies was not unduly depressed by the presence of methionine, glycine, and purines in the media. Indeed, the resistance mechanism of the plasmid described by Fling et al. has since been shown to be due to the plasmid-encoded production of a new dihydrofolate reductase (type III) (11). We have now shown that the Tp^r mechanism of the unusual plasmid pUK1123 described here is due to the plasmid production of yet another dihydrofolate reductase (type IV), the synthesis of which is inducible (21a).

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