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Prevalence and genetic location of non-transferable trimethoprim resistant dihydrofolate reductase genes in South African commensal faecal isolates

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SUMMARY

In a recent survey of trimethoprim resistance, 357 Gram-negative aerobic organisms were isolated from healthy volunteers from rural and urban populations in South Africa. Trimethoprim resistance did not transfer to an *Escherichia coli* J62-2 recipient strain by conjugation in a liquid mating in 161 (45·1%) of the isolates. These isolates which did not transfer their resistance were probed with intragenic oligonucleotide probes for the types Ia, Ib, IIIa, V, VI, VII, VIII, IX, X and XII dihydrofolate reductase genes. Contrary to all previous data, the most prevalent dihydrofolate reductase gene in this group of non-transferable isolates which hybridized, was the type VII (38%) followed by the type Ia (25%), Ib (12%), V (1·7%) and VIII (1·2%). None of the strains hybridized to the types IIIa, VI, XI, X and the XII dihydrofolate reductase probes. Southern blots of plasmid and chromosomal DNA from selective isolates revealed that the type VII dihydrofolate reductase genes were located on the chromosome and were associated with the integrase gene of Tn*21*. However, the type Ib and V dihydrofolate reductase genes were all found on plasmids which could not be mobilized. The type Ia dihydrofolate reductase genes were found on both non-transferable plasmids and on the chromosome. The nature of the genetic structures associated with a dihydrofolate reductase gene strongly affects the means of spread of the gene in a population.

INTRODUCTION

Trimethoprim is a broad-spectrum antimicrobial agent used on its own or in combination with sulphamethoxazole in the treatment of Gram-negative infections. Trimethoprim selectively inhibits the bacterial dihydrofolate reductase (DHFR) thus preventing reduction of dihydrofolate to tetrahydrofolate [1]. The most common mechanism of resistance to trimethoprim in Enterobacteria is the production of an additional plasmid mediated DHFR which unlike the chromosomal enzyme, is less sensitive to inhibition by trimethoprim [2]. Fifteen of these trimethoprim resistant enzymes have been identified in Enterobacteria

and have been characterized according to their biochemistry and DNA sequence. They are the Ia [3], Ib [4], IIa [5], IIb [6], IIc [7], IIIa [8], IIIb [9], IIIc [10] renamed the type VIII [11], IV [12], V [13], VI [14], VII [15], IX [16], X [17] and XII [18].

Since the introduction of trimethoprim to South Africa, high levels of resistance (48·5%) have been recorded among hospital isolates of Gram-negative aerobic bacteria [19]. The high incidence of trimethoprim resistance is cause for concern as it increases the number of treatment failures and limits the available choice of antibiotics for therapy. The problem of antimicrobial resistance is further complicated by the presence of plasmids bearing resistance factors to more than one therapeutic agent.

It has been suggested that the faecal flora may act as a reservoir for antibiotic resistance genes with the potential for transfer to human pathogens [20]. Levy and colleagues [21] suggested that resistance genes may possibly be transferred to pathogens via bacteriophage or transposable elements. Trimethoprim resistant DHFR genes have also been found as cassettes capable of site-specific insertion into integrons [22]. This mechanism has the potential for the development of multiple resistance on a single mobile genetic element. In some individuals resistant organisms constitute a major fraction of the gut flora [23]. Resistance in faecal flora is important since these organisms are thought to be implicated in infection of the urinary tract [24].

Trimethoprim resistance at first world centres has been dominated by the Tn*7* mediated type Ia DHFR [16, 25–27]. Much of the success of the type 1a DHFR is based on its ability to integrate as part of Tn*7* into the attTn*7* site on the chromosome of *E. coli* and other bacteria [28]. This promiscuous transposon has also been found integrated into random sites on the chromosome and on plasmids albeit at a lower frequency [29]. The type Ia DHFR is found as part of a cassette like structure thus giving it the potential to be inserted by integrase activity into integron like structures of different genetic elements. The type Ia DHFR has been found within integron structures of Tn*7* and the antimicrobial resistance integron of the predominantly plasmid located transposon Tn*21* [30].

A study by Heikkilä and colleagues [26] found the type Ia DHFR on the chromosome in 75 out of 76 isolates. Nine of these isolates carried the type Ia DHFR on both the plasmid and chromosome and only one isolate carried the gene on a plasmid only. Of these isolates, 61 (80%) probed positive for the *tns*C gene suggesting an active Tn*7*. Fifty of these isolates (82%) had Tn*7* located in the attTn*7* site. The type II, V, VII and XII DHFRs have all been found as cassette structures on self transmissible plasmids in the integron of Tn21 like elements [13, 31]. The following other DHFRs have been observed to contain the characteristic repeats that suggest that the gene forms part of a cassette: VI [15] and the type Ib of Tn 4132 [32].

Little is known about the epidemiology or genetic location of trimethoprim resistance determinants in third world countries. Surveys from Sri Lanka [33] and South Africa [19] showed there to be a low prevalence of the type Ia DHFR. A study of commensal flora from South India showed that the most prevalent DHFR was the type V [34]. In Taiwan the frequency of the type Ia and type V DHFRs in urine isolates was 45·4% and 10·4% respectively [35].

A recent study of antimicrobial resistance showed that the occurrence of trimethoprim resistance in Gram-negative aerobic faecal flora isolated from rural and urban black populations in South Africa was 74·2% [36]. The trimethoprim-resistant isolates from this survey were further studied [37] and in these isolates, the prevalence of transferable plasmid mediated DHFRs detected with oligonucleotide probes was as follows: type Ib (30%), VIII (23%), V (13%), Ia (6%), VII (3%) and XII (0·5%) [38]. This paper reports the non-transferable DHFRs that were identified with oligonucleotide probes and investigates the genetic location of some of these genes.

METHODS

Conjugation

Conjugation experiments were performed in liquid media by the method of Amyes and Gould [39]. *Escherichia coli* K-12 strain J62-2 (*his trp pro rif*^{r}) was used as the recipient. Isolates resistant to rifampicin were mated with *E. coli* K-12 strain J62-2 (*his trp pro nal*^{r}). The selective medium used was Davis Mingioli minimal medium [40] with 2·8 g/l glucose, 10 mg/l trimethoprim (Wellcome Medical Division, Crewe, UK), 25 mg/l rifampicin (Gruppo Lepetit, Milan, Italy) or 20 mg/l nalidixic acid (Sanofi Winthrop, Gilford, UK), supplemented with 50 mg/l histidine, 50 mg/l proline and 50 mg/l tryptophan. Colonies were purified on selective medium and their auxotrophic requirements checked on Davis Mingioli minimal agar supplemented with 2·8 g/l glucose. With those strains that did not transfer their trimethoprim resistance, a triple mating was set up with an X^+ factor as the mobilizing plasmid [41].

Filter matings were performed with a rifampicin resistant mutant of *E. coli* K802 (*hsd*R, *met*B) [42]. Matings were prepared by passing 1 ml of 1:10 mixture of an overnight culture of donor and recipient strain respectively through sterile 13 mm cellulose nitrate filters (pore size 0·45 μm). The filters were placed onto the surface of isosensitest agar and incubated overnight at 22 °C or 37 °C. The filters were placed in 10 ml of Davis Mingioli minimal medium and shaken vigorously. A 100 μl of this mixture was plated out onto isosensitest agar with 10 mg/l of trimethoprim and 25 mg/l of rifampicin. The auxotrophic requirements of the transconjugants were confirmed on Davis Mingioli minimal agar supplemented with 2·8 g/l glucose.

DNA isolation

Plasmid DNA was isolated as described previously [37]. Total DNA was isolated by the following method. All manipulations were carried out at room temperature in 1·5 ml micro centrifuge tubes unless otherwise stated. Cells were harvested from 0·5 ml of overnight Luria broth culture and resuspended in 350 μl of HTE buffer (50 mM-Tris-Cl, pH 8·0, 20 mM-EDTA). An equal volume of 2% *N*-lauroylsarcosine in HTE buffer was added and incubated at 55 °C for 30 min to lyse the cells. The lysate was cooled then extracted with one volume of phenol:chloroform (1:1, equilibrated with 0·5 M-Tris-Cl, pH 8·0). One volume of isopropanol was added to the supernatant and the DNA was precipitated for 10 min prior to

Table 1. *Oligonucleotides for DHFR gene probes*

DHFR	Oligonucleotide sequence	Origin/Reference
Type Ia	5′-CAAGTTTTACATCTGACAATGAGAACGTAT-3′	DNA sequence: 429–459 bp [3]
Type Ib	5′-GTTGGACATCAAATGATGACAATGTAGTTG-3′	DNA sequence: 430–460 bp [32]
Type IIIa	5′-ATCCCCAATGGCAGGCCGAAGGGGTGGAGG-3′	DNA sequence: 298–328 bp [8]
Type VIII	5′-AAGCGCTGGAGCTTCCGGGTGTTCGTGACG-3′	DNA sequence: 779–809 bp [11]
Type V	5′-CCTGGACGGCCGATAATGACAACGTAATAG-3′	DNA sequence: 1501–1531 bp [13]
Type VI	5′-CTAAAATTATCTCGAATGACCCTGATGTTG-3′	DNA sequence: 528–558 bp [14]
Type VII	5′-GAATTTCAAGCTCAAATGAAAATGTATTAG-3′	DNA sequence: 789–819 bp [15]
Type IX	5′-CAGTACCACCCACCCAGAACACTGATCAAG-3′	DNA sequence: 924–954 bp [16]
Type X	5′-CAACTATCACAGAGCACGAAGTGCTCAACA-3′	DNA sequence: 848–878 bp [17]
Type XII	5′-AAGCTAACTACCGCGCCACTGGCTGCGTAG-3′	DNA sequence: 414–444 bp [18]

centrifugation. The pellet was resuspended in 50 μl of H_2O with 20 μg/ml of RNase.

Probe construction

Probes to distinguish between the type Ib and V DHFRs were used as described by Young and colleagues [32]. The region used is very heterogeneous between these two genes. Considerable heterogeneity occurs in the same region throughout all resistant DHFRs and this region was selected for the construction of 30 bp oligonucleotide probes for the type Ia, Ib, IIIa, VIII, V, VI, IX, X, and XII DHFR genes. The nucleotide sequence of the 30mer oligonucleotide probes for the DHFRs appear in Table 1. All of the oligonucleotide probes were tested for homology with other DNA sequences on the GenBank database. The probe for the integrase gene of Tn*7* was previously described [26]. The probe for the integrase gene of Tn*21* was a 22mer oligonucleotide probe 5′-GTCAAGGTTCTGGACCA-GTTGC-3′ from the integrase gene of Tn*21* [11]. The oligonucleotide probes were labelled using the ECL Oligo labelling and detection kit (Amersham, UK). The type II gene probe consisted of a 280 bp *Eco*R I, *Sau*3A I intragenic fragment of pWZ820 [43] and was labelled using the ECL Randomprime labelling and detection kit (Amersham, UK). Table 2 shows the control strains that were used with the probes.

Preparation of Dot Blots

Total DNA isolated from the transconjugants was denatured by the addition of one volume of 1 M-NaOH followed by a 10 min incubation at 37 °C. DNA concentrations were determined by agarose gel electrophoresis and 500 ng of DNA was spotted onto nitro-cellulose membranes (DuPont Genescreen, Germany). The membranes were dried then baked at 80 °C for 2 h.

Table 2. *Control plasmids used in hybridization protocols*

DHFR	Plasmid	Reference or origin
Type Ia	pFE506	[3]
Type Ib	pUK163	[32]
Type IIa	R67	[44]
Type IIb	R388	[6]
Type IIc	R751	[7]
Type IIIa	pAZ1	[8]
Type IIIb	pBH600	[10]
Type VIII	pBH700	[10]
Type IV	pUK1123	[45]
Type V	pLMO20	[33]
Type VI	pUK672	[46]
Type VII	pLMO226	[33]
Type IX	pCJO01	[16]
Type X	pMAQ41	[17]
Type XII	pBEM155	[18]

Southern hybridization

Plasmids were isolated as described previously [37]. Plasmid and total DNA was run on 0·8% agarose gels and Southern blotted onto nitrocellulose membranes (Amersham, Hybond C-Extra). Total DNA that was restricted with *Eco*R I or *Bam*H I was run on 1% agarose gels before Southern blotting.

Hybridization with oligonucleotide probes

Hybridization was carried out with the ECL Oligo labelling and detection kit (Amersham, UK). The hybridization was performed for 2 h at 53 °C according to the manufacturer's recommendations. Stringency washes were performed as described previously [26].

Hybridization with gene probes

Hybridization was carried out with the ECL Randomprime labelling and detection kit (Amersham, UK). The blots were hybridized overnight at 62 °C according to the manufacturer's recommendations with the following stringency washes: two 15 min washes with 1 × SSC and 0·1% SDS followed by two 15 min washes with 0·05 × SSC and 0·1% SDS at 62 °C. The type IIb and IIc were used as positive controls.

RESULTS

The oligonucleotide probes discriminated well between the DHFRs and no cross hybridization was observed with any of the negative controls. Out of 357 isolates of trimethoprim resistant Gram-negative commensal faecal flora isolated from healthy volunteers from rural and urban populations, resistance was transferable to a recipient strain (*E. coli* J62-2) in a liquid mating in 184 (51·5%) of the isolates. A further 12 (3·4%) plasmids could be mobilized into *E. coli* J62-2 in a triple mating with an X^+ factor. Total DNA from the 161 (45·1%) of the isolates which

Table 3. *Frequency of resistant DHFRs in South African commensal faecal flora*

DHFR	Transferred by conjugation (%)	Non-transferable (%)	Total (%)
Type Ia	11 (3·1)	41 (11·5)	52 (14·6)
Type Ib	58 (16·2)	20 (5·6)	78 (21·8)
Type VIII	45 (12·6)	1 (0·3)	46 (12·9)
Type V	25 (7·0)	3 (0·8)	28 (7·8)
Type VII	5 (1·4)	62 (17·4)	67 (18·8)
Type XII	1 (0·3)	0	1 (0·3)
Unknown	52 (14·6)	38 (10·6)	90 (25·2)

Table 4. *Frequency of non-transferable DHFRs and the organism of origin*

DHFR	Hybridization (%)	Species
Type Ia	41* (11·5)	*E. coli* (32)
		Klebsiella spp. (3)
		Enterobacter spp. (4)
		Citrobacter spp. (1)
		Proteus vulgaris (1)
Type Ib	20 (5·6)	*E. coli* (14)
		Klebsiella spp. (6)
Type VIII	1 (0·3)	*Citrobacter* sp. (1)
Type V	3 (0·8)	*E. coli* (1)
		Klebsiella spp. (2)
Type VII	62 (17·4)	*E. coli* (57)
		Klebsiella spp. (3)
		Enterobacter spp. (2)

* Of the isolates which hybridized to the type Ia probe, four also hybridized with the type VII probe and one hybridized with the type VIII probe.

did not transfer their resistance to a recipient strain, was probed for the presence of the types Ia, Ib, IIb, IIc, IIIa, VIII, V, VI, VII, IX, X and XII DHFR genes. A comparison of the hybridization frequencies of the DHFR genes, which were transferable by conjugation and those which did not transfer to *E. coli* J62-2, is shown in Table 3.

Of the isolates which did not transfer their resistance, the most prevalent DHFR type was the type VII which occurred in 62 (17·4%) of the isolates. The second most prevalent DHFR was the type Ia 41 (11·5%), followed by the type Ib 20 (5·6%), the type V, three isolates and the VIII two isolates. The species distribution of the isolates, which hybridized to each probe is shown in Table 4. Five isolates hybridized to more than one DHFR probe, four of which hybridized to both to the type Ia and VII DHFR probes and one isolate to the types Ia and VIII DHFR probes. Since there was no cross hybridization with the control strains, it appears that two resistant DHFR genes are present in these isolates. None of the isolates hybridized to the probes for the types IIb, IIc, IIIa, VI, IX and XII DHFR genes. Thirty-eight isolates did not hybridize to any of the DHFR probes.

Representative isolates carrying each DHFR type were randomly selected. Plasmid and total DNA from each isolate was separated on a 0·8% agarose gel, Southern blotted then probed to determine the genetic location of the DHFR

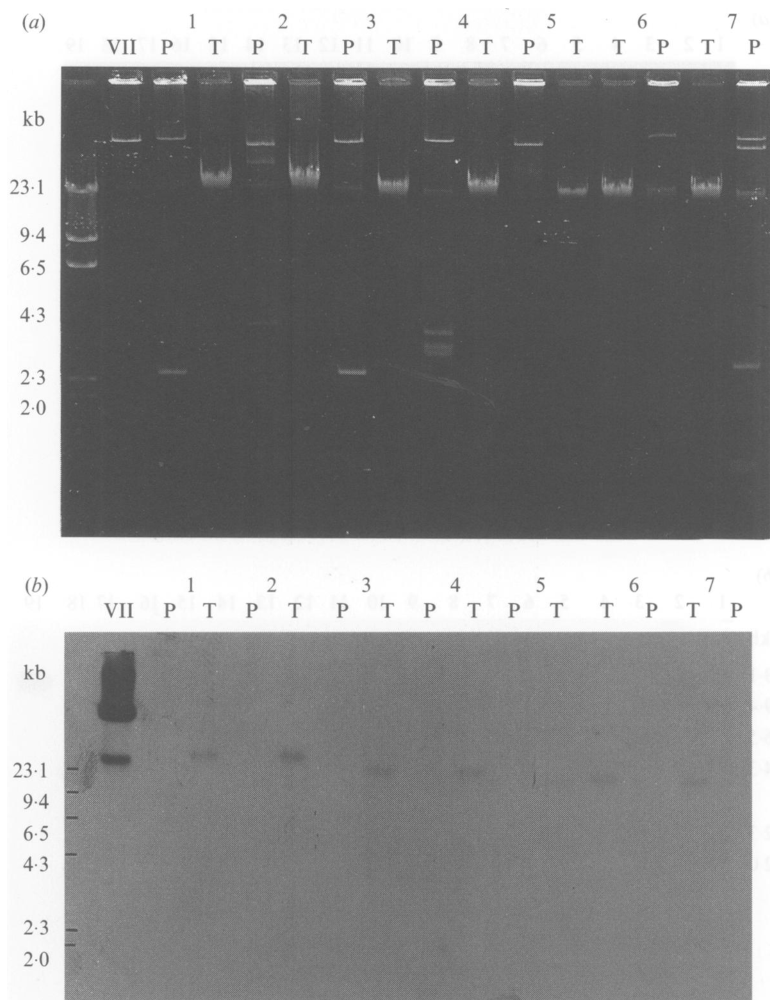

Fig. 1. (*a*) 0·8% Agarose gel of plasmid and total DNA preparations from seven isolates which hybridized to the probe for the type VII DHFR. (*b*) Southern blot showing the chromosomal location of the type VII DHFR gene. VII, Plasmid pLMO226 control; P, Plasmid DNA; T, Total DNA.

gene. The type VII DHFR gene was found located on the chromosome in all the isolates tested ($n = 9$). Figure 1 shows the agarose gel and the Southern blot of seven of these isolates. Sixteen isolates harbouring a non-transferable type VII DHFR gene including these nine isolates were further analysed by Southern blotting a *Bam*H I restriction of the chromosomal DNA and probing it with oligonucleotide probes for the type VII DHFR and the integrase gene of Tn*21* (Fig. 2). For each isolate both probes hybridized positively to fragments of the same size.

The chromosomal DNA probed positive in 8 out of 9 isolates which hybridized to the type Ia DHFR probe. In the remaining isolate the type Ia DHFR was

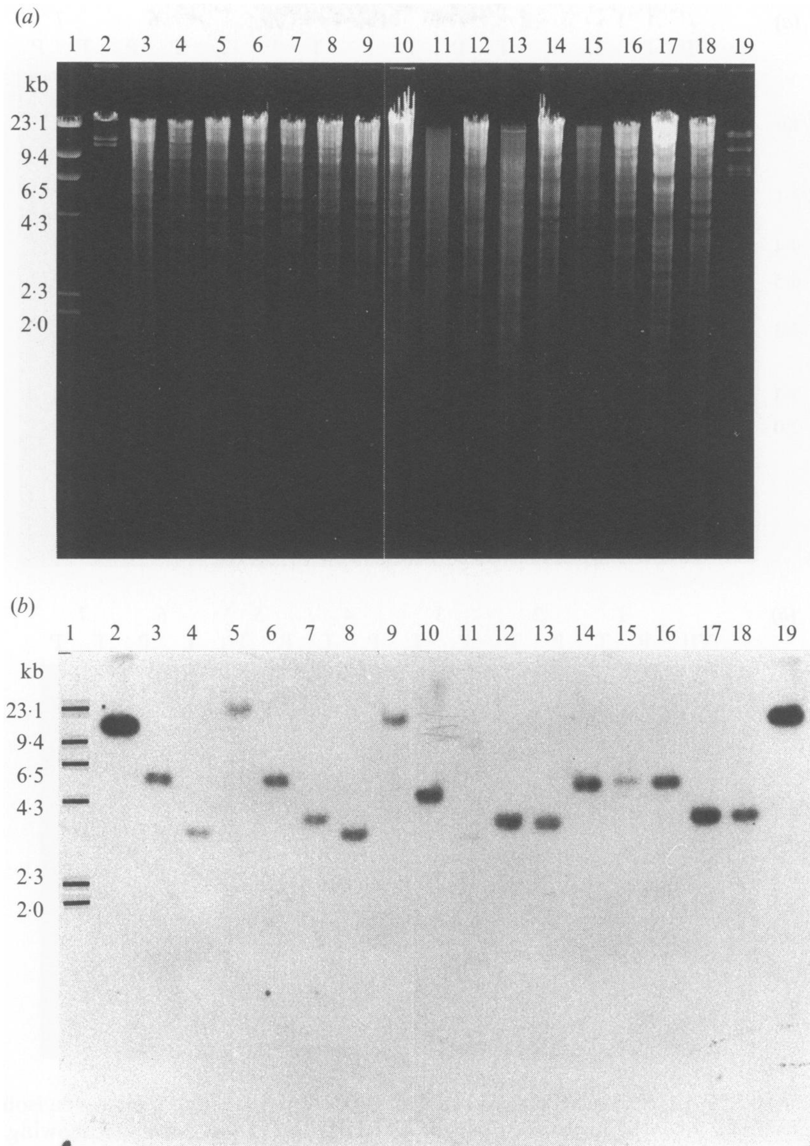

Fig. 2. (*a*) 1% Agarose gel of restricted total DNA preparations from isolates which hybridized to the probe for the type VII DHFR. (*b*) Southern blot showing the fragment sizes which hybridized to the type VII DHFR probe. Lanes 1, *Hind* III restricted λ DNA; 2, *Bam*H I restricted pLMO226 control DNA; 3–18, *Bam*H I restricted total DNA from isolates which hybridized to the type VII DHFR probe; 19, *Eco*R I restricted pLMO226 control DNA.

Table 5. *Genetic location of DHFR genes and associated integrase genes*

DHFR	(*n*)	Location of DHFR	Associated transposon
Type Ia	9	Chromosome (8)	Tn*7* (7)
		Plasmid (1)	Tn*21* (1)
Type Ib	7	Plasmid (7)	Tn*21* (2)
Type V	3	Plasmid (3)	Tn*21* (3)
Type VII	9	Chromosome (9)	Tn*21* (9)

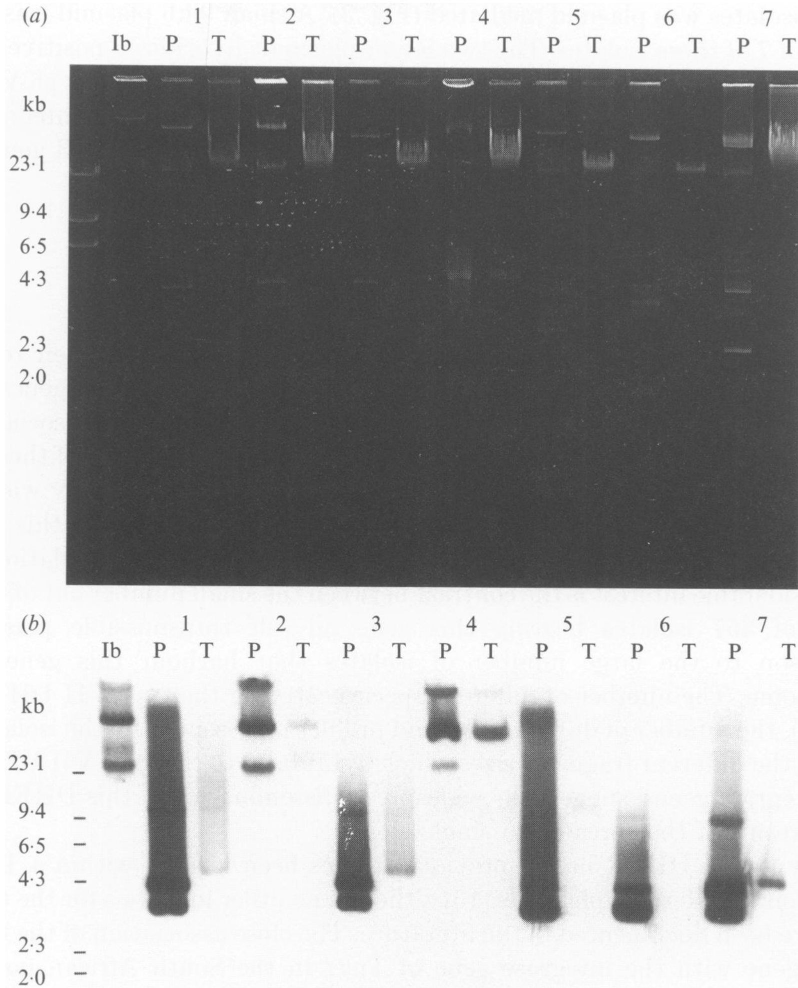

Fig. 3. (*a*) 0·8% Agarose gel of plasmid and total DNA preparations from seven isolates which hybridized to the probe for the type Ib DHFR. (*b*) Southern blot showing the plasmid location of the type Ib DHFR gene. Ib, Plasmid pUK163 control; P, Plasmid DNA; T, Total DNA.

located on a plasmid. A southern blot of *Eco*R I restricted chromosomal DNA from these isolates revealed a number of fragments of variable size which hybridized to the type Ia DHFR probe. The probe for the integrase gene of Tn*7* hybridized to fragments of the same size in 7 out of 8 chromosomal mediated *dhfrIa* bearing isolates. The plasmid carrying the type Ia DHFR gene probed positive for the integrase gene of Tn*21* (Table 5). Attempts to mobilize this plasmid into a restriction endonuclease deficient recipient (*E. coli* K802) in a filter mating at 22 °C and 37 °C were not successful.

Southern hybridizations of plasmid and total DNA from seven isolates that probed positive for the type Ib DHFR gene revealed that the type Ib DHFR gene

in these isolates was plasmid mediated (Fig. 3). A small 9 kb plasmid was present in 5 out of 7 of these isolates. The two larger plasmids hybridized positively to the integrase gene of Tn*21*. All three isolates which hybridized to the type V DHFR probe were found to be plasmid mediated and associated with the integrase gene of Tn*21*. None of these plasmids bearing the type of Ib and V DHFR genes could be mobilized into *E. coli* K802 in a filter mating at 22 °C and 37 °C.

DISCUSSION

The distribution of the DHFR genes which could not transfer their resistance by conjugation is completely different from the distribution of these genes located on transferable plasmids. This suggests that the genetic structures associated with these genes play an important role in the dissemination and spread of these genes.

The type VII DHFR gene has been reported to be geographically widespread [15]. The incidence of the type VII DHFR that was recorded in this study is considerably higher than that found in any previously studied population. What is of considerable interest is the contrast between the small number out of the total sample of 357 isolates bearing this gene on self transmissible plasmids in comparison to the large number of isolates that harbour this gene on the chromosome. The number of different species carrying the type VII DHFR gene (Table 4), the number of different plasmid profiles represented by the isolates (Fig. 1*a*), and the different fragment sizes which hybridized to the type VII DHFR and Tn*21* integrase genes, suggest the widespread dissemination of this DHFR gene is not the result of the spread of a single clone.

The type VII DHFR has in previous studies been located within a Tn*21* like element on transferable plasmids [15] although no other locations for the type VII gene have been documented in the literature. The close association of the type VII DHFR gene with the integrase gene of Tn*21* in the South African population suggests that the type VII DHFR gene is integrated into an integron-like structure. The number of different sized restriction fragments that showed this combination of these two genes suggest that the integrase may be incorporated into a number of different Tn*21* like elements such as Tn*8056* [15] which may insert at random sites into the chromosome. It is unclear why the integrase gene of Tn*21* which is normally associated with DHFR genes on plasmids [13, 15, 26, 31] should show a preference for integration into the chromosome.

Although the genetic structures associated with the spread of the type Ia DHFR appear to be similar to those reported by Heikillä and colleagues [26], the frequency of the type Ia DHFR in South African populations is considerably lower than that reported in other populations.

In contrast to the type Ia and VII DHFRs, the DHFR genes which probed positive for the type Ib and V DHFRs were located on plasmids in the isolates that were tested. These plasmids appear to be stable and could not transfer their resistance by conjugation under a variety of conditions. The origins and mechanisms of dispersal of these plasmids is difficult to determine. However it appears that these phenomena may account for a larger proportion of non-transferable resistance factors than previously expected.

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